UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Sorocaba

PROGRAMA DE PÓS-GRADUAÇÃO em
Ciências Ambientais

MÔNICA PASCOLI

AVALIAÇÃO DA TOXICIDADE E ATIVIDADE BIOLÓGICA DE NANOPARTÍCULAS DE ZEÍNA CONTENDO O BIOCIDA AZADIRACTINA

Sorocaba
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Tese apresentada como requisito para a obtenção do título de Doutor em Ciências Ambientais da Universidade Estadual Paulista "Júlio de Mesquita Filho" na Área de Concentração Diagnóstico, Tratamento e Recuperação Ambiental

Orientador: Prof. Dr. Leonardo Fernandes Fraceto
Coorientadora: Profª. Drª. Renata de Lima

Sorocaba
2019

P281a

Pascoli, Mônica
Avaliação da toxicidade e atividade biológica de nanopartículas de zeína contendo o biocida azadiractina / Mônica Pascoli. -- Sorocaba, 2019
124 f. : il., tabs., fotos

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia, Sorocaba
Orientador: Leonardo Fernandes Fraceto
Coorientadora: Renata de Lima

1. Nanobiopesticida. 2. Óleo de Neem. 3. Nanotoxicidade. 4. Atividade Biológica. I. Título.

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: Avaliação da toxicidade e atividade biológica de nanopartículas de zeína contendo o biocida azadiractina.

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COORIENTADORA: RENATA DE LIMA

Aprovada como parte das exigências para obtenção do Título de Doutora em CIÊNCIAS AMBIENTAIS, área: Diagnóstico, Tratamento e Recuperação Ambiental pela Comissão Examinadora:

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Sorocaba, 02 de setembro de 2019

Á minha mãe Neidiana, sempre.

AGRADECIMENTOS

Agradeço a Deus pela proteção constante, saúde, fé e força. Acredito em sua vontade, que ela sempre prevaleça frente todas as minhas inseguranças, meus medos e fraquezas.

Agradeço a FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo - 2015/17120-4) pela bolsa de doutorado concedida e pelo apoio financeiro para a realização deste trabalho. As opiniões, hipóteses e conclusões ou recomendações expressas neste material são de responsabilidade do(s) autor(es) e não necessariamente refletem a visão da FAPESP.

Ao Prof. Dr. Leonardo Fernandes Fraceto pela oportunidade única de orientação, pela confiança e dedicação. Pela fundamental paciência, respeito, incentivo e esforço para o crescimento de todos os integrantes do nosso grupo de pesquisa. Que você seja grandemente realizado e feliz.

À Profª. Drª. Renata de Lima por participar desse trabalho como coorietadora, pela prontidão, apoio e auxílio imensurável no desenvolvimento de toda essa pesquisa.

À Profª. Drª Daiana Silva Ávila pela oportunidade de trabalhar e aprender com *Caenorhabditis elegans* e com você, por toda a sua colaboração e amizade.

Ao Prof. Dr. Daniel Júnior de Andrade pelos ensaios realizados com *Tetranychus urticae*, por toda prontidão e conhecimento.

Ao Prof. Dr. Halley Caixeta de Oliveira pela prontidão em auxiliar e transmitir gigante quantidade e qualidade de conhecimento em fisiologia vegetal.

Ao Prof. Dr. Jayme Augusto Souza-Neto pela receptividade e auxílio para aquização de imagens dos *Acanthoscelides obtectus.*

Ao Prof. Dr. Marcelo Bispo de Jesus, pela colaboração com os ensaios em culturas de células.

Ao Prof. Dr. Ricardo Antônio Polanczyk pelos ensaios com *Bemisia tabaci* e apoio com nosso trabalho.

À Felícia Pereira de Albuquerque, por toda dedicação e auxílio com os experimentos com *Acanthoscelides obtectus,* por nossas conversas e amizade.

Ao Dr. Anderson do Espírito Santo Pereira, pela colaboração com as imagens adquiridas no microscópio de força atômica.

A todos os meus companheiros de grupo de pesquisa, pelo bom convívio, companherismo e aprendizado. A todos os grupos de pesquisa dos colaboradores citados acima, pelo fundamental apoio no desenvolvimendo dos experimementos, sobretudo aos integrantes dos laboratórios quais passei, você foram muito importantes no meu dia-a-dia, para meu crescimento profissional e pessoal, obrigada por todas as experiências.

Aos membros da banca examinadora, pela disponibilidade e conhecimento para avaliar e enriquecer o trabalho desenvolvido.

Ao Programa de Pós-Graduação em Ciências Ambientais e a ao ICTS-UNESP (Instituto de Ciência e Tecnlologia de Sorocaba) pela oportunidade, pelo espaço e pelo conhecimento proporcionado.

Á UNISO (Universidade de Sorocaba), UNIPAMPA (Universidade Federal do Pampa - Campus Uruguaiana), UEL (Universidade de Londrina) e UNESP (Univerdidade Estadual Paulista “Júlio de Mesquita Filho - Campus Lageado Botucatu e Jaboticabal) pela parceria, uso dos laboratórios e equipamentos, sobretudo pelo real acolhimento.

Agradeço a minha melhor e mais importante pessoa, que é o meu anjo da guarda, Neidiana. Obrigada mãe, por ser tão incrível e iluminada. Obrigada pelo amor incondicional e acima de tudo pela compreensão, positividade e alegria infinita.

À minha avó, Claide, obrigada pelo exemplo de força, determinação e garra que você é. Obrigada por todo apoio e amor. Sem você, nós nada seríamos.

Ao meu pai, João Erasmo, por todo apoio, amor, carinho e preocupação. Por toda simplicidade de onde eu vim, por tudo que me ensinou a ser.

Ao meus familiares, pela curiosidade e torcida constante.

À Nala, por ser minha paz e meu refúgio cheio de amor e carinho.

Ao meu namorado, Eduardo, obrigada por ser um grande companheiro e dividir comigo todos os momentos. Por ser admirável e me acrescentar uma segunda forma de ver e viver a vida.

Aos meus amigos de vida pelo incentivo, cuidado e felicidade proporcioada.

A tudo e a todos que de alguma forma tenham contribuído, apoiado e compartilhado esses anos em que foi desenvolvido este trabalho.

PASCOLI, M. Toxicity evaluation and biological activity of zein nanoparticles containing the biocide azadirachtin. 2019. 124 p. Qualification Thesis (Doctoral Degree in Environmental Sciences - Institute of Science and Technology, UNESP - Universidade Estadual Paulista, Soracaba, 2019.

ABSTRACT

For pest control, large amounts of agrochemicals are continuously used which often have adverse effects on the environment and human health. This fact has motivated the search for less impacting alternatives. In this scenario, several mechanisms are being studied in order to minimize these damages, such as the development of carrier systems using biodegradable polymers and proteins. In addition, the use of botanical insecticides has also shown potential for pest control, due to the possible lower impacts caused by these products of natural origin. Therefore, the present work describes the preparation and characterization of a nanobiopesticide based on neem oil-loaded zein nanoparticles, together with evaluation of their toxicity towards nontarget organisms (using *Allium cepa*, soil nitrogen cycle microbiota, and *Caenorhabditis elegans*) and of their phytotoxicity (*Phaseolus vulgaris*). In addition the biological activity of this new system on agricultural pests (*Acanthoscelides obtectus*, *Bemisia tabaci* and *Tetranychus urticae*) were also carried out. The spherical nanoparticles showed an average diameter of 278 ± 61.5 nm, a high encapsulation efficiency (>80%) and a good stability during the experiments. In the toxicity assays with *A. cepa*, the neem oil-loaded zein nanoparticles mitigated the increase in the DNA relative damage index caused by the neem oil. Molecular genetic analysis of the soil nitrogen cycle microbiota revealed that neem oil-loaded zein nanoparticles did not change the number of genes which encode nitrogen-fixing enzymes and denitrifying enzymes. In *C. elegans*, the neem oil-loaded zein nanoparticles had no toxic effect, while neem oil interfered with pharyngeal pumping and GST-4 protein expression. Pre- and post-emergence treatments using this new system did not cause any phytotoxic effects towards *P. vulgaris*. The neem oil nanobiopesticide exhibited insecticidal effects on *B. tabaci* and *T. urticae*, while the effect against *A. obtectus* was significantly increased, compared to plain neem oil. Using labeled nanoformulations, it was possible to identify the exposure between nanobiopesticide and *A. obtectus* mainly by their ventral region with prominence to the legs and mouthparts. These neem oil-loaded zein nanoparticles showed promising results as a nanobiopesticide developed with the "safer by design" strategy which gives it great potential for use in pest control in sustainable agriculture, causing less impacts on the environment and human health.

Keywords: Nanobiopesticide. Neem oil. Nanotoxicity. Phytotoxicity. Biological activity. Pest control. Sustainable agriculture.

PASCOLI, M. Avaliação da toxicidade e atividade biológica de nanopartículas de zeína contendo o biocida azadiractina. 2019. 124 f. Tese de Doutorado em Ciências Ambientais - Instituto de Ciência e Tecnologia, UNESP - Universidade Estadual Paulista, Soracaba, 2019.

RESUMO

Para o controle de pragas, contínuamente se faz o uso de elevadas quantidades de agroquímicos que muitas vezes possuem efeitos adversos tanto para o ambiente quanto para a saúde humana. Este fato tem motivado a busca por alternativas menos impactantes. Neste cenário, diversos mecanismos estão sendo estudados a fim de minimizar estes danos, como por exemplo, o desenvolvimento de sistemas carreadores utilizando polímeros biodegradáveis e proteínas. Aliado a isto, a utilização de inseticidas botânicos também tem demonstrado potencialidade para o combate a pragas, devido aos possíveis menores impactos causados por esses produtos de origem natural. Portanto, o presente trabalho descreve o preparo e caracterização de um nanobiopesticida baseado em nanopartículas de zeína carreadas com óleo de neem junto com a avaliação da sua toxicidade contra organismos não alvos (usando *Allium cepa*, microbiota do solo envolvida no ciclo do nitrogênio e *Caenorhabditis elegans*) e da sua fitotoxicidade (usando *Phaseolus vulgaris*). Ademais, a atividade biológica desse novo sistema em pragas agrícolas (*Acanthoscelides obtectus*, *Bemisia tabaci* and *Tetranychus urticae*) também foi investigada. As nanopartículas esféricas apresentaram diâmetro médio de 278 ± 61,5 nm, alta eficiência de encapsulação (> 80%) e boa estabilidade durante os experimentos. Nos ensaios de toxicidade com *A. cepa*, as nanopartículas de zeína carregadas com óleo de neem mitigaram o aumento do índice de danos relativo ao DNA causado pelo óleo de neem. A análise genética molecular da microbiota do solo envolvida no ciclo do nitrogênio revelou que as nanopartículas de zeína carreadas de óleo de neem não alteraram o número de genes que codificam enzimas fixadoras de nitrogênio e enzimas desnitrificantes. Em *C. elegans*, as nanopartículas de zeína carreadas com óleo de neem não tiveram efeito tóxico, enquanto o óleo de neem interferiu no bombeamento faríngeo e na expressão da proteína GST-4. Este novo sistema não causou nenhum efeito fitotóxico em *P. vulgaris* em tratamentos pré e pós-emergentes. O nanobiopesticida e o óleo de neem exibiram efeito inseticida sobre *B. tabaci* e *T. urticae* e o nanobiopesticida aumentou significativamente o efeito inseticida em *A. obtectus* comparado ao óleo de neem. Utilizando nanoformulações marcadas, foi possível identificar a exposição entre o nanobiopesticida e *A. obtectus,* principalmente por sua região ventral, com destaque para as pernas e partes bucais. Estas nanopartículas de zeína carreadas com óleo de neem mostraram resultados promissores como nanobiopesticida desenvolvido com a estratégia "safer by design" o que lhe confere grande potencialidade de uso no controle de pragas na agricultura sustentável, causando menores impactos ao meio ambiente e à saúde humana.

Palavras-chave: Nanobiopesticida. Óleo de Neem. Nanotoxicidade. Fitotoxicidade. Atividade Biológica. Agricultura sustentável.

SUMÁRIO

INTRODUÇÃO GERAL

A expectativa em relação ao aumento da população mundial é alarmante. De acordo com estimativas realizadas pelas Nações Unidas, a população mundial atingiu 7,7 bilhões no ano de 2017, e a previsão é que este número cresça para perto de 9,8 bilhões até 2050. Consequentemente, haverá uma maior demanda por alimentos, e a previsão é que seja necessário um aumento de 50% na produção de alimentos (FAO 2017). De acordo com Zhang et al. (2018), 187 milhões de toneladas métricas de fertilizantes e quase 4 milhões de toneladas de pesticidas são consumidos anualmente pela agricultura. O uso intensivo de pesticidas apresenta sérios riscos à saúde ambiental e humana, como contaminação da água e solo, desenvolvimento de pragas resistentes, acúmulo de resíduos tóxicos nos alimentos, bem como efeitos em organismos não-alvos (Sankoh et al. 2016). Com isso, outras estratégias devem ser adotadas para suprir a necessidade agrícola evitando causar prejuízos a outros setores, como a utilização de defensivos agrícolas a base de compostos naturais (biopesticidas), que podem ser pós e extratos botânicos e/ou óleos essenciais de origem vegetal. Os biopesticidas podem apresentar diversos mecanismos de ação como inibição de ovoposição, alimentação, alteração no desenvolvimento, movimento, podem causar infertilidade e afetar hormônios, e geralmente apresentam baixa à moderada toxicidade para organismos não alvos e prejuízos ao ambiente (Isman and Grieneisen, 2014; Rehman et al. 2014; Benelli and Pavela, 2018; Oliveira et al., 2018b).

O óleo de neem tem sido usado há séculos como planta medicinal na Ásia. Atualmente, o uso de seus extratos se destaca como inseticida natural, prática que se intensificou nos últimos 30 anos, quando seu principal composto, a azadiractina, foi isolado (Mossini and Kemmelmeier, 2005). Além da azadiractina, o óleo de neem é constituído por mais de 300 compostos ativos, a maioria triterpenos, que apresentam eficiência contra uma vasta gama de pragas pelo seu amplo espectro de ação (Campos et al., 2016; Chandramohan et al., 2016). Porém, apesar da potencialidade destes compostos, a sua utilização pode ser limitada na agricultura pois possuem alta sensibilidade à luz, umidade, temperatura e também à degradação por microrganismos (Campos et al. 2016).

A utilização da nanotecnologia para o desenvolvimento de sistemas nanoestruturados tem emergido como uma estratégia promissora e necessária a ser empregada na agricultura, uma vez que pode conferir aumento de solubilidade, eficácia, estabilidade e biodisponibilidade ao composto bioativo, bem como redução da sua degradação precoce, da dose necessária e da toxicidade a organismos não alvos, assim maximizando a produção agrícola e aumentando a sustentabilidade (Borgatta et al., 2018; Kah et al., 2019; Lowry et al., 2019). Os sistemas nanoestruturados podem ser produzidos a partir de diversas matrizes poliméricas e proteínas através de diversas metodologias de sínteses. A quitosana e a zeína são exemplos de matrizes usadas na produção de nanomateriais para aplicação na agricultura e têm apresentado resultados promissores (Mittal et al., 2013; Campos et al., 2014; Bautista-Banos et al., 2016; Oliveira et al., 2018a, 2018b, 2019).

A limitada quantidade de informações sobre a segurança do uso de nanopartículas gera preocupações à medida que vários nanomateriais são atualmente sintetizados em larga escala, comercializados, manipulados e descartados no ambiente sem controle ou regulamentação. Não são definidos os potenciais riscos desses nanomateriais aos seres humanos e ao ecossistema. Uma vez que a nanoencapsulação é capaz de modificar as propriedades e o comportamento dos compostos, é necessário que todo novo sistema nanoestruturado seja investigado para garantir segurança em seu uso. Assim, devem ser realizados ensaios para avaliar seu comportamento no ambiente e possível toxicidade utilizando organismos alvos e não alvos, visando a regulamentação do uso de nanobiopesticidas na agricultura sustentável (Campos et al., 2018a, 2018b; Fraceto et al., 2016; Pascoli et al., 2018a, 2018b; Kah et al., 2019; Prajitha et al., 2019). O novo sistema deve ser sempre comparado com os pesticidas comerciais e os novos riscos e benefícios emergentes devem ser muito bem investigados. Nesse viés, se faz necessário destacar que para a aplicabilidade da nanotecnologia na agricultura ser bem-sucedida é imprescindível um trabalho multidisciplinar no desenvolvimento da formulação, bem como o envolvimento do próprio agricultor para que se alcance seu objetivo, e da própria sociedade para a aceitação da nova tecnologia (Kah et al., 2019; Pascoli et al., 2019).

Desta forma, o objetivo principal da presente tese foi preparar e caracterizar um novo sistema carreador baseado na matriz natural zeína para carrear o

composto óleo de neem. Também foram investigados os possíveis efeitos tóxicos do nanobiopesticida a organismos não alvos (*Allium cepa*, microbiota do solo envolvida no ciclo do nitrogênio, *Caenorhabditis elegans* e *Phaseolus vulgaris*) e sobretudo avaliada a atividade biológica deste novo sistema a diferentes pragas agrícolas (*Acanthoscelides obtectus*, *Bemisia tabaci* and *Tetranychus urticae*). A tese está estruturada em quatro capítulos, os quais são compostos por artigos publicados e/ou submetidos à periódicos científicos internacionais.

Os dois primeiros capítulos constituem a revisão bibliográfica da tese. No ***primeiro capítulo*** é apresentada uma revisão de literatura onde se aborda o potencial uso do óleo de neem. O artigo descreve os compostos ativos do óleo de neem, bem como seu amplo espectro de aplicação que vai da saúde à agricultura. A respeito do seu uso na agricultura, é apresentado um levantamento dos produtos comerciais que contém o óleo de neem como principal composto ativo, seu modo de ação contra diversas pragas e as limitações de seu uso em campo. Contudo, essas limitações abrem perspectivas de que a nanotecnologia possa ser uma potente aliada em seu uso.

Já no ***segundo capítulo***, é encontrado um “mini-review” que visa descrever as diversas metodologias de síntese de nanopartículas de zeína, sobretudo estratégias para melhorar a estabilidade coloidal destas formulações. Composto por trabalhos ciêntíficos recentes, este trabalho pontua uma lacuna nos artigos a respeito de nanopartículas de zeína uma vez que muitos deles não avaliam a estabilidade destas nanopartículas em função do tempo, ou demonstram baixa estabilidade coloidal do sistema. As estratégias para se obter um sistema de nanopartículas de zeína com boa estabilidade coloidal vão desde tratamento térmico da proteína, até recobrimento da partícula e condições de armazenamento.

Os dois últimos capítulos constituem de artigos com os resultados do nosso trabalho. O ***terceiro capítulo*** mostra o desenvolvimento do nanopesticida à base de óleo de neem como uma formulação ambientalmente amigável para aplicações na agricultura sustentável. O artigo descreve o preparo e caracterização do novo sistema, bem como são apresentados ensaios de toxicidade em organismos não alvos (*Allium cepa*, microbiota do solo envolvida no ciclo do nitrogênio, *Caenorhabditis elegans*). Os ensaios com *A. cepa* mostraram redução no índice de

danos relativos causado pelo composto ativo devido a nanoencapsulação e em *C. elegans*, o nanobiopesticida não apresentou efeito tóxico ao nematóide enquanto o óleo de neem interferiu de maneira negativa no bombeamento faríngeo e na expressão da proteína GST-4.

No ***quarto capítulo***, é apresentada a síntese e caracteização das formulações de nanopartículas de zeína em função do tempo de estocagem, bem como a avaliação dos possíveis impactos em organismos alvos e não alvos. A fitotoxicidade foi investigada em *Phaseolus vulgaris* e a atividade biológica foi avaliada contra as pragas agrícolas *Acanthoscelides obtectus*, *Bemisia tabaci e Tetranychus urticae*. O nanobiopesticida aumentou significativamente o efeito inseticida em *A. obtectus* comparado ao óleo de neem. Utilizando nanoformulações marcadas com rodamina, foi possível identificar a exposição entre o nanobiopesticida e os besouros, principalmente pelo seu tegumento.

Por fim, o ítem ***conclusão geral e perspectivas*** completam a tese.

CHAPTER I

NEEM OIL AND CROP PROTECTION: FROM NOW TO DE FUTURE

Frontiers in Plant Science, v. 7, 2016.

doi: 10.3389/fpls.2016.01494.

frontiers in Plant Science

PERSPECTIVE
published: 13 October 2016
doi: 10.3389/fpls.2016.01494

Neem Oil and Crop Protection: From Now to the Future

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A major challenge of agriculture is to increase food production to meet the needs of the growing world population, without damaging the environment. In current agricultural practices, the control of pests is often accomplished by means of the excessive use of agrochemicals, which can result in environmental pollution and the development of resistant pests. In this context, biopesticides can offer a better alternative to synthetic pesticides, enabling safer control of pest populations. However, limitations of biopesticides, including short shelf life, photosensitivity, and volatilization, make it difficult to use them on a large scale. Here, we review the potential use of neem oil in crop protection, considering the gaps and obstacles associated with the development of sustainable agriculture in the not too distant future.

Keywords: neem oil, nanoparticles, sustained release, sustainable agriculture

OPEN ACCESS

Edited by:
Susana Araújo, Instituto de Tecnologia Química e Biológica (ITQB, Nova), Portugal

Reviewed by:
Anandham Rangasamy, Tamil Nadu Agricultural University, India
Yuksel Tuzel, Ege University Faculty of Agriculture, Turkey

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Specialty section:
This article was submitted to Crop Science and Horticulture, a section of the journal Frontiers in Plant Science

Received: *28 June 2016*
Accepted: *20 September 2016*
Published: *13 October 2016*

Citation:
Campos EVR, de Oliveira JL, Pascoli M, de Lima R and Fraceto LF (2016) Neem Oil and Crop Protection: From Now to the Future. Front. Plant Sci. 7:1494. doi: 10.3389/fpls.2016.01494

INTRODUCTION

Attention is increasingly being paid to the use of natural compounds (such as essential oils) as a promising option to replace agrochemicals in agricultural pest control. These odoriferous substances are extracted from various aromatic plants, which are rich sources of biologically active secondary metabolites such as alkaloids, phenolics, and terpenoids (Esmaeili and Asgari, 2015), using extraction methods employing aqueous or organic solvents, or steam distillation. Their mechanisms of action can vary, especially when the effect is due to a combination of compounds (de Oliveira, 2011; Esmaeili and Asgari, 2015).

Neem oil is extracted from the neem tree, *Azadirachta indica* Juss., a member of the *Meliaceae* family that originates from the Indian subcontinent and is now valued worldwide as an important source of phytochemicals for use in human health and pest control. *Azadirachta* is a fast-growing small-to-medium sized evergreen tree, with wide and spreading branches. It can tolerate high temperatures as well as poor or degraded soil. The young leaves are reddish to purple, while the mature leaves are bright green, consisting of petiole, lamina, and the base that attaches the leaf to the stem and may bear two small lateral leaf-like structures known as stipules (Norten and Pütz, 1999; Forim et al., 2014).

Neem oil contains at least 100 biologically active compounds. Among them, the major constituents are triterpenes known as limonoids, the most important being azadirachtin (**Figure 1**), which appears to cause 90% of the effect on most pests. The compound has a melting point of 160°C and molecular weight of 720 g/mol. Other components present include meliantriol, nimbin, nimbidin, nimbinin, nimbolides, fatty acids (oleic, stearic, and palmitic), and salannin. The main neem product is the oil extracted from the seeds by different techniques. The other parts of the neem tree contain less azadirachtin, but are also used for oil extraction (Nicoletti et al., 2012). It has

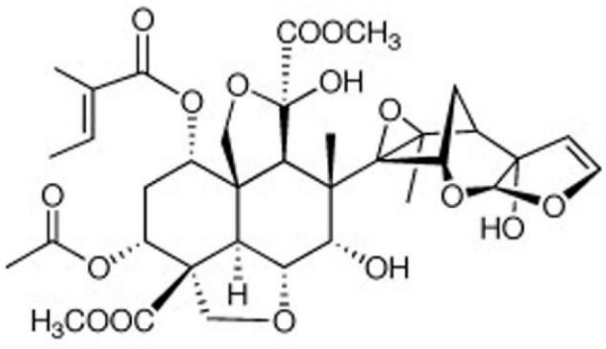

FIGURE 1 | Chemical structure of azadirachtin, the main component of neem oil.

been suggested that the content of azadirachtin in the seeds can be increased by artificial infection with arbuscular mycorrhiza (Venkateswarlu et al., 2008).

Among the botanical insecticides currently marketed, neem oil is one of the least toxic to humans and shows very low toxicity to beneficial organisms, so it is, therefore, very promising for the control of many pests. Target insect species include the following: *Anopheles stephensi* (Lucantoni et al., 2006), *A. culicifacies* (Chandramohan et al., 2016), *Ceraeochrysa claveri* (Scudeler et al., 2013, 2014; Scudeler and dos Santos, 2013), *Cnaphalocrocis medinalis* (Senthil Nathan et al., 2006), *Diaphorina citri* (Weathersbee and McKenzie, 2005), *Helicoverpa armigera* (Ahmad et al., 2015), *Mamestra brassicae* (Seljåsen and Meadow, 2006), *Nilaparvata lugens Stal* (Senthil-Nathan et al., 2009), *Pieris brassicae* (Hasan and Shafiq Ansari, 2011), and *Spodoptera frugiperda* (Tavares et al., 2010). Arachnid targets include *Hyalomma anatolicum excavatum* (Abdel-Shafy and Zayed, 2002) and *Sarcoptes scabie* var. cuniculi larvae (Xu et al., 2010).

The oil is considered a contact insecticide, presenting systemic and translaminar activity (Cox, 2002). It has a broad spectrum of action, inhibiting feeding, affecting hormone function in juvenile stages, reducing ecdysone, deregulating growth, altering development and reproduction, suppressing fertility, sterilizing, repelling oviposition, and disrupting molting processes (Brahmachari, 2004). Little is known about the mode of action of azadirachtin as a feeding inhibitor, although it is possible that it stimulates cells involved in feeding inhibition, causing weakness and pest death (Brahmachari, 2004).

Azadirachtin, salannin, and other limonoids present in neem oil inhibit ecdysone 20-monooxygenase, the enzyme responsible for catalyzing the final step in conversion of ecdysone to the active hormone, 20-hydroxyecdysone, which controls the insect metamorphosis process. However, these effects are probably secondary to the action of azadirachtin in blocking microtubule formation in actively dividing cells (Morgan, 2009). Moreover, azadirachtin can inhibit the release of prothoracicotropic hormone and allatotropins from the brain-corpus cardiacum complex, resulting in problems of fertility and fecundity (Mulla and Su, 1999). Meliantriol and salannin also act to inhibit the feeding of insects, while nimbin and nimbidin mainly present antiviral activity (EMBRAPA, 2008).

Azadirachtin can also interfere in mitosis, in the same way as colchicine, and has direct histopathological effects on insect gut epithelial cells, muscles, and fatty tissues, resulting in restricted movement and decreased flight activity (Wilps et al., 1992; Mordue (Luntz) and Blackwell, 1993; Qiao et al., 2014).

Several studies have described the action of neem oil in specific groups of insects. Among the major insect groups, neem oil has shown action against (i) Lepidoptera: antifeeding effect and increased larvae mortality (Mancebo et al., 2002; Michereff-Filho et al., 2008; Tavares et al., 2010); (ii) Hemiptera: early death of nymphs in due to inhibition of development and ecdysis defects (Weathersbee and McKenzie, 2005; Senthil Nathan et al., 2006; Formentini et al., 2016); (iii) Hymenoptera: food intake decrease, reduced larval and pupal development, larvae death during the molting process (Li et al., 2003); (iv) Neuroptera: severe damage in the midgut cells of larvae, injury and cell death during the replacement of midgut epithelium, and changes in cocoons, with increased porosity and decreased wall thickness affecting pupation (Scudeler et al., 2013, 2014; Scudeler and dos Santos, 2013). In another class, the Arachnida, exposure of the Ixodidae group to neem oil decreased egg hatching and caused malformation, deformities, and death of larvae and adults (Abdel-Shafy and Zayed, 2002).

NEEM APPLICATIONS

For centuries, neem has been used in folk medicine for the treatment of conditions such as malaria, ulcers, cardiovascular disease, and skin problems. Despite the limited existence of clinical trials to support therapeutic claims, the use of neem has expanded over time, and it is an important component of Ayurvedic medicine (medical knowledge developed in India about 7000 years ago; Girish and Shankara Bhat, 2008; Ogbuewu et al., 2011).

In addition to its medical applications, neem has aroused interest in many other areas (**Figure 2**). In the cosmetics and hygiene sector, neem is used in the composition of face masks, lotions, sunscreens, soaps, and toothpastes (Mathur and Kachhwaha, 2015). Products derived from neem can contribute to sustainable development and the resolution of pest control problems in agriculture (Lokanadhan et al., 2012). These products benefit from the natural properties of neem as a powerful insect growth regulator (IGR) that also affects many other organisms (such as nematodes and fungi) and can act as a plant fertilizer (Brahmachari, 2004).

The use of neem in agriculture is not a new practice. In India, the traditional farming system employed neem extracts for pest management and to supply nutrients to plants (Mossini and Kemmelmeier, 2005; Sujarwo et al., 2016). Scientific research has shown that neem is safe for workers, with no handling risks, and can be used throughout the entire crop production cycle (Boeke et al., 2004).

Neem has proven use as a fertilizer, with the organic and inorganic compounds present in the plant material acting to

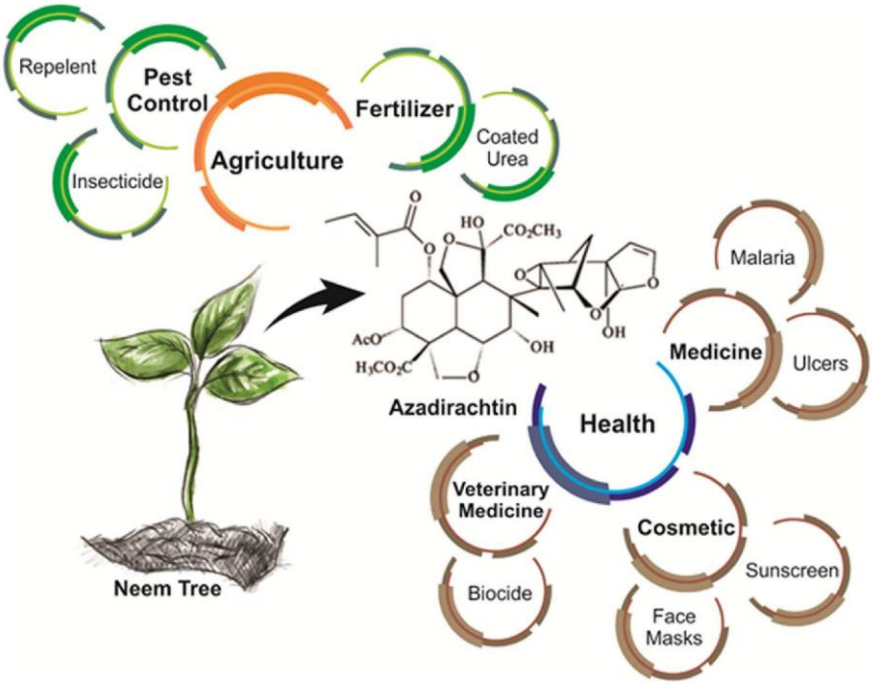

FIGURE 2 | Potential applications of azadirachtin in different areas.

improve soil quality and enhance the quality and quantity of crops. The waste remaining after extraction of the oil from neem seeds (neem seed cake) can be used as a biofertilizer, providing the macronutrients essential for plant growth (Ramachandran et al., 2007; Lokanadhan et al., 2012).

Nitrogen is one of the main nutrients required by plants for their development, and urea is the main source of nitrogen fertilizer used worldwide to supply the nitrogen demand of crops. The control of urea hydrolysis and nitrification is one of the principal strategies employed to avoid nitrogen losses in agriculture (Ni et al., 2014). Neem has demonstrated activity as a nitrification inhibitor, helping to slow the bacterial activity that is responsible for denitrification, hence decreasing the loss of urea from the soil (Musalia et al., 2000; Mohanty et al., 2008).

Due to their compositional complexity, neem-based products can act as antifeedants, growth regulators, sterilants, anti-oviposition agents, and repellents (Gonzalez-Coloma et al., 2013). Other factors that have stimulated the use of neem-based products for pest control in agriculture are ecological and toxicological aspects (low toxicity to non-target organisms), as well as economic aspects (small amounts of the product can provide effective pest control; Ogbuewu et al., 2011).

These features of neem support its contribution to organic agricultural production systems that are more sustainable and do not generate chemical residues (plants and crops are grown without the use of any agrochemicals). This method also helps to maintain soil productivity, ensuring longer production times. Organic agriculture can be a viable alternative production method for farmers, but there are numerous challenges to be overcome. A key to success is to be open to new approaches, and in this respect neem products can effectively contribute to organic agriculture, being used as organic pesticides and as soil fertilizers. In addition, growing concerns about conventional agriculture and the demand for products that do not generate waste justify increased adoption of the use of biopesticides by farmers, which contributes to the growth of organic agriculture (Dubey et al., 2010; Seufert et al., 2012; Gahukar, 2014).

COMMERCIAL PRODUCTS DERIVED FROM NEEM (*Azadirachta indica*)

Neem has acquired commercial recognition due to its various beneficial properties, which have been extensively investigated over time. Compared to conventional chemicals, which are generally persistent in the environment and highly toxic, botanical pesticides are biodegradable and leave no harmful residues. Most botanical pesticides are non-phytotoxic and are also more selective toward the target pest. In terms of commercial applications, biopesticides can provide substantial economic advantages, since the infrastructure required is inexpensive, compared to conventional pesticides (Pant et al., 2016).

This has resulted in the publication of numerous scientific research articles and books, as well as the organization of international conferences to discuss the benefits of the plant (Girish and Shankara Bhat, 2008).

Several patents related to processes and products based on neem have been deposited in the United States, India, Japan, Australia, and elsewhere. Many of the products derived from

neem are manufactured by crushing the seeds and other plant parts, followed by the use of solvents to extract the active ingredients possessing pesticide activity. The different methods and techniques employed to obtain neem products can result in different concentrations of the active compounds, as well as different biological effectiveness (Roychoudhury, 2016). **Table 1** lists some of the main commercial products based on neem.

Despite its many promising properties, there are limitations that hinder effective large-scale use of neem. These impediments must be overcome and many uncertainties clarified so that the full potential of neem can be exploited. One of the main problems facing the commercial development of neem is a lack of industrial interest, largely due to the difficulty of patenting natural products, as well as a shortage of scientific evidence to support claims regarding the benefits of these substances. As a results, the products are not widely publicized in the farming community and elsewhere (Pant et al., 2016).

Disadvantages of neem are its low stability under field conditions, due mainly to a high rate of photodegradation, as well as a short residence time and slow killing rates, compared to conventional pesticides (Isman, 2006; de Oliveira et al., 2014; Miresmailli and Isman, 2014). Genetic factors are mainly responsible for determining the chemical composition of neem oil. However, environmental factors and the type of extraction method can lead to significant differences in composition. As a result, there is no standard active ingredient in the composition of this botanical insecticide, which limits its application in the control of agricultural pests (Ghosh et al., 2012; Tangtrakulwanich and Reddy, 2014; Siegwart et al., 2015).

Neem oil contains a group of active ingredients with different chemical characteristics. It was therefore believed that the development of insect resistance would be virtually impossible. However, as studies have progressed, it has been observed that due to the low residual power of botanical insecticides, multiple applications are required in order to control pests, which can increase selection pressure on the pest population, possibly leading to resistance (Ghosh et al., 2012; Tangtrakulwanich and Reddy, 2014; Siegwart et al., 2015).

Currently, most of the botanical insecticides that are being studied and that are effective against many pests are those with feeding deterrent action, so their indiscriminate use could result in the development of resistance (Tangtrakulwanich and Reddy, 2014; Mpumi et al., 2016). Feng and Isman (1995) evaluated the behavior of two lines of *Myzus persicae*, which were exposed to pure azadirachtin or to refined neem seed extract at the same concentration as azadirachtin. It was found that after forty generations, the line treated with azadirachtin had developed ninefold greater resistance to azadirachtin, compared to a control line, whereas the line treated with the extract did not show resistance.

TABLE 1 | Neem applications and commercial products available worldwide.

Application	Product	Manufacturer
Fertilizer	Ozoneem Cake®	Ozone Biotech (India)
	Plan "B" Organics – Neem Cake®	Plan "B" Organics (USA)
	Fortuneem Cake®	Fortune Biotech (USA)
	Bio Neem Oil Foliar®	FUSA – Fertilizers of the USA
	Neem Cake®	Unibell Corporation (Russia)
	Ozoneem Coat®	Ozone Biotech (India)
	Parker Neem Coat®	Parker Neem (India)
	Neem Urea Guard®	Neemex (India)
	Fortuneem Coat®	Fortune Biotech (USA)
	Azadirachtin-based products	
Agrochemical	AZA-Direct®	Gowan Company (USA)
	Neemix 4.5®	Certis (USA)
	Fortune Aza 3% EC®	Fortune Biotech (USA)
	Azamax®	UPL Ltda. (Brazil)
	Neemazal Technical®	E.I.D. Parry Ltd. (India)
	Ecosense®	Agro Logistic Systems Inc. (USA)
	Safer Brand 3 in 1 Garden Spray®	Woodstream Corp. (Canada)
	Azatin XL®	OHP Inc. (USA)
	Azact CE®	EPP Ltda. (Brazil)
	Neem oil	
	Triact 70 EC®	Certis Company (USA)
	BioNeem®	Woodstream Corporation (USA)
	Shubhdeep Neem Oil®	King Agro Food (India)
	DalNeem®	Dalquim Ltda. (Brazil)
	OzoNeem Oil®	Ozone Biotech (India)
	NeemDrop®	Neem India Products Ltd. (India)

FUTURE TRENDS

Biological control is defined as the action of natural enemies on a population of pests in order to keep it at a population density that does not cause economic damage to crops (Pal and McSpadden Gardener, 2006). Natural enemies have been known since the third century BC, when the Chinese used predatory ants for pest control in citrus. However, after 1939, with the synthesis of the chlorinated pesticide dichlorodiphenyltrichloroethane (DDT) and organophosphorus pesticides, research on synthetic chemical pesticides and their use increased greatly, while the opposite occurred with biological control methods (Doutt, 1964; Niu et al., 2014). Currently, with the emergence of the concept of Integrated Pest Management (IPM), there is a resurgence of research with emphasis on biological control techniques. Such systems seek to harmoniously integrate various forms of control, with emphasis on biological control, in order to gain economic, social, and environmental improvements (Kogan, 1998; Ehler, 2006; EPA, 2016).

The biological control of insects and mites in agriculture can be achieved using small wasps or flies, known as parasitoids, which parasitize eggs, small caterpillars, and even adults. It can also be performed using predators such as ladybugs, bugs, predatory mites, and spiders, as well as parasitism by entomopathogenic microorganisms including fungi, bacteria, and viruses (Landis et al., 2000; Ehler, 2006; Smith and Capinera, 2014). Although biological control will not control all pests all of the time, it is a key component of integrated pest management.

The purpose of biological control is not to eradicate pests, but to keep them at tolerable levels at which they cause no appreciable harm (Orr and Lahiri, 2014).

There has recently been increased interest in the application of plant-based materials (botanical insecticides), such as neem oil, in pest control. Although these products are safer for the management of pests, compared to synthetic chemicals, their effects in IPM must be evaluated. Several studies have investigated the relationships between botanical insecticides and natural enemies of agricultural pests (Islam et al., 2011; Mamoon-ur-Rashid et al., 2011; Islam and Omar, 2012; Tunca et al., 2012; Usman et al., 2012). Sahayaraj et al. (2011) evaluated the use of different neem-based products in colonies of *Beauveria bassiana*, *Isaria fumosoroseus*, and *Lecanicillium lecanii*, and the results showed that these entomopathogenic fungi were compatible with most products tested. Raguraman and Kannan (2014) conducted a review in order to score the impact and safety of different botanical insecticides in the presence of parasitoids and predators (beneficial arthropods), with the aim of standardizing strategies and application methods to achieve better management of agricultural pests.

The integrated use of botanical insecticides associated with biological control (synergism) in IPM is becoming increasingly widespread in the farming and research communities. The advantage of this approach is that it offers the potential to control agricultural pests, without serious impacts on the environment, non-target organisms, and animal and human health.

Botanical insecticides must meet the same criteria as conventional insecticides. In other words, they must be selective for the target pest and provide sufficient residual activity to protect the plant during the period of vulnerability. Over the past decade, there has been a significant increase in the number of publications concerning the use of neem oil to control agricultural pests (Montes-Molina et al., 2008; War et al., 2012; da Costa et al., 2014; Gahukar, 2014; Rehman et al., 2014; Bakry et al., 2016). However, many studies have only involved testing at the laboratory level (*in vitro*), due to the instability of this substance under field conditions. From these studies, it is not possible to draw firm conclusions concerning the in vivo biological efficacy of the formulations, due to the effects of numerous environmental variables.

In order to overcome the above-mentioned limitations, nanotechnology has emerged as a novel tool to address the problems of agricultural sustainability and food security (Khot et al., 2012; Kah and Hofmann, 2014; Kookana et al., 2014; Kah, 2015; Kashyap et al., 2015; Fraceto et al., 2016). Many studies have shown that the encapsulation of agrochemicals in nanoparticulate systems can enhance the efficacy of the active ingredient, decrease toxicity toward the environment and humans, and reduce losses due to volatilization, leaching, and photobleaching (Kulkarni et al., 1999; Riyajan and Sakdapipanich, 2009; Devi and Maji, 2010; de Oliveira et al., 2014; Bakry et al., 2016; Giongo et al., 2016).

From the point of view of sustainable agriculture, nanotechnology can help in the development of environmentally friendly agricultural inputs, improving the safety and stability of active agents, enhancing their activity in pest control, and, consequently, increasing their acceptance by producers (Nair et al., 2010; Srilatha, 2011; Khot et al., 2012; Agrawal and Rathore, 2014; Ram et al., 2014). The use of nanoparticles provides an effective means of protecting neem oil against premature degradation, resulting in prolongation of its effect on the target pest. Sustained release of the active agent is achieved, and environmental damage is minimal because the polymers employed are biodegradable. Furthermore, the number of applications of neem oil can be reduced, bringing substantial economic benefits (Kulkarni et al., 1999; Isman et al., 2001; Isman, 2006; de Oliveira et al., 2014; Isman and Grieneisen, 2014; Miresmailli and Isman, 2014).

Although studies have demonstrated the beneficial effects of nanoencapsulation of neem oil, some issues need to be resolved so that the synergistic effect of nanoparticles associated with this botanical insecticide can significantly contribute to the control of insect pests. These issues include the need for: (a) regulation of the use of nanomaterials in agriculture; (b) nanoformulations that are easily scalable; (c) comparative studies employing neem formulations available commercially to prove the cost/benefit of nanoformulations; (d) detailed studies of the degradation and behavior of these nanopesticides in the environment; and (e) evaluation of toxicity toward non-target organisms (De Jong and Borm, 2008; Joint Research Centre, 2015; Servin and White, 2016).

Given the importance of neem oil and its worldwide use for combating numerous pests in different crops, the nanoencapsulation of this oil should enable the production of more stable formulations for the control of insects that damage crops, especially those that are essential for human consumption. In addition, the use of nanotechnology is an excellent way to combat the development of resistance in insects due to the indiscriminate use of neem oil.

AUTHOR CONTRIBUTIONS

EC, JdO, and MP wrote the manuscript. LF and RdL contributed to the discussion and revised the manuscript. All authors approved the final manuscript.

ACKNOWLEDGMENTS

Financial support for this research was provided by the São Paulo State Research Foundation (FAPESP, processes #2014/20273-4, #2013/12322-2, #2014/20286-9, #2015/15617-9, and #2015/17120-4).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER II

ZEIN NANOPARTICLES AND THE STRATEGIES TO IMPROVE THE COLLOIDAL STABILITY: A MINI REVIEW

Frontiers in Chemistry, v. 6, 2018.

doi: 10.3389/fchem.2018.00006.

frontiers in Chemistry

MINI REVIEW
published: 25 January 2018
doi: 10.3389/fchem.2018.00006

Zein Nanoparticles and Strategies to Improve Colloidal Stability: A Mini-Review

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Zein, a protein extracted from maize, can be employed to easily produce nanoscale particles suitable for use as carrier systems. This review investigates the main methods for obtaining zein nanoparticles, as well as the problems and options available in the development of stable colloidal suspensions. Considerable gaps were identified in the literature concerning this topic, with studies being unclear about the factors that affect the stability of zein particles. In the vast majority of cases, no data are presented in relation to the stability of the formulations over time. It could be concluded that in order to produce a high quality system, detailed evaluation is required, considering factors including the zein concentration, pH, ionic strength, thermal treatment of the protein prior to preparation of the nanoparticles, strategies employing other materials as coatings, and the storage conditions. It is extremely important that these aspects should be considered during product development, prior to commercial-scale manufacture.

Keywords: colloidal stability, thermal treatment, ionic strength, zein, nanoparticles

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Nanoscience, a section of the journal Frontiers in Chemistry

Received: *15 August 2017*
Accepted: *10 January 2018*
Published: *25 January 2018*

Citation:
Pascoli M, de Lima R and Fraceto LF (2018) Zein Nanoparticles and Strategies to Improve Colloidal Stability: A Mini-Review. Front. Chem. 6:6. doi: 10.3389/fchem.2018.00006

INTRODUCTION

Zein is the main protein present in maize, accounting for around 50% of the total protein content. It belongs to the prolamin class and is composed of lipophilic amino acid residues. The α-zein form accounts for over 70% of the total zein protein and is the type that is commercially available (Paliwal and Palakurthi, 2014). It is not used for direct human consumption, due to its negative nitrogen balance and low solubility in water. However, it can be easily converted to spherical colloidal nanoparticles (Patel et al., 2010). Due to its high coating capacity, biodegradability, and biocompatibility, zein has been used in modified release systems for the delivery of enzymes, drugs, and essential oils, among other substances (Lee et al., 2013; da Rosa et al., 2015; Park et al., 2015; Wang et al., 2017).

The purpose of the present work is to provide an overview of the main methods of preparation of zein nanoparticles, as well as the main problems related to the temporal stability of these systems. Possible options for increasing the colloidal stability of zein nanoparticles are presented, together with future perspectives for the development of these carrier systems.

Preparation of Zein Nanoparticles

There are many methodologies described in the literature for the preparation of zein nanoparticles used for loading with different active compounds (Supplementary Table 1), including nanoprecipitation, liquid-liquid dispersion, phase separation, and electrospraying. Encapsulation techniques are attractive methods based on precipitation processes (Tarhini et al., 2017).

Antisolvent Nanoprecipitation, Liquid-Liquid Dispersion, and Phase Separation Techniques

The antisolvent nanoprecipitation technique for the synthesis of nanoparticles has been widely described in the literature (**Figure 1A**). It is based on the differences in solubility of a protein in different solvents, as a function of pH, ionic strength, and electrolytes. The method involves the addition of a non-solvent to a solution in order to induce supersaturation, leading to precipitation of the solute and the formation of nanoparticles. It is important to select a suitable solvent and antisolvent, considering their miscibility in the concentration range in which they will be used (Li et al., 2013). In this methodology, the nanoparticles formed are dependent on the method and rate of injection of the organic phase into the aqueous phase, the agitation speed, and the volume ratio. There is no need for an emulsifier for particle formation, although its nature and concentration can influence the nanoparticle size (Rao and Geckeler, 2011).

Liquid-liquid dispersion methods consist of the same antisolvent nanoprecipitation method, with the different solubilities of zein in ethanol and water being exploited in order to produce nanoparticles. The interaction of the alcohol and water acts to decrease the concentration of ethanol, hence reducing the solubility of the zein and causing its production in the form of nanoparticles (Zou et al., 2012).

The method described in the literature as phase separation consists of the same procedures described for antisolvent precipitation and liquid-liquid dispersion, followed by centrifugation, separation, and purification of the nanoparticles (Lee et al., 2013).

These different zein nanoparticle production methods involve evaporation of the solvent, which can be achieved by magnetic stirring at room temperature, rotary evaporation, or placing under a flow of nitrogen (Hu and McClements, 2015; Chuacharoen and Sabliov, 2016).

Advantages of these methodologies are that they do not require complex equipment and involve straightforward preparation conditions. They are low cost and provide satisfactory encapsulation efficiencies for active ingredients. However, while these techniques are useful for optimization of formulations using small volumes of samples, the same results may not necessarily be obtained at larger scales.

Electrohydrodynamic Atomization Method

The electrohydrodynamic atomization method (**Figure 1B**), also known as electrospraying, is based on the separation of a liquid into charged droplets under the influence of an electric field. The liquid passes through a fine metal tube, such as a capillary or a needle, and the liquid meniscus at the tip of the tube is electrically stressed. Nanoparticles with different characteristics can be obtained by varying the electric field strength, the properties of the liquid, and the injection rate. Multiple solutions can be used, with injection of one solution into another, which has the important advantage of producing monodispersed nanoparticles with high encapsulation efficiency (Gomez-Estaca et al., 2012). This method offers faster production of nanoparticles in a single step, making scale-up feasible. However, a disadvantage is the high cost of the production process.

COLLOIDAL STABILITY OF ZEIN NANOPARTICLES

Despite the effectiveness of the methods used to prepare zein nanoparticles, considerable challenges remain concerning the temporal chemical stability of these systems under different storage conditions (Li et al., 2013; Park et al., 2015). Chen and Zhong (2014) studied dispersions of zein nanoparticles and concluded that they presented poor colloidal stability, readily forming aggregates and precipitates in the formulations, hence losing their functionality.

At higher pH, formulations of zein nanoparticles have been found to exhibit aggregation and precipitation (Cheng and Jones, 2017), due to the fact that in solutions with pH above 5, zein is close to its isoelectric point (pH 6.2) (Hu and McClements, 2015).

In the case of ionic strength and pH, these nanoparticles have been shown to be highly liable to aggregation at a low concentration of sodium chloride (NaCl), and to be unstable at pH above 5. The salt added to formulations increases the ionic strength, with consequent increases in van der Waals interactions and hydrophobic effects among the protein chains, favoring aggregation and precipitation of the proteins (Dai et al., 2016). Similar findings have been reported in other studies (Patel et al., 2010).

Given that the technology of nanoencapsulation in zein particles offers considerable potential for the development of formulations capable of improving the properties of the encapsulated compounds, it is essential to develop strategies to improve the chemical stability and extend the shelf life of these systems. Aspects to consider include the particle size, polydispersity index, encapsulation efficiency, and release of the active agent over an extended period. These issues are often not discussed or presented in the published studies, but they are vital for the development of commercial products based on zein nanoparticles.

STRATEGIES TO IMPROVE THE STABILITY OF ZEIN NANOPARTICLES

Several strategies have been reported for improving the stability of zein nanoparticles (**Table 1**). However, it should be stressed that there have been few relevant published studies concerning this issue.

Storage Conditions

Studies of zein nanoparticles have found that their stability varies according to the way that the formulation is stored. Gomez-Estaca et al. (2012) reported that a formulation of zein nanoparticles with curcumin remained stable for 3 months when stored in the dark. The stability was not evaluated over an extended period or in the presence of light, which would be very important due to the photosensitivity of the active compound. Lai and Guo (2011) and da Rosa et al. (2015) produced zein nanoparticles containing active agents such as 5-fluoracil, thymol, and carvacrol and found that formulations kept at 20°C presented precipitation and aggregation after 2 and 6 months of storage. However, nanoparticles kept at 6°C remained stable over

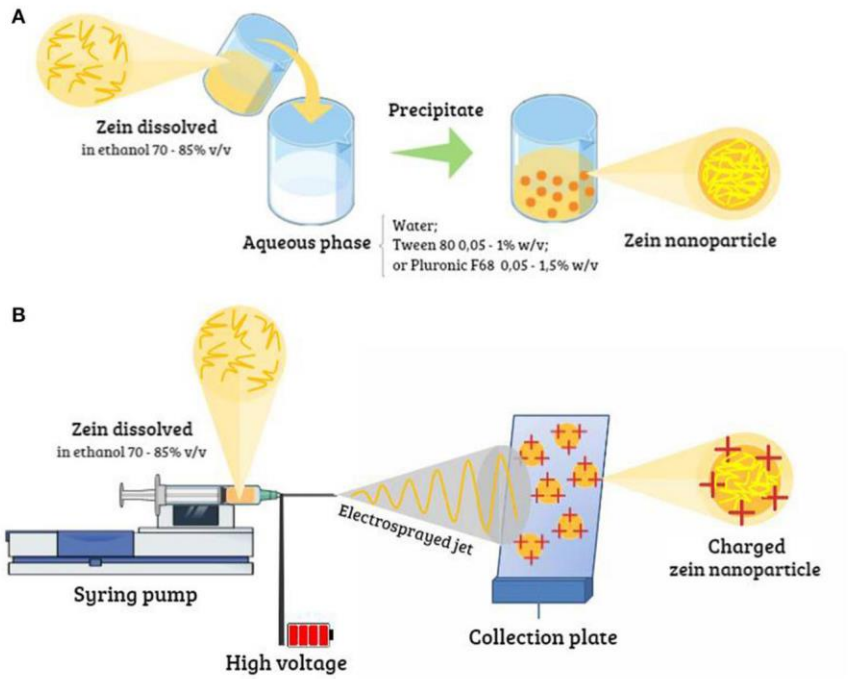

FIGURE 1 | Different methodologies for preparation of zein nanoparticles: **(A)** antisolvent precipitation/liquid-liquid dispersion/phase separation techniques, and **(B)** electrohydrodynamic atomization.

TABLE 1 | Studies found in the literature concerning strategies to improve the stability of zein nanoparticles, and the results obtained.

Zein NPs (ZNPs)	Strategies to improve stability	Stability results	References
5-fluorouracil ZNPs	Formulation stored at 4°C	ZNPs aggregated after 6 months at 25°C, but not at 4°C.	Lai and Guo, 2011
Thymol ZNPs	Coated with caseinate and chitosan	Coating the ZNPs increased the encapsulation efficiency. At low concentrations of emulsifiers, ZNPs agregated.*	Zhang et al., 2014
Mint oil ZNPs	Coated with gum arabic	Coated ZNPs were stable at pH 3 to 8; uncoated ZNPs released the oil faster.*	Chen and Zhong, 2015
Thymol and carvacrol ZNPs	Formulation stored at 4°C	ZNPs precipitated after 2 months at 20°C, but not at 4°C.	da Rosa et al., 2015
Resveratrol ZNPs	Coated with sodium caseinate	Coating the ZNPs improved their stability, considering the effects of ionic strength, pH, and temperature during storage for 28 days.	Joye et al., 2015
Lutein ZNPs	Coated with lecithin and Pluronic	Coating the ZNPs improved their chemical stability during 30 days, compared to uncoated ZNPs.	Chuacharoen and Sabliov, 2016
Hollow ZNPs	Thermal treatment in a thermostatic water bath	ZNPs with treatment at 75°C for 15 min presented a smaller mean diameter and lower polydispersity index.*	Sun et al., 2016
Hollow ZNPs	Coated with carrageenan	Coated ZNPs maintained a constant average diameter during storage at pH between 5.25 and 6.75 for 30 days; uncoated ZNPs precipitated.	Cheng and Jones, 2017
Resveratrol ZNPs	Coated with pectin	The stability was influenced by the pectin concentration.*	Huang et al., 2017

(*) No evaluation was made of the temporal stability of these particles.

the same periods of time. No explanations were provided for the differences in behavior according to temperature.

Thermal Treatment

Sun et al. (2016) subjected zein to thermal treatment in a thermostatic water bath before synthesis of the nanoparticles, using different conditions of time and temperature, in order to modify the characteristics of the material and increase its denaturation temperature. Zein particles produced with treatment at 75°C for 15 min presented a smaller mean diameter and lower polydispersity index. However, when longer periods of time and higher temperatures were employed, the mean diameter and polydispersity index of the nanoparticles increased. No evaluation was made of the stability of the formulations as a function of time. Selling et al. (2007) investigated the effects of temperatures in the range 25–70°C on the secondary and tertiary structures of zein. It was found that treatment at 70°C for 15 min caused changes in the primary structures and decreased the alpha-helix content of the secondary structure. These alterations were reversed when the temperature returned to 25°C. In

contrast, Cabra et al. (2006) reported irreversible changes in the alpha-helix structures of zein proteins after treatment at 90°C.

These results suggest that a short heat treatment (15 min) partially unravels the tertiary structures of zein molecules, resulting in a monodisperse formulation with smaller nanoparticle size. Heat treatment for longer times and at higher temperatures leads to complete unraveling of the zein molecules, which can then aggregate, hence increasing interactions among the polypeptide chains of different protein molecules (Sun et al., 2016). However, there are limited studies about the heat-induced structural and physicochemical changes of alcohol-soluble proteins.

Coatings

Solutions proposed for overcoming the problems of aggregation and precipitation of zein nanoparticles have involved coating the particles with emulsifiers such as carrageenan, gum arabic, lecithin, Pluronic, sodium caseinate, pectin, and chitosan, in order to maintain repulsion among the particles (Luo et al., 2011; Chuacharoen and Sabliov, 2016; Cheng and Jones, 2017; Huang et al., 2017). As an example, the use of carrageenan was found to maintain a constant average diameter of zein nanoparticles stored at pH between 5.25 and 6.75 during 30 days, while uncoated nanoparticles showed significant precipitation (Cheng and Jones, 2017). Coating with gum arabic resulted in stability of zein nanoparticles loaded with mint oil in an extended pH range from 3 to 8, while release of the oil was faster at lower pH (Chen and Zhong, 2015). However, no evaluation of the temporal stability of these particles was made. Chuacharoen and Sabliov (2016) coated nanoparticles composed of zein and lutein with lecithin and Pluronic. The coated particles presented a larger average diameter, compared to particles not coated with the emulsifiers, together with improved performance in the chemical stability parameters evaluated during 30 days in the presence of ultraviolet light. Joye et al. (2015) reported that the coating of zein nanoparticles with sodium caseinate was effective in improving the stability of nanoparticles loaded with resveratrol, considering the effects of ionic strength, pH, and temperature during storage for 4 weeks. In a study of synthesized zein nanoparticles loaded with resveratrol and coated with pectin, Huang et al. (2017) reported an important influence of the pectin concentration on the stability of the formulation, although no data were provided for the chemical stability of the particles according to time, temperature, or pH. The average diameter and zeta potential of the nanoparticles were shown to be dependent on the pectin concentration, with the nanoparticles aggregating and forming precipitates at lower pectin concentrations. This effect of emulsifier concentration on the chemical stability was reported previously by Zhang et al. (2014), who used sodium caseinate and chitosan to increase the encapsulation efficiency and improve the antimicrobial activity of thymol contained in the formulation. The substantial aggregation and sedimentation of the nanoparticles at low concentrations of emulsifiers could be due to the low electrical potentials on the particles, resulting in weak electrostatic repulsion among them. It is also possible that an emulsifier molecule could bind to two or more zein nanoparticles, forming bridges and causing precipitation of the formulation (Hu and McClements, 2015).

Protein and Emulsifier Concentrations

Zhang et al. (2014) and Huang et al. (2017) described directly proportional relations between the zein concentrations used to produce the particles and their mean diameters, while inversely proportional relations were obtained between the emulsifier concentrations and the mean particle diameters, with the smallest nanoparticles being produced using a combination of Pluronic and lecithin.

Ionic Strength and pH

The results obtained for the influence of ionic strength disagreed with the findings of Joye et al. (2015), since there was no evidence of any effect of ionic strength on the synthesis of the particles. When the pH of the aqueous phase was increased from 2 to 7.4, the mean diameter and polydispersity index values decreased, while when the pH was further increased from 7.4 to 10, the diameter and polydispersity of the particles increased. However, no information was provided concerning the final pH values or the temporal stabilities of the formulations.

Considering the findings of the published studies, we had summarized how the factors affects the colloidal stability of zein nanoparticles (Supplementary Figure 1). Also, Supplementary Figure 2 (see supplementary material) recommended a strategy for improving the stability of zein nanoparticles such as thermal treatment as well as the use of coatings employing other biopolymers such as pectin, chitosan, caseinate, and others, or lipids such as lecithin. However, each case should be analyzed individually, since if one of the objectives of preparing the nanoparticles is to produce a carrier system for bioactive compounds, it is necessary to consider possible interactions of the active agent with the components of the coating.

CONCLUDING REMARKS

This article presents and describes the methods used to produce zein nanoparticles, as well as the main issues concerning the colloidal stability of these particles and ways to improve their stability. It was found that there have been few detailed studies of the temporal stability of these particles present in solution. It is notable that a considerable number of studies have reported low stabilities of the formulations, which limits the production of zein nanoparticles on a large scale for commercial purposes. Several techniques have been employed to overcome this difficulty, in order to benefit from the advantages offered by encapsulation employing zein nanoparticles.

Therefore, it is strongly recommended that a detailed study should be undertaken for each type of particle that it is intended to prepare. Experimental design tools can be employed to optimize preparation conditions in order to produce nanoparticulate systems with good colloidal characteristics. A number of important factors always must be considered during this optimization, in order to maximize the temporal stability of the system. These include polymer and emulsifier concentrations, pH, ionic strength, thermal treatment, coatings, temperature, and the presence of illumination during storage. Furthermore, it is essential to evaluate and publish the colloidal stability over extended periods. Such procedures are of great

importance for the feasibility and reproducibility of processes used to produce zein nanoparticles with the aim of developing scalable processes for their commercial manufacture. The marketing of products with shelf lives that are in accordance with commercial requirements can bring benefits for various purposes, justifying the investment in research carried out on this subject.

AUTHOR CONTRIBUTIONS

MP, RdL, and LF proposed and wrote the manuscript.

ACKNOWLEDGMENTS

Financial support for this research was provided by the São Paulo State Research Foundation (FAPESP, processes #2015/17120-4 and #2015/15617-9).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2018.00006/full#supplementary-material

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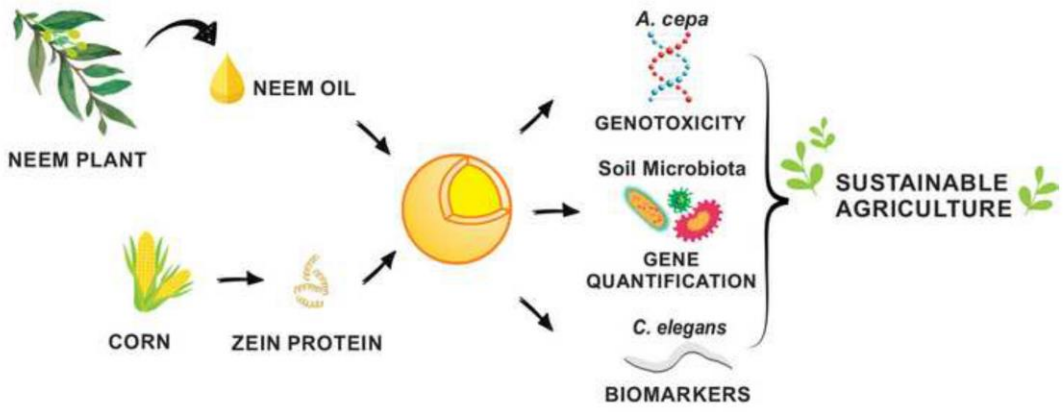
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER III

NEEM OIL BASED NANOPESTICIDE AS AN ENVIRONMENTALLY-FRIENDLY FORMULATION FOR APPLICATIONS IN SUSTAINABLE AGRICULTURE: AN ECOTOXICOLOGICAL PERSPECTIVE

Science of The Total Environment, v. 677, 2019.
doi: 10.1016/j.scitotenv.2019.04.345.

*Graphical Abstract



NEEM OIL BASED NANOPESTICIDE AS AN ENVIRONMENTALLY-FRIENDLY FORMULATION FOR APPLICATIONS IN SUSTAINABLE AGRICULTURE: AN ECOTOXICOLOGICAL PERSPECTIVE

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Abstract

Sustainable agriculture encourages practices that present low risks to the environment and human health. To this end, zein (corn protein) can be used to develop nanocarrier systems capable of improving the physicochemical properties of biopesticides, reducing their possible toxicity. Neem oil extracted from the *Azadirachta indica* tree contains many active ingredients including azadirachtin, which is the active ingredient in multiple commercially available biopesticides. In this study, we describe the preparation and characterization of neem oil-loaded zein nanoparticles, together with evaluation of their toxicity towards nontarget organisms, using *Allium cepa*, soil nitrogen cycle microbiota, and *Caenorhabditis elegans* aiming to achieve the safer by design strategy. The spherical nanoparticles showed an average diameter of 278 ± 61.5 nm and a good stability during the experiments. In the toxicity assays with *A. cepa*, the neem oil-loaded zein nanoparticles mitigated the increase in the DNA relative damage index caused by the neem oil. Molecular genetic analysis of the soil nitrogen cycle microbiota revealed that neem oil-loaded zein nanoparticles did not change the number of genes which encode nitrogen-fixing enzymes and denitrifying enzymes. In *C. elegans*, the neem oil-loaded zein nanoparticles had no toxic effect, while neem oil interfered with pharyngeal pumping and GST-4 protein expression. This neem oil-loaded zein nanoparticles showed promising results in the toxicity studies, opening perspectives for its use in crop protection in organic agriculture.

Keyworks: Zein nanoparticle, nanopesticide, biopesticide, azadirachtin, safer by design.

1 Introduction

Biopesticides include essential oils which are complex mixtures of substances typically containing more than sixty volatile and lipophilic compounds derived from secondary metabolites in plants, involving terpenoids such as monoterpenes, sesquiterpenes, and phenols (Campos et al., 2018; Chellappandian et al., 2018). Essential oils can be extracted from the whole plant or from isolated parts in order to obtain higher concentrations of a specific compound. Since antiquity, essential oils have been used due to their repellent, insecticidal, fungicidal, nematicidal, and bactericidal activities. They are considered safer than synthetic pesticides, having been used for human consumption and as medicines for thousands of years. They might be less toxic to nontarget organisms, such as humans, and have low impacts in the environment. Therefore, essential oils are a promising option for substituting the synthetic pesticides used in agriculture (Benelli and Pavela, 2018; de Oliveira et al., 2018; Ponsankar et al., 2016). Neem oil, which is extracted from the Indian neem tree (*Azadirachta indica Juss.*), is valued worldwide for use in the areas of human health and pest control (Lokanadhan et al., n.d.). Neem oil contains more than 300 biologically active compounds, with the major constituents being triterpenes known as limonoids (Figure 1), the most important of which is azadirachtin (Chandramohan et al., 2016; Gupta et al., 2017; Nicoletti et al., 2012). Neem oil is effective against a wide range of pests, exhibiting a broad spectrum of action due to its systemic and transmembrane activities. It inhibits feeding, reduces ecdysone, motion, and flight activity, deregulates development, suppresses fertility and reproduction, and acts as a repellent (Campos et al., 2016). In addition, neem oil can act as a fertilizer, improving the

quality of soil for crop production, hence contributing to sustainable organic agriculture. However, its use in the field is limited by its short persistence in the environment (Kumar et al., 2018; Shah et al., 2017).

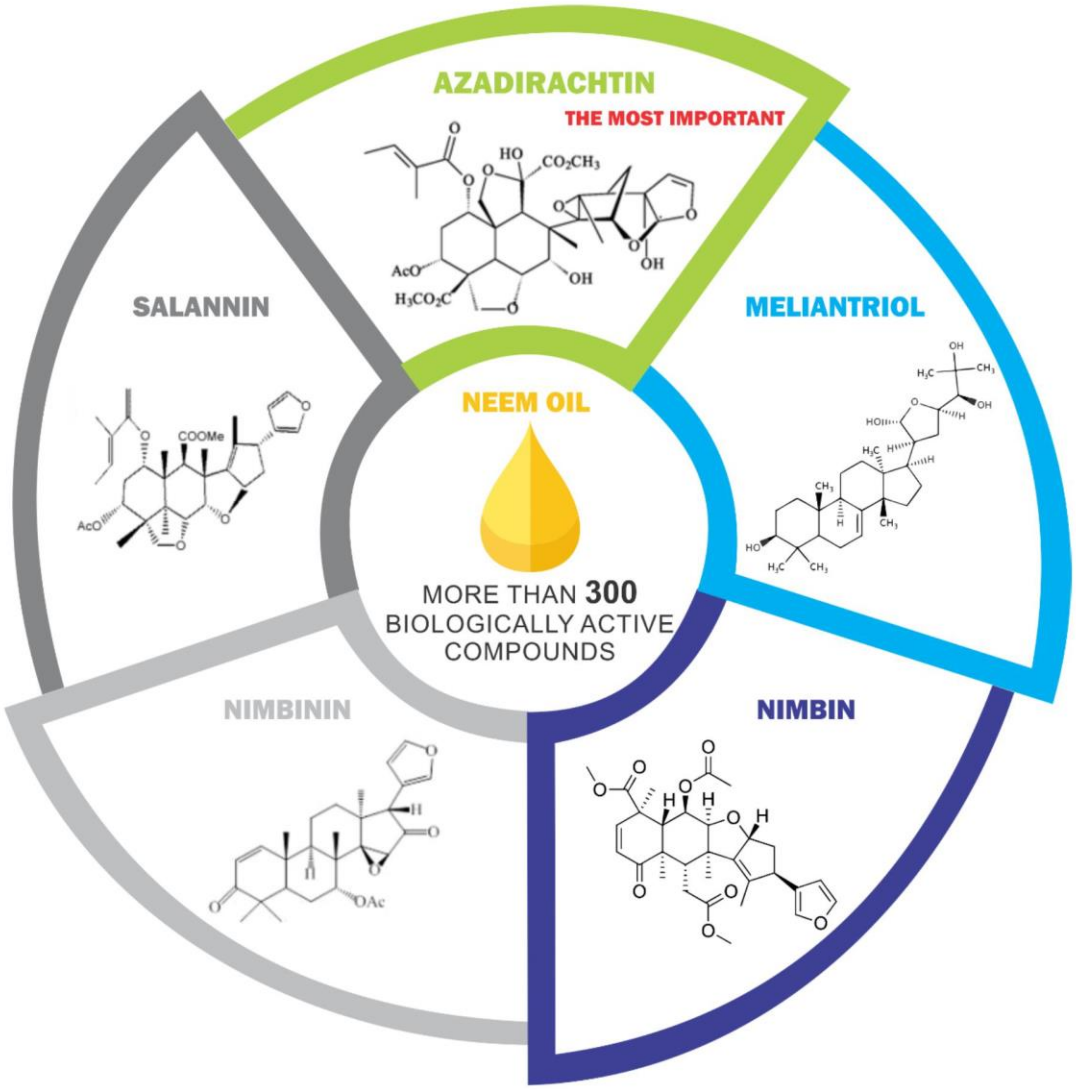


Figure 1. Chemical structures of the main active compounds of neem oil.

The application of nanotechnology in agriculture emphasizes the goal of the development of clean, safe, and environmentally friendly nanomaterials, using biocompatible and nontoxic solvents, biodegradable and biocompatible natural matrices, and energy-efficient and sustainable processes (Ashoka et al.,

2017; Bai et al., 2018; Saratale et al., 2018). Nanocarriers are capable of increasing the solubility of active compounds, while protecting them from volatilization and from degradation. The improvements in efficiency can generate better results, using lower doses and numbers of applications, hence contributing to the reduction of both environmental contamination and risks to human health (Campos et al., 2018; Choudhary et al., 2017; Oliveira et al., 2018). Different types of nanoparticle formulations are used in agriculture as herbicides, insecticides, fungicides, acaricides, fertilizers, and growth regulators, among others (Pascoli et al., 2018b). The use of polymeric nanoparticles as sustained release systems in agriculture has shown excellent results, due to their biocompatibility, biodegradability, and low toxicity (Campos et al., 2018; de Oliveira et al., 2018; de Oliveira et al., 2018b; Oliveira et al., 2018). Several studies have demonstrated the potential of formulations of biopesticides associated with polymeric nanoparticles (Campos et al., 2018; de Oliveira et al., 2018; de Oliveira et al., 2018b; Maruyama et al., 2016; Oliveira et al., 2018c; Pascoli et al., 2018). Zein nanoparticles meet the requirements of environmentally friendly nanotechnology, since zein is a naturally product that is biodegradable and biocompatible. It represents the main protein content of corn, is composed of lipophilic amino acid residues, and is not used for direct human consumption, due to its negative nitrogen balance and low water solubility (Paliwal and Palakurthi, 2014). Due to its high coating capacity, zein is used in the production of nanocarrier systems, employing a low toxicity solvent, such as ethanol, which is evaporated during the synthesis, hence causing no harm to the environment when the formulation is used in the field. Nanoparticles are capable of modifying the properties of the active substances that

106 encapsulated, so, it is necessary to re-screen the material in order to ensure its
107 safe use. This involves assays using target and nontarget organisms, as well as
108 evaluation of the behaviors of new formulations in the environment, aiming at
109 regulation of the use of biopesticides associated with nanomaterials in crop
110 protection (Campos et al., 2018; Dere et al., 2015; Fraceto et al., 2016; Pascoli
111 et al., 2018; Sola et al., 2014).

112 Given this background, the innovation of this study was to develop neem
113 oil-loaded zein nanoparticles. In addition to preparation and characterization of
114 the nanocarriers, using the novel safe by design strategy their potential toxicity
115 was evaluated by investigating their effects on nontarget organisms (*Allium*
116 *cepa*, nitrogen cycle bacteria, and *Caenorhabditis elegans*). The choice was
117 because they are model organisms, all are used in the research of toxicity of
118 materials making possible a broad investigation of the possible action of zein
119 nanoparticles loaded with neem oil, since they are in different classes of
120 organisms (plant, nematodes and microorganisms) that can come into contact
121 with this new biopesticide in the crops. The work opens perspectives for the use
122 of nanobiopesticides based on neem oil in crop protection, contributing to
123 sustainable organic agriculture as well as improved food safety.

124

125 **2 Materials and Methods**

126 **2.2 Materials**

127 Zein and Pluronic F-68 were purchased from Sigma-Aldrich. Neem oil
128 (Azamax) containing 12g/mL of azadirachtin was acquired from UPL Brazil.
129 Ethanol was obtained from Labsynth. *Allium cepa* seeds were purchased from

130 Isla seeds (Brazilian company). The soil used was obtained from a local
131 agricultural supplier. *C. elegans* N2 (wild type) and CL2166 (dvIs19 [(gst-
132 4p::gfp::nls] III) strains were purchased from the Caenorhabitis Genetics Center,
133 Minnesota, USA. Other chemicals and solvents used were analytical grade and
134 were purchased from local suppliers.

135

136 **2.3 Preparation of neem oil-loaded zein nanoparticles**

137 Zein nanoparticles were prepared by the environmentally-friendly
138 antisolvent precipitation method (Hu and McClements, 2014) with some
139 modifications (Pascoli et al., 2018a). The antisolvent method is a principle
140 where the active compound is dissolved in a solvent; the solution is then
141 injected with an antisolvent solution (in which the compound is insoluble). The
142 compound precipitates as a consequence of the change of supersaturation
143 caused by mixing the solution and the antisolvent solution. In this way, zein
144 powder (2% w/v) was added to an aqueous solution of ethanol (85% v/v) and
145 kept under magnetic stirring overnight. The zein solution was adjusted to pH
146 5.8, heat-treated at 75 °C for 15 min for protein densification, centrifuged, and
147 filtered through a 0.45 µm membrane (Millipore) to remove insoluble particles. A
148 100 mg quantity of neem oil (in the form of an emulsifiable concentrate
149 containing 12 g/L of azadirachtin) was added to the zein solution. An aqueous
150 solution of Pluronic F-68 (a block-copolymer of ethylene oxide and propylene
151 oxide ($C_3H_6O.C_2H_4O)_x$) extensively used as surfactant, wetting agents and
152 emulsifiers) (2% v/v) was prepared and was adjusted to pH 4. The presence of
153 Pluronic F-68 decreased the surface tension of the nanoparticles and maintain

154 the stability of the nanoparticles in suspension. Using a syringe, the zein
155 solution was rapidly injected into the Pluronic solution, under stirring. The
156 resulting colloidal dispersion was stirred for 12 h, at room temperature, in order
157 to evaporate the ethanol, and water (pH 4.0) was then added to make up the
158 original volume. The final concentration of neem oil in the nanoformulation was
159 5 mg/mL. This concentration was chosen since in agriculture, neem oil is used
160 at concentrations of between 4 and 6 mg/mL as recommended by the
161 manufacturer UPL Brazil.

162

163 **2.4 Nanoparticle physicochemical characterization**

164 The nanoparticle mean size distribution and polydispersity index (an
165 indicator of the homo/heterogeneity of the size distribution of particles
166 calculated by the square of the standard deviation divided by the square of the
167 mean size) were determined by the dynamic light scattering technique (DLS).
168 The zeta potential was measured by the microelectrophoresis method. These
169 analyses were performed using a ZetaSizer Nano ZS90 system (Malvern
170 Instruments, UK) at a fixed angle of 90° and 25 °C. The nanoparticle
171 concentrations, size distributions, and Span values (an additional parameter to
172 show the width of the size distribution calculated as Span = $(D_{90} - D_{10})/D_{50}$
173 being that D_{10}, D_{50} and D_{90} refer, respectively, to the diameters where 10%,
174 50% and 90% of the particle population) were also measured by nanoparticle
175 tracking analysis (NTA), using a NanoSight LM 10 cell (green laser with
176 wavelength of 532 nm) and a sCMOS camera, controlled by NanoSight v. 3.2
177 software (Grillo et al., 2012; 2014).

For these analyses, the samples were diluted 1000x in ultrapure water and in liquid medium (0.5% saline solution), at the highest concentration used in the *C. elegans* assay. Stability analyses were performed using sample aliquots removed after 0, 10, 20, 30, 40, 50, 60, 90, and 120 min of incubation in the saline solution. Each result was expressed as the average of three determinations.

Aliquots of zein nanoparticles and neem oil-loaded zein nanoparticles were collected and diluted in ultrapure water. Samples were dripped onto a silicon plate AFM sampler and kept in a desiccator for complete drying. The samples were analyzed using an Easy Scan 2 Basic BT02217 atomic force microscope (Nanosurf, Switzerland) operated in noncontact mode with TapAl-G cantilevers (BudgetSensors, Bulgaria) and tip voltage of 90 Hz. The acquired images were analyzed using Gwyddion software.

2.5 Toxicity studies

2.5.1 *Allium cepa* assay

Based on the procedure described by de Lima et al., (2010) germinated *A. cepa* seeds were exposed to the nanoformulations (zein nanoparticles, neem oil-loaded zein nanoparticles), neem oil (at a concentration of 5 mg/mL), Pluronic F-68 surfactant, and ultrapure water (negative control) in 10 mL glass beaker, in dark conditions for periods of 24 h. 10 roots were exposed to each treatment. The roots were fixed in Carnoy's reagent (methanol:acetic acid, 3:1 v/v), followed by acid hydrolysis with 1 mol/L HCl at 60 °C during 9 min. The roots were stained with Schiff reagent for 2 h. For preparation of the slides, the

meristematic region was crushed in one drop of 2% acetic-carmine, using a cover slip. Three roots exposed for each treatment were used to prepared the slides and all the cells were analyzed. This assay was repeated three independent times in different days. Calculations were made of the mitotic index (MI), the damage index (DI), and the relative index (RI) which are indicators of the presence of cytotoxic, mutagenic or carcinogenic potential agents in the environment. MI was calculated by dividing the number of cells in division by the total number of cells. DI was calculated by dividing the number of cells showing DNA alterations during the mitosis by the total number of cells in division. RI was calculated by dividing the values obtained for the treatments by the values for the negative control.

2.5.2 Molecular analysis of the effects of the nanoparticles on soil microbiota

We investigated the changes in all genes from the N cycle due the importance of this cycle for the nitrogen fixation in soil making the soil fertile by converting nitrogen into bioavailable forms that can be assimilated by living beings for production of organic molecules such as amino acids proteins and and nucleic acids. Therefore, the quantification of these functional genes involved in N transformation performed in this work improves our understanding of N-cycling soil microbiota responses to environmental impact (Hirsch and Mauchline, 2015; Fang et al., 2019).

Before use, the fertilized commercial soil, (14% organic matter, pH 6.80) was sieved using a 0.2 micrometer sieve, dried and separated into vessels with

226 surface area of 0.025 m^2 each, and kept moist in a heated cabinet at 25 °C for
227 15 days. Two untreated soil samples were used as the negative control. Each
228 treatment (zein nanoparticles, neem oil-loaded zein nanoparticles, and neem
229 oil) were tested in duplicates (two vessels containing soil for each treatment).
230 The applications of the formulations (using sprays) were based on the dosage
231 and number of applications of neem oil employed in the field (three applications
232 were performed on the same sample at 7-day intervals, using a 5 mg/mL
233 solution at a dosage of 100 L/ha).

234 The extraction of DNA from soil microorganisms was performed 7, 14,
235 21, and 30 days after the first application of the treatments, using a Power Soil
236 DNA Isolation Kit (MoBio Laboratories). Quantification of the genetic material
237 was performed by fluorescence, using a Qubit 3.0 fluorometer with the Qubit
238 dsDNA BR Assay Kit (Invitrogen). All the samples were diluted to final
239 concentrations of 1000 ng/mL.

240 Real-time polymerase chain reactions (qPCR) were performed for
241 specific genes from nitrogen cycle bacteria: nifH (nitrogen fixation), nirK, nirS,
242 narG, cnorB, and nosZ (denitrification). The bacterial 16S RNA gene was used
243 as a reference. The reactions were performed using 1 µL of DNA sample, 12.5
244 µL of Planium SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen), 1 µL
245 of each primer (sense and antisense), and sufficient ultrapure water to complete
246 the final volume to 25 µL. The amplifications were conducted according to a
247 procedure adapted from Jung et al., (2011) using a StepOne thermocycler
248 (Applied Biosystems), with an initial denaturation at 95 °C for 3 min, followed by
249 40 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s. The SYBR Green
250 fluorescence emitted was measured at the end of each incubation at 72 °C.

The results were analyzed using relative quantification, with calculation of ΔΔCt ($2^{-\Delta\Delta Ct}$), employing 16S rRNA as the reference gene and the initial soil as the reference sample (Yuan et al., 2008). The calculations were performed using the StepOne Plus v2. 3 software of the equipment.

2.5.3 *Caenorhabditis elegans* assays

C. elegans trains N2 (wild type, established as valuable experimental model due to the high level of genetic homology with humans, fast life cycle, easy maintenance and handling) and CL2166 (genetically equal to wildtype and tagged to green fluorescent protein, GFP, fused to the promoter of the detoxifying enzyme glutathione- S- transferase-4) were maintained in plates containing NGM (nematode growth media) enriched with salts and seeded with the bacterium *E. coli* OP50, at 20 °C. The fertilized nematodes were synchronized by lysing them with a bleaching mixture (1% NaOCl, 0.25 M NaOH). The eggs obtained were washed with M9 buffer (0.02 M KH_2PO_4, 0.04 M Na_2HPO_4, 0.08 M NaCl, and 0.001 M $MgSO_4$) and were kept in plates containing M9 without bacteria, during 14 h, until the larvae hatched in stage L1.

Chronic exposure of the L1 worms to the negative control (0.5% NaCl) and the different formulations (zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil) was performed for 30 min with 0.05, 0.25, 0.5, and 0.75 mg/mL of the test material, using 1500 worms per replicate (per microtube), in a liquid medium (0.5% NaCl), with stirring to ensure contact of the nematodes with the treatments. Concentrations were tested in duplicates, in every experiment using *C. elegans*, a procedure that was repeated in three

275 independent experiments (in different days and different batch of worms). After
276 exposure, the worms were placed with the treatment on NGM plates with *E. coli*
277 OP50, and were kept at 20 °C for 48 h.

278 The wild type strain nematodes were evaluated in terms of their survival,
279 reproduction, body size, and pharyngeal pumping. For GST-4 enzymatic
280 expression, CL2166 strain that has GST-4 tagged to a GFP was used and the
281 labeled xenobiotic detoxification protein was determined according to its
282 fluorescence (Rathor et al., 2017).

283 For survival evaluation, 48 h after exposure a transparent grid was
284 placed beneath the NGM plate and 18 quadrants were analyzed under a
285 dissection microscope, obtaining a score according to the number of living
286 animals. After scoring survival, reproduction was determined by counting the
287 hatched larvae daily from three individual worms from each treatment
288 transferred to NGM plate covered with *E. coli* OP50, during 4 reproductive days.
289 Body size was evaluated by images acquired 48 h after the exposures, using an
290 inverted microscope (MEDILUX MDL-INV-1) connected to a digital camera
291 (SAMSUNG ST64). ImageJ software was used to measure the body lengths of
292 10 worms per group, in each experiment. Pharyngeal pumping was counted for
293 1 min using 10 worms submitted to each treatment, in order to assess the
294 intake of the treatments. Individuals of the CL2166 transgenic strain were
295 exposed to the different treatments (0.5% NaCl as negative control, zein
296 nanoparticles, neem oil-loaded zein nanoparticles and neem oil) and were then
297 transferred to microscope slides containing levamisole (1 mM) as an anesthetic.
298 Images were acquired using an epifluorescence microscope (Nikon Eclipse 50i)

299 with a GFP filter (with excitation at 365 nm and emission at 420 nm), and the
300 fluorescence was measured using ImageJ software.

301

302 **2.6 Statistical analysis**

303 The molecular analysis of the effects of the nanoparticles on soil
304 microbiota and *C. elegans* assays were performed in duplicate, and all other
305 experiments were performed in triplicate, however, these replicates are
306 considered one independent experiment and were repeated at least three
307 times, and the data were expressed as average of three independent
308 experiments ± standard deviations, represented by error bars. Statistical
309 analyses were performed with GraphPad Prism v. 6 software, using two-way
310 ANOVA followed by the Tukey post-hoc test, at a significance level of $p < 0.05$.

311

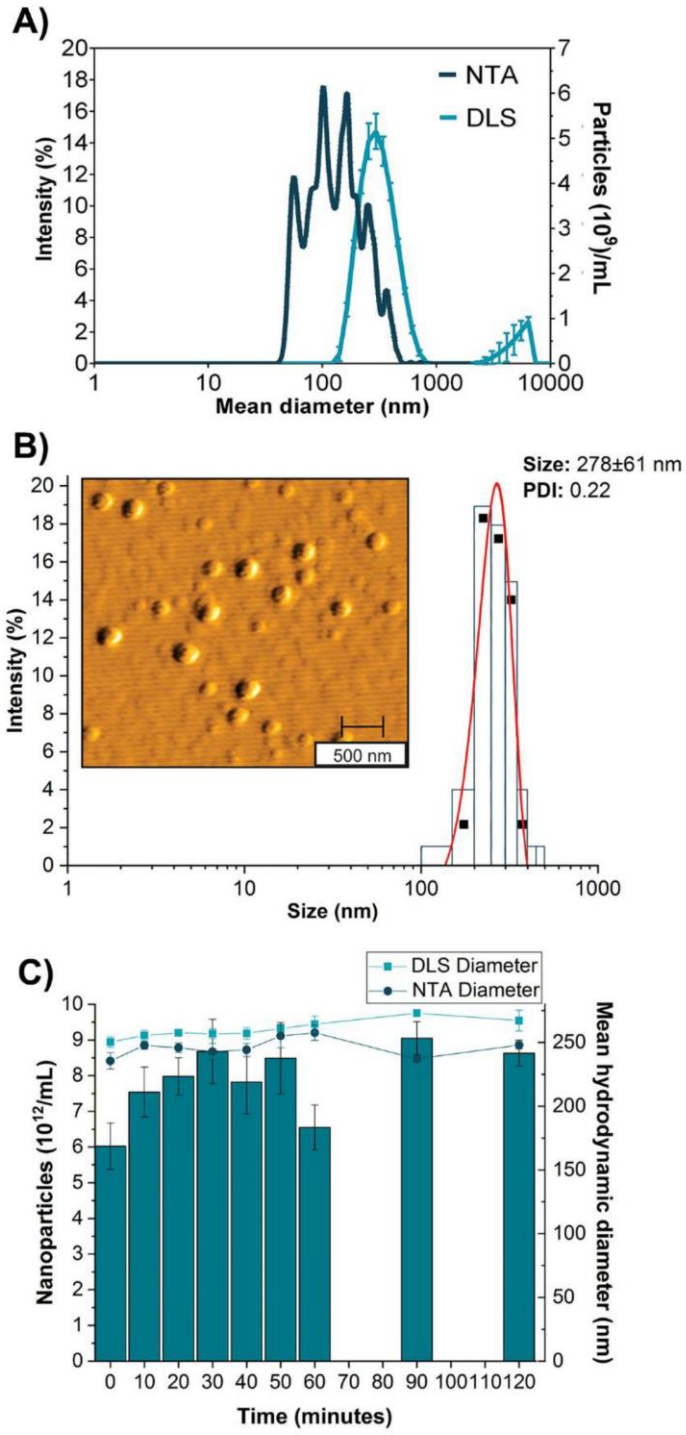
312 **3 Results and Discussion**

313 **3.1 Physicochemical characterization of the neem oil-loaded zein**
314 **nanoparticles**

315 In order to characterize the neem oil-loaded zein nanoparticles we have
316 measured the mean hydrodynamic diameters of the nanoparticles dispersed in
317 water using DLS and NTA. The results obtained by DLS and NTA were 288 ± 6
318 and 198 ± 16 nm, respectively (Figure 2A). These results indicated that during
319 the zein nanoparticles formation in presence the surfactant showed a range of
320 size as described by other authors. Wu et al., (2012), using zein nanoparticles

containing thymol and carvacrol showed the mean size distribution by DLS in a range of 52 to 328 nm. In pursuance of size distribution, using atomic force microscopy (Figure 2B), the results showed that the neem oil-loaded zein nanoparticles were spherical, with a mean diameter of 278 ± 61 nm (with a concentration of nanoparticles/mL of 1.13 x 10^{12}). Using AFM, Chen et al., (2013) observed that zein nanoparticles were spherical, with sizes of around 100-200 nm. Cheng et al., (2019) reported the same size for spherical zein nanoparticles containing lutein. Oliveira et al., (2018) showed that zein nanoparticles containing geraniol and citronellal were spherical, with smooth surfaces and mean size of 90-250 nm.

However, Figure 2A and Figure 2B showed a broad size distribution curves, indicating that the particles were not monodisperse. This information was confirmed by the measurement of the polydispersity index. The value obtained for neem oil-loaded zein nanoparticles was 0.313 ± 0.005. Also, determined by NTA, the Span value calculated as described by Bender et al., (2012) was 1.3 ± 0.005. Based on both parameters a formulation is defined as monodisperse when the polydispersity index and span presented values lower than 0.2 and 1 respectively. However, in literature was described that nanoparticles prepared with matrices of natural origin (such as zein) was not monodisperse (Chuacharoen and Sabliov, 2016; Oliveira et al., 2018).



341

342 **Figure 2.** Characterization and stability of the neem oil-loaded zein
343 nanoparticles: A) Mean hydrodynamic size distribution curves obtained using
344 the DLS and NTA techniques applied to a suspension of the nanoparticles in
345 water; B) Micrograph and size distribution obtained using the AFM technique in

noncontact mode with TapAl-G cantilevers and tip voltage of 90 Hz. The image obtained was treated using Gwyddion software; C) Mean hydrodynamic size (lines) and concentration (bars) of the nanoparticles in saline medium (0.5% NaCl), as a function of time. The spherical nanoparticles showed an average diameter of 278 ± 61.5 nm with no aggregates of 288 ± 6 nm. The nanoparticles were stable over 120 minutes, under the experimental conditions. Data are expressed as average of three independent experiments (n=3) and the error bars represent the standard deviations. A significance level of $p < 0.05$ was adopted.

Also, in order to investigate the stability, we have been used the microelectrophoresis technique to measure the zeta potential of neem oil-loaded zein nanoparticles. The results showed that the zeta potential of this system was -36 ± 1 mV, which was close to the values characteristic of a stable formulation (+/-30 mV). Furthermore, in the case of this zein nanoparticles, during the preparation process we used Pluronic F-68 that provided steric hindrance, which was another factor that contributing to the stability of the zein nanoparticles in solution (Chuacharoen and Sabliov, 2016). Just in order to compare, negative zeta potential values (determined by microelectrophoresis) have been reported previously for zein nanoparticles loaded with 5-fluorouracil (-45 ± 0.3 mV) (Lai and Guo, 2011), zein nanoparticles loaded with thymol (from -34 to -40 mV) (Li et al., 2013), and zein nanoparticles stabilized with carrageenan (from -40 to -50 mV) (Cheng and Jones, 2017).

369 Moreover as we investigated the effect of the toxicity of these particles in
370 models (such as *C. elegans*) that used saline medium (0.5% NaCl), the Figure
371 2C showed that they maintained the same mean hydrodynamic size,
372 polydispersity, and concentration during 120 min (exposition time in *C.*
373 *elegans*), while the zeta potential decreased significantly. The zeta potential
374 was significantly lower in the saline environment, reaching -7.4 mV. This
375 decreasing in zeta potential value in the presence of saline medium was
376 reported in literature (de Oliveira et al., 2015; Grillo et al., 2014, 2012; Jacques
377 et al., 2017) and explained due the greater ionic strength of the saline medium
378 altered the ionic balance, leading to changes in the nanoparticle surface charge.
379 It is important to pointed out that even with the low values of zeta potential (-7.4
380 mV) the particles kept stable in solution, showing in this way, the importance of
381 the steric hindrance of Pluronic F-68 in neem oil-loaded zein nanoparticles.

382

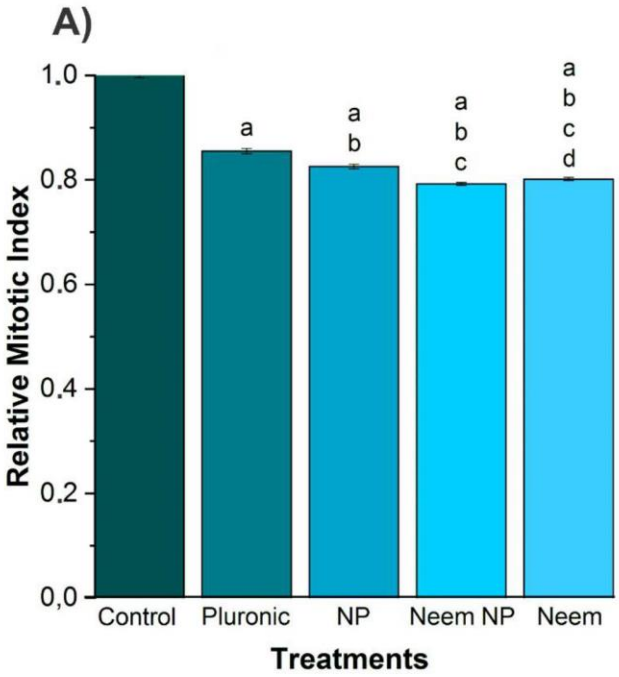
383 **3.2 Toxicity studies**

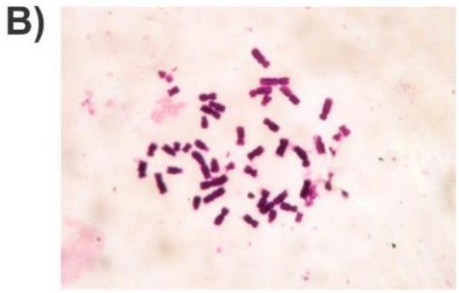
384 **3.2.1 *Allium cepa* chromosome aberration assay**

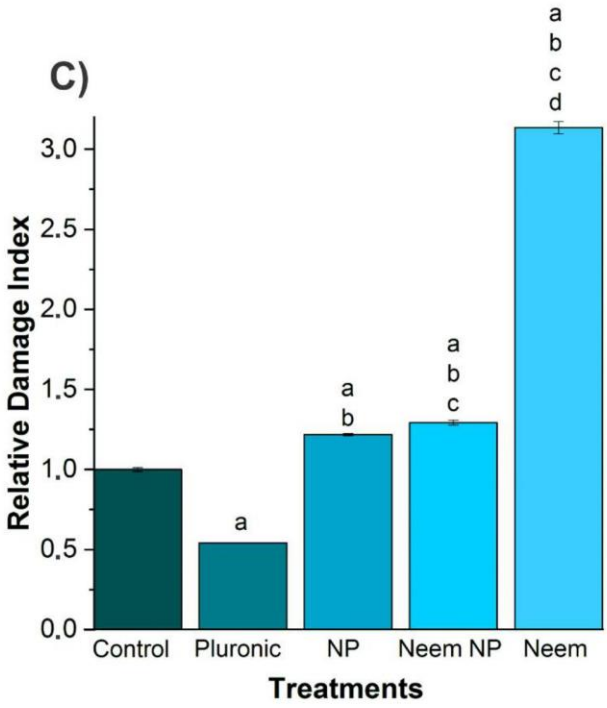
385 The results obtained in the *A. cepa* assay (Figure 3) showed significant
386 differences between the control and all treatments, for both parameters
387 evaluated (mitotic index and relative damage index). The treatments with
388 Pluronic, zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil
389 decreased the relative mitotic index (Figure 3A). Use of the neem oil-loaded
390 zein nanoparticles resulted in a greater decrease in the mitotic index, compared
391 to all other treatments. Then, our results confirm the ability of the treatments to
392 interfer in cell mitosis, as reported by Kwankua et al., (2010) and Pasquoto-

393 Stigliani et al., (2017), that showed that neem oil extract caused a significant
394 decrease in the mitotic index of *Allium cepa* roots. The decreases in the mitotic
395 index caused by neem oil, together with the presence of chromosome
396 alteration, c-metaphase (Figure 3B), could be attributed to the azadirachtin
397 ability to interfere in mitosis usually a consequence of changes in the spindles,
398 similar to that seen with colchicine treatment, which prevents the formation of
399 spindle fibers, impairing the cell cycle progress (Soliman, 2001).

400 The results obtained for the relative damage index (Figure 3C) showed
401 that the surfactant used in production of the zein nanocarriers caused fewer
402 chromosomal changes, compared to the control. For the other treatments (zein
403 nanoparticles, neem oil-loaded zein nanoparticles, and neem oil), the
404 chromosomal changes were significantly increased. The zein nanoparticles and
405 the neem oil-loaded zein nanoparticles caused increases of 25% in the damage
406 index. However, it should be noted that neem oil alone (in the absence of
407 nanoparticles) increased the number of chromosomal aberrations by 200%. A
408 similar result was reported by Kwankua et al., (2010) who found that neem oil
409 caused a 400% increase in chromosomal aberrations in *Allium cepa*, that
410 solidifies our findings that indicate the zein nanoparticles are promising carriers
411 for neem oil, since they are able to decrease over the genotoxicity towards
412 nontarget organisms.


A)
Relative Mitotic Index
1.0
0.8
0.6
0.4
0.2
0,0
a
a b
a b c
a b c d
Control
Pluronic
NP
Neem NP
Neem
Treatments


B)


C)
Relative Damage Index
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a b
a b c
a b c d
Control
Pluronic
NP
Neem NP
Neem
Treatments

413

414

415 **Figure 3.** Results of the *Allium cepa* aberration assay: A) Relative mitotic index
416 values for the different treatments; B) Presence of c-metaphases in the neem oil
417 treatment; C) Relative damage index of roots submitted to treatments for 24 h
418 with Pluronic F-68 surfactant (280 mg/mL), zein nanoparticles (NP), neem oil-
419 loaded zein nanoparticles (Neem NP), and neem oil (Neem), using neem oil
420 concentrations of 5 mg/mL. Data are expressed as average of three
421 independent experiments (n=3) and the error bars represent the standard
422 deviations. Letters a, b, c, and d indicate a significant difference relative to the
423 control, Pluronic, NP, and Neem NP, respectively. The significance level
424 adopted was $p < 0.05$.

425

426 **3.2.2 Effects of the nanoparticles on soil bacteria involved in the nitrogen**
427 **cycle**

428 Soil microbiota are considered soil quality parameters once they are
429 responsible for regulating several important soil processes such as organic
430 matter decomposition, degradation of organic pollutants and transformation of
431 nutrients (Fang et al., 2019). The Nitrogen (N) cycle consists of several N
432 transformation processes which are performed by bacteria that have specific
433 genes to encode enzymes involved in each stage of the cycle including *nifH*
434 (encoding nitrogenase reductase, nitrogen-fixing enzyme: reduction of nitrogen
435 gas in ammonia), *amoA* (encoding ammonia monooxygenase, nitrification
436 enzyme: conversion of ammonia to hydroxylamine), *haO* (encoding
437 hydroxylamine oxidase, nitrification enzyme: oxidation of hydroxylamine to

438 nitrite) *narG* (encoding nitrate reductase, first two denitrification steps: reduction
439 of nitrate to nitrite) *nirK* and *nirS* (encoding Cu-containing nitrite reductase and
440 nitrite reductase, respectively, first two denitrification steps: catalyze the
441 reduction of nitrite to nitric oxide), *cnorB* (encoding nitric oxide reductase,
442 second two denitrification steps: reduces nitric oxide to nitrous oxide) and *nosZ*
443 (encoding nitrous oxide reductase; second two denitrification steps: reduction of
444 nitrous oxide to molecular nitrogen) (Hirsch and Mauchline, 2015; Ouyang et al.,
445 2018).

446 In this context, soil analysis should be evaluated based on control
447 sample that exists for each period evaluated. The percentages of nitrogen cycle
448 genes (Figure 4B) show that after 7 days there is a small amount of bacteria
449 that present the cnorB gene, but this also presents small amount in the control,
450 indicating a homogeneity between the samples and the non-alteration of the
451 genes compared to the control (possible observation in 5A and 5B, referring to
452 7 days after exposure). The results in time of 14 days after exposure it is
453 possible to observe the presence of bacteria that have the *cnorB* gene, being
454 the proportions similar to those found in control soil. The concentration of
455 bacteria (time 14 days) presents a greater variation in relation to the control, but
456 the existing proportion of each type of bacteria responsible for the maintenance
457 of the nitrogen cycle is similar between the treatments and the control. It is also
458 possible to observe an increase the *nifH* gene, responsible for the nitrogen
459 fixation, especially in control sample, and in other treatments this still remains
460 with a low relative quantification. After 21 days the quantification shows that in
461 relation to the number of genes that participate in the cycle the treatments are
462 matched in a smaller quantity to control, it is possible to observe a decrease in

463 the quantification of cnorB and a slight increase in the amount of *nirS* gene,
464 responsible for the second step of denitrification. In the end experiment (after 30
465 days of exposure) the increase *nosZ* and *cnorB* genes indicates an increase in
466 final steps of the nitrogen cycle, being observed in all the samples evaluated,
467 including in control. It was possible to observe greater homogeneity between
468 the samples in relation to both the quantification and the distribution have great
469 similarity indicating that the soil, in relation to the bacteria responsible for the
470 nitrogen cycle do not seem to suffer changes in the presence of the evaluated
471 compounds.

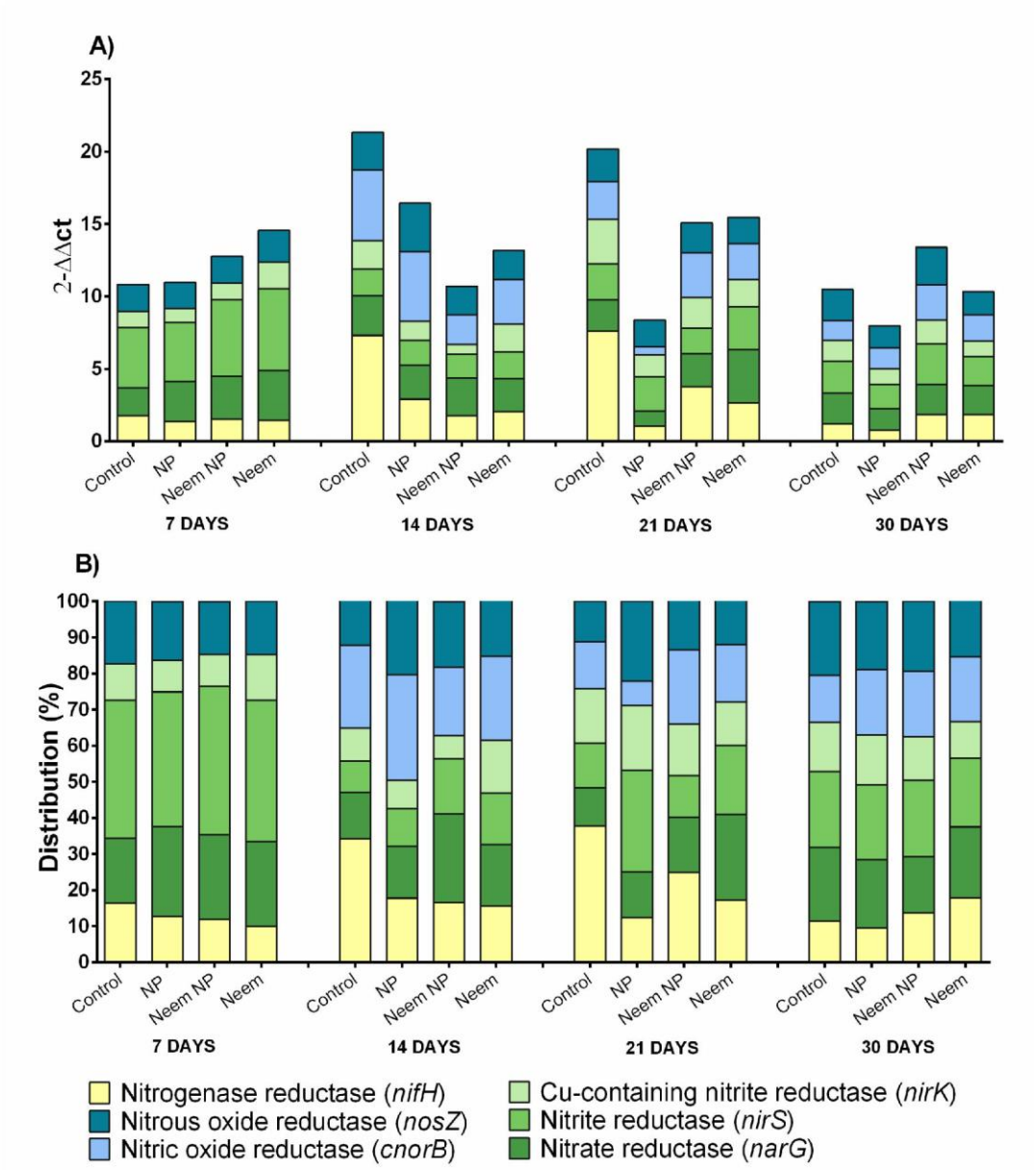


Figure 4. Molecular analysis of the genes of bacteria associated with the nitrogen cycle (*nifH, nosZ, cnorB, nirK, narG, and nirS*). A) Relative quantification of genes by qPCR and B) proportions of genes in the control soil and soils exposed to the zein nanoparticles (NP), neem oil-loaded zein nanoparticles (Neem NP), and neem oil (Neem), at 7, 14, 21, and 30 days after the initial treatment. Data are expressed as average of three independent experiments (n=3).

In according to our results, Pasquoto-Stigliani et al., (2017) investigated the behavior of bacteria involved in the nitrogen cycle when exposed to poly(ε-caprolactone) nanocapsules loaded with neem and showed that the differences in the proportions of these bacteria, compared to the control, varied during the experiment, with no significant difference after 300 days. Maruyama et al., (2016) evaluated atrazine and imazethapyr nanocapsules, showed lower effects on the bacterial profile associated with the nitrogen cycle, in the soil displayed in comparison with control. Yang et al., (2013) and Guilger et al., (2017) analyzed the effects of silver nanoparticles on nitrogen-fixing, nitrifying, and denitrifying bacteria, and found that the nitrifying bacteria were significantly affected, while the nitrogen-fixing and denitrifying organisms were not.

The use of molecular analysis of the genes of soil microbiota involved in the nitrogen cycle to investigate the possible toxicity of new materials, especially nanoparticles, is still recent and the literature is very limited. Further detailed studies are needed and are essential to ensure the safe use of newly emerging technologies.

3.2.3 Effects of the formulations on the nematode *C. elegans*

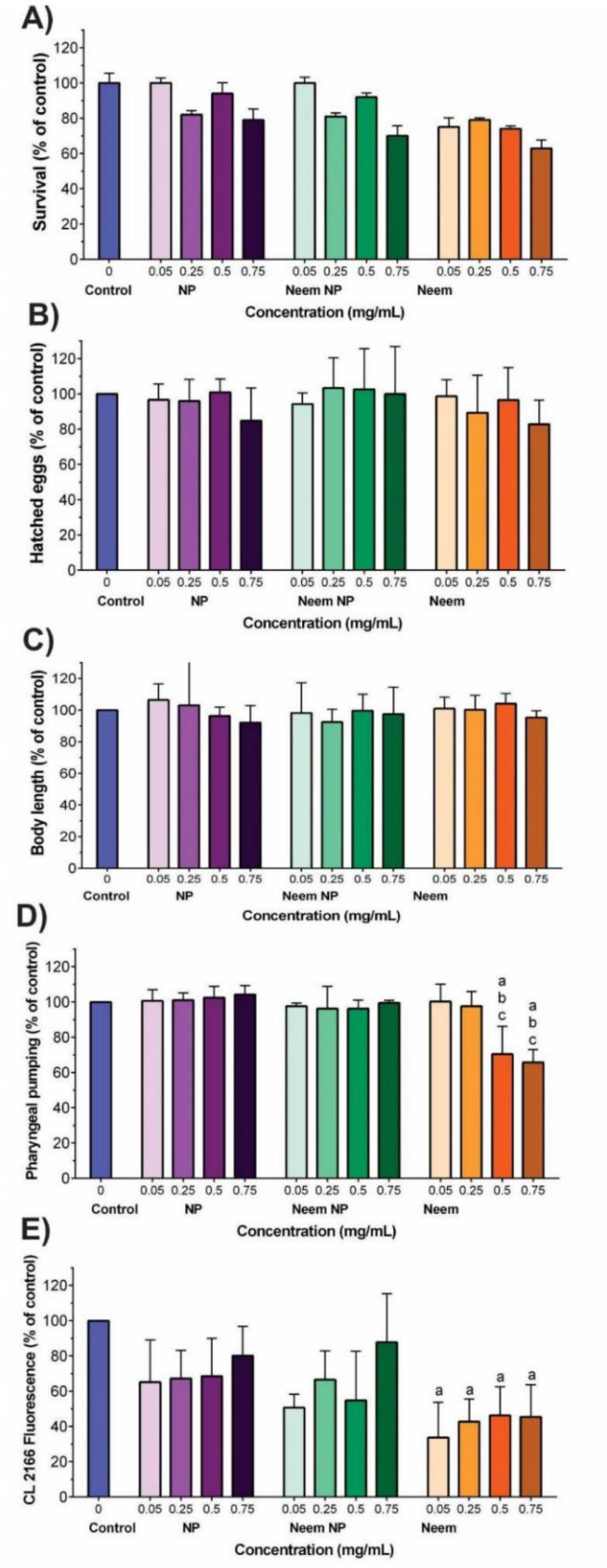
The results of toxicity assays performed with *C. elegans* (Figure 5) showed that the survival, reproduction, and body length of the worms did not present significant differences after exposure to the zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil (using neem oil concentrations of 0.05, 0.25, 0.5, and 0.75 mg/mL). This lack of toxicity for a nontarget organism

504 is very promising for the advance of neem oil-loaded zein nanoparticle
505 research. These endpoints have been validated as the basic triad for safety
506 assessment. Even if mortality rate does not increase following exposure to a
507 toxicant, the reproductive system and the development of the worms are very
508 sensitive and may show tenuous signs of cellular damage (Tejeda and Olivero,
509 2016). That because during the larval stages, mitosis and meiosis are in fast
510 speed and it has been demonstrated that toxicants, pesticides included, can
511 disrupt cell cycle, elevate DNA double-strand break formation, activate
512 apoptosis and increase embryonic lethality (Shin et al., 2019). Of note, another
513 study evaluating the toxicity of zein nanoparticles loaded with the antidiabetic
514 drug glibenclamide (with an average size of 190 nm and a surface charge of
515 −37 mV) and showed that the formulation exerted significant hypolipidemic
516 activity in *C. elegans*, without causing any toxic effect (Lucio et al., 2017). In
517 contrast, nanoparticles toxicity can be detected in this animal model. Jacques et
518 al. (2017) have shown that different NPs interfered in the survival and vital
519 parameters of *C. elegans*. Solid lipid nanoparticles with or without atrazine and
520 simazine (sizes of 293 ± 3 and 288 ± 6 nm, respectively) and polymeric
521 nanoparticles with or without atrazine (sizes of 367 ± 13 and 305 ± 12 nm,
522 respectively) depicted dose-dependent increases of lethality and decreases of
523 *C. elegans* body length. Chitosan/tripolyphosphate nanoparticles, produced
524 using a natural biopolymer, with or without paraquat (sizes of 262 ± 14 and 246
525 ± 7 nm, respectively), caused increased mortality, but did not alter reproduction
526 or worm length in the surviving animals, therefore providing evidences that
527 natural biopolymers can be more compatible to nontarget organisms (Jacques
528 et al., 2017). Using another nontarget organism Deng et al., (2013) and Wang

529 et al., (2013) investigated the subacute, acute, and subchronic toxicity of neem
530 oil towards mice and the only significant result was after 90 days, when the
531 mice treated with neem oil at a dose of 1600 mg/kg/day presented several
532 degrees of lesions in the testes, liver, and kidneys. However, the lesions were
533 decreased or eliminated after a 30-day recovery period not demonstrating
534 critical toxicity to the organism studied, in the same way that it happened in our
535 research. (Wang et al., 2013).

536 In the present work, the pharyngeal pumping of the worms (Figure 5D)
537 decreased significantly in the treatments with neem oil at concentrations of 0.5
538 and 0.75 mg/mL, compared to the control group, while the neem oil-loaded zein
539 nanoparticles caused no significant alterations in the worms. Pharyngeal
540 pumping is an indicator of a healthy worm and is mainly controlled by
541 cholinergic and glutamatergic innervation, as well as by dopamine and
542 serotonin (Raizen, 2012). Reduced pharyngeal pumping can lead to dietary
543 restriction (Powolny et al., 2011). The results suggested that the zein
544 nanoparticle formulation was able to decrease the toxicity of neem oil in this
545 organism. These findings were in agreement with the work of Sanches Moraes
546 et al., (2016) who reported the ability of polymeric nanocapsules to decrease
547 the toxic effects of clozapine in *C. elegans*.

548

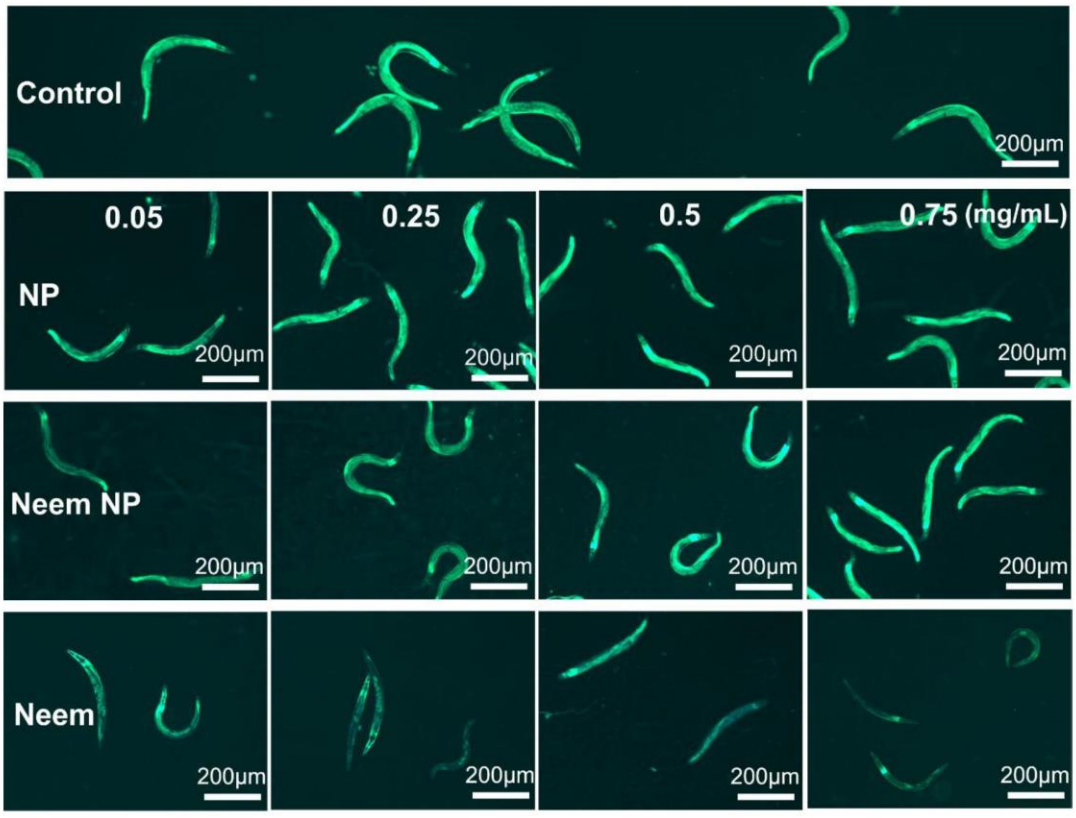

A)
Survival (% of control)
B)
Hatched eggs (% of control)
C)
Body length (% of control)
D)
Pharyngeal pumping (% of control)
a b c
a b c
E)
CL 2166 Fluorescence (% of control)
a a a a
0 0.05 0.25 0.5 0.75
Control
NP
Neem NP
Neem
Concentration (mg/mL)

549

Figure 5. Toxicity assay using *C. elegans* exposed for 48 h to 0.05, 0.25, 0.5, and 0.75 mg/mL of zein nanoparticles (NP), neem oil-loaded zein nanoparticles (Neem NP), and neem oil (Neem). The wild type strain was evaluated for A) survival rate, B) brood size, C) body length, and D) pharyngeal pumping. The transgenic CL2166 strain was evaluated for E) fluorescence intensity, which indicates levels of GST-4 expression. The neem oil caused decreases in pharyngeal pumping and GST-4 expression. Data are expressed as average of three independent experiments (n=3) normalized to % and the error bars represent the standard deviation. Letters a, b, and c indicate a significant difference relative to the control, NP, and Neem NP, respectively. A significance level of $p < 0.05$ was considered.

As shown in Figure 5E, the treatments with neem oil at all concentrations caused significant decreases in fluorescence intensity, indicating reduced GST-4 expression, compared to the untreated animals. The zein nanoparticles and neem oil-loaded zein nanoparticles did not affect the GST-4 enzyme levels. GST-4 is involved in cellular detoxification and cell defense, so the reduction induced by neem oil could lead to oxidative stress and cell death (Lindblom and Dodd, 2006). The results showed that the neem oil decreased GST-4 levels in *C. elegans* by up to 66%, compared to the control, representing a threat since this protein is regulated by protective transcription factors, promoting longevity and resistance to stress (Rathor et al., 2015). It should be highlighted that the neem oil-loaded zein nanoparticles did not affect this parameter, providing further evidence that the new zein nanoparticle system was capable of reducing toxicity towards nontarget organisms (Figure 6). In previous studies, it has been

575 found that treatments using extracts of *Lavandula latifolia*, *Melissa officinalis*,
576 *Origanum vulgare* (Gayoso et al., 2018), *Ginkgo biloba* (Kampkotter et al.,
577 2007) and antioxidant compounds such as quercetin (Büchter et al., 2015) led
578 to reduced GST-4 expression. It should be noted that the antioxidant capacity of
579 pure neem oil has been demonstrated in several previous studies (Mattos et al.,
580 2017; Rinaldi et al., 2017; Sithisarn et al., 2005).



581

582 **Figure 6.** Images of the CL2166 transgenic strain exposed to the zein
583 nanoparticles, neem oil-loaded zein nanoparticles, and neem oil (using neem oil
584 concentrations of 0.05, 0.25, 0.5, and 0.75 mg/mL) for 48 hours acquired using
585 an epifluorescence microscope (Nikon Eclipse 50i) with a GFP filter. It is
586 possible to observe the decrease in the intensity of the fluorescence emitted by
587 worms treated with neem oil at all concentrations used, indicating a decrease in
588 GST-4 expression.

589

590 **4 Conclusions**

591 The neem oil-loaded zein nanoparticles developed in this work presented good
592 colloidal characteristics and stability in different media. In the *A. cepa* analysis,
593 the use of zein nanoparticles decreased the relative damage index caused by
594 neem oil. In relation to the microbiota of the soil nitrogen cycle, the response to
595 the neem oil-loaded zein nanoparticles was similar to that observed for control.
596 In tests using C. elegans, the organism was susceptible to the effects of neem
597 oil, while the nanoparticles did not show potential toxicity. In this way, more
598 studies must be carried out to guarantee the effects of this nanopesticide before
599 its application in agriculture. It is therefore extremely important to recognize its
600 mechanisms of action (for both, nanopesticides and neem), as well as their
601 possible effects at the cellular level, their efficacy and their toxicity to target
602 organisms. A final consideration is that the definition of the risks associated with
603 nanobiopesticides requires a multidisciplinary approach and that, in order to be
604 sustainable and safe, it is crucial to ensure the awareness and use of correct
605 management practices between farmers and the wider population.

606

607 **Abbreviations**

608 GST-4, glutathione S-transferase 4; CL2166, transgenic fluorescence *C.*
609 *elegans* type; AFM, atomic force microscopy; qPCR, real-time polymerase
610 chain reactions; L1, first *C. elegans* larval stage; GFP, green fluorescent
611 protein; DLS, dynamic light scattering; NTA, nanoparticle tracking analysis; NP,

612 zein nanoparticles; Neem NP, neem oil-loaded zein nanoparticles; Neem, neem
613 oil.

614

615 **Acknowledgments**

616 The authors are grateful for the financial support for this research provided by
617 the São Paulo State Research Foundation (FAPESP) [grants #2017/21004-5
618 and #2015/17120-4] and the National Council for Scientific and Technological
619 Development (CNPq).

620

621

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CHAPTER IV

NANOBIOPESTICIDE BASED ON ZEIN NANOPARTICLES AND NEEM OIL: A STUDY USING TARGET AND NON-TARGET ORGANISMS

Submitted to Journal of Pest Science

NANOBIOPESTICIDE BASED ON ZEIN NANOPARTICLES AND NEEM OIL: A STUDY USING TARGET AND NONTARGET ORGANISMS

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Abstract

Nanotechnology has been widely explored with the aim of achieving a new revolution in crop protection, especially considering the development of improved biopesticides that offer increased stability and efficiency of the natural active compounds, while reducing the possible adverse effects on nontarget organisms. Nanomaterials composed of natural matrices associated with biopesticides have promising applications in sustainable agriculture. In this study, neem oil was encapsulated in zein nanoparticles in order to improve its stability and efficiency. Assays of phytotoxicity (using *Phaseolus vulgaris*) and biological activity against three pests (*Acanthoscelides obtectus*, *Bemisia tabaci*, and *Tetranychus urticae*) were also performed. The neem oil-loaded zein nanoparticles presented satisfactory physicochemical stability, together with high encapsulation efficiency (>80%). Pre- and post-emergence treatments using this new system did not cause any phytotoxic effects towards *P. vulgaris*. The neem oil nanobiopesticide exhibited insecticidal effects on *B. tabaci* and *T. urticae*, while the effect against *A. obtectus* was significantly increased, compared to plain neem oil. The results of the characterization, toxicity, and biological activity studies showed the promising potential of these neem oil-loaded zein nanoparticles for use in pest management in sustainable agriculture.

Keywords: Nano-scale, sustainable development, azadirachtin, phytotoxicity, biological activity, pest control.

Key Message

- To maximize agricultural protection and overcome adverse effects caused by synthetic pesticides, the utilization of nanobiopesticides can be recommended in sustainable agriculture.
- Nanobiopesticide based on zein nanoparticles and neem oil is an environmentally-friendly formulation and non-phytotoxic to *Phaseolus vulgaris*.
- Encapsulation of neem oil increased significantly its insecticidal effects against store pest *Acanthoscelides obtectus*.
- This nanobiopesticide are effective against worldwide pests such as *Bemisia tabaci* and *Tetranychus urticae*.

Author contributions

MP and LFF designed research. MP produced and characterized the nanobiopesticide. MP, FPA, AKC, KCG, JFDV and STSM conduced biological assays. BTN and WHCO contributed in analyzes of interactions between nanoparticles and organisms. MP, FPA and DJA analyzed data. LFF and RL supervised the research. MP wrote the manuscript. FPA, HCO, DJA, RAP, JASN, RL and LFF reviewed it. All authors read and approved the manuscript.

1 Introduction

Crops are attacked by about 67,000 species of organisms, including insects and mites, which are estimated to cause production losses ranging from 10 to 16% (Bradshaw et al. 2016; Savary et al. 2019). In order to reduce these losses, new systems have been developed using nanotechnology to protect crops from pests such as weeds, insects, fungi, and mites, as well as to detect and treat plant diseases, deliver fertilizers and other active agents, increase nutrient and water absorption, and allow genetic exploration and transformation (Verma 2017; Koul 2019).

In sustainable agriculture, nanotechnology can be used to produce new systems developed from clean materials and technologies, which are effective for field application (Fraceto et al. 2016; Campos et al. 2018b; Oliveira et al. 2018c; Kah et al. 2019; Lowry et al. 2019). These new systems should be extensively evaluated in terms of their possible risks to public health and the environment, especially where there is direct interaction between food products and nanomaterials (Fraceto et al. 2016; Pascoli et al. 2018; Prajitha et al. 2019).

In the area of crop protection, nanocarriers have been widely studied for the production of nanobiopesticides, consisting of nanostructured carriers with pesticidal activity or that can be loaded with active biological compounds. Such formulations can provide greater protection of the active agent, with improved stability, absorptive capacity, and effectiveness against the target organism, while minimizing adverse effects (Prasad et al. 2014; Grillo et al. 2016; Malaikozhundan et al. 2017; Oliveira et al. 2018c; Borgatta et al. 2018; Oliveira et al. 2019).

92 The production of nanobiopesticides that comply with the principles of
93 sustainable agriculture can be achieved using polymer matrices such as
94 chitosan and zein, which have shown promising results in the synthesis of
95 nanomaterials for application in agriculture (Mittal et al. 2013; Campos et al.
96 2014; Bautista-Banos 2016).

97 The biological activity of chitosan nanoparticles synthesized from
98 *Metarhizium rileyi* (Farlow) was evaluated against *Spodoptera litura* (Fabricius),
99 with insecticidal activity observed towards all the larval stages (Namasivayam et
100 al., 2018). No toxic effects were found towards the developmental stages of
101 zebrafish, while treatment with the nanocomposite did not cause hemolysis in *in*
102 *vitro* assays. Hasheminejad et al. (2019) produced chitosan nanoparticles
103 loaded with clove oil, which prolonged the release of the active agent and
104 increased its antifungal activity against *Aspergillus niger* (van Tieghem).
105 Campos et al. (2018a) encapsulated carvacrol and linalool in β-
106 cyclodextrin/chitosan nanoparticles, which led to higher insecticidal activity
107 against *Helicoverpa armigera* (Hübner) (corn earworm) and *Tetranychus urticae*
108 (Koch) (two-spotted spider mite), together with lower cytotoxicity in 3T3
109 fibroblasts and V79 lung cells.

110 Oliveira et al. (2018a, 2019) used zein to encapsulate combinations of
111 geraniol and R-citronellal, as well as geraniol, eugenol, and cinnamaldehyde. In
112 the first study, encapsulation increased the biological activity of the compounds
113 against *T. urticae*. In the second study, enhanced effects were observed against
114 the same pest and *Chrysodeixis includens* (Walker). In both cases, there were
115 decreased toxic effects towards nontarget organisms. In other work, Kamaraj et
116 al. (2018) demonstrated potential antifeedant activity of neem gum-loaded

117 nanoparticles against *H. armigera* and *S. litura* larvae and pupae, while this
118 nanoformulation did not affect the nontarget organism *Eudrilus eugeniae*
119 (Kinberg).

120 Adopting the same approach, Pascoli et al. (2019) prepared neem oil-
121 loaded zein nanoparticles with a mean diameter of 278 ± 6.1 nm, which were
122 stable under the experimental conditions. The use of *in vitro* ecotoxicological
123 assays showed that the new system decreased or eliminated the toxic effects of
124 the active compound against nontarget organisms such as *Allium cepa* L. and
125 *Caenorhabditis elegans* (Maupa). In addition, the formulation did not affect soil
126 bacteria involved in the nitrogen cycle. However, there have not yet been any
127 tests of the biological activity of this nanoformulation towards target insects, or
128 its potential phytotoxicity under realistic *in vivo* conditions.

129 The aim of the present study was to investigate the effects of neem oil-
130 loaded zein nanoparticles on target organisms, in order to evaluate the potential
131 of this system as a nanobiopesticide. Its biological efficacy was evaluated
132 against three species of agricultural pest: i) the bean weevil *Acanthoscelides*
133 *obtectus* (Say), ii) the whitefly *Bemisia tabaci* (Gennadius), and iii) the two-
134 spotted spider mite (*T. urticae*). In addition, the potential phytotoxicity of this
135 nanoformulation was evaluated using common bean plants (*Phaseolus vulgaris*
136 L.). The stability of the nanoparticles was investigated during 90 days, using
137 measurements of mean hydrodynamic diameter, polydispersity index, span
138 index, zeta potential, nanoparticle concentration, and encapsulation efficiency.
139 This innovative study opens perspectives for the use of nanobiopesticides
140 based on neem and zein nanoparticles in pest control, especially for sustainable

141 agriculture, since it uses only a natural active component and a naturally-
142 occurring polymer in its composition.

143 **2 Materials and Methods**

144 **2.1 Materials**

145 Zein and Pluronic F-68 were obtained from Sigma-Aldrich. Neem oil
146 (Azamax) was acquired from UPL Brazil. Ethanol was purchased from
147 Labsynth. The 18:1 Liss Rhod PE fluorophore (1,2-dipalmitoyl-sn-glycero-3-
148 phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt))
149 was acquired from Avanti Polar Lipids. Seeds of common bean (*P. vulgaris*
150 cultivar IPR Curió, Carioca group, register 30616, protection 20130167) were
151 kindly supplied by the Agronomic Institute of Paraná (IAPAR, Londrina, Parana,
152 Brazil). Bean weevils (*A. obtectus*) were obtained from a colony maintained at
153 the Biology Laboratory of São Paulo State University (UNESP, Sorocaba, São
154 Paulo, Brazil). Whitefly (*B. tabaci)* and two-spotted spider mite (*T. urticae*) were
155 obtained from colonies maintained at São Paulo State University (UNESP,
156 Jaboticabal, São Paulo, Brazil). Other chemicals, reagents, and solvents used
157 were purchased from local suppliers.

158 **2.2 Preparation of neem oil-loaded zein nanoparticles**

159 Zein nanoparticles were prepared by the anti-solvent precipitation
160 method, described by Hu and McClements (2014), after treatment of zein as
161 performed by Pascoli et al. (2019). First, zein (2% w/v) was solubilized in a

hydroethanolic solution (85% v/v), under magnetic stirring overnight. The pH of the zein solution was adjusted to 5.8, followed by centrifugation for 30 min at 85750*g, heat treatment at 75 °C for 15 min, and filtering through a 0.45 µm membrane (Millipore). A 100 mg aliquot of neem oil (containing 12 g/L of azadirachtin) was added to the zein solution. An aqueous solution of Pluronic F-68 (2% v/v) was prepared and the pH was adjusted to 4. The zein solution containing neem oil was rapidly injected into the Pluronic solution, under magnetic stirring. The colloidal formulation was stirred at room temperature, in order to evaporate the ethanol, and water (pH 4.0) was added to complete to 20 mL. The final concentration of neem oil in the nanoformulation was 5 mg/mL. This concentration was chosen since in agriculture, neem oil is used at concentrations of between 4 and 6 mg/mL. Control nanoparticles were prepared without neem oil. Labeled nanoparticles, with and without neem oil, were also prepared with addition of rhodamine (18:1 Liss Rhod PE) in the zein solution (0.05% m/m, relative to the polymer), in order to investigate the interaction between the bean weevils and the formulation.

2.3 Physico-chemical stability of the nanoparticles

Physico-chemical characterization of the formulations was performed as a function of time, in order to evaluate their colloidal stability up to 90 days. Determinations of the mean hydrodynamic diameter and the polydispersity index of the nanoparticles were performed by photon correlation spectroscopy, using a ZetaSizer Nano ZS 90 analyzer (Malvern Instruments) at a fixed angle of 90° and temperature of 25 °C. The same equipment was used to determine the zeta potential, according to the microelectrophoresis method. The mean

186 nanoparticle diameter was also determined using the span index (an indicator of
187 the stability of the formulation, showing the width of the size distribution),
188 calculated as follows:

189 $$Span = \frac{(D90 - D10)}{D50} \qquad \text{(Equation 1)}$$

190 where D10, D50, and D90 are the mean diameters corresponding to 10, 50, and
191 90% of the particle population, respectively. The particle concentrations in the
192 formulations were measured using a NanoSight LM10 instrument (Malvern
193 Panalytical) equipped with a 532 nm laser. The images were collected using a
194 sCMOS camera and were processed using NanoSight v. 2.3 software Grillo et
195 al. (2014). For these analyses, the samples were diluted 1000 times. The
196 efficiency of encapsulation of the neem oil in the zein nanoparticles was
197 quantified using the ultrafiltration/centrifugation method, with analysis using a
198 UV-Vis spectrophotometer (Cary 50, Varian). The samples were centrifuged
199 using Microcon 10 kDa regenerated cellulose ultrafilters (Millipore), which only
200 allowed passage of the unencapsulated neem. The analytical curve
201 concentration range was from 10 to 200 µg/mL and detection employed a
202 wavelength of 225 nm (Dubhashi et al., 2013). The difference between the
203 amount of neem initially added and the filtered amount provided the
204 encapsulation efficiency.

205 **2.4 Phytotoxicity evaluation using bean plants**

206 The substrate used for plant growth was clay soil and sand, in a ratio of
207 1:1 (v:v). The pots and growing trays were kept in the greenhouse of the Center

208 of Biological Sciences of Londrina State University (Londrina, Paraná, Brazil),
209 under natural conditions of air relative humidity and temperature, with 75% of
210 total environmental photosynthetic photon flux density (PPFD). The soil was
211 enriched with the nutrient solution of Hoagland and Arnon (1950) and was
212 regularly watered. Pre- and post-emergence assays were performed, with the
213 following treatments: water (negative control), zein nanoparticles, neem oil-
214 loaded zein nanoparticles, and neem oil. The concentration adopted in each
215 application of these treatments was the same as that recommended for the
216 commercial product: 5 mg/mL applied at 100 liters per hectare.

217 For the post-emergence assay, three applications to the leaves of bean
218 plants were performed, with intervals of 7 days. Each treatment was applied to
219 seven pots, each with three seedlings. At the beginning of the experiment, only
220 the first pair of leaves was fully expanded; hence, all the chlorophyll *a*
221 fluorescence, gas exchange, and oxidative stress analyses were performed
222 using these leaves. In the pre-emergence assay, the treatments were applied
223 once, directly to the soil of five pots (each with 25 seeds), using amounts
224 equivalent to the three applications of the post-emergence test.

225 Chlorophyll *a* fluorescence was measured at the adaxial surfaces of the
226 leaves, using an OS1p fluorometer (Opti-Sciences, Hudson, USA). The
227 maximum quantum yield of photosystem II photochemistry (F_v/F_m) was
228 determined as follows:

229 $$\frac{F_v}{F_m} = \frac{F_m - F_0}{F_m} \qquad \text{(Equation 2)}$$

where F_0 refers to the minimum, F_m to the maximum, and F_v to the variable fluorescence of dark-adapted leaves after receiving a saturating pulse of actinic light (Baker, 2008). Gas exchange analyses were performed to determine the light-saturated net photosynthesis (A_{max}), using a portable infrared gas analyzer (Model 6400 XT, LI-COR Biosciences, Lincoln, USA) connected to a 6 cm^2 chamber. The saturating PPFD inside the chamber during the analyses was 1,500 $\mu mol.m^{-2}.s^{-1}$, as determined previously using a light-curve analysis. In the post-emergence assay, the analyses were always carried out two days after application of the treatments to the plants, at the same times (07:30 a.m. for F_v/F_m and 08:30 a.m. for A_{max}). In the pre-emergence assay, the analyses were performed only at the end of the experiment, at the same times described.

Hydrogen peroxide and lipid peroxidation were measured as markers of oxidative stress. For these analyses, 100 mg portions of fresh leaves and roots were ground to a powder in liquid nitrogen, followed by extraction with 1.8 mL of methanol + 0.2% trichloroacetic acid (TCA). After centrifugation (13700*g for 5 min at 4 °C), the supernatant was used for measurement of the hydrogen peroxide content by reaction with potassium iodide, in phosphate buffer (Alexieva et al. 2001), and for the determination of thiobarbituric acid reactive substances (TBARS) (Camejo et al. 1998). For determination of hydrogen peroxide, the supernatant was subjected to reaction for one hour with 1 M potassium iodide (KI), in pH 7.5 phosphate buffer (PBS), keeping the mixture on ice and in the dark. A hydrogen peroxide standard curve was used, with the absorbance measured at 390 nm, using a 96-well plate and a microplate reader (Model Victor TM 3, PerkinElmer, Turku, Finland). For determination of TBARS, the supernatant was subjected to reaction with 0.02% butylated hydroxytoluene

(BHT) in pH 7.4 PBS buffer, together with 1.3% thiobarbituric acid (TBA) and 0.3% sodium hydroxide (NaOH), in the presence of 50% TCA, at 60 °C for 60 min. The concentration was determined using a malondialdehyde standard curve (MDA) constructed from fluorescence readings obtained at excitation and emission wavelengths of 535 and 590 nm, respectively, employing the Victor TM 3 reader (Camejo et al. 1998).

Finally, for dry mass determination, the plants were harvested (after 13 days in the pre-emergence assay and after 24 days in the post-emergence assay), individually packed in paper bags, and dried in an oven at 60 °C until reaching constant mass.

2.5 Biological activity assays

2.5.1 Evaluation of mortality of *Acanthoscelides obtectus* and its interaction with the nanobiopesticide

The bioassays using *A. obtectus* were conducted in the Biology Laboratory of UNESP/ICTS, in controlled climate chambers with constant aeration, absence of light, temperature of 27 ± 2 °C, and maximum and minimum humidity of 73 and 52%, respectively, based on the studies of Jumbo et al. (2014), Soares et al. (2014), and and Janković-Tomanić et al. (2015). The colony was maintained under the same conditions. The *P. vulgaris* (Qualitá®) used to maintain the culture and to carry out the experiments was previously kept in a freezer for 14 days and dried, in order to prevent possible infestation from the field and to reduce any potential effect of insecticide residue, as proposed by Jumbo et al. (2014).

The biocidal activity was analyzed according to the method described by Jumbo et al. (2014), using an acute mortality assay (96 h) to determine the mean lethal concentration (LC_{50}). Masses of 25 g of beans were placed in 145 mL plastic bottles with small holes in the cap for aeration, followed by application of the treatments (zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil) and shaking the vials manually for 60 s to ensure complete distribution of the material in the beans. Ten 1 to 5 day old adults of *A. obtectus* (unsexed) were placed in each vial. The experiment was carried out using concentrations equivalent to 1.35, 2.7, 5.4, 10.8, and 21.6 mg of azadirachtin per kg of beans. These concentrations were based on the work of Tofel et al. (2017), who obtained LC_{50} of around 9 mg of azadirachtin per kg of corn, using *Callosobruchus maculatus* (Fabricius) as the target organism. After the exposure period, mortality was evaluated using a stereomicroscope (Model XTB-2B, Coleman), with the weevils being considered dead when they did not show movement, even when stimulated by touching with a fine-bristle brush for 4 min. Two replicates were performed for each dose and for the control treatment, and the experiment was repeated three times. The LC_{50} values were estimated as proposed by Hamilton et al. (1977), using the Trimmed Spearman-Karber method.

The treatments with the rhodamine-labeled nanoparticles were performed in the same way, under the same experimental conditions as described for the *A. obtectus* biological activity assay, using the LC_{50} concentration for the neem oil-loaded nanoparticles and the same volume for the zein nanoparticles without the active agent. The weevils were analyzed at the Central Multiusers Laboratory of the School of Agricultural Sciences

303 (UNESP) after 96 h of exposure, using a Carl Zeiss SteREO Discovery v. 12
304 microscope fitted with a red filter for fluorescence, in order to identify the
305 presence of the nanoformulation in the bodies of the insects. The images were
306 acquired with an Axiocam 2.0 Zen Blue camera and were treated using the
307 equipment software. The images of the bodies of *A. obtectus* were merged with
308 the fluorescence evaluation images, enabling visualization of the interactions
309 between the weevils and the treatments. A total of 10 specimens were analyzed
310 for each treatment. Untreated control specimens were used to evaluate any
311 possible natural fluorescence emitted by the body of the insect.

312 **2.5.2 *Bemisia tabaci* mortality assay**

313 The whitefly (*B. tabaci*) mortality experiments were conducted in the
314 Microbial Control of Pest Arthropods Laboratory (UNESP/FCAV). The *B. tabaci*
315 used in this assay were reared on bean plants in a greenhouse and were
316 collected in flat bottom glass tubes, using manual suction. A total of 480 insects
317 were collected in 48 tubes (10 insects per tube). These tubes were transferred
318 to the previously treated bean plants in pots (24 pots, each with 2 plants) and
319 were left open until the flies had emerged from the tubes. Prior to the transfer of
320 the whiteflies, the treatments were applied to the bean plants by manual
321 spraying, as recommended by the manufacturer of the commercial neem oil (3
322 applications, spaced at intervals of 7 days). Three scenarios with different
323 concentrations were simulated: concentration of 5 mg/mL, 100 L/hectare (also
324 as recommended by the manufacturer), concentration estimating overdosage
325 (15 mg/mL, 100 L/hectare), and concentration representing lower use of the
326 active compound (1 mg/mL, 100 L/hectare). Six replicates were performed for

each treatment and the dead insects found on the floors of the cages were counted daily.

2.5.3 Biological effects on *Tetranychus urticae*

The assays of biological effects against the *T. urticae* mite were conducted in the Acarology Laboratory (UNESP/FCAV), using mites obtained from jack bean plants (*Canavalia ensiformes* L.). The plants were cultivated in 2 L pots containing soil, sand, and bovine manure (1:1:1, v:v:v) as the substrate. The mites were kept in a temperature-controlled climate chamber at 25 ± 1 ºC, relative humidity (RH) of 60 ± 10%, and 12 h light: 12 h dark photoperiod. The experiments were performed using arenas (2.5 cm diameter) of *C. ensiformes* leaves obtained using a circular metal cutter. The arenas were placed in Petri dishes (9 x 2 cm) containing a moistened foam and a hydrophilic cotton layer (1.0 cm), in order to maintain the turgidity of the arenas, and were surrounded with hydrophilic cotton to avoid escape of the mites.

Evaluations of biological activity were performed using the larvae, nymphs, and adults of *T. urticae*. The treatments (water as the negative control, zein nanoparticles, zein nanoparticles with neem oil at 5 mg/mL, neem oil at 5 mg/mL, and the commercial synthetic acaricide Oberon® as a positive control) were evaluated for direct and residual action. For evaluation of the direct action, the mites in the different stages of development (larvae, nymphs, or adult females) were transferred to the arenas (10 mites per arena). The treatments were then sprayed under a Potter tower calibrated at 4 $lbf.in^{-2}$, using 2 mL of treatment solution per arena, corresponding to 1.56 $mg.cm^{-2}$ of dry residue.

Each treatment was repeated 8 times. After the applications, the arenas were transferred to a climate-controlled chamber, as described above. For the residual evaluation of the formulations, jack bean (*C. ensiformes*) seeds were planted in 5 L pots containing soil, sand, and bovine manure (1:1:1, v:v:v) as substrate. Approximately 30 days after germination, the plants were separated into 5 groups of three plants to receive the applications of the different treatments. The products were applied with a 500 mL capacity manual sprayer, until complete coverage of the plants. An average of 15 mL of treatment solution was required per plant. After 1, 6, and 12 days following the applications, leaves of the bean plants were collected and arenas were prepared in Petri dishes, as described above, followed by the transfer of 10 larvae, nymphs, or adults to each arena. Each assay employed 8 replicates. The numbers of mites that were alive, dead, or trapped in the cotton barrier were counted daily during 5 days, using a stereomicroscope (40x magnification). Mites that did not react to the touch of a fine brush were considered dead.

2.6 Statistical analysis

The results of the biological activity assays were treated as proposed by Abbott (1925) for corrected mortality. The statistical analyses of the stability, phytotoxicity, and biological activity assays were performed with GraphPad Prism v. 6 software, using two-way ANOVA followed by the Tukey post-hoc test, at a significance level of $p<0.05$.

3 Results

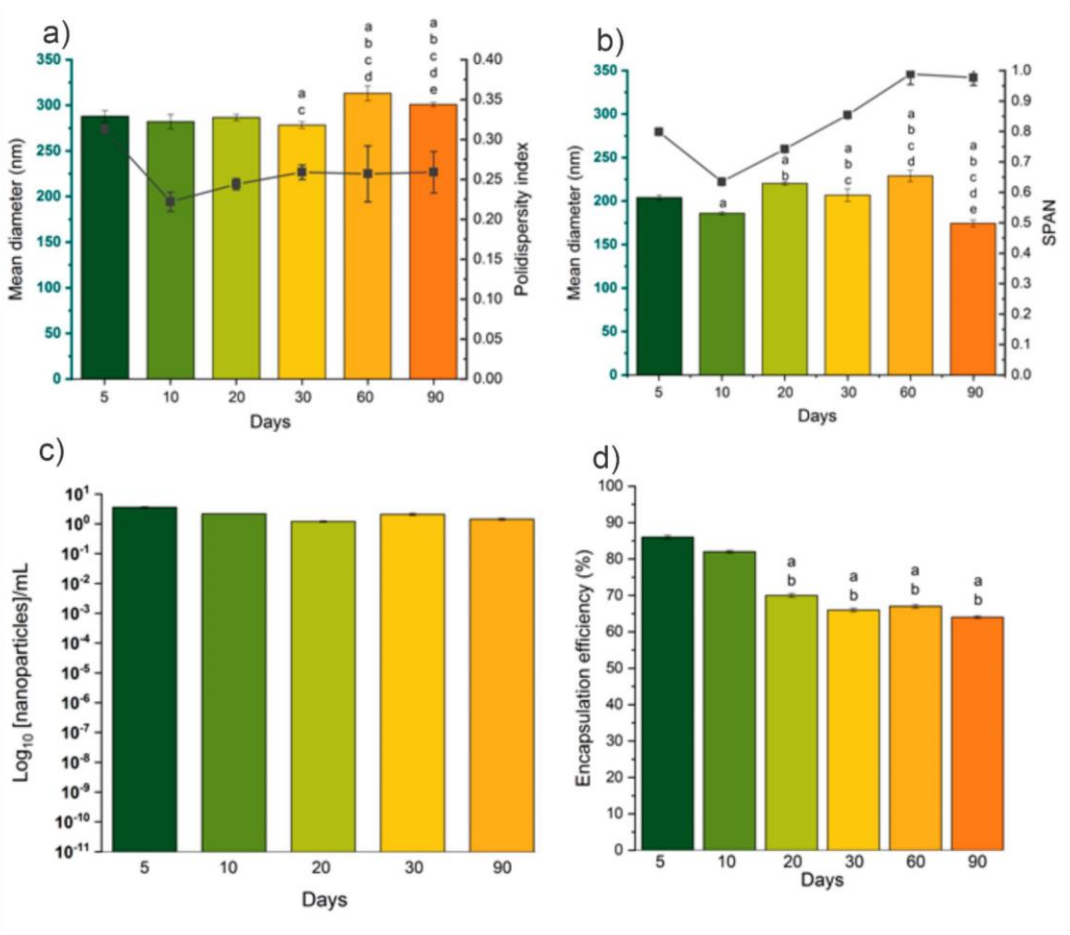
373 **3.1 Physico-chemical stability of the nanoparticles**

374 In this study, the physico-chemical stability of the neem oil-loaded zein
375 nanoparticles was evaluated by determination of several parameters during
376 storage of the formulations for 90 days. Initially, mean diameters were obtained
377 by DLS and NTA with values of 288 ± 6 and 198 ± 16 nm, respectively. The
378 mean hydrodynamic diameter obtained by the DLS technique showed a
379 significant decrease on day 30, with an average diameter of 278 ± 3.6 nm,
380 followed by a significant increase in mean size, reaching 300 ± 2.5 nm on day
381 90. Using the same technique, the polydispersity index (Figure 1A) was found to
382 remain at around 0.2, without any significant differences, indicating good
383 physicochemical stability of the polymer system (Mohanraj and Chen 2006).
384 Use of the NTA technique, which enables determination of the hydrodynamic
385 diameter of the particles by directly measuring their diffusion coefficients when
386 they are in Brownian motion, resulted in nanobiopesticide particle sizes that
387 were smaller than obtained by DLS (Figure 1B). Using this technique, the mean
388 diameters oscillated significantly, increasing and decreasing throughout the
389 storage time, which could have been because the technique is more sensitive
390 and analyzes each particle individually. The span index values (Figure 1B) were
391 less than 1 and showed no significant differences during the 90 days of storage,
392 which was also characteristic of a stable formulation (Bender et al. 2012).

393 Evaluation of the nanoparticle concentration by NTA (Figure 1C) showed
394 no significant differences during the 90 days of storage.

395 Determination of the efficiency of encapsulation of neem oil in the zein
396 nanoparticles (Figure 1D) showed that the highest encapsulation efficiency of

397 86 ± 0.5% was obtained on day 5, followed by a significant gradual decrease to
398 70 ± 0.7% after 20 days, which remained constant until day 90. This decrease
399 could be explained by the release of the active agent from the nanoparticles
400 over time.



401

402 **Fig. 1** Stability of the neem oil-loaded zein nanoparticles during 90 days: A)
403 Mean hydrodynamic size (bars) and polydispersity index (line), obtained using
404 DLS. B) Mean hydrodynamic size (bars) and span index (line), obtained using
405 NTA. C) Concentration of nanoparticles in the formulation, obtained by NTA. D)
406 Encapsulation efficiency of neem oil in the zein nanoparticles, obtained by UV-
407 Vis spectroscopy. The data are expressed as the average of three independent
408 experiments (n = 3) and the error bars represent the standard deviations. The

409 letters a, b, c, d, and e indicate significant difference relative to days 5, 10, 20,
410 30, and 60, respectively. A significance level of $p < 0.05$ was adopted.

411

412 The zeta potential values (data not shown) fluctuated significantly during
413 the 90 days of storage (-36 ± 1 mV on day 1, 15.5 ± 2.5 mV on day 60, and -
414 22.5 ± 0.7 mV on day 90), indicating a lack of stability. However, Pluronic F-68
415 was used during the nanoparticles preparation process, which provided steric
416 hindrance and was responsible for the stability of the system (Chuacharoen and
417 Sabliov 2016).

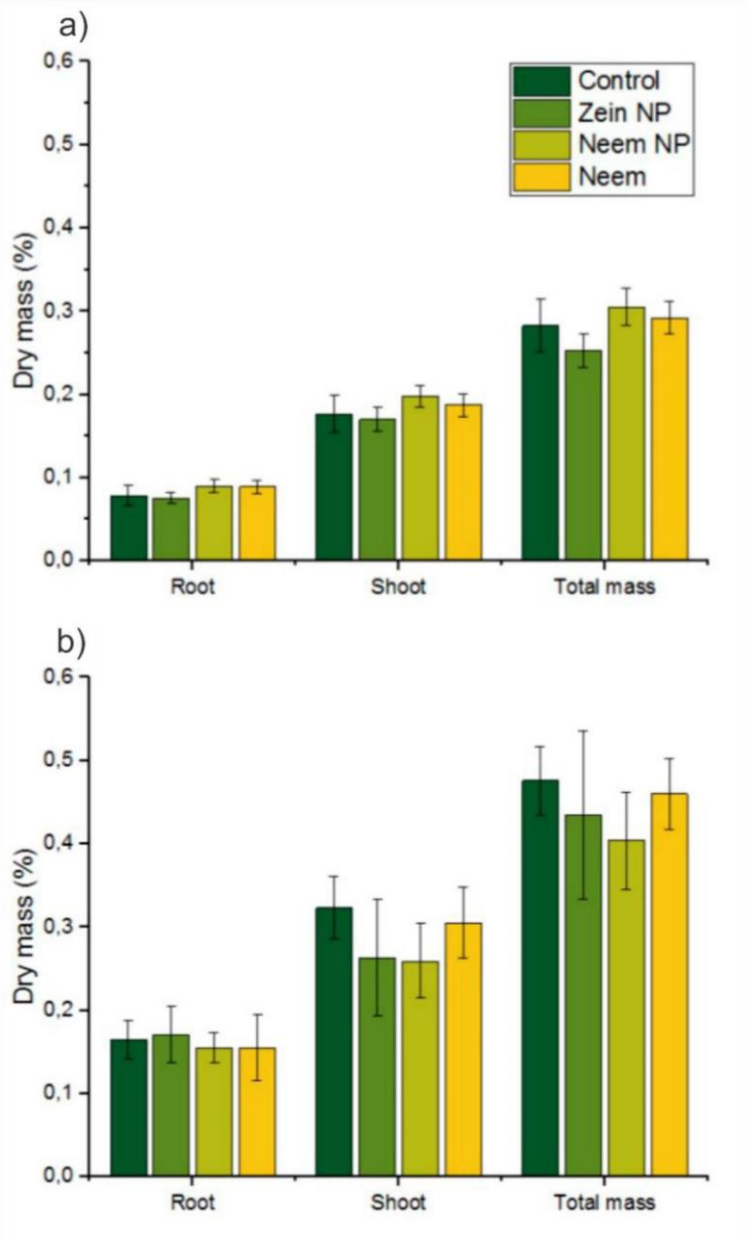
418 **3.2 Phytotoxicity evaluation using bean plants**

419 The F_v/F_m ratio, which indicates the maximum quantum efficiency of
420 electron transport in photosystem II, was not affected by any of the formulations
421 tested, regardless of the type of treatment (Table 1). All the leaves presented
422 F_v/F_m values near 0.8, characteristic of healthy, unstressed plants (Klughammer
423 and Schreiber 2008). Similarly, the A_{max} values for the treated plants showed no
424 significant differences, compared to the corresponding controls, evidencing that
425 the formulations did not affect photosynthetic activity in the leaves. In the third
426 evaluation of the plants in the post-emergence test, there was a significant
427 decrease of A_{max}, relative to the first evaluation of the same plants. However,
428 this feature, which was observed for all the treatments (including the control),
429 could be explained by the senescence of the leaves used for the analyses.

430 Similar to the photosynthetic parameters, lipid peroxidation and H_2O_2
431 levels in the roots and leaves showed no significant differences between the

control and the treatments (Table 1), demonstrating that the formulations did not induce oxidative stress in common bean plants.

In accordance with the lack of phytotoxic effects detected in the previous analyses, the dry mass of the bean plants did not show any significant difference among the control and the treatments in the pre- and post-emergence experiments, showing that the biopesticide and the neem oil did not affect the growth of the plants (Figure 2).



440

441 **Fig. 2** Results of phytotoxicity assays using common bean plants: Dry masses
442 of plants treated with water (control), zein nanoparticles (Zein NP), neem oil-
443 loaded zein nanoparticles (Neem NP), and neem oil (Neem). A) Pre-emergence
444 assay; B) post-emergence assay. The data are expressed as averages of ten (n
445 = 10) and fourteen (n = 14) plants for the pre- and post-emergence assays,
446 respectively. The error bars represent the standard deviations. A significance
447 level of $p < 0.05$ was adopted.

448

449 **Table 1** Maximum quantum yield of photosystem II photochemistry (F_v/F_m),
450 light-saturated net photosynthesis (A_{max}), and oxidative stress parameters of the
451 bean plants. 1st, 2nd, and 3rd represent the analyses after the first, second, and
452 third treatment applications, respectively. The data are expressed as average ±
453 standard deviation for ten (n = 10) and fourteen (n = 7) plants in the pre- and
454 post-emergence assays, respectively. The symbols † and ϕ indicate significant
455 difference relative to the 1st and 2nd analyses, respectively. A significance level
456 of $p < 0.05$ was adopted.

	F_v/F_m	A_{max} (µmol CO_2 m^{-2} s^{-1})	Lipid peroxidation (nmol MDA g^{-1})		H_2O_2 (µmol g^{-1})	
Treatments			**root**	**leaf**	**root**	**leaf**
Pre-emergence assay						
Control	0.774±0.011	15.8±3.3	12.7±2.8	29.2±5.8	31.0±2.3	332.2±12.3
Zein NP	0.760±0.021	16.5 ±2.0	9.6±3.2	36.6±3.0	35.3±3.4	356.1±19.5
Neem NP	0.753±0.015	17.5 ±2.3	7.2±4.4	32.8±9.1	25.3±4.8	334.9±40.4
Neem	0.767±0.019	16.2 ±2.0	12.6±8.8	33.8±5.9	27.9±4.2	356.7±33.0
Post-emergence assay						
1st Control	0.826±0.008	25.9±3.1	-	-	-	-
1st Zein NP	0.827±0.007	25.6±3.0	-	-	-	-
1st Neem NP	0.829±0.006	23.5±1.6	-	-	-	-
1st Neem	0.830±0.005	26.5±2.5	-	-	-	-
2nd Control	0.794±0.015	16.7±2.4	-	-	-	-
2nd Zein NP	0.792±0.019	17.3±1.1	-	-	-	-
2nd Neem NP	0.788±0.008	17.1±2.2	-	-	-	-
2nd Neem	0.791±0.020	16.75±1.7	-	-	-	-
3rd Control	0.790±0.017	4.1±2.1† ϕ	12.7±4.6	47.5±5.8	28.2±16.2	362.7±39.8
3rd Zein NP	0.785±0.019	5.2±2.8† ϕ	12.3±5.5	48.3±4.8	18.3±12.5	373.9.1±40.2
3rd Neem NP	0.808±0.005	6.6±3.1† ϕ	14.2±4.3	50.1±3.0	25.60±20.5	450.9±48.9
3rd Neem	0.797±0.014	5.8±2.8† ϕ	7.8±3.4	51.0±5.5	12.3±10.3	422.8±44.4

457

458 **3.3 Biological activity**

459 **3.3.1 *Acanthoscelides obtectus*: mortality and interaction between the**
460 **nanobiopesticide and the target organism**

461 In the *A. obtectus* acute assays, the concentration range investigated
462 was based on the work of Tofel et al. (2017), who reported the azadirachtin
463 LC_{50} (9 mg of azadirachtin per kg of corn) for another species of bruchine (*C.*
464 *maculatus*). Here, the LC_{50} values defined by the Trimmed Spearman-Karber
465 method, according to the confidence interval of the results, were 6.65 mg of
466 azadirachtin per kg of beans for the neem oil-loaded zein nanoparticles and
467 11.22 mg of azadirachtin per kg of beans for the neem oil.

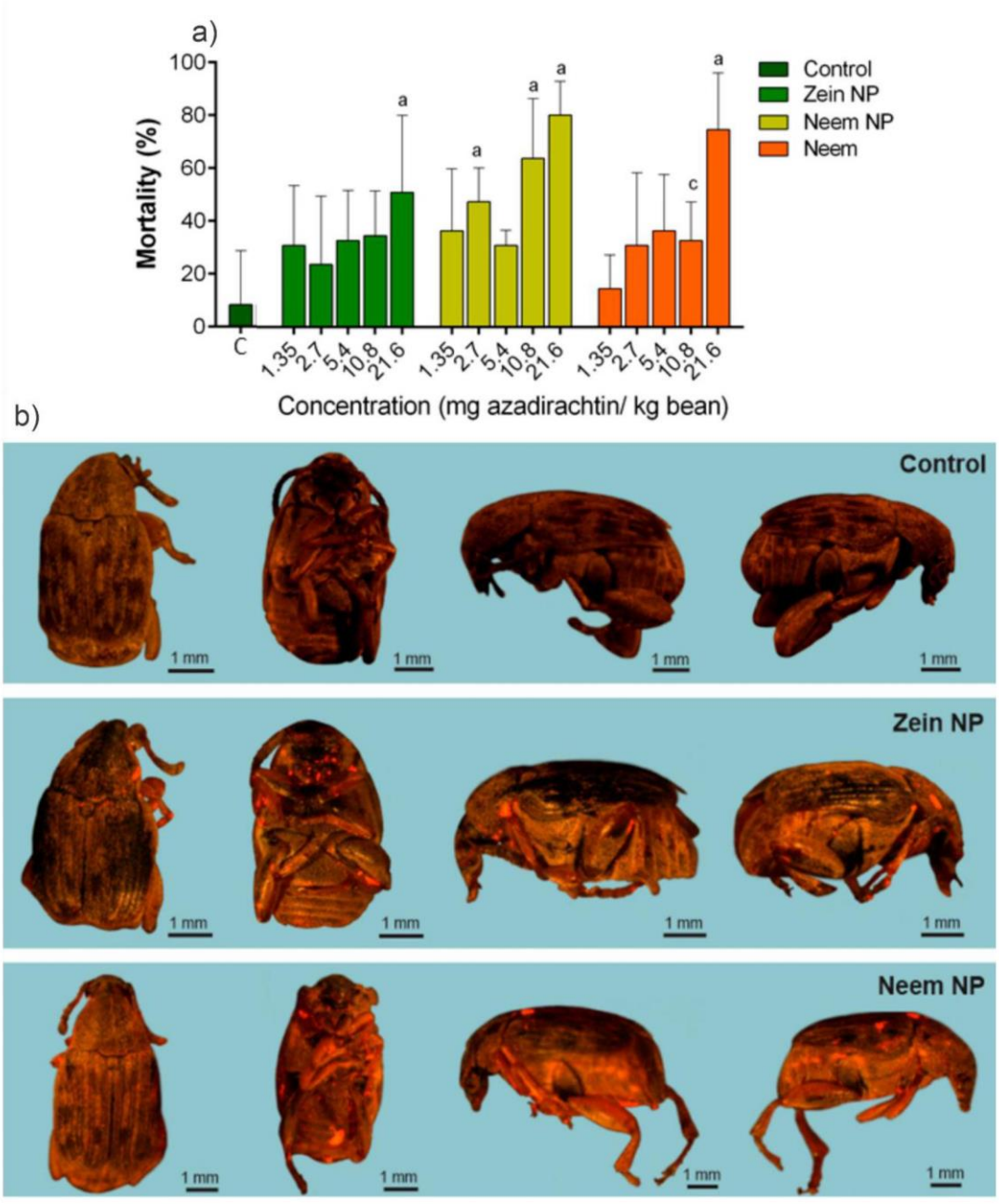
468 The results (Figure 3A) showed that the neem oil nanobiopesticide
469 caused significant mortality of the pest from the second lowest concentration
470 tested, while the neem oil only caused significant mortality at the highest
471 concentration evaluated. Therefore, the new system provided greater efficiency
472 against this bean pest, compared to the traditional neem oil. It was also
473 observed that the zein nanoparticles without the active compound only had an
474 effect at the highest concentration employed, in agreement with the work of
475 Pascoli et al. (2019), where it was found that empty zein nanoparticles were not
476 very toxic. The greatest effect was therefore due to the nanoencapsulated neem
477 oil.

478 In order to evaluate the contact between the nanobiopesticide and the
479 insects, the nanobiopesticide was labeled with the 18:1 Liss Rhod PE

480 fluorophore. The resulting material had the same physical chemical
481 characteristics as the unlabeled nanobiopesticide (data not shown).

482 Using fluorescence microscopy, it was possible to observe that the
483 exposure of the *A. obtectus* individuals to the nanoformulations was mainly via
484 the integument (Figure 3B), with the greatest exposure occurring in the ventral
485 region, especially the legs and mouthparts. Nanoparticles could also be seen on
486 the antennae and the abdomen. These results suggested that the increased
487 mortality of *A. obtectus* (Figure 3A) was probably due to direct contact and
488 interaction with the nanobiopesticide, with better adhesion facilitating entry of
489 the nanostructures into the body of the insect.

490



491

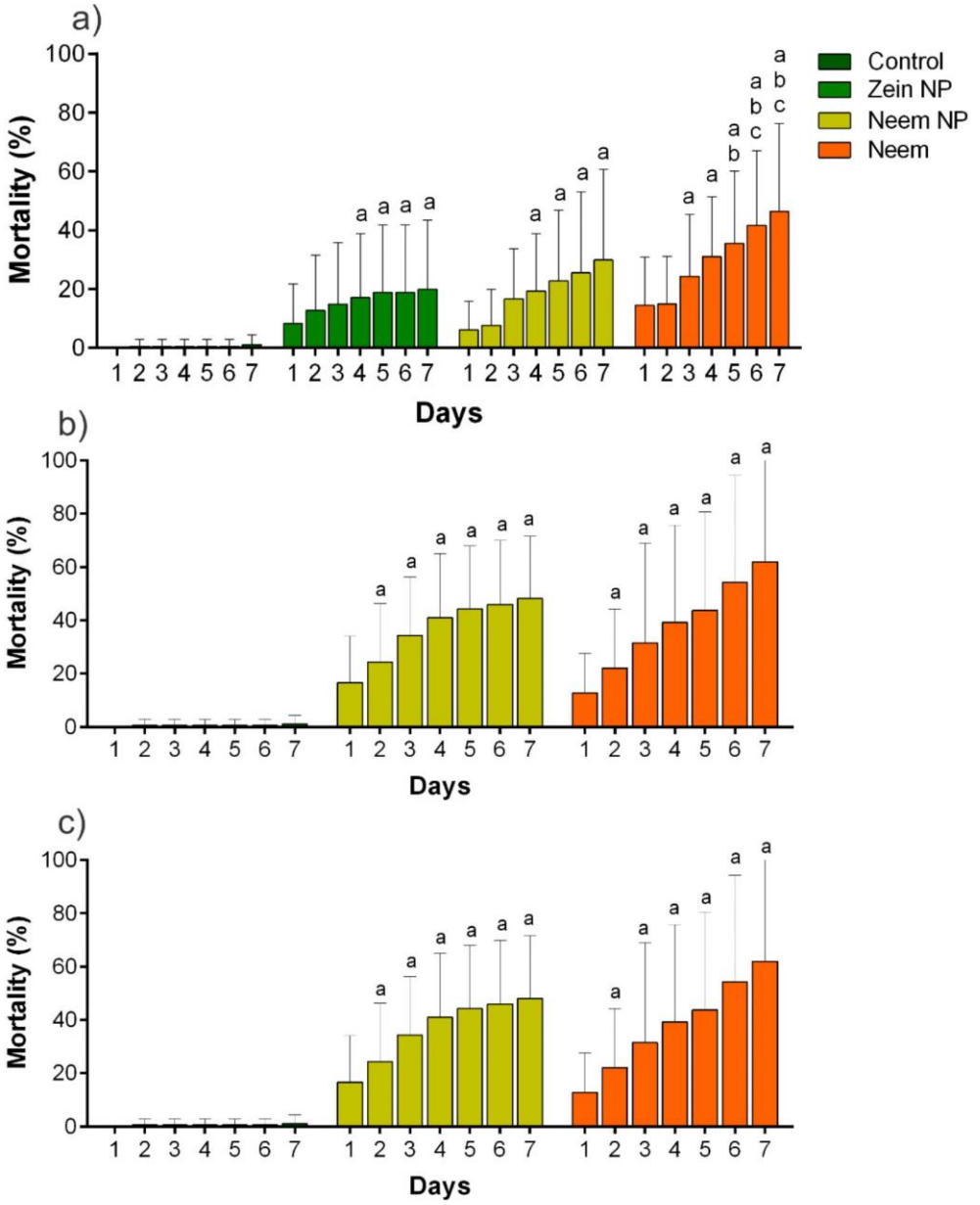
492 **Fig. 3** Results of assays using *Acanthoscelides obtectus*: A) Mortality of *A.*
493 *obtectus* following acute exposure (96 h) to beans treated with the zein
494 nanoparticles (Zein NP), the neem oil-loaded zein nanoparticles (Neem NP),
495 and the neem oil (Neem), at concentrations of 1.35, 2.7, 5.4, 10.8, and 21.6 mg
496 of azadirachtin per kg of beans. The zein nanoparticle treatment was used as a
497 control, at the same volume as the treatments containing the active agent. B)

Images of *A. obtectus* exposed for 96 h to beans treated with neem oil-loaded zein nanoparticles labeled with rhodamine (Neem NP), at a concentration of 6.64 mg of azadirachtin per kg of beans. Labeled zein nanoparticles and untreated bruchines were used as a control. The data are expressed as the average of three independent experiments (n = 3), normalized to %. The error bars represent the standard deviation. The letters a, b, and c indicate significant difference relative to the control, NP, and Neem NP treatments, respectively. A significance level of $p < 0.05$ was adopted.

3.3.2 Biological effect on *Bemisia tabaci*

Figure 4 shows the results of the mortality assays using the nanoformulations and neem oil against *B. tabaci*. The treatments were performed at concentrations of 5 mg/mL, as recommended by the manufacturer of commercial neem oil, 15 mg/mL, representing overdosage, and 1 mg/mL, representing less use of the bioinsecticide.

In the assay performed under the use conditions recommended by the manufacturer (Figure 4A), the mortality of the pest presented significant increases, compared to the control, starting on the 3rd day (for the neem oil) and on the 4th day (for the zein nanoparticles with and without neem oil). In this case, the commercial neem oil showed higher efficiency than the neem oil-loaded zein nanoparticles (with significant differences on days 6 and 7).



520

521 **Fig. 4** Mortality of whiteflies treated with zein nanoparticles (Zein NP), neem oil-
522 loaded zein nanoparticles (Neem NP), and neem oil (Neem), at A) the
523 recommended concentration (5 mg/mL), B) overdosage concentration (15
524 mg/mL), and C) lower dosage (1 mg/mL). The data are expressed as averages
525 of three independent experiments (n = 3), normalized to %. The error bars
526 represent the standard deviation. The letters a, b, and c indicate significant

527 difference relative to the control, NP, and Neem NP, respectively (on the same
528 day). A significance level of $p < 0.05$ was adopted.
529
530 In the overdosage scenario (Figure 4B), the treatments presented
531 significantly higher mortality, compared to the control, with no significant
532 difference between the treatments. Considering the capacity of *B. tabaci* to
533 develop resistance to pesticides, the increase in mortality could be attributed to
534 the increase of the concentration of the applied active compound.

535 In the assay using lower concentrations of the bioinsecticide (Figure 4C),
536 the mortality results were again similar for the neem oil and the neem oil-loaded
537 nanoparticles. However, calculation of the areas under the curves (Table 2)
538 revealed that in the experiment carried out using the neem oil at a concentration
539 of 1 mg/mL, the nanobiocide was more effective than the neem oil, with areas of
540 207.7 and 179, respectively, showing the potential for using a lower
541 concentration of the pesticide to control whitefly.

542

543 **Table 2** Area under the curve values for the biological activity assays using the
544 control and the nanobiopesticide at concentrations of 5, 15, and 1 mg/mL: water
545 (Control), zein nanoparticles (Zein NP), neem-loaded zein nanoparticles (Neem
546 NP), and neem oil (Neem). The data are expressed as the average ± standard
547 deviation of three independent experiments (n = 3). The letters a, b, and c
548 indicate significant difference relative to the control, Zein NP, and Neem NP,
549 respectively. A significance level of $p < 0.05$ was adopted.

Treatments	Area under the curve (mortality x days)
5 mg/mL assay	
Control	3.33±4.06
Zein NP	96.37±36.72[a]
Neem NP	110.00±36.61[a]
Neem	178.10±38.39[abc]
15 mg/mL assay	
Neem NP	222.40±39.45[a]
Neem	228.10±60.02[a]
1 mg/mL assay	
Neem NP	207.70±48.28[a]
Neem	179.00±44.36[a]

550

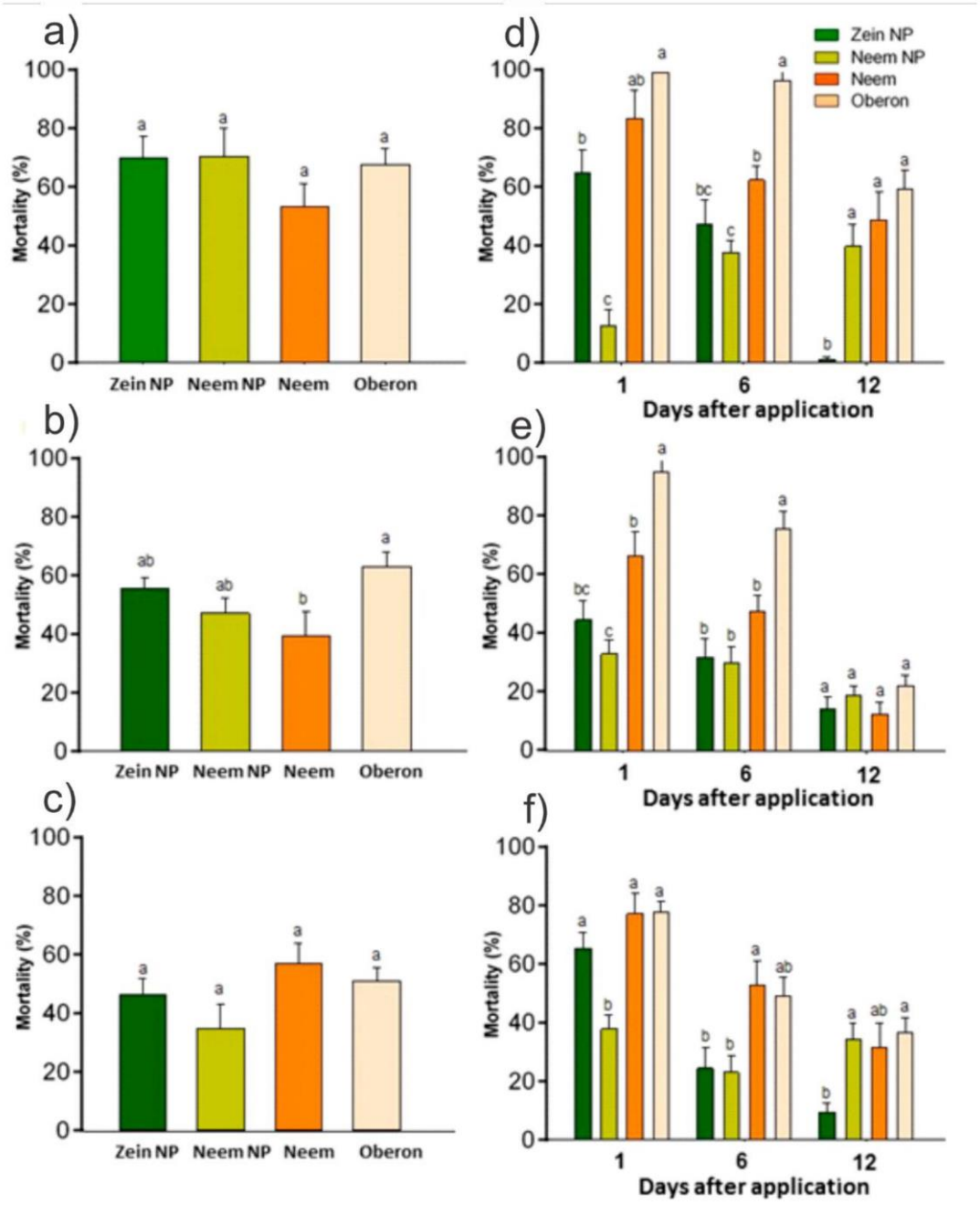
551 **3.3.3 *Tetranychus urticae* mortality**

552 The effects of the formulations on the mites (larvae, nymphs, and adult
553 females) were evaluated considering the mortality rates after direct or residual
554 treatments. Figure 5 shows the mortality rates following direct application of the
555 treatments (at a neem oil concentration of 5 mg/mL) to the larvae (Figure 5A),
556 nymphs (Figure 5B), and adults (Figure 5C). For the larvae and nymphs, use of
557 the neem oil-loaded nanoparticles led to a slightly higher mortality rate,
558 compared to use of the neem oil, although the differences were not significant.
559 However, both neem oil and the neem oil-loaded zein nanoparticles showed
560 acaricide potential against *T. urticae*, exceeding 50% mortality, with a similar
561 result for the positive control. It was interesting to note that the zein
562 nanoparticles caused mortality of the mites, especially when applied to the
563 larvae, where the mortality rates were similar to those observed for the
564 insecticide.

565 The residual treatments resulted in similar response profiles for the
566 larvae (Figure 5D), nymphs (Figure 5E), and adults (Figure 5F), with the
567 mortality rates generally decreasing over time. The best overall results were
568 observed on the first day after application, which were comparable to the results
569 obtained in the direct treatment (Figures 5A, 5B, and 5C). A possible
570 explanation for this was that in the case of the residual treatment (Figures 5D,
571 5E, and 5F), the leaves were attached to the plants at the time of application, so
572 the active metabolism could have led to the treatments reaching the leaves,
573 resulting in the mites ingesting more of the active ingredient. However, over
574 time, the treatments were degraded and their efficiencies decreased.

575 An exception to the reduction in mortality over time in the residual effect
576 assays was observed for the effect of the neem nanoparticles on the larvae
577 (Figure 5D), where larval mortality increased on the 12^{th} day. This could be
578 attributed to the ability of the nanoparticles to protect the active agent, hence
579 prolonging its effectiveness, under the experimental conditions employed.

580



581

582 **Fig. 5** Results of biological activity assays using *Tetranychus urticae*. Mortality 5
583 days after direct applications on the A) larvae, B) nymphs, and C) adults, using
584 zein nanoparticles (Zein NP), neem oil-loaded zein nanoparticles (Neem NP),
585 neem oil (Neem), and Oberon® (acaricide as positive control). Residual effects
586 on the D) larvae, E) nymphs, and F) adults, 1, 6, and 12 days after application
587 of the treatments. The data are expressed as the averages of eight repetitions

588 (n = 8), normalized to %. The error bars represent the standard deviation.
589 Different letters denote significant differences. A significance level of $p < 0.05$
590 was adopted.

591 **4 Discussion**

592 In a recently published study by our research group, we provided a
593 characterization of the zein nanoparticles containing neem oil, which presented
594 a spherical shape with mean diameter of 278 ± 6 nm and polydispersity index of
595 0.22 obtained using atomic force microscopy (Pascoli et al. 2019). In this work,
596 the mean diameter obtained by DLS was higher than that obtained using NTA.
597 A similar result was reported by Oliveira et al. (2018a), who showed that
598 nanoparticles loaded with geraniol and R-citronellal presented sizes from 142.5
599 ± 9.3 to 172.3 ± 3.8 nm, respectively, using DLS, and from 145.6 ± 4.5 to 159.1
600 ± 7.5 nm, using NTA.

601 In relation to encapsulation efficiency, Wu et al. (2012) and da Rosa et al.
602 (2015) reported encapsulation efficiencies of 90 and 91.9%, respectively, for
603 thymol in zein nanoparticles. da Rosa et al. (2015) found an encapsulation
604 efficiency of 99.9% for carvacrol in zein nanocarriers. These results show that
605 novel zein nanocarrier systems have promising potential for the encapsulation
606 and protection of active compounds.

607 The potential zeta results were in agreement with the findings of
608 Podaralla and Perumal (2012) and Oliveira et al. (2019), who used Pluronic F-
609 68 to obtain zein nanoparticles that presented zeta potential values of -11.3 ±
610 1.8 and -12 to -25 mV, respectively.

611 The physico-chemical stability results showed that although the
612 nanoparticles in suspension presented oscillations of the mean diameter, the
613 polydispersion and span indices remained similar to the values characteristic of
614 stable formulations. The nanoparticle concentration also showed no significant
615 alterations, while the encapsulation efficiency decreased, as expected since the
616 nanocarrier released the active compound as a function of time. Nonetheless,
617 despite the release, the loading still remained at 70%, which could be
618 considered high. Therefore, it could be concluded that the presence of Pluronic
619 F-68 as a surfactant was effective in maintaining the stability of the
620 nanobiopesticide.

621 Several studies have reported that encapsulation can provide benefits
622 including decreased phytotoxicity of active agents (Grillo et al. 2012; Pereira et
623 al. 2014; Clemente et al. 2014; Oliveira et al. 2018a; Campos et al. 2018a;
624 Bombo et al. 2019). On the other hand, Rai et al. (2018) reviewed several
625 studies that reported phytotoxic effects induced by nanoparticles, such as
626 decreased chlorophyll content, lower growth, reduced biomass, loss of the
627 normal shape of the plant, disruption of physiological processes, and induction
628 of oxidative stress. The penetration of nanoparticles into plants can occur
629 through water pores and stomata, as well as by endocytosis, with the particles
630 being translocated by means of the apoplastic and symplastic pathways
631 (Schwab et al. 2015; Karny et al. 2018). Therefore, it is very important that the
632 phytotoxicity of new nanotechnological systems should be carefully
633 investigated, given that pest control would lead to plants being exposed to high
634 concentrations of nanoformulations. The analysis of chlorophyll fluorescence
635 and gas exchange parameters enables investigation of the physiological

636 behavior of plants against possible stress in a non-destructive way (Ashraf and
637 Harris 2013; Murchie and Lawson 2013; Kalaji et al. 2014). Lipid peroxidation
638 and reactive oxygen species (ROS) are indicators of oxidative stress suffered
639 by the plant. Lipid peroxidation has attracted considerable interest as a
640 response to stress, especially since lipids are among the most important
641 constituents of biological membranes in all plant tissues. The peroxidation
642 products cause the permeability of the lipids to increase, in addition to inducing
643 oxidative changes in proteins, inhibiting nitrogen and protein metabolism, and
644 reducing mineral uptake (Pospíšil and Yamamoto 2017; Zhou et al. 2017; Rems
645 et al. 2019; Liu et al. 2019). Plants have evolved protective enzymatic and non-
646 enzymatic mechanisms to prevent ROS accumulation. However, if the
647 production of ROS is high and elimination is ineffective, ROS can lead to
648 uncontrolled oxidation of membranes, proteins, and DNA, causing oxidative
649 stress and death (Yin et al. 2016; Gutteridge and Halliwell 2018; Fichman et al.
650 2019). The dry mass of the plant is a parameter that reflects any possible
651 interference caused by morphological, biochemical, molecular, and/or
652 physiological injuries.

653 Taken together, the results of the phytotoxicity analyses indicated that
654 this new nanobiopesticide is safe for application to *P. vulgaris*.

655 Yu et al. (2015) and Oliveira et al. (2015) showed that polymeric
656 nanoparticles loaded with herbicides did not cause the same adverse effects as
657 the free herbicides in nontarget plants. However, other studies have observed
658 reductions of root length and dry mass of plants exposed to nanoparticles
659 (Pereira et al. 2014; Nakasato et al. 2017). The present results corroborated the
660 work of Sridharan et al. (2015) and Pasquoto-Stigliani et al. (2017) showing that

neem oil and polymeric nanoparticles containing neem oil could be used as potential tools in pest control in agriculture, since they did not present phytotoxicity. Pasquoto-Stigliani et al. (2017) found that polymeric nanoparticles containing mixtures of neem oil and oleic acid inhibited light-saturated net photosynthesis (A_{max}) and stomatal conductance in corn. These findings show that the mechanism of action of nanoparticles is related to their chemical composition, size, and morphology. They can interact with plants through the roots and leaves, and if absorbed can cause various effects including interference in the absorption of water and nutrients, which highlights the importance of evaluating the phytotoxicity of nanoformulations (Ruttkay-Nedecky et al. 2017; Pasquoto-Stigliani et al. 2017; Rai et al. 2018).

Acanthoscelis obtectus is one of the most important pests of *P. vulgaris* dry beans, multiplying in the field and post-harvest (Mutungi et al. 2015; Vuts et al. 2018). This bruchine has a wide variety of host plants (*Cajanus indicus*, *Cicer arietinum*, *Lathyrus odoratus*, *Lens esculenta*, *Mucuna pruriens*, *Phaseolus glabellus*, *P. mungo*, *P. acutifolius latifolius*, *P. coccineus*, *P. lunatus*, *P. vulgaris*, *Sesbania sesban*, *Vicia faba*, *V. sativa*, *Vigna caracalla*, *V. sesquipedalis*, *V. caracalla*, *V. umbellata*, *V. unguiculata*, *Voandzeia subterranea*). The weevil is considered to be a pest of stored products and has a cosmopolitan distribution (Pérez et al. 2013).

This pest reduces the mass, volume, physiological quality, and germination index of beans, while increasing the temperature and water content, leading to losses of around 7-40% (Mbogo et al. 2009). Bean producers and distributors control *A. obtectus* using insecticides including pyrethroids, organophosphates, and aluminum phosphide fumigant (Corrêa et

686 al. 2011; Pimentel et al. 2012). However, the use of these compounds has led
687 to concerns regarding environmental contamination, pest resistance, and
688 impacts on human health. Hence, it is important to study new technologies for
689 the control of *A. obtectus* that can contribute to safety in agriculture (Shelef et
690 al. 2018; Pellegrini and Fernández 2018).

691 This new nanobiopesticide increased insecticidal effects against store
692 pest *Acanthoscelides obtectus*.

693 Paul et al. (2009) reported that the use of 8.3 kg of whole or powdered
694 leaves of *Azadirachtina indica* A. Juss per 100 kg of beans increased the
695 mortality of *Zabrotes subfasciatus* (Boheman) and *A. obtectus*. Paulraj et al.
696 (2017) observed higher mortality of larvae using Ponneem® against *H. armigera*
697 when the active agent was encapsulated in chitosan nanoparticles. However, it
698 should be noted that evaluation of the biological activity of neem-based
699 pesticides should not be based only on the amount of azadirachtin, as there are
700 other active ingredients present (such as aflatoxins, salinin, and nimbandiol),
701 which are also potential insecticides (Campos et al. 2016). This was confirmed
702 by (Roy and Gurusubramanian 2011), who compared the efficiencies of five
703 formulations containing from 300 to 50,000 ppm of azadirachtin against three
704 tea pests (*Helopeltis theivora* (Waterhouse), *Scirtothrips dorsalis* (Hood), and
705 *Empoasca flavescens* (Fabricius)), with the LC_{50} value decreasing when the
706 amount of azadirachtin was increased.

707 Also, the findings with the images of *A. obtectus* exposed for neem oil-
708 loaded zein nanoparticles labeled with rhodamine open perspectives for
709 improving understanding of the effects of nanoformulations.

710 The sweet potato whitefly, *B. tabaci*, is one of the most serious
711 polyphagous pests of field and greenhouse crops (Li et al. 2011; Abd-Rabou
712 and Simmons 2015). The *B. tabaci* species complex includes at least 43
713 morphologically indistinguishable species worldwide (Tay et al. 2017). Its
714 damage is caused by direct feeding (it feeds on over 600 host plants), the
715 transmission of viruses (more than 150 viruses), the excretion of honeydew,
716 and plant physiology disorders (Stansly and Naranjo, 2010; Elfekih et al., 2018).
717 The control of this pest is largely dependent on chemical pesticides. However,
718 the high levels of application of insecticide have resulted in *B. tabaci*
719 populations developing resistance to most classes of active ingredients,
720 including pyrethroids and neonicotinoids (Wang et al. 2010; Liang et al. 2012).
721 Therefore, new strategies to protect crops against *B. tabaci* need to be
722 developed in order to manage the evolution of resistance, as well as to reduce
723 toxic effects towards nontarget organisms, the environment, and human health
724 (Kazak et al. 2015; Guo et al. 2017; Karut et al. 2018).

725 An important point was that although the neem oil commercial product
726 was recommended for use against this pest, the mortality shown was lower than
727 expected (not reaching 50%), which could have been due to the great ability of
728 *B. tabaci* to develop resistance to pesticides. In addition, the different
729 populations of *B. tabaci* present genetic differences that could be responsible
730 for important biological differences among them, in terms of symbionts, feeding
731 behavior, virus transmission, host plant variety, and resistance to insecticides
732 (Harish et al. 2019; Hussain et al. 2019; Wang et al. 2019).

733 In these experiments, was observed the potential for using a lower
734 concentration of the pesticide to control whitefly. Different to the assay

735 performed with *A. obtectus* (which showed a directly proportional relationship
736 between concentration increase and insecticidal effect), a possible explanation
737 for this result was that at the lower concentration, the nanoparticles presented
738 greater dispersion, which reduced the possibility of aggregation and enhanced
739 the capacity of the nanoparticles to enter into contact with the organism, even
740 penetrating its integument.

741 Kumar (2008) reported mortality in *B. tabaci* using commercial neem oil
742 (NeemAzal-U 17%) under semi-field conditions, with greater effectiveness of
743 the product when it was applied to the soil. Boursier et al. (2011) found that
744 neem plant extract had the same effect on whitefly as commercial neem oil.
745 Campos et al. (2018[a]) and Oliveira et al. (2019) studied the effects of polymeric
746 nanoparticle formulations containing essential oils against *H. armigera* and *C.*
747 *includens*, respectively, and in both cases, a greater sublethal effect was
748 obtained using the encapsulated compounds, compared to commercial
749 compounds. However, Oliveira et al. (2018b) found that chitosan/gum arabic
750 nanoparticles loaded with eugenol had an attractive effect for *B. tabaci*. It can
751 be seen from these results that the effect of the active agent can vary according
752 to its form and the experimental conditions, which emphasizes the need to carry
753 out an extensive evaluation of any new system.

754 The two-spotted spider mite, *T. urticae*, is a polyphagous pest that is
755 among the most damaging agricultural pests worldwide, attacking more than
756 1,100 species including vegetables, fruits, corn, cotton, ornamentals, and
757 weeds. It causes damage to the plants by puncturing the leaf, stems, and fruits,
758 before feeding on the leaking cell contents. Chlorophyll synthesis is inhibited in

759 the plant, causing leaf chlorosis, necrotic brown spots, and even plant death
760 (Grbić et al. 2011; Azandémè-Hounmalon et al. 2014; Ilias et al. 2014).

761 Due to the fast reproductive capacity of *T. urticae* and the development
762 of pesticide resistance, heavy applications of non-selective pesticides may be
763 required for its control, leading to high levels of pesticide residues in agricultural
764 products, which can have adverse human health and environmental effects
765 (Attia et al. 2013). So far, resistance to more than 90 compounds belonging to
766 different chemical classes has been reported worldwide (Van Leeuwen et al.
767 2013; Sparks and Nauen 2015; Piraneo et al. 2015). Therefore, there is an
768 urgent need to develop alternatives to the use of synthetic acaricides for the
769 control of phytopathogenic mites. For this purpose, the use of essential oils has
770 shown promising results (Moharramipour and Negahban 2014; Oliveira et al.
771 2018b, 2019; Seifi et al. 2018; Abdelgaleil et al. 2019).

772 Considering that *T. urticae* is a pest that exhibits resistance to a wide
773 range of active agents, this nanobiopesticide may be promising for field
774 application, since it can confer protection of the active agent and consequently
775 reduce the need for reapplication of the product on the larvae.

776 In recent work, Ahmadi et al. (2018) and Campos et al. (2018a) also
777 showed the ability of nanoencapsulation to increase the acaricidal activities of
778 natural compounds against *T. urticae*. Ahmadi et al. (2018) encapsulated the
779 essential oil of *Satureja hortensis* L. in chitosan/TPP nanoparticles, while
780 Campos et al. (2018a) produced β-cyclodextrin-grafted chitosan nanoparticles
781 co-loaded with carvacrol and linalool. In both studies, it was found that the
782 acaricidal activities of the compounds increased due to the protection provided

783 by the nanocarriers, which led to prolonged effects of the active agents,
784 indicating the potential benefits of these nanotechnological products in
785 agricultural applications.

786 In this context, zein nanoparticles containing neem oil showed good
787 physicochemical stability during 90 days, with encapsulation efficiency above
788 80% and maintaining particle size between 170 and 290 nm. Up to 30 days,
789 there were no significant changes of any of the colloidal parameters evaluated.
790 This new system had no phytotoxic effects on bean plants and presented
791 biological activity similar to that of neem oil against whitefly (*B. tabaci*) and two-
792 spotted spider mite (*T. urticae*). Encapsulation of neem oil in zein nanoparticles
793 increased its effectiveness against the bruchine *A. obtectus*. Fluorescence
794 labeling of the nanoparticles enabled visualization of the interaction of the
795 nanomaterial with the test organism. In previous work, the loading of neem oil in
796 zein nanoparticles was found to decrease or eliminate the damage caused by
797 commercial neem oil in nontarget organisms such as *A. cepa* and the nematode
798 *C. elegans*.

799 Therefore, the present findings provide further support for the excellent
800 potential of this nanobiopesticide for use in pest control in sustainable
801 agriculture.

802 **Acknowledgments**

803 The authors are grateful for the financial support for this research provided by
804 the São Paulo State Research Foundation (FAPESP, grants #2017/21004-5,

805 #2015/17120-4, and #2013/11343-6) and the National Council for Scientific and
806 Technological Development (CNPq).

807 **Conflicts of interest**

808 There are no conflicts of interest to declare.

809 **Human and animal rights**

810 This article does not contain any studies with human participants or animals
811 (vertebrates) performed by any of the authors.

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CONCLUSÕES GERAIS E PERSPECTIVAS

O modelo agrícola atual tem capacidade de gerar alimentos para quase oito bilhões de pessoas, contudo, com o esperado aumento populacional e consequentemente o aumento pela demanda da produção agrícola, esse sistema não será capaz de suprir as necessidades da população. Novos sistemas agrícolas produtivos e sustentáveis serão necessários para maximizar a produção de alimentos e outros recursos primordiais, e um setor promissor para alcançar esse objetivo é a associação da nanotecnologia a biopesticidas. O termo nanobiopesticida vem sendo utilizado para definir essa nova tecnologia, e embora existam diversos estudos relacionados a eles, a expansão de seu uso ainda é limitada por ausência de marcos regulatórios, de padronização da composição dos biopesticidas bem como da definição do risco do uso das nanoformulações. Desta forma, é importante destacar a importância deste trabalho onde além do desenvolvimento e caracterização de um novo sistema nanoparticulado foi avaliada a sua atividade biológica contra pragas agrícolas, bem como investigado seu potencial risco tanto para células vegetais, micróbios e nematóides não alvos.

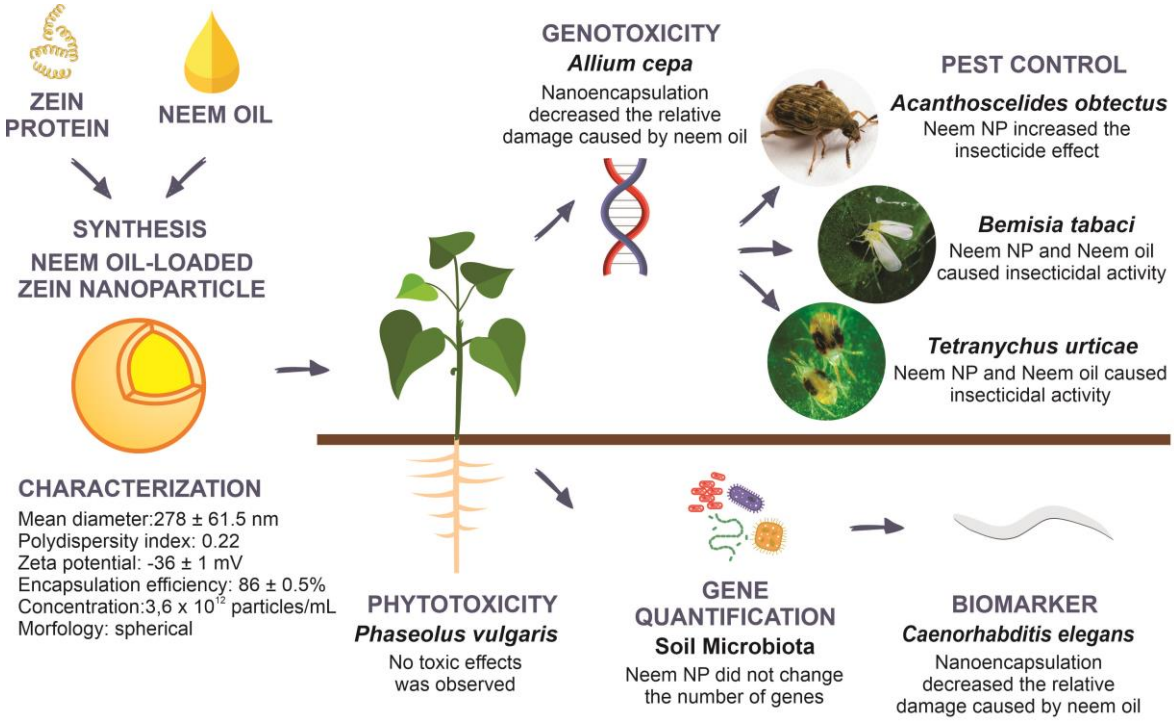
As formulações de nanopartículas de zeína carreadas com óleo de neem obtidas neste trabalho apresentaram formato esférico, com diâmetro médio de 278 ± 61,5 nm, índices de polidispersão e SPAN característicos de formulações estáveis (abaixo de 0,2 e próximo de 1 respectivamente), alta eficiência de encapsulação (> 80%) e sobretudo boa estabilidade nas condições experimentais e em função do tempo de armazenamento.

Pela estratégia “safer by design”, iniciamente os possíveis impactos ecotóxicos deste novo sistema foram avaliados em organismos não alvos e apresentaram resultados bastante oportunos. As nanopartículas de zeína demonstraram capacidade em diminuir esses efeitos tóxicos causados pelo óleo de neem em *A. cepa*, e cessar esses efeitos em *C. elegans* bem como não apresentaram nenhum efeito de fitotoxicidade na germinação, morfologia, clofrofila *a*, trocas gasosas ou estresse oxidativo em *P. vulgaris* e no número de genes que codificam enzimas fixadoras de nitrogênio e enzimas desnitrificantes. Posteriormente a essa avaliação de risco do nanobiopesticida, seu efeito e o do óleo de neem foram mostrados nas pragas agrícolas *B. tabaci* e *T. urticae*, e a atividade biológica mais promissora correu contra *A. obtectus*, onde o

nanobiopesticida aumentou significativamente a mortalidade da praga em comparação ao óleo de neem. Por fim, com nanoformulações marcadas com rodamina, foi possível identificar a exposição entre o nanobiopesticida e *A. obtectus,* com destaque para a região ventral, pernas e partes bucais

Desta forma, esse sistema com base em nanopartículas de zeína carreadas com óleo de neem apresentaram boas características coloidais, baixa toxicidade a organismos não alvos e eficiência no controle de pragas agrícolas e assim demonstram potencialidade de seu uso na proteção de culturas na agricultura sustentável, auxiliando no progresso da produção agrícola, sobretudo na segurança ambiental e alimentar. Entretanto, é importante ainda destacar que mais trabalhos devem ser desenvolvidos para que esse nanobiopesticida possa ser comercialmente utilizado com segurança e estes devem apresentar uma abordagem multidisciplinar que reconheçam seus mecanismos de ação, seus possíveis efeitos a nível celular e divulguem de forma clara seus benefícios, riscos e correta forma de manejo aos agricultores e população.

Figura 1: Esquema representativo dos principais resultados da tese. Fonte das fotografias dos organismos alvos *A. obtectus, B. tabaci e T. urticae*: google imagens.



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