



**PRISCILA DE CASTRO E SILVA**

**DESENVOLVIMENTO DE NANOBIOCOMPÓSITOS  
ANTIFÚNGICOS POLIMÉRICOS PARA  
REVESTIMENTO DE SEMENTES**

**LAVRAS – MG  
2019**

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POLIMÉRICOS PARA REVESTIMENTO DE SEMENTES**

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Engenharia de Biomateriais, área de concentração em Produtos e Nanoprodutos Alimentícios, para a obtenção do título de Doutor.

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**DEVELOPMENT OF ANTIFUNGAL NANOBIOCOMPOSITS  
POLYMERS FOR SEED COATING**

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***Dedico***

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## RESUMO

Filmes biodegradáveis são confeccionados com materiais biológicos e atuam como barreira a elementos externos, protegendo o produto e adicionando sua vida útil. São preparados a partir de proteínas, polissacarídeos, lipídeos ou da combinação desses compostos. Um sistema promissor para a modificação das características desses filmes é a incorporação de nanoestruturas aos mesmos. Contudo, no Brasil ainda não há uma legislação para nanomateriais. Logo, com o maior uso dessa tecnologia, torna-se importante avaliar o impacto destes novos materiais, visando direcionar melhor as pesquisas. Diante disso, objetivou-se desenvolver um revestimento ativo de pectina com nanoemulsão de cera de carnaúba e óleo de Neem e avaliar a sua atividade antimicrobiana. Nanoemulsão de lipídeos serão produzidas por processo de ultrassonicação e incorporadas em pectina, via solução, obtendo-se filmes por casting. As seguintes técnicas de caracterização dos filmes/soluções filmogênicas foram utilizadas: Análise do Tamanho das Partículas, Reologia, Potencial Zeta, Permeabilidade ao Vapor de Água, Espectrofotometria de Infravermelho, Ensaio Mecânicos de Tração e Punctura, Microscopia Eletrônica de Varredura (MEV/FEG) e germinação de sementes revestidas com os nanocompósitos. A elaboração de filmes nanocompósitos compostos foi viável. A estabilidade do tamanho da partícula foi observada para as nanoemulsões produzidas e foi essencial durante a polimerização de modo a levar à elaboração de filmes uniformes. A nanoemulsão de óleo de Neem com o menor tamanho de partícula foi a mais efetiva no controle antifúngico, tanto nos testes *in vitro* quanto sobre as sementes contaminadas com fungos *Aspergillus Flavus* e *Penicillium Citrinum*. A presença de fases oleosas acarretou um aumento do alongamento destes filmes, melhorando suas propriedades mecânicas. As propriedades de barreira de água dos filmes de pectina foram afetadas positivamente com a incorporação das nanoemulsões de óleo de Neem. Confirmou-se que a nanoemulsão à base de óleo de Neem foi eficiente no controle dos fungos estudados sem causar efeitos fitotóxicos para as sementes. Logo, este estudo mostra que os nanocompósitos desenvolvidos possuem grande potencial de aplicação na agricultura, em especial para o revestimento de grãos e sementes.

**Palavras-chave:** Polímeros. Pectina. Cera de carnaúba. Nanoemulsão. Óleo de Neem.

## ABSTRACT

Biodegradable films are made with biological materials with the role of acting as a barrier to external elements, protecting the product and adding its useful life. They are prepared from proteins, polysaccharides, lipids or from compressors. Thus, new packaging has been developed in recent years to meet the consumer preferences on products with an extended shelf life. However, there are several challenges in the adequacy of barrier properties and incorporation of new functionalities (such as the antimicrobial propertie) into pectin films to make their use suitable for packaging. A promising system to modify the films charecteristics is the nanostructures incorporation to them. However, in Brazil there is still no legislation for nanomaterials and for this technology usage, being important to evaluate the impact of these new materials to better target the research. This study aimed to develop an active pectin package with nanoemulsion of carnauba wax and neem oil and to evaluate its antimicrobial activity and toxicity. Lipid nanoemulsion was produced through the ultrasonication process and it was incorporated into pectin, via solution, obtaining films by casting. Analysis of Particle Size, Rheology, X-Ray Diffraction, Zeta Potential, Water Vapor Permeability, Infrared Spectrophotometry, Thermogravimetry, Differential Scanning Calorimetry, Mechanical Traction and Puncture Testing, Film Solubility in Water, Scanning Electron Microscopy (MEV / FEG). The composite nanocomposite films elaboration was feasible. A particle size stability was observed for the nanoemulsions produced and it was essential during the polymerization to prepare uniform films. The Neem oil nanoemulsion with the smallest particle size was the most effective in the antifungal control, both in in vitro and contaminated seed tests. The oily phase presence increased these films elongation, improving their mechanical properties. The water barrier properties of the pectin films were positively affected by the Neem oil nanoemulsions incorporation. It was confirmed that the Neem oil-based nanoemulsion was efficient in controlling the studied fungi without causing phytotoxic effects on the seeds. Therefore, this study showed that the developed nanocomposites have a great potential of application in agriculture, especially for the grains and seeds coating.

**Keywords:** Polymers. Pectin. Carnauba wax. Nanoemulsion. Neem oil.



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## LISTA DE ABREVIATURAS E SIGLAS

AFM	Microscopia de força atômica
Astm	American Society for Testing and Materials
DMA	Dynamic Mechanical Analysis
DRX	Difração de Raio-X
DSC	Calorimetria exploratória diferencial
FTIR	Infravermelho por transformada de Furrier
KBr	Brometo de potássio
MEA	Malt Extract Agar
MEV	Microscopia eletrônica de varredura
NMP	Número mais provável
PET	Politereftalato de etileno
PVA	Permeabilidade a vapor de água
rpm	Rotações por minuto
TG	Termogravimetria
Tm	Temperatura de fusão
UFC	Unidades formadoras de colônia
UFLA	Universidade Federal de Lavras
UR	Umidade relativa

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## PRIMEIRA PARTE

### 1 INTRODUÇÃO GERAL

O Brasil é destaque no comércio internacional como exportador de *commodities* agrícolas devido à sua produção de sementes. Dentre as *commodities* produzidas no país, a soja (*Glycine max (L.) Merrill*) é considerada a de maior importância mundial. Isto, porque o país ocupa o primeiro lugar como maior exportador e segundo lugar maior produtor (SMANIOTTO et al., 2014). Nesse contexto, a armazenagem de produtos agrícolas é de extrema importância para atender a logística de comercialização. Entretanto, avalia-se que cerca de 20% da produção anual de sementes seja perdida durante o armazenamento (KUMAR; KALITA, 2017).

A semente é um dos principais insumos na agricultura moderna. Constitui-se, portanto, como a base de todo o avanço para o cultivo da soja. Entretanto, a baixa qualidade configura uma das razões da redução da produtividade em lavouras no Brasil. Isto ocorre, sobretudo, em virtude da maior parte dos agricultores empregarem as suas próprias “sementes”, as quais são expostas, em geral, a elevados graus de umidade, baixa germinação e vigor. Além disso, grande parte são atacadas por insetos e contaminadas, principalmente, pela presença de patógenos associados (SMANIOTTO et al., 2014).

Esse processo de deterioração é inevitável. Contudo, pode ser retardado dependendo das condições de armazenamento e das características das sementes (CARDOSO; BINOTTI; CARDOSO, 2012). Em função disso, torna-se indispensável adotar tecnologias com o intuito de preservar a qualidade e o potencial de armazenamento das mesmas. Principalmente quando se trata de sementes contaminadas por microrganismos que transmitem e disseminam várias doenças para o campo (DERESSEGN; MEKBIB, 2016).

Nesse contexto, o enlace cada vez mais relevante entre ciência e nanotecnologia proporciona modificações no âmbito da agricultura e na indústria agroalimentar. Assim, novas técnicas de manejo no campo e inovações na produção de insumos agroquímicos têm surgido. Uma das tecnologias propostas para o controle de doenças consiste na aplicação de revestimentos com polímeros biodegradáveis em sementes. Todavia, a aplicação destes materiais com substâncias ativas ainda é incipiente.

Revestimentos biopoliméricos são produzidos a partir de macromoléculas capazes de formar matrizes contínuas e coesas. Esse sistema faz com que possam atuar como um obstáculo a fatores exteriores, tais como umidade, óleo e gases. Por conseguinte, atribuem

uma maior proteção ao produto revestido, prolongando assim seu período de armazenamento (RUBILAR et al., 2016).

O emprego de recobrimentos em sementes a base de polímeros biodegradáveis pode promover a obtenção de um conjunto de particularidades indispensáveis ao estabelecimento das plântulas no campo. Além de proporcionarem proteção às sementes, os recobrimentos poliméricos oferecem ainda uma série de aplicações adicionais como a habilidade de trabalhar como suportes de substâncias ativas, tais como nutrientes ou agentes antimicrobianos. Além disso, proporcionam a redução da exposição dos operadores aos agrotóxicos e facilitam o manejo do tratamento, reduzindo as quantidades de produtos utilizados (CHEN; LIU, 2016).

Dessa forma, o interesse no desenvolvimento de filmes e revestimentos de polímeros biodegradáveis está adjunto à probabilidade de diminuição do uso de materiais sintéticos, que degradam no meio ambiente. Além disso, sua aplicação contribui para a agregação de valor do produto, acondicionado por meio do emprego de matérias-primas renováveis, especialmente derivadas de produtos agrícolas. Como consequência, evitam-se problemas com as legislações dos países importadores que estão cada vez mais rígidas quanto à questão ambiental (REDDY, 2015). Assim, a aplicação de tecnologias, como recobrimento ativo utilizando produtos biodegradáveis, às sementes de soja constitui uma alternativa interessante. Tendo em vista um mercado cada vez mais competitivo e primoroso por atitudes de sustentabilidade ambiental, isso se torna efetivo.

Logo, considerando a atual busca por tecnologias comercialmente viáveis para agregação de valor à cadeia produtiva, associados a ausência de trabalhos científicos sobre formulação de revestimentos poliméricos aplicados a sementes, objetivou-se esta pesquisa, formular revestimentos à base de matérias-primas nacionais (pectina, óleo de Neem e cera de carnaúba), e avaliar suas propriedades químicas, físico-mecânicas e biodegradáveis; bem como seus efeitos sobre a fisiologia de sementes de soja.

## **1.1 Objetivos**

### **1.1.1 Objetivo geral**

Diante da busca por tecnologias comercialmente viáveis e da ausência de trabalhos científicos sobre a formulação de revestimentos, compostos à base de nanoemulsões antifúngicas e biodegradáveis de cera de carnaúba e óleo de Neem em matriz polimérica de pectina, objetivou-se com a presente pesquisa investigar formulações de revestimentos à base dessas matérias-primas nacionais.

### **1.1.2 Objetivos específicos**

I. Estabelecer um protocolo de produção das nanoemulsões lipídicas de óleo de Neem e Cera de carnaúba pelo método de alta temperatura. Inclui-se a concentração dos tensoativos empregados e tempo de ultrassonificação dos sistemas. Isto, de modo a garantir a sua estabilidade, além da concentração ideal para o efeito microbiológico.

II. Selecionar as variáveis (concentração de pectina e a razão cera de carnaúba x óleo de Neem) que influenciam na estabilidade física dos revestimentos emulsionados e da resistência ao vapor de água;

III. Selecionar formulações de revestimentos nanoemulsionados que promovam nanoemulsões estáveis e revestimentos com melhores propriedades mecânicas, microbiológicas e de barreira ao vapor de água e aos gases;

IV. Estudar o efeito dos revestimentos selecionados sobre propriedades mecânicas, grau de umidade, qualidade sanitária, bem como a germinação de sementes de soja. O intuito é minimizar a influência externa sobre a qualidade fisiológica da semente.



## **2 REFERENCIAL TEÓRICO**

### **2.1 Nanociência e nanotecnologia**

Durante as últimas décadas, o desenvolvimento tecnológico tem revolucionado o setor industrial. A mais notável e recente área é a nanotecnologia (MARÍAPÉREZ-PAGE et al., 2016). Um nanomaterial é definido como um material com uma ou mais dimensões externas em nanoescala (1 a 100 nm). Por consequência apresenta variações em relação ao seu tamanho, forma, área superficial e composição química (OJA et al., 2016). Estes nanomateriais se diferem do tamanho convencional pela modificação na magnitude superfície-volume. Minimizando o volume, por meio do tamanho, as proporções dos átomos na superfície das partículas aumentam. Por conseguinte, as características de superfície podem sobrepor as propriedades do material em macroescala (MARÍAPÉREZ-PAGE et al., 2016).

Neste contexto, a nanotecnologia pode ser aplicada na produção de alimentos como a ampliação de novos materiais funcionais, projeto de instrumentação e práticas para melhoramento da segurança alimentar (WEISS; TAKHISTOV; MCCLEMENTS, 2006). Deste modo, a descoberta de materiais com inovações, comparado propriedades convencionais, acenderam um grande campo de aplicações tanto industriais, quanto tecnológicas e científicas. Isto se torna cada vez máxima a facilidade no alcance de nanomateriais e no domínio de suas propriedades.

#### **2.1.1 Nanotecnologia aplicada em embalagens: benefícios x riscos**

O enlace cada vez mais relevante entre ciência e nanotecnologia tem proporcionado modificações no âmbito da agricultura e na indústria agroalimentar. Nessa vanguarda, a admissão de novas técnicas de manejo no campo, inovações na produção de insumos agroquímicos e avanços nos processos de embalagens de produtos têm sido observados (RAM; VIVEK; KUMAR, 2014). Isso resultou em grandes investimentos na área de nanotecnologia, atingindo cerca de 1,5 bilhões de dólares em 2016/2017 (KOSHOVETS; GANICHEV, 2016).

Nesse contexto, a influência das características do material em nanoescala e a biodisponibilidade destas nanopartículas têm sido largamente pesquisados (YUE; WANG, 2012). Várias tecnologias estão sendo empregadas, tais como a produção de nanodispersões, nanocápsulas, nanocompósitos e nanofibras. Dentre estes, os primeiros nanocompósitos que

surgiram no mercado são os polímeros contendo nanopartículas de argila. Eles oferecem atributos térmicos e mecânicos, estabilidade à umidade e resistência à água e ao calor (NANDY; SHOROWORDI, 2015). Não obstante, o uso dessa tecnologia incorporando materiais naturais para a embalagem dos alimentos torna-se uma escolha ambientalmente adequada. Estes minimizam a exigência de plásticos sintéticos como materiais de embalagem e protegem o alimento, aumentando a sua vida útil (UMARAW; VERMA, 2017).

No entanto, se por um lado, as nanotecnologias podem contribuir para avanços no desempenho industrial, por outro lado ainda há poucas informações quanto ao seu efeito sobre à saúde humana e ao meio ambiente (MARÍAPÉREZ-PAGE et al., 2016). Isso ocorre, uma vez que as propriedades físicas, químicas e biológicas dos materiais em tamanho nanométrico podem ser diferentes dos convencionais. Assim, seus efeitos e impactos não podem ser antevistos a partir do conhecimento já existente sobre os materiais convencionais (AW-YONG et al., 2018). Outra questão ainda em aberto trata-se do uso de nanomateriais e nanotecnologias em embalagens de alimentos, já que há a possibilidade da migração de algum composto para o produto, podendo promover um impacto negativo sobre a segurança ou qualidade do alimento (SADEGHI et al., 2017).

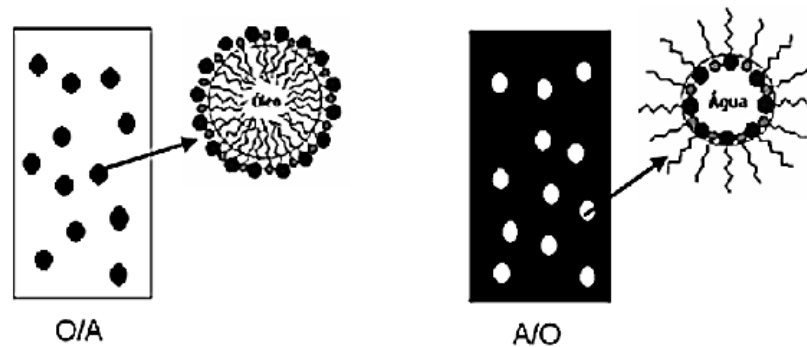
## **2.2 Sistemas emulsionados**

### **2.2.1 Emulsão**

Emulsões são definidas como sistemas coloidais heterogêneos e termodinamicamente estáveis. Tem sua formação a partir da combinação de dois líquidos imiscíveis (em geral óleo e água). Um dos seus componentes encontra-se disperso (interno), em configuração de partículas esféricas pequenas, no outro (fase contínua). São característica de emulsões apresentar diâmetro das partículas em geral, entre 0,1 e 100  $\mu\text{m}$  (DEBNATH, B. K., SAHA, U. K., SAHOO, 2015).

As emulsões são estabelecidas pela fase oleosa, fase aquosa e tensoativos. O líquido menos polar caracteriza a fase oleosa e o mais polar representa a fase aquosa. Segundo a hidrofília e lipofília da fase dispersante, estes sistemas qualificam-se em óleo em água (O/A), no qual, a fase interna é oleosa e a fase externa aquosa; ou água em óleo (A/O), constituindo-se de uma fase interna aquosa e a uma fase externa oleosa (ZAFEIRI et al., 2017). Isso pode ser observado na Figura 1.

Figura 1 – Representação esquemática dos glóbulos de emulsões óleo/água (o/a) e água/óleo (a/o).



Fonte: Oliveira (2008).

Estes sistemas apresentam-se estabilizados cineticamente por meio da adição de tensoativos adequados. O papel destes tensoativos é minimizar a tensão interfacial. Para tanto, constitui-se um filme interfacial esférico com características eletrostáticas em contorno dos glóbulos da fase interna (BERRY et al., 2015). Assim, como uma molécula de tensoativo é adicionada na interface ar-água, ocorre a absorção de suas moléculas na superfície da água, enfraquecendo sua tensão superficial.

Os agentes tensoativos podem ser classificados conforme seu balanço hidrofílico – lipofílico (HLB). Estes valores variam entre 0 e 20. Maiores valores refletem uma máxima hidroflicidade. De acordo com o valor do HLB, o tensoativo será então classificado em lipofílico (HLB < 9) ou hidrofílico (HLB > 11). Consequentemente, será empregado em emulsões do tipo água/óleo ou óleo/água, respectivamente. Aqueles na faixa de 9 - 11 são classificados como intermediários (LIN et al., 2016).

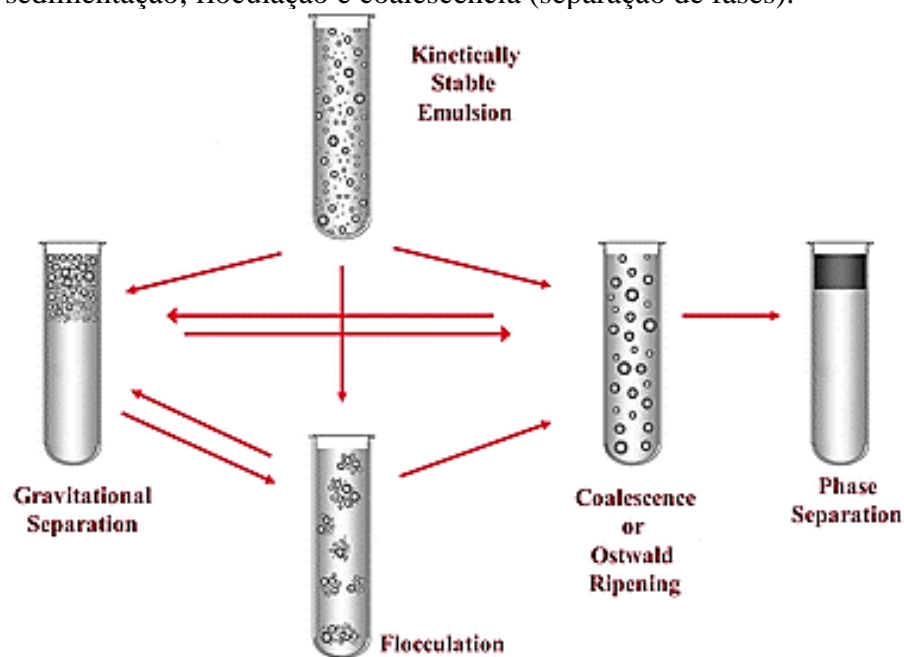
A estabilidade das emulsões está relacionada intrinsecamente com a influência mútua interfásica proporcionada pelos tensoativos entre as fases imiscíveis que as compõem. Não obstante, a instabilidade física do sistema revela-se, sobretudo por meio dos consequentes fenômenos descritos abaixo e que podem ser visualizados também na Figura 2 (MCCLEMENTS, 2011):

- Floculação (ocasionada pelas forças de atração de Van der Waals): ação onde ocorre a agregação reversível das gotículas, com manutenção do filme interfacial, compondo uma rede bidimensional, ausente de coalescência;
- Cremeação ou sedimentação (movidada pela gravidade): processo em que as gotículas apresentam tendência de se separar da fase externa da emulsão, ocorrendo cremeação ou

sedimentando (sedimentação), dependendo da variação de densidade entre as duas fases. A emulsão resultante incluirá duas porções: uma apresentando maior volume de fase externa e outra com maior volume de fase interna;

- Coalescência (ocasionada pelo afinamento e rompimento do filme entre as gotículas): ação onde duas ou mais gotículas da fase dispersa tendem a se aproximar uma da outra com energia necessária para fundirem-se e constituírem uma gotícula maior. Este procedimento é irreversível e derivará no isolamento de fases da emulsão.

Figura 2 – Representação dos fenômenos de instabilidade física de emulsões: cremação; sedimentação; floculação e coalescência (separação de fases).



Fonte: McClements (2011).

Devido a estes fatores relacionados à instabilidade do sistema emulsionado, atualmente, muito se concentra em desenvolver melhorias para otimização deste processo. Neste sentido, é ascendente os avanços da nanotecnologia, sobretudo no desenvolvimento de nanoemulsões, as quais apresentar estabilidade superior aos sistemas emulsionados.

### 2.2.2 Nanoemulsão

Nanoemulsões constituem sistemas coloidais compostos de partículas de dimensão reduzida, com tamanho de 20 a 100 nm. Em comparação com emulsões que são bastante instáveis, as nanoemulsões são cineticamente metaestáveis, ou seja, estáveis por um extenso

período de tempo. Tal estabilidade pode ser explicada devido à natureza dinâmica das nanoemulsões e influência mútua entre as partículas que as compõe. Por apresentarem essa natureza dinâmica, compreende-se que as partículas se conservam numa condição contínua de oscilação. Volta e meia vão ao encontro umas com as outras, o que ocorre em virtude ao movimento browniano ser menor que a taxa de cremação induzida pela gravidade. Assim o processo de sedimentação das nanopartículas apresenta baixa probabilidade de ocorrer (AGRAWAL; MADDIKERI; PANDIT, 2017). Os mecanismos de estabilidade das nanoemulsões podem ser classificados como eletrostático, estérico e eletroestérico (HOLMBERG, 2010). A estabilidade da nanoemulsão é controlada pelas características do filme interfacial desenvolvido entre a fase aquosa e oleosa, e pelas propriedades físico-químicas da camada de adsorção constituída na superfície das partículas dispersas. Esse filme pode induzir forças estéricas ou eletrostáticas repulsivas em meio as partículas próximas. Essas propriedades abrangem a redução do valor da tensão interfacial. São dependentes da composição e da concentração do agente tensoativo empregados. (MCCLEMENTS; JAFARI, 2018). O amplo interesse é evidenciado devido às múltiplas conveniências que estas formulações proporcionam (RAHN-CHIQUÉ et al., 2012; ULUATA; DECKER; MCCLEMENTS, 2016):

- I – O tamanho reduzido previne resultados de coalescência, desde que não exista modificação da superfície. Além disso, a espessura do filme de tensoativo em relação ao tamanho das partículas faz com que não aconteça ruptura do filme líquido entre as gotas.
- II – O tamanho reduzido das gotas faz com que o movimento browniano seja capaz de superar a força da gravidade, diminuindo fenômenos de instabilidade como cremação e sedimentação.
- III – Nanoemulsões são sistemas apropriados para distribuição eficientes de ativos. A grande área superficial admite maior superfície de contato, podendo facilitar a penetração dos ativos.
- IV – Demanda uma menor concentração de tensoativos quando comparado às microemulsões.

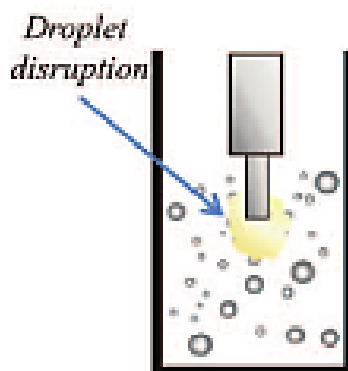
Devido aos fenômenos descritos, confirma-se que quanto menor o tamanho das gotas, maiores os benefícios quanto à sua estabilidade cinética. As vantagens das nanoemulsões incluem: mais transparência, melhores propriedades físico-químicas, maior estabilidade,

disfarçando o sabor ou cheiro do material do núcleo. Portanto, evidencia menor efeito nas propriedades sensoriais dos produtos e melhora a atividade biológica, aumentando a área de superfície, permitindo o uso de doses mais baixas de composto ativo (NOORI; ZEYNALI; ALMASI, 2018a).

Deste modo, o principal objetivo da produção de nanoemulsões é produzir gotas menores possíveis, garantindo a estabilidade cinética em função do tempo para aplicações industriais (OLIVEIRA et al., 2017). Contudo, nanoemulsões não se constituem espontaneamente, sendo indispensável o provimento de energia ao sistema. Esta energia pode ser obtida por métodos de baixa ou alta energia de emulsificação. Os métodos designados de baixa energia estão associados à modificação espontânea de curvatura das moléculas do tensoativo. Essa etapa é a mais importante do processo de emulsificação e pode ser alcançada pela alteração da temperatura com o método denominado temperatura de inversão de fase, *Phase Inversion Temperature – PIT* (SETYA; TALEGAONKAR; B.K.RAZDAN, 2014). Já as tecnologias de alta energia empregam dispositivos mecânicos, apropriados para gerar forças de cisalhamento intensas capazes de quebrar estruturas da ordem de micrômetros em partículas nanométricas (MISHRA; BHARGAVA, 2017). Em geral, nos processos de nanoemulsificação por alta energia, acontece primeiro a combinação das fases oleosa e aquosa, derivando em uma emulsão grosseira. Em seguida, esta emulsão é forçada a incidir por um sistema com introdução de forças de cisalhamento, diminuindo o tamanho médio de gota (SANTANA; PERRECHIL; CUNHA, 2013). Exemplos de dispositivos empregados para a fabricação de nanoemulsões pelo método de alta energia são: homogeneizadores, microfluidizadores e ultrassonicadores (MCCLEMENTS; RAO, 2011).

Os homogeneizadores ultrassônicos, atualmente, são os principais métodos usados para produzir nanoemulsões de qualidade superior. A tecnologia de nanoemulsificação ultrassônica é livre da maioria de inconvenientes e repetidas vezes, usada em estudos de laboratório (PESHKOVSKY; BYSTRYAK, 2014). Estes empregam elevada magnitude de ondas ultrassônicas para provocar a força de cisalhamento indispensável, induzindo a quebrar a emulsão em pequenas gotículas. Tais ondas são provocadas por uma *probe* colocada sobre os líquidos a serem homogeneizados. A mesma promove sobre os líquidos, vibrações que permitem a constituição, desenvolvimento e quebra de pequenas gotículas (BURGER et al., 2017), como ilustrado na Figura 3.

Figura 3 – Representação do mecanismo mecânico ultrassônico para produção de nanoemulsão.



### **Ultrasonic Probe Homogenizer**

Fonte: McClements e Rao (2011).

A aquisição de um pequeno tamanho de gotas pelo o método de alta energia está sujeito às características do equipamento como força, fluxo e intensidade de energia. Ao mesmo tempo, outros fatores como temperatura, composição da amostra, tipo e concentração de tensoativo, além de fatores físico-químicos como tensão interfacial e viscosidade também influenciam no processo (MCCLEMENTS; RAO, 2011; WOOSTER; GOLDING; SANGUANSRI, 2008).

Nesta vanguarda, associados a produção de nanoemulsões por meio de sistemas ultrassônicos, é crescente a priorização do desenvolvimento de materiais que, além de apresentar características funcionais e propriedades superiores aos de materiais convencionais, também sejam uma fonte ambientalmente amigável. Tudo isto, utilizando-se de matérias primas provindas de fontes biodegradáveis. Vale pontuar que nos últimos anos, os biopolímeros têm sido muito pesquisados, devido à versatilidade química e elevado potencial de aplicação em diversas áreas como biomédica, agroindustrial e alimentícia (SHELKE; JAMES; LAURENCIN, 2014).

### **2.3 Polímeros biodegradáveis**

Polímero biodegradável “é um plástico degradável, em que a degradação deriva da atuação de microrganismos naturais tais como fungos, bactérias e algas”. Estes são transformados em compostos mineralizados simples. Em sequência, remanejados no meio

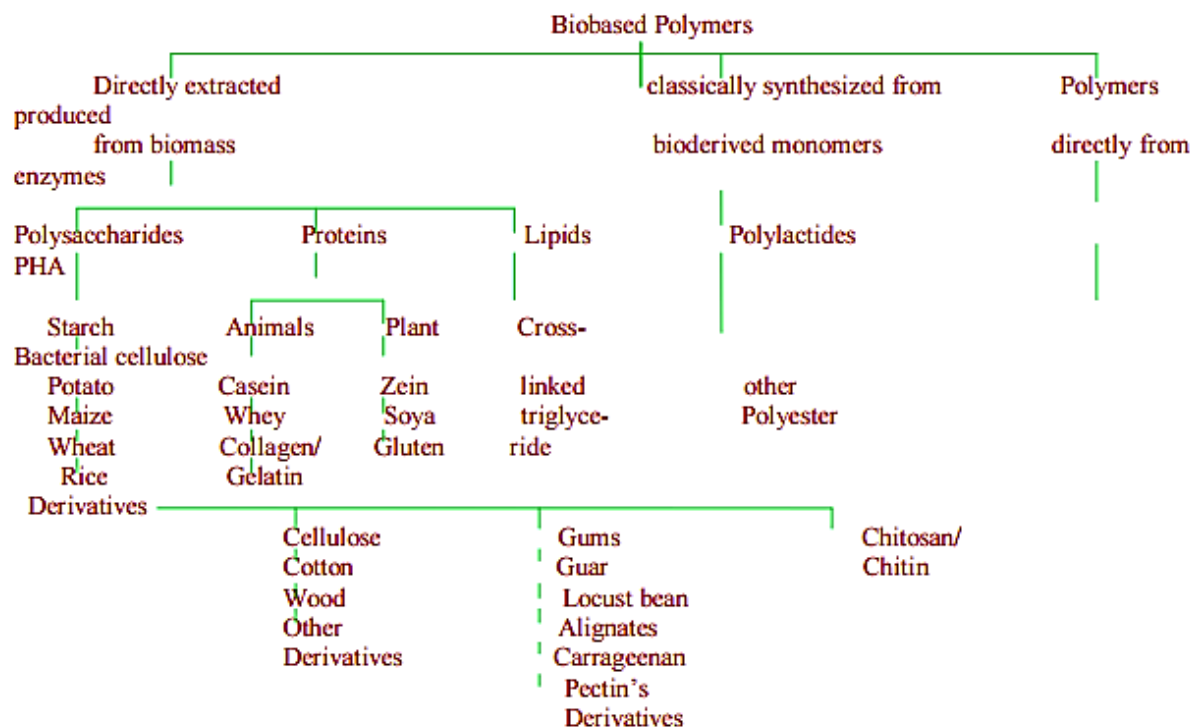
ambiente, por meio do ciclo elementar, tal como o do carbono, nitrogênio e enxofre. O processo de biodegradação está sujeito de múltiplos fatores, como a velocidade microbiana no ambiente, temperatura, pH, peso molecular e cristalinidade do polímero (BADIA; GIL-CASTELL; RIBES-GREUS, 2017).

Os polímeros de base biológica podem ser divididos em três categorias principais com base na sua origem e produção:

- Categoria 1: Polímeros diretamente extraídos ou removidos da biomassa como polissacarídeos (amido e celulose e proteínas como caseína e glúten).
- Categoria 2: Polímeros produzidos por síntese química utilizando monômeros renováveis de base biológica como o ácido polilático - um biopoliéster polimerizado a partir de monômeros de ácido lático.
- Categoria 3: Polímeros produzidos por microrganismos ou bactérias geneticamente modificadas como polihidroxialcanoatos (VALDÉS et al., 2014).

Tais categorias podem ser ilustradas na Figura 4.

Figura 4 – Categorias de classificação dos biopolímeros.





Os polímeros biodegradáveis são uma alternativa importante, sobretudo, aqueles desenvolvidos a partir de recursos renováveis. São mais compatíveis com a preservação ambiental e desenvolvimento sustentável (LOREVICE et al., 2016). No final do seu período útil, materiais poliméricos biodegradáveis são enviados para aterros sanitários ou compostagem. Enquanto a reciclagem é energeticamente difícil, a compostagem permite a eliminação das embalagens no solo, contrário aos plásticos provenientes da indústria petroquímica. Neste aspecto ainda, os biomateriais poliméricos são candidatos preferidos devido a autenticidade de suas propriedades físicas, químicas e mecânicas. Estas propriedades podem ainda, ser facilmente variadas pela composição do material e pela arquitetura do polímero. Suas taxas de degradação podem ainda, ser ajustadas conforme necessário para um processo eficiente. Na última década, polímeros biodegradáveis, foram amplamente utilizados em dispositivos industriais, bem como para indústria alimentícia. Em particular, polímeros como a pectina são candidatos promissores para tais aplicações. Suas propriedades abrangem uma gama de aplicações, sobretudo por meio de sua característica de atuar como emulsificante.

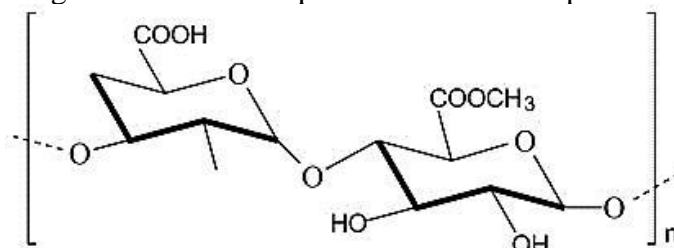
### **2.3.1 Pectina**

A pectina é um polissacarídeo aniônico de elevado peso molecular, combinado de diferentes unidades subestruturais. Distingue sua estrutura com a metodologia de extração, matéria prima, localização, e outros fatores ambientais. O termo geral pectina indica ácidos pectínicos altamente hidrofílico devido à presença de grupos polares. Estes são capazes de produzir soluções viscosas em água com grau variável de grupos metil éster e um grau de neutralização adequado de formar gel (KARAKI et al., 2016). É um dos principais componentes da parede celular vegetal e uma das mais complexas macromoléculas em natureza.

A matéria-prima empregada na fabricação da pectina são cascas de frutas cítricas, sendo o produto comercial é um pó branco ou creme que pode ser diluído em água compondo uma solução viscosa. A aplicação mais conhecida desta matéria-prima é como agente espessante e gelificante na fabricação de diversos alimentos (EINHORN-STOLL, 2017). Todavia, características como biocompatibilidade e a não-toxicidade também admitem que a pectina seja crescentemente utilizada nas áreas farmacêutica e biotecnológica. Dentre estes, destaca-se o uso em sistemas de liberação controlada de princípios ativos (WANG et al., 2018).

Quimicamente a pectina é classificada como um polímero heterogêneo. Estruturalmente é composta por uma cadeia principal linear de unidades repetidas de ácido D-galacturônico que estão ligados covalentemente por ligações  $\alpha$  - (1, 4). Os grupos carboxílicos podem ser metil esterificados parcialmente com metanol em diferentes extensões. Essa cadeia pode ser interrompida por unidades de L - ramnose através de ligações  $\alpha$  - (1, 2), às quais estão ligadas cadeias laterais por açúcares neutros, sobretudo unidades de galactose e arabinose, conforme ilustrado na Figura 5 (PÉREZ et al., 2015). Estas cadeias laterais são responsáveis pela união das moléculas da pectina à matriz da parede celular vegetal e sua presença depende principalmente da fonte e do método de extração utilizado (MISHRA; BANTHIA; MAJEED, 2012).

Figura 5 – Estrutura química da cadeia da pectina.



A razão entre os grupos ésteres e os grupos ácidos galacturônicos, é conhecido como grau de esterificação (DE). Esse teor de metoxilação altera com a idade e a localização dentro do tecido da planta, método de extração e possui influência sobre as propriedades da pectina, de maneira especial, na solubilidade de filmes (KAYA et al., 2014).

No geral, as pectinas extraídas de vegetais exibem teores de metoxilação compreendidos entre 10 e 12%. As pectinas comerciais na maioria das vezes apresentam um DE de 50%, e podem ser divididas em duas classificações: alto grau de metoxilação (HM) e baixo grau de metoxilação (LM) (VITYAZEV et al., 2017). Pectinas do tipo HM formam géis em pH baixos em torno de 3,0 ou menor. Já a pectinas do tipo LM requerem a presença de uma quantidade controlada de cálcio, ou outros cátions divalentes, para formarem gel entre as moléculas poligalacturônicas. Esta determinada quantidade de cálcio é responsável por um acoplamento das cadeias originando um complexo do tipo “egg-box”, reduzindo sua solubilidade e induzindo a associações de ligação não covalentes das cadeias polissacarídicas adjacentes (KERMANI et al., 2015; MEHMOOD, 2015). Além disso, os grupos carboxil podem ser convertidos em carboxamidas. Isso ocorre quando a amônia é utilizada no processo de desesterificação, originando a pectina amidada. Em ambos os casos, os grupos carboxilas

remanescentes estão presentes como uma mistura na forma de ácidos livres (-COOH) e sais (-COO<sup>-</sup> Na<sup>+</sup>). Os graus de metoxilação e de amidação influenciam intimamente as propriedades funcionais, tais como: solubilidade, capacidade de geleificação, temperatura e condições de geleificação das pectinas (CHRISTIAENS et al., 2016).

Em estudos de Moreira et al. (2017) pode se concluir que o uso de revestimentos comestíveis à base de pectina enriquecidos com folhas de maçã e sua aplicação, têm sido viáveis para estender a vida de prateleira de maçãs minimamente processadas. A abordagem de preservação baseada na combinação de ambas as tecnologias levou ainda a uma redução na contagem de microorganismos de deterioração.

Mais adiante, Chang et al. (2017) pesquisando sobre revestimento de pectina, verificou que o revestimento foi aplicado com sucesso à superfície de nanopartículas de NaCas / zeína. O processo envolvia adsorção eletrostática induzida por pH e aquecimento. O encapsulamento da curcumina nas nanopartículas de NaCas / zeína revestidas de pectina também melhoraram drasticamente a sua atividade antioxidante em meio aquoso. Também alimentou a liberação sustentada em fluidos gástricos e intestinais simulados.

### 2.3.2 Cera de canaúba

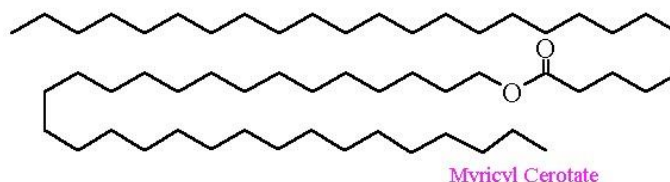
A cera de carnaúba é um exsudado vegetal da palmeira brasileira (*Copernicia cerifera*). A carnaubeira permite a extração do pó de suas folhas, constituindo quatro tipos: o tipo I conhecido, como “Pó - olho”, é removido das folhas ainda fechadas da carnaúba (amarela - clara). Apresenta características de melhor qualidade, mais pureza e, por conseguinte, mais valor. Já o tipo II, “Pó - palha”, é extraído das folhas abertas e determina a cera rotulada como do tipo II. Os tipos III e IV constituem a “Bagana” que é o resíduo da palha seguida da extração do pó. O tipo IV é escuro e menos puro, enquanto que a do tipo III é de tonalidade e pureza intermediária, sendo adquirida a partir da cera tipo IV (CRESPO; GOMES, 2007).

A cera é composta, quase inteiramente, de ésteres, ácidos carboxílicos C<sub>24</sub> e C<sub>28</sub> e álcoois mono-funcionais saturados de cadeia longa (RODRIGUES et al., 2014). Trata-se de uma mistura complexa de alto peso molecular. É composta de ésteres, álcoois livres, ácidos aromáticos, hidrocarbonetos e triterpeno dióis. Destacam-se os ésteres, como componentes principais. Eles correspondem a mais de 80% da composição e consistem em ésteres alifáticos e diésteres, como ilustra a Figura 6. Dentre estes, os principais constituintes são o cerotato de miricila, (C<sub>25</sub>H<sub>51</sub>CO<sub>2</sub>C<sub>30</sub>H<sub>61</sub>), responsável pelas características de formação de pasta e

emulsões. Também merece destaque, ácido cinâmico que possui propriedades antioxidantes e antifúngicas (FREITAS et al., 2015; GONÇALVES et al., 2010).

Figura 6 – Estrutura química da cera de carnaúba.

Carnauba Wax



Devido à sua composição química, a cera de carnaúba apresenta temperatura de fusão de 85 °C. Seu comprimento médio da cadeia alterna-se em 50 átomos de carbono. Portanto, apresenta-se muito superior à de outras ceras vegetais e animais. Dessa forma, é classificada como uma cera vegetal mais complexa. Apresenta-se combinada por baixa quantidade de ácido palmítico e esteárico, que contém pares de alcoóis livres (C32 principalmente) e por ésteres de cadeia muito longa (C56 é o composto majoritário). Isso faz com que a cera possua baixa permeabilidade ao vapor de água devida sua grande hidrofobicidade (BRZOSKA et al., 2018).

Os empregos da cera de carnaúba são variados nos setores industriais, como os setores farmacêutico e alimentício. Na indústria de alimentos, é empregado para recobrimentos de frutas com o intuito de aumentar a vida útil e conservar a qualidade pós-colheita do produto (ZHANG et al., 2016). A camada de cera aplicada na superfície do produto vegetal exhibe diferentes taxas de permeabilidade ao O<sub>2</sub>, CO<sub>2</sub> e ao vapor de água. Isso se dá, de acordo com as propriedades da matéria prima, de sua concentração e da espessura da película (DANG; SINGH; SWINNY, 2008). Assim, as ceras são aplicadas para reduzir a perda de matéria fresca dos produtos. Além disso, confere brilho e não são tóxicas (ZHANG et al., 2016).

Yeon, Sea e Min (2018), trabalharam com revestimento de tangerina, usando extrato de semente de toranja – cera de carnaúba, incorporadas para a sua preservação e concluíram que o revestimento GSE – CW pode prolongar a vida útil pós-colheita das tangerinas, inibindo o crescimento de *P. italicum*.

Em estudos de SINGH et al., 2016 sobre revestimento comestível à base de cera de carnaúba, objetivando aumentar a vida de prateleira e qualidade das frutas de berinjela (*Solanum melongena*), concluiu-se que a eficácia dos revestimentos de emulsão CW, pode aumentar o tempo de armazenamento. Por consequência, aumentar a atividade antioxidante de berinjelas embaladas no período máximo de armazenamento durante a temperatura ambiente.

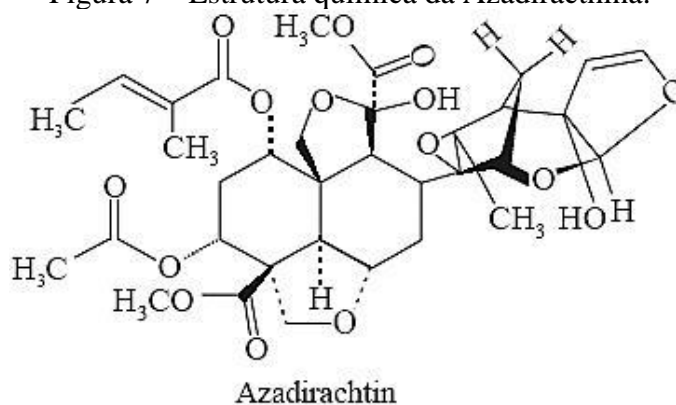
## 2.4 Óleo de Neem

A árvore Neem é uma planta milenar pertencente à família *Meliaceae*, conhecida pelo nome botânico *Azadirachta indica* A. Juss. É originária da Índia, nativa das zonas áridas do subcontinente. Pode atingir cerca de 30 metros de altura. A planta apresenta folhas verdes, raízes profundas, flores pequenas e brancas e frutos lisos. Nessas regiões, o Neem é considerado uma planta medicinal. Apresenta ainda relevante importância pelos seus efeitos positivos na saúde das plantações, animais e do próprio homem (BERNARDI; MEURER; ARANTES, 2012).

A espécie chegou ao Brasil em 1993, por iniciativa da Empresa Brasileira de Pesquisa (EMBRAPA). Em razão de seu rápido crescimento e de sua resistência excepcional à seca, torna-a propícia ao clima tropical brasileiro. Além disso, todos os produtos à base de Neem são completamente naturais e atóxicos aos seres humanos e meio ambiente (CHAUDHARY, 2017).

O óleo de Neem é extraído por prensagem a frio das sementes. É altamente eficaz contra insetos e pragas, devido à presença de dissulfureto em sua composição, que confere sua bioatividade. O óleo de Neem contém ainda triterpenóides, incluindo nimbina e salanina. Todavia, o principal contribuinte para sua atividade é a Azadiractina, ilustrada na Figura 7 (CHAUDHARY, 2017).

Figura 7 – Estrutura química da Azadiractina.



A Azadiractina, o principal limonóide isolado do Neem, apresenta várias atividades biológicas, altamente eficientes no controle de pragas (BERNARDI; MEURER; ARANTES, 2012; KOONA; BUDIDA, 2011). Também é utilizado para a produção de sabão, como matéria-prima para proteção de plantas, estoque e proteção têxtil, medicina e cuidados com

animais. Além disso, a Azadiractina é totalmente biodegradável e seu tempo de meia vida no solo é de cerca de 20 dias (NDE; BOLDOR; ASTETE, 2015).

Nesta vanguarda, a Organização das Nações Unidas para Agricultura e Alimentação, estima que um quarto das culturas alimentares mundiais são afetadas por micotoxinas. A produção e a contaminação de micotoxinas em sementes são inevitáveis. Dependem de uma variedade de fatores ambientais no campo e / ou durante o armazenamento, o que promove um desafio exclusivo para a alimentação (ELIAS, 2016). Por conseguinte, a presença de fungos toxigênicos do gênero *Aspergillus*, *Penicillium* em alimentos apresentam um perigo potencial à saúde (PALACIO; BETTUCCI; PAN, 2016). Para tanto, vários inibidores fúngicos químicos, têm sido empregados na preservação de sementes armazenadas. Entretanto, muitas desvantagens são pertinentes com seu uso. Destacam-se dentre elas, geração de resíduos tóxicos, desenvolvimento de resistência e auto custo. Nesse sentido, pesquisas têm evidenciado a correspondência entre crescimento micelial, produção de micotoxinas em fungos toxigênicos e compostos derivados do Neem (MOHANTY; RAGHAVENDRA; DASH, 2008).

Neste conjunto, produtos industriais à base de Neem têm sido comercializados, exibindo boa eficácia na agricultura para controle de doenças fúngicas. O óleo de Neem tem sido aplicado em cultivo contra fungos patogênicos do solo. Como resultado, vem promovendo a inibição do crescimento de algumas espécies dos gêneros *Aspergillus* e *Penicillium* (ALAM; HAMIM; ASHRAFUZZAMAN, 2014; MAHMOUD et al., 2011). Ademais, o composto Azadiractina, oferece mínimo ou nenhum impacto sobre os organismos não-alvo. É ainda compatível com outros agentes de controle biológico e mostra boa adaptação aos programas de manejo integrado de pragas.

Estudos empregando o extrato de folhas de Neem contra *Aspergillus flavus* também comprovaram o bloqueio da biossíntese de aflatoxina. Porém o extrato não foi efetivo sobre o crescimento fúngico (ALAM; HAMIM; ASHRAFUZZAMAN, 2014). Pesquisas com *Penicillium expansum*, relatam que o extrato bloqueia a produção da micotoxina patulina, produzida pelo fungo (MOSSINI; DE OLIVEIRA; KEMMELMEIER, 2004). Em trabalhos avaliando o potencial do Neem na inibição da produção de citrinina, por *Penicillium citrinum*, houve diferenças entre as características macroscópicas de colônias nos controle e tratamentos (MOSSINI; KEMMELMEIER, 2008). Hirose et al. (2001), avaliaram o efeito fungitóxico do óleo de Neem, juntamente com outros três biofertilizantes, contra dois fungos: entomopatogênicos, *Metarhizium anisopliae* e *Beauveria bassiana*. Foi observado um efeito

significativo do óleo de Neem sobre a germinação, produção de conídios e crescimento vegetativo dos dois fungos.

Assim, diante da necessidade continuada de estratégias no controle de fungos fitopatogênicos, utilizando extratos naturais que preservem o meio ambiente, o emprego do óleo de Neem demonstra-se como uma alternativa interessante. Este bioativo fornece um controle mais eficiente. Como melhoria tem-se a qualidade e rendimento de cultivos, principalmente com a elaboração de filmes para o revestimento de sementes.

## **2.5 Revestimento de sementes**

Os preceitos contemporâneos da cultura agrícola dirigem-se em passo acelerado para uma agricultura de precisão. Dessa forma, é necessário o aperfeiçoamento dos sistemas de cultivo. Conquista-se assim, de fato, o êxito técnico e econômico das práticas agrícola. No entanto, determinados fatores podem atrapalhar a agricultura de precisão. Estas, muitas das vezes, ainda estão fora de domínio dos pesquisadores. Como exemplo tem-se os problemas de armazenagem e uniformização de todos os estádios bem-sucedidos das plantas, desde a germinação até a colheita (IAVICOLI et al., 2017). Em meio as soluções indicadas para sanar os problemas de produção dessas espécies, o emprego da técnica de revestimento de sementes apresenta-se como uma alternativa interessante (FINCH-SAVAGE; BASSEL, 2016).

O conceito de recobrir sementes não é inovador. O recobrimento de sementes, desde a década de 90, evolui de maneira que atualmente se encontra no topo da indústria sementeira. Dessa forma, tem sido exigência crescente do mercado cada vez mais concorrente e com foco em agregação de valor às sementes (SANTOS, 2016). No entanto, no Brasil, para as amplas culturas, o revestimento de sementes ainda é considerado uma tecnologia nova. Isto, uma vez que, faltam conhecimentos técnico-científicos sobre o assunto (IAVICOLI et al., 2017). O revestimento de sementes trata-se de um tema de grande relevância, pois proporciona potencial para solucionar problemas essenciais como a proteção das sementes aos ataques de insetos e patógenos. Tais ações impulsionam progressos contínuos na sanidade das sementes e no estabelecimento das plântulas (SHARMA et al., 2015). Garantem também, segurança no uso desses produtos, pois cria um obstáculo entre a pele do operador e o produto. Por consequência, eliminam-se contratempos pertinentes ao tratamento, a embalagem e a semeadura. Além disso, resguarda a semente contra danos mecânicos decorridos do manuseio e transporte. Mais à frente, protege sementes armazenadas sob condições de umidade elevada. Em se tratando do controle de patógeno, o revestimento de sementes estabelece uma das

técnicas mais promissoras. Sua eficácia no tratamento de sementes está associada ao tipo do patógeno. Também vincula-se a disponibilidade da substância que está sendo empregada como revestimento (VERMA; VERMA, 2014).

O revestimento de sementes se dá através da deposição de uma camada fina e invariável de um polímero na superfície da mesma. Esta prática auxilia o manuseio e a distribuição das sementes, principalmente daquelas menores, pilosas e rugosas. As sementes com esse revestimento têm forma e tamanho semelhantes à semente sem cobertura (VERMA; VERMA, 2014). Nos últimos anos, a velocidade com que as pesquisas aperfeiçoaram as técnicas de revestimento fez com que tudo mudassem rapidamente. Isso, tanto sobre os produtos, como sobre as técnicas disponíveis na literatura, para as distintas espécies. O material polimérico passou a ser utilizado conjuntamente com o tratamento químico e biológico. Logo, produzindo um impacto mínimo sobre o meio ambiente. Assim, o interesse no desenvolvimento de revestimentos de polímeros biodegradáveis, empregando matérias-primas renováveis, principalmente as derivadas de produtos agrícolas têm crescido muito. Tudo isso, associado à possibilidade de redução do uso de materiais sintéticos que degradam o meio ambiente.

A incidência de microrganismos em sementes é responsável pela destruição das plântulas no campo. Neste contexto, engloba a ocorrência da perda de vigor e poder germinativo no armazenamento, acarretando redução do estande final no campo (KOPPAD; PANNEERSELVAM, 2016). Nesse sentido, o revestimento também tem sido aplicado com o intuito de proteger as sementes da ação de fungos (patógenos) que se encontram presentes tanto na semente como no solo. Deste modo há garantia de germinação e o desenvolvimento das plântulas no campo (ACCINELLI et al., 2016).

Vários estudos comprovaram a eficácia do revestimento de sementes. Rivas, McGee e Burris (1998), destacaram que o bom emprego de películas de revestimento em sementes de milho pôde restringir a invasão das mesmas, por fungos de armazenamento. Isto ocorreu por diminuição da absorção de umidade. Pastor et al. (2010), pesquisaram a atividade antifúngica de filmes ativos a base de hidroxipropilmetilcelulose (HPMC), contendo própolis, contra *Penicillium italicum* e *Aspergillus niger*. Os filmes apresentaram efetividade na ação antifúngica, sendo mais intensa contra *Penicillium sp.* Corrobora-se, nesse contexto que as características das substâncias ativas naturais da própolis estiveram conservadas. Os autores concluíram ainda que esses filmes podem ser aproveitados para o controle do ataque de fungos, em gêneros alimentícios e sugeriram novas pesquisas para um melhor aproveitamento desses filmes.



Mais recentemente, Accinelli et al. (2016) avaliaram uma formulação de bioplástico líquido pulverizável para aplicação de biocontrole em culturas agrícolas e hortícolas. A germinação de ambas as espécies não foi afetada pelo revestimento bioplástico fino. Os revestimentos bioplásticos contendo esporos do fungo promotor de crescimento de plantas, *Trichoderma harzianum*, estimularam significativamente o crescimento de sementes de milho e canola. Verma e Verma (2014) também concluíram que o revestimento de sementes com polímeros melhora a eficácia do fungicida. Logo, mantém-se uma germinação mais elevada da semente de soja após o armazenamento.

## 2.6 Revestimentos biopoliméricos

Os filmes e revestimentos biodegradáveis são aqueles desenvolvidos por meio do uso de polímeros naturais. Podem ser provenientes de origem animal ou vegetal. Apresentam a capacidade de compor uma matriz contínua e de coesão adequada, definido como uma macromolécula como polissacarídeos, lipídios e proteínas (NUR HANANI; ROOS; KERRY, 2014). Estes, quando lançados no meio ambiente, convertem-se em compostos simples, mineralizados. Logo, quando redistribuídos através dos ciclos de carbono, nitrogênio e enxofre, não agridem o biosistema (ILES; MARTIN, 2013). Recobrimentos são aplicados diretamente sobre o produto ou alimento a ser protegido. Já os filmes são estruturas independentes que podem ser utilizadas para envolvê-los, após sua fabricação (AGUIRRE-JOYA et al., 2017; FALGUERA et al., 2011).

A técnica de fabricação de recobrimentos e filmes pode ser alcançada por duas metodologias principais: o "processo seco" que se fundamenta no desempenho termoplástico que determinadas proteínas e polissacarídeos oferecem. Isso, a baixos níveis de umidade na extrusão e moldagem por compressão; e o "processo por via úmida", que engloba uma primeira etapa de solubilização da macromolécula em um solvente (água, etanol, solução de ácido acético, etc.) ao qual podem ser incorporados aditivos (plastificantes, agentes reticulantes, etc.). Na segunda etapa, a solução formadora do filme é espalhada sobre um suporte e submetida à secagem até desprendimento do molde ou aplicada no recobrimento de um produto. Este processo também é conhecido como *casting* (OLIVEIRA et al., 2015). O revestimento pode adaptar à irregularidade da superfície e prevenir a perda de elementos essenciais. Podem ainda proporcionar a fortificação de camadas naturais. Conseqüentemente impedem perdas de umidade. Além disso, tolera seletivamente a troca de gases, tais como oxigênio, dióxido de carbono e etileno. Ambos, envolvidos em processos de respiração. Não

obstante, podem atuar como barreiras ao soluto, gás e vapor d'água. Isso torna mínima a deterioração da qualidade dos alimentos e contribui para a vida útil dos mesmos (ELSABEE; ABDOU, 2013).

Além disso, revestimentos à base de biopolímeros podem atuar como transportes de diversos aditivos, abrangendo agentes antimicrobianos, antioxidantes, agentes corantes, e nutrientes (MONTAZER; HARIFI, 2017). Do mesmo modo, essa tecnologia pode ser empregada para tolerar condições de estocagem das sementes. Por meio do recobrimento, minimiza-se o acréscimo de umidade das mesmas e garante sua qualidade. Isto porque, até uma pequena elevação do grau de umidade da semente é suficiente para restringir sua capacidade de germinação (CHOI; SINGH; LEE, 2016; KAVAK; ESER, 2009).

No entanto existem alguns requisitos específicos para o uso deste biopolímeros, tais como: boa aceitabilidade sensorial, propriedades mecânicas e de barreira adequadas, estabilidade bioquímica, físico-química e microbiológica, ser não-polvente, apresentar processamento simples e baixo custo (SALGADO et al., 2015). Os materiais aproveitados para a fabricação destes revestimentos biopoliméricos podem ser divididos em quatro categorias: hidrocolóides, lipídios, resinas e compósitos. Os lipídios compreendem ceras, glicerídeos e ácidos graxos. Como exemplos de resinas têm a goma-laca e a resina de madeira. Hidrocolóides compreendem polissacáridos tais como amido, derivados de celulose, pectinas, alginatos e as proteínas, tais como gelatina, colágeno, caseína, proteína do soro de leite, zeína do milho. Mais adiante, a associação de elementos lipídicos sob a forma de dupla camada ou emulsão, juntamente com hidrocolóide, resulta na formação de um revestimento compósito (GÓMEZ-ESTACA et al., 2016). Além de biodegradáveis, é vantajoso o emprego destes polímeros, uma vez que são de ampla disponibilidade na natureza, vasta abundância de estruturas, biocompatibilidade, atoxicidade e apresentam elevado leque de propriedades funcionais (LESKINEN et al., 2017).

### **2.6.1 Propriedades funcionais de revestimentos e filmes biodegradáveis**

As propriedades dos revestimentos biopoliméricos podem ser destacadas com base na funcionalidade das mesmas. Dentre as inúmeras funcionalidades destaca-se: domínio da migração de água; permeabilidade ao oxigênio e migração lipídica em um sistema alimentício multifásico (GUILLARD; GASTALDI; PEYRON, 2018). Todavia, as funcionalidades destas embalagens são alteradas pela estrutura molecular do polímero. Por sua vez, estão, portanto, sujeitas à composição de sua formulação, do plastificante empregado, entre outros aspectos

(OLIVAS; BARBOSA-CÁNOVAS, 2008; VERSINO et al., 2016). Tomando como exemplo, os polissacáridos são hidrocolóides de elevada massa molar. São solúveis em água e constituem ligações de hidrogênio ativas com a mesma. Conforme a configuração molecular apresenta a capacidade de se espessar e/ou desenvolver um gel em solução aquosa. Isto acontece, devido ao efeito da ligação de hidrogênio entre as cadeias dos polímeros (LOVEGROVE et al., 2017). Por consequência apresentam-se barreiras eficazes aos gases, aroma, e lipídios e conferem boa proteção mecânica. Contudo, são bastante permeáveis ao vapor d'água. Devido à sua natureza hidrofílica proporcionam pobre barreira ao vapor d'água (MATTER, 2017). Assim, para melhorar as propriedades dos filmes à base de polissacarídeos, a adição de material lipídico constitui-se de uma alternativa interessante. Isto, uma vez que seria possível o desenvolvimento de material com boas propriedades mecânicas e de barreira ao vapor de água, em virtude de sua característica hidrofóbica.

Por outro lado, filmes de lipídeo puro atuam como barreira à umidade, mas possuem propriedades mecânicas deficitárias. A barreira à umidade dos filmes baseados em lipídios também se altera com a polaridade do lipídio. Sua polaridade está sujeita ao grupo químico, comprimento da cadeia alifática e grau de instauração. Quanto maior a polaridade, mais hidrofílico e menor a propriedade de barreira à umidade (CAZÓN et al., 2017; MORILLON et al., 2002).

As propriedades mecânicas e de barreira destes revestimentos compostos são alterados pela constituição e homogeneidade das substâncias hidrofóbicas na matriz. Assim sendo, os revestimentos compostos visam associar lipídios e polissacarídeos ou proteínas para aprimorar suas propriedades de barreira. Os polissacarídeos promovem a integridade da película, compondo uma matriz para os lipídios, enquanto estes formam uma barreira à umidade (PASCALL; LIN, 2013).

Diversas tecnologias como compósitos, nanocompósitos dentre outras são empregadas para adicionar lipídios em filmes. Estes podem ser constituintes dos filmes, compondo uma camada sobre a matriz polimérica ou desenvolver uma emulsão com o polímero (FABRA et al., 2018). Neste cenário, Yang e Paulson (2000) ressaltaram que a adição de ceras, ácidos graxos saturados de cadeia longa e álcoois graxos apresentaram-se eficaz na redução da PVA de filmes à base de gelatina. As particularidades de um filme adicionado destes compostos estão sujeitas à ligação dos lipídios à matriz polimérica. Sartori e Menegalli (2016), produziram filmes constituídos por amido isolado de bananas da variedade "Terra" incorporada com micropartículas lipídicas contendo ácido ascórbico. Como resultado da fração lipídica que envolve as partículas presentes no material de embalagem, as películas

adicionadas com micropartículas lipídicas contendo ácido ascórbico apresentaram menor permeabilidade e alongamento ao vapor de água, bem como, uma maior resistência à tração em comparação com um filme de controle que não contém aditivos.

Por hora, cabe mencionar que as funcionalidades dos biopolímeros ainda podem ser aplicadas aos revestimentos, aproveitando determinadas características que podem estar em contato com o produto envolvido ou ainda apenas atuando como sistema de embalagem bioativa. Na última década, uma pesquisa emergente sobre filmes ativos de embalagem, combinando as boas propriedades gerais do polímero (mecânica, barreira, óptica e térmica) com a inclusão de aditivos com propriedades antioxidantes/antimicrobianas (aromas, especiarias e corantes) foram destaque.

## **2.7 Embalagens bioativas**

As embalagens constituem um importante instrumento para indústrias alimentícias, pois conferem, de tal modo, proteção e garantem qualidade ao produto. Além disso, auxiliam durante o armazenamento, propiciando uma maior vida de prateleira, principalmente em alimentos susceptíveis a oxidação e deterioração microbiana (BANERJEE et al., 2016).

Nesse contexto, embalagem ativa é um dos conceitos mais inovadores de embalagem de alimentos. Logo, o desenvolvimento de filmes ativos de embalagens antifúngicas com óleos essenciais é de grande interesse, e o presente estudo é baseado nesta idéia (LEITES et al., 2017). As principais vantagens de usar essa tecnologia para o aplicativo de agentes antifúngicos naturais em alimentos são a liberação controlada do compostos bioativos para o produto durante o tempo de armazenamento. Além disso, menor possibilidade de desenvolvimento de sabores indesejáveis pela adição direta em alimentos (PEREIRA DE ABREU; CRUZ; PASEIRO LOSADA, 2012; VALDÉS et al., 2017).

Dentre inúmeras aplicações de filmes à base de biopolímeros com adição de um composto ativo natural são demonstrados alguns exemplos na Tabela 1. As embalagens ativas têm se desenvolvido cada vez mais com os progressos da nanotecnologia. Essas embalagens são capazes de conferir amplos benefícios para indústria com o intuito de aperfeiçoar o frescor e a vida útil. Bem como, buscam admitir monitoramento do produto desde o local de fabricação, condições de armazenamento, até o consumo pelo consumidor final (WILSON et al., 2017).

Tabela 1 – Aplicações de filmes à base de macromoléculas naturais com adição de compostos ativos.

<b>Macromolécula</b>	<b>Composto ativo</b>	<b>Alimento</b>	<b>Resultado</b>	<b>Fonte</b>
Gengibre de jujuba	Óleo essencial de urtiga	Esturjão	Novo material antimicrobiano e antioxidante, menor peso e deterioração de texturas e cor.	(MOHAMMAD; GHARIBZAHEDI; MOHAMMADNABI, 2017)
Gelatina	Extrato aquoso de henna	Carne de bovino	Excelente proteção para evitar a deterioração da carne.	(JRIDI et al., 2018)
Caseinato de sódio	Óleo essencial de gengibre	Filé de peito de frango	Diminuição significativa da bactéria aeróbica total em frango refrigerados durante 12 dias.	(NOORI; ZEYNALI; ALMASI, 2018b)

Fonte: Do autor (2019).

Neste contexto, o emprego de sistemas de liberação em nanoescala estimula potencialmente a atividade antimicrobiana quando comparado aos sistemas convencionais (DONSÌ et al., 2012). O uso de nanoemulsões como antimicrobianos ainda é uma ciência nova e não integralmente elucidada. No entanto, acredita-se que quanto menor o tamanho médio da partícula, máxima sua biodisponibilidade (DONSÌ et al., 2014). Pequenas partículas com elevada área superficial podem fundir-se com células e membranas eucarióticas e procarióticas rompendo-as, com forte agilidade antimicrobiana. Diminui-se a amostra necessária de princípio ativo, induz à redução dos custos e menor contato de trabalhadores com agrotóxicos (BURANASUKSOMBAT et al., 2011). Desta forma, optar pela utilização de embalagens biodegradáveis em nanoescala ajusta benefícios financeiros a proteção e segurança direta de trabalhadores.

Em meio aos compostos ativos empregues para fabricação de filmes à base de fontes renováveis como pectina, tanto o óleo de Neem, como a cera de carnaúba, merecem atenção em virtude de suas propriedades funcionais, tais como suas propriedades antimicrobianas (MISHRA et al., 2014). Todavia, estudos com a incorporação destes em filmes à base de pectina ainda não foram descritos na literatura.

Logo, com a busca por tecnologias comercialmente viáveis e da ausência de trabalhos científicos sobre a formulação de revestimentos compostos à base de nanoemulsões antifúngicas e biodegradáveis de cera de carnaúba e óleo de Neem em matriz de pectina, objetivou-se com a pesquisa formular revestimentos à base dessas matérias-primas nacionais, visando minimizar a influência da umidade sobre a qualidade fisiológica de sementes de soja.

### 3 CONCLUSÃO GERAL

Observou-se no estudo I que as nanoemulsões produzidas provaram ser estáveis por forças de dispersão de origem estérica. O tamanho médio de partículas permaneceu em torno de  $69 \pm 0,63$  nm e índice de polidispersividade de  $0,3 \pm 0,01$ . Os resultados mostram que a melhor formulação foi conseguida por agitação da nanoemulsão durante 10 min com uma concentração de tensoativos de 1,5% (w/w) na proporção Tween/lecitina (100:0 w/w). No estudo II, observou-se que a formulação de nanoemulsão contendo o óleo Neem, foi otimizada com êxito pelo método de alta energia. A nanoemulsão de óleo de Neem com o menor tamanho de partícula foi encontrada como sendo mais efetiva no controle antifúngico dos gêneros *Aspergillus flavus* e *Penicillium citrinum*. Em nosso estudo, confirmou-se que a nanoemulsão à base de óleo de Neem é eficiente no controle dos fungos estudados e não apresenta efeitos fitotóxicos para as sementes. Já no estudo III, verificou-se que os filmes de pectina HDM com as diferentes concentrações de nanoemulsões foram produzidos com sucesso, apresentando características de materiais homogêneos, compactos e contínuos. Estes materiais apresentaram boa estabilidade térmica e com interações intermoleculares entre as nanoemulsões e a matriz polimérica, resultando no aumento da barreira ao vapor de água e materiais com aumento na elongação. A incorporação do óleo de Neem nos filmes de pectina, causou aumento na resistência à tração quando comparado a cera de carnaúba e redução no módulo elástico. No processo de biodegradação, os materiais nanocompósitos, principalmente com óleo de Neem, apresentaram excelentes resultados quanto a não formação de macro-resíduo no solo. Por fim, o estudo IV confirmou-se que a elaboração de filmes nanocompósitos compostos de nanoemulsão de óleo de Neem à base de pectina é viável. Considerando as imagens de SEM, permitiram visualizar que a adição de Noil não alteraram substancialmente as matrizes de pectina. Isto, aliada ainda a atividade fungicida fornecida pelo óleo de Neem contra patógenos atuantes no armazenamento de sementes. Notavelmente esta ação foi melhorada pelo aumento da concentração de nanoemulsão. O processo de revestimento das sementes não altera o grau de umidade das mesmas. Os filmes de nanoemulsões são, portanto, um material antimicrobiano ecologicamente promissor para aplicações nos revestimentos de sementes.

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**SEGUNDA PARTE – ARTIGOS****ARTIGO 1 – PRODUCTION AND STABILITY OF CARNAUBA WAX  
NANOEMULSION**

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**ABSTRACT:** The study and manufacture of nanoemulsions from regional products has the potential to develop materials with optimized properties and add value to them. In this context, we evaluated the process of obtaining and analyzing nanoemulsions of carnauba wax, a Brazilian semiarid species. The method employed was high energy by ultrasound. The objective was to contribute to a better understanding of the relationships between production parameters and the properties of the nanoemulsions obtained. For this, they were characterized by measurements of particle size distribution, polydispersity index, zeta potential and pH. Nanoemulsions were synthesized at different concentrations and proportions of surfactants. The mean particle size remained around  $69 \pm 0.63$  nm and polydispersity index of  $0.3 \pm 0.01$ . It was observed that the increase in surfactant concentrations and reduction in the emulsification time provided the nanoemulsions with smaller diameters. For pH values, the mean values of 7.5 to 8.5 were close in all treatments over time, confirming the absence of acids derived from surfactant hydrolysis. Stability monitoring after 10 days of production of the obtained nanoformulations confirmed good homogeneity of the formulations that remained stable, restricting the effects of coalescence on the system. These results open new opportunities compatible with industrial needs, contributing to cost reduction, thus leading to more sustainable treatments.

**Keywords:** Nanoemulsion, carnauba wax, high energy, stability, particle size.

## 1. INTRODUCTION

Nanotechnology has emerged as one of the most promising fields of research. Thus, new natural source nanomaterials have been originated since they are biodegradable and non-toxic.<sup>1</sup> As an example, we have carnauba wax, *Copernicia cerifera*, an endemic species of the Brazilian semiarid region. It consists of a complex mixture of esters, alcohols, aliphatic acids, hydrocarbons and triterpene diols. The esters corresponding to 80% of the chemical composition consist of aliphatic esters and diesters of cinnamic acid, which has antimicrobial properties.<sup>2,3</sup> It is commonly used as a coating to preserve food quality due to the high hydrophobicity related to long chain acids and alkanes.<sup>4,5</sup>

In this scenario, there is a growing interest in the use of nanoemulsions due to changes in physical-chemical properties and performance associated with changes in the size of these materials.<sup>6</sup> The reduction of particle size has favorable consequences such as greater particle stability and gravitational separation; greater clarity; and enhanced bioavailability of hydrophobic and antimicrobial substances.<sup>7</sup> Conceptually, nanoemulsions are colloidal dispersions containing reduced particles (20-100 nm diameter) dispersed in aqueous medium, dependent on the production process.<sup>8</sup>

High energy emulsifying methods using ultrasound exhibit advantages such as reduced size, lower energy consumption and minimum use of surfactant. More homogeneous samples are also obtained compared to conventional mechanical processes.<sup>9</sup> The process of acoustic cavitation in the liquid medium due to ultrasonic intensity is responsible for the production of turbulence.<sup>10</sup> These intense shear forces induce excitations at the interface of immiscible phases, facilitating the production of stable nanoemulsions.<sup>6</sup> Droplet sizes can be controlled by optimization of parameters such as potency, nature and proportion of surfactants and emulsification time. Other parameters such as viscosity and lipid volume also affect nanoscale particle formation.<sup>9,11</sup> However, the ability to create nanoscale emulsion and subsequent Ostwald maturation are important challenges in the formulation of nanoemulsions.<sup>12</sup>

In this context, the objective of this work was to evaluate the effect of the variables on carnauba wax nanoemulsions. The implication on particle size distribution and its stability was evaluated in terms of operational variables such as the time of application of the ultrasound, the concentration and the proportion of surfactants used.

## 2. EXPERIMENTAL DETAILS

### 2.1. Materials

Carnauba wax type 1, Megh Wax ECF-124 (melting point 82-85 °C) produced by Megh Indústria e Comércio Ltda, Brazil. The surfactants were soybean lecithin (Imcopa S / A, Brazil) and Tween 80 (Polysorbate 80) (Sigma-Aldrich). In addition, ultrapure water of one Milli Q, 18.2 MΩ.cm at 25°C was used in the preparation of the nanoemulsions. All chemicals used were of analytical grade unless otherwise noted.

### 2.2. Preparation of the Nanoemulsion

Nanoemulsions were prepared by the technique with adaptations proposed by Jadhav et al.<sup>13</sup>, formulated using 5% w/w carnauba wax as the lipid phase, nonionic surfactants Tween 80, soy lecithin and water as the aqueous phase. Initially the mixture of the surfactant solution and water was heated to 65-70°C, then the molten wax added at a rate of 1 ml/min in vigorously magnetic stirred aqueous solution (Model RQ1210, Remi Metals Ltd. Gujarat, India) at 1000 rpm. After complete addition of the required amount of wax, the emulsion prepared was sheared using a Branson Digital-Model 450 sonicator, with a maximum power of 400 W. The homogenization processes were performed for different time at the maximum power density. After preparation, the nanoemulsions were packed in Falcon tube (15 mL) and stored at 20 °C for up to 10 days in the absence of light.

#### 2.2.1. Experimental Design

A complete factorial design was selected to optimize the production of nanoemulsions. Concentration of surfactants (**X1**), tween 80 / soybean lecithin ratio (**X2**) and homogenization time (**X3**) were considered critical factors (Dependent Variables), as presented in Table I. These variables were selected based on the literature.<sup>14-17</sup> The particle size (**Y1**) and polydispersity index (**Y2**) were considered the independent variables.

Table I – Experimental design to production nanoemulsion using full factorial.

Trials	Coded variables – Dependent variables			Real variables		
	X1	X2	X3	Surfactants (%)	Ratio tween/ soy lecithin (%)	Time (min)
1	-1.00	-1.00	-1.00	0.5	60/40	10
2	1.00	-1.00	-1.00	1.5	60/40	10
3	-1.00	1.00	-1.00	0.5	100/0	10
4	1.00	1.00	-1.00	1.5	100/0	10
5	-1.00	-1.00	1.00	0.5	60/40	15
6	1.00	-1.00	1.00	1.5	60/40	15
7	-1.00	1.00	1.00	0.5	100/0	15
8	1.00	1.00	1.00	1.5	100/0	15

## 2.3. Characterization of Nanoemulsion

### 2.3.1. Physical and Chemical Characteristics

The FTIR spectrum of the raw material carnauba wax was obtained in the Shimadzu FT-IR (Prestige) spectrophotometer. The spectrum was collected in the region of 400 - 4000  $\text{cm}^{-1}$  with an average of 32 scanning with spectral resolution of 2  $\text{cm}^{-1}$ . About 15 mg of sample was mixed with about 500 mg of KBr (potassium bromide) by trituration in a mortar. The sample was placed in a cell with a nitrogen atmosphere.

The thermal behavior of the raw material was characterized using differential scanning calorimetry (DSC). DSC curves were obtained using a DSC-60 Shimadzu. Approximately 3 mg of sample was placed in a sealed aluminum pan. The analysis was performed at 30 to 300  $^{\circ}\text{C}$  at a heating rate of 10 $^{\circ}\text{C}/\text{min}$ . These samples were heated in an inert atmosphere of nitrogen at a rate of 50  $\text{ml}/\text{min}$ .<sup>18</sup>

### 2.3.2. Determination of Particle Size, Polydispersity Index (PDI), and Zeta Potential ( $\zeta$ )

Particle size distributions were measured using a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). This equipment determines the size of the particles from the relative intensity of the fluctuations dispersion of the sample with a laser beam (633 nm) at a 90 $^{\circ}$  angle. Each sample was evaluated with an average of 5 replicates. Prior to measurements to avoid multiplication of dispersion effects and to ensure free Brownian motion of the particles, the samples were diluted (x 100) using a buffer

solution (pH 3.0). The mean particle diameter (DLS) and polydispersity index (PDI) were calculated from the particle size distribution. The surface charge of the nanoemulsion particles was determined by measuring the electrophoretic mobility at 25 °C and zeta potential values were expressed in mV. All measurements were performed at room temperature.<sup>13</sup>

#### **2.4. Emulsion Stability Tests**

The effect of stress in nanoemulsion centrifuge was evaluated. Both were centrifuged (Biofuge, Heraeus, Germany) at room temperature, 2000 rpm for 15 min. This process was performed after nanoemulsion production and after 10 days of storage. Macroscopic changes such as sedimentation, cremation and separation of phases were observed and reported. Samples were stored in Falcon tube containing 15 ml at  $20 \pm 2$  °C.

Changes in the size, polydispersity index and zeta potential of the particles were measured at designated time points, that is, on the day and after 10 days of production, with NanoZS Zetasizer (Malvern Instr., Malvern, UK).

PH was determined every 24 hours using a Tecnal pH meter calibrated with a buffer solution of 4.0 and 7.0. The determination was made directly on the nanoemulsions. All measurements were made in triplicate and the results were reported as mean results.<sup>19</sup>

#### **2.5. Statistical Analysis**

All analyzes were performed with three or five replicates and the results are presented as the calculated mean and standard deviation of these measurements. Statistical analysis was performed using Statistica Analysis Systems 8.0 software. ANOVA and Tukey test were used to determine statistically significant differences between means ( $p \leq 0.05$ ).

### **3. RESULTS AND DISCUSSION**

#### **3.1. Physical and Chemical Characteristics**

Infrared absorption spectroscopy analysis of carnauba wax (Figure 1A) identified the presence of characteristic broad bands at  $3446 \text{ cm}^{-1}$  for the intermolecular -OH group elongation vibration; Absorption peaks at 2918 and  $2849 \text{ cm}^{-1}$  assigned to the symmetrical stretching vibrations of the -CH<sub>3</sub> and -CH<sub>2</sub> groups, respectively; Also ester groups ( $1738$ ,  $1717$ ,  $1169 \text{ cm}^{-1}$ ), unsaturated ( $1630$ ,  $930 \text{ cm}^{-1}$ ); Peaks at  $1463$  and  $719 \text{ cm}^{-1}$  representing absorption characteristics for the bending vibration of -OH groups and p-methoxy aromatic

functional group ( $1020\text{ cm}^{-1}$ ). This spectrum is similar to that obtained by Xia et al.;<sup>20</sup> Andréa et al.,<sup>3</sup> and is characteristic of p-methoxycinnamic diester.

In the thermogram in Figure 1B, the wax sample exhibits two endothermic peaks in the heat flow curve located at  $78\text{ }^{\circ}\text{C}$  and  $82\text{ }^{\circ}\text{C}$  due to  $\Delta H_m$  fusion of  $-6.27\text{ Jg}^{-1}$  and  $-12.07\text{ Jg}^{-1}$  respectively. This melting value ensures that the wax nanoemulsions produced are not undergoing thermal degradation events during the ultrasonic process, whose maximum temperature observed during the process was equivalent to  $62\text{ }^{\circ}\text{C}$ . Similar values were observed by Xia et al.;<sup>20</sup> Andréa et al.<sup>3</sup> Pantelli et al.<sup>21</sup> show melting points at  $62^{\circ}\text{C}$  and  $88^{\circ}\text{C}$  for methyl ferulic cinnamic acid and methyl sinapinic acid esters, respectively.

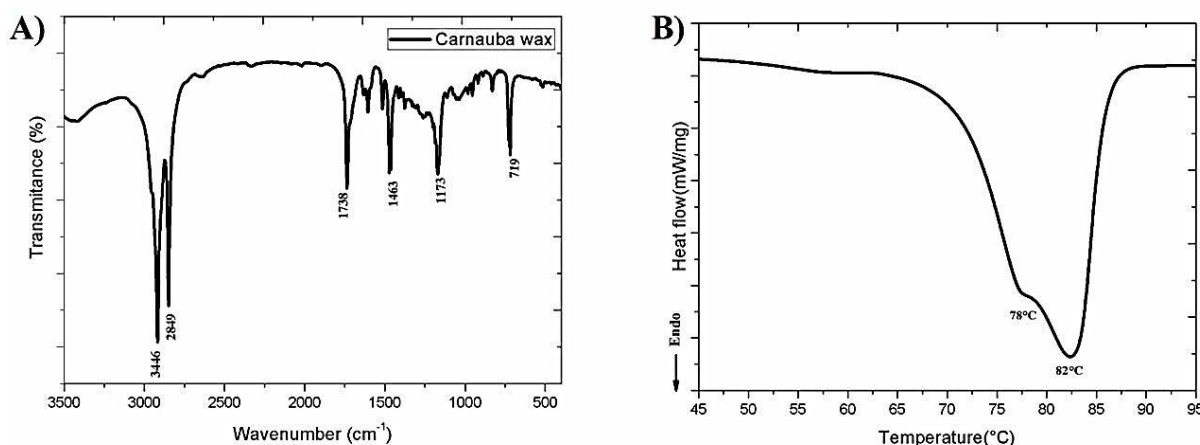


Figure 1 – Physical-chemical wax characteristics: (A) infrared spectrum; (B) DSC thermograms.

### 3.2. Fitting the Response Surface Models

In this work, the potential of ultrasound application to obtain stable nanoemulsions, minimizing the need for energy was studied. To this end, an emulsion containing 5.0% (w/w) carnauba wax and 0.5% and 1.5% (w/w) of a mixture of Tween 80 and soy lecithin was considered. This emulsifying mixture has previously been reported to form highly stable emulsions, due to the formation of a blend layer of nonionic surfactants on the emulsion particles.<sup>14,22,23</sup> Non-ionic emulsifiers, such as Tweens, are known to be absorbed rapidly on the particle surface during homogenization without undergoing structural modifications.<sup>24</sup> The evaluation was performed based on the lower, intermediate and higher levels of the independent variables.

Table II shows the experimental data for each response variable under the different independent variables according to the complete factorial.

Table II – Particle size (nm), polydispersion index (PDI), and zeta potential ( $\zeta$ ) for each trial.

Trials	Real variables			Independent variables		
	Surfactants (%)	Ratio tween/soy lecithin (%)	Time (min)	Particle size (nm)	Polydispersion index (PDI)	Zeta potential ( $\zeta$ )
1	0.5	60/40	10	78.57 $\pm$ 1.47b	0.36 $\pm$ 0.01ab	0.0 $\pm$ 0.30b
2	1.5	60/40	10	72.78 $\pm$ 2.87a	0.4 $\pm$ 0.02ab	-0.05 $\pm$ 0.41b
3	0.5	100/0	10	69.18 $\pm$ 0.73a	0.28 $\pm$ 0.02a	-0.14 $\pm$ 0.31b
4	1.5	100/0	10	69.28 $\pm$ 0.63a	0.3 $\pm$ 0.00a	-0.1 $\pm$ 0.34b
5	0.5	60/40	15	90.08 $\pm$ 1.18abc	0.39 $\pm$ 0.02bc	-0.07 $\pm$ 0.29b
6	1.5	60/40	15	68.54 $\pm$ 1.98a	0.45 $\pm$ 0.03b	-0.09 $\pm$ 0.28b
7	0.5	100/0	15	100.55 $\pm$ 14.41c	0.45 $\pm$ 0.10b	-0.61 $\pm$ 0.13a
8	1.5	100/0	15	84.46 $\pm$ 3.26b	0.5 $\pm$ 0.07c	-0.17 $\pm$ 0.13b

Note: Values in columns labeled with different letters are significantly different ( $p \leq 0,05$ ).

The results show that the lower particle sizes and polydispersity were achieved by agitating the nanoemulsion for 10 min at a surfactant concentration of 1.5% (w/w) in the Tween / lecithin ratio (100/0 w/w). Response surface models (Figures 2 and 3) allowed prediction of variations in particle size and polydispersity index as a function of the nanoemulsion composition of carnauba wax.

Response surface analyzes showed significant relationships ( $P \leq 0.05$ ) between the independent variables and the response variables. Analysis of variance (ANOVA) was used to evaluate coefficients and that large  $f$ -values and small  $p$ -values showed significant effects on the respective response variables. From the data obtained, the emulsification time (x3) had a more significant effect ( $p \leq 0.0001$ ) on the particle size with a high value (63.51), followed by the linear term of the surfactant concentration (x1) ( $p$  - value 0.0001 e - value 41.14). However, for the proportion of surfactants (x2) a non-significant effect ( $p \geq 0.05$ ) on particle size was found, since it showed the lowest  $f$ -value (3.98).

ANOVA showed similar behavior on the significant effect on the PDI of the nanoemulsion, mainly influenced by the emulsification time (x1) with a significant scale effect ( $p$ -value 0.000001,  $f$  - value 36.72), again followed by the concentration of surfactant

(x1) ( $p$ -value 0.001089,  $f$ -value 12.81), while the proportion of surfactants (x2) remained insignificant ( $p \geq 0.05$ ) as for the other variable.

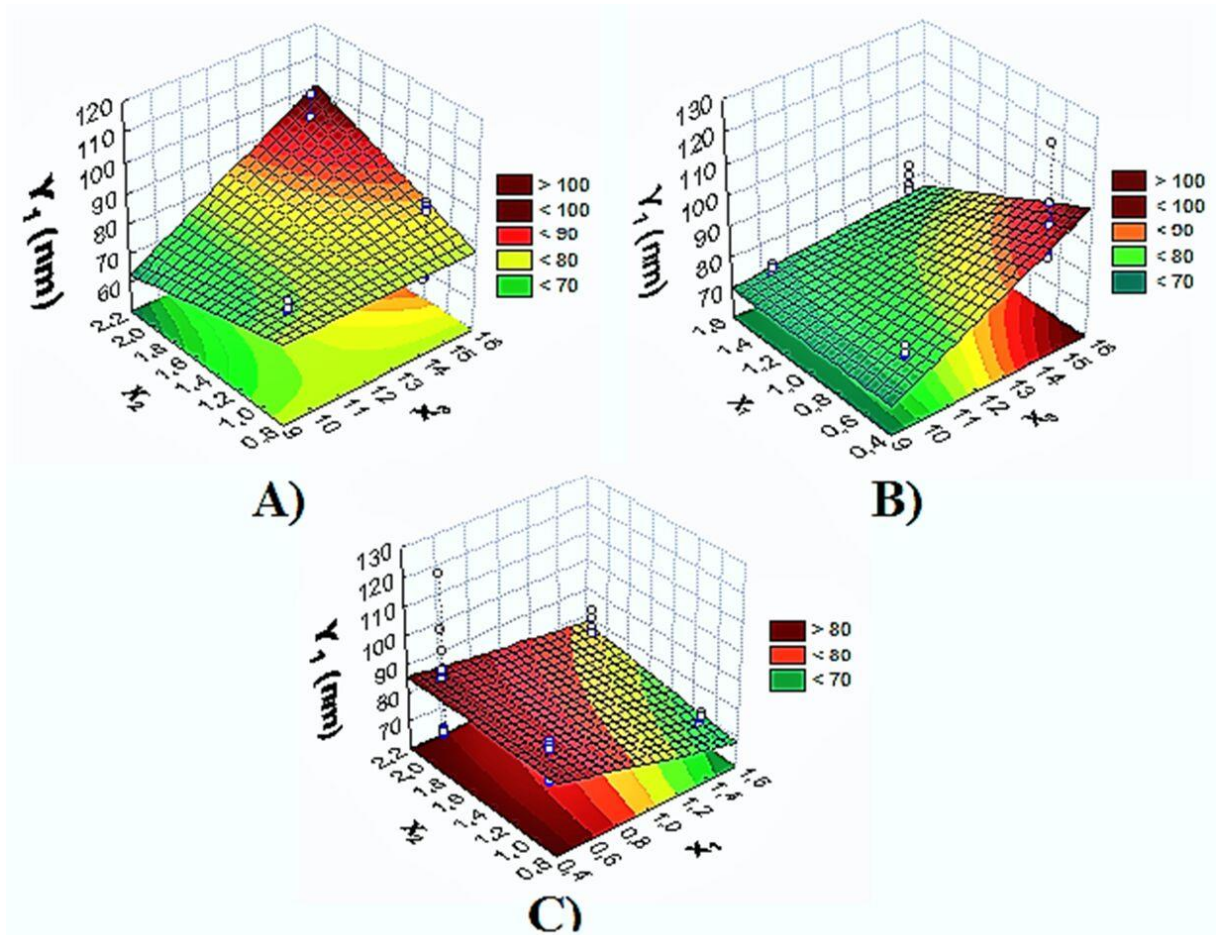


Figure 2 – Response surface variation DLS on the proportion of the surfactants (x2) and time (x3) (A), the concentration of surfactant (x1) and time (x3) (B) and the proportion of tensitivos (x2) and surfactant concentration (x1) (C) in the formulation of lipid particles.



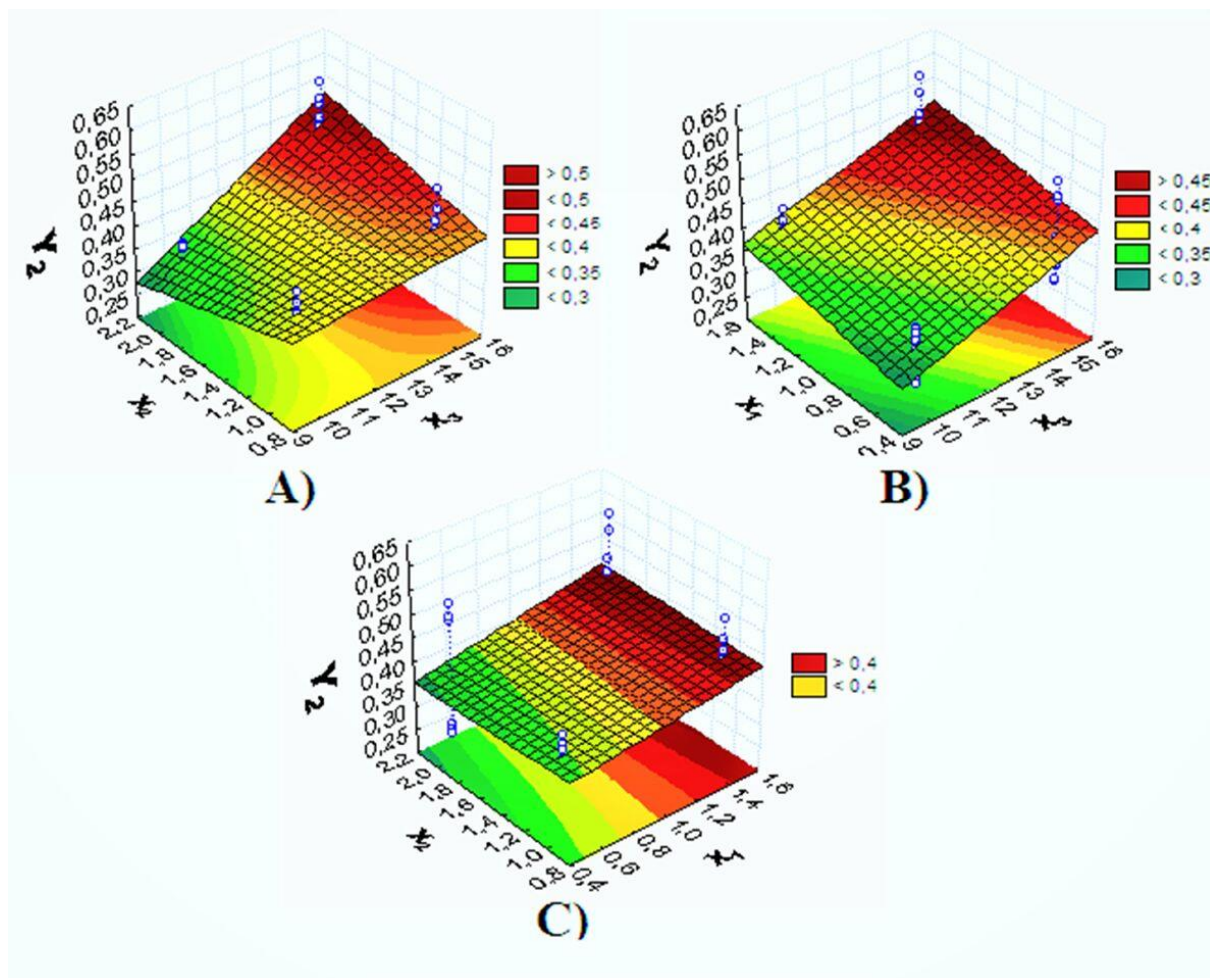


Figure 3 – Response surface variation of the PDI based on the proportion of the surfactants (x2) and time (x3) (A), the concentration of surfactant (x1) and time (x3) (B) and the proportion of tensiatives (x2) and surfactant concentration (x1) (C) in the formulation of lipid particles.

### 3.3. Response Surface Analysis

#### 3.3.1. Particle Size and Polydispersity Index (PDI)

The effects of the concentration and proportion of surfactants on the mean diameter and the polydispersity index were shown (Figures 2 and 3). It was observed that the average particle diameter of the systems measured by the dynamic light scattering presented smaller particle size, the higher the amount of surfactant used (1.5% w/w) and the optimum tween / lecithin ratio (100:0 w/w), which has been reported in other studies.<sup>14</sup> This tendency can be attributed to the fact that there were more surfactant molecules available to cover the oil-water interfaces, which allowed smaller particles (larger surface area) to be formed.<sup>25</sup> In addition, a higher concentration of surfactant typically leads to rapid coverage of the surfaces of the oil particles. As a consequence, there is a reduction in interfacial tension (facilitating the rupture

of the droplet) and leading to a faster formation of a protective coating (inhibiting coalescence).<sup>26</sup> These results indicate nanoemulsions containing small diameters ( $D < 70\text{nm}$ ) as shown in Table 2. It should be noted that even those emulsions prepared using lower concentrations of surfactant (0.5% w/w) obtained small average particle diameters ( $D < 100\text{nm}$ ) and narrow distributions ( $\text{PDI} < 0.5$ ).

In relation to the proportion of surfactants used there were no significant differences between the particle sizes of the nanoemulsions produced, however, systems with tween/lecithin ratio (100:0 w/w) presented smaller diameters. Surfactants, such as polysorbates (tweens), are very effective in reducing the size of the oil particles.<sup>27</sup> This observation can probably be explained by the hydrophilicity of the different emulsifiers. As demonstrated by Tan et al.,<sup>28</sup> emulsifiers with higher hydrophilicity can wrap and stabilize the O/W emulsion particles more efficiently, resulting in smaller particles. The hydrophilicity of emulsifiers is measured in terms of hydrophilic-lipophilic balance (HLB) values, with higher numbers indicating greater hydrophilicity. The HLB values of the emulsifiers used in this study were 15.0 and 8.0 for Tween 80 and lecithin, respectively.<sup>29</sup> The higher HLB value of Tween 80 may be responsible for smaller particle sizes in the emulsions produced with this emulsifier. Relatively small differences in the HLB values between the Tween/lecithin combination (60/40 w/w) may explain the lack of a consistent pattern in the particle size of the emulsions prepared with both.<sup>30</sup>

The polydispersity index is a measure of the particle size distribution, thus measures the homogeneity of the Nanoemulsion.<sup>31</sup> It is an important factor for stability assessment.<sup>19</sup> In this study, the PDI did not follow the same trend as the particle diameter, exhibiting relatively small change with the reduction of the concentration of surfactants. This fact was also observed in Saberi et al.<sup>25</sup> In relation to the proportion of surfactants employed, the behavior similar to that observed for particle diameter was observed. Lower indices, as seen in Table II, were achieved using only Tween as a surfactant. However, PDI values remained in the range of 0.2 to 0.5 for all formulations studied. This reflects on the homogeneous distribution of the nanoparticles.<sup>32</sup>

Regarding the time variable, both for particle size and for PDI it was reported that homogenization times of 10 min. presented lower values for both. Similar studies to this in the preparation of nanoemulsion employing ultrasound also obtained time as the best for the nanoemulsion production process.<sup>19</sup> However, higher homogenization at this time (15 min) contributed to the instability of the colloidal particles by the high energy input which leads to coalescence.<sup>33,34</sup> Prolonged energy intensity by ultrasound is generated enough impact forces

to initiate collision of the droplets. These collision rates will disrupt the oil-water interfacial layer.<sup>35</sup> Consequently, the larger particles are formed, as shown in Fig. In conclusion, 10 min homogenization time optimizes emulsification due to the smaller nanoemulsion size, 69 nm.

Similar to this study, Tadros et al.,<sup>36</sup> found small particle diameters employing lower concentrations of surfactants associated with reduced energy consumption. These nanoemulsions were also polydispersed and more stable.

### ***3.3.2. Zeta potential***

The zeta potential is used to predict the stability of colloidal systems since it indicates the magnitude of the surface charge in particles and thus the degree of repulsion that prevents coalescence.<sup>37</sup> The zeta potential of nanoemulsions is shown in Table II. Low values in all prepared nanoemulsions may be due to the greater amount of Tween-80 used in the formulations. The surface charge is attributed only by lecithin.

Electrostatic and steric repulsion are the major stabilizing forces for nanoemulsions.<sup>38</sup> In this case, the dispersion forces are of steric origin and generated by the adsorption of this neutral molecule and have high affinity for the surface of the carnauba wax. These non-ionic surfactants have a long chain, which physically prevents the particles from clumping, counterbalancing the van der Waals attractive force, restricting the effects of coalescence on the system.<sup>37</sup> The advantage of dispersion by the steric mechanism is that it does not depend on the ionic concentration of the liquid medium and has no restriction on the pH of the medium, since the surface potential is not involved.<sup>39</sup>

## **3.4. Stability of Nanoemulsion**

### ***3.4.1. Effect of Centrifugation***

For commercial applications, it becomes important that nanoemulsions remain physically stable during storage, transport, and use.<sup>31</sup> The physical stability of the nanoemulsions obtained by ultrasound was studied during storage at 20 °C for up to 10 days. They were subjected to accelerated centrifugation for 15 min, none of which showed any phase separation. In fact, all formulations showed excellent physical stability (Figure 4). The combination of emulsifiers used did not appear to be important either. There were no significant changes in the particle sizes of any of the formulations. This finding pointed to the fact that nanoemulsions generally have a high kinetic stability.<sup>24</sup> To ensure the long-term stability of a nanoemulsion, control of particle size distribution and selection of suitable emulsifier is of paramount importance.<sup>40</sup>

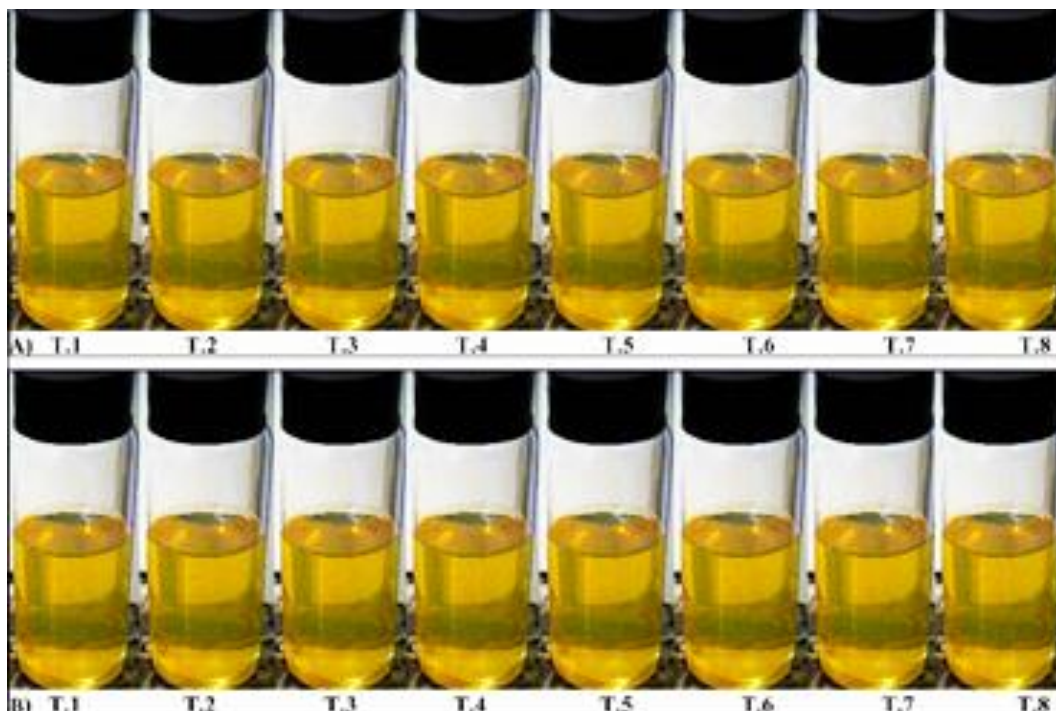


Figure 4 – Visual appearance of nanoemulsions after production (A) and after storage (B).

### 3.4.2. Effect of pH

pH is an important parameter to consider because large variations in the pH values of the formulations can cause chemical incompatibilities, besides being a parameter related to the intrinsic stability of the formulation.<sup>41,42</sup> As expected, there was little variation in the pH of samples containing nonionic surfactants. The pH of the nanoemulsions remained in the basic pH range of  $7.5 \pm 0.02$  to  $8.5 \pm 0.04$  during storage. In addition, no observed changes were observed in all emulsions stored for 10 days at 20 °C (Figure 5). This fact indicates stability of the formulations. Oscillations in the pH value may indicate the presence of free fatty acids in the formulation. These would come from the hydrolysis of the surfactant system, and triglycerides from the core oils,<sup>43,44</sup> which would affect the mechanical barrier forces between the oil particles and the aqueous phase (formed by the emulsifiers) and the forces of repulsion between the particles.<sup>44</sup> However, the small pH variations of the emulsions did not affect the mean particle size. Furthermore, it is worth mentioning that Li et al.,<sup>45</sup> in a follow-up study of the effects of pH and ionic strength on the final diameters of nanoemulsions particles, did not observe any significant difference in droplet size between nanoemulsions prepared at various pH values (3-10). This is because the stabilizing effect of non-ionic surfactants is independent of pH and the ionic strength of the continuous phase.<sup>46</sup>

### ***3.4.3. Particle Size, Polydispersity Index and Zeta Potential During Storage***

Nanoemulsions may become unstable through different physical-chemical mechanisms, including gravitational separation, flocculation, coalescence, and maturation of Ostwald.<sup>47</sup> The rate at which a nanoemulsion decomposes and the mechanism by which this process occurs depends on its composition and microstructure.<sup>30</sup> Therefore, it is worth noting that there was no significant increase in the mean particle diameter (Figure 5a) and polydispersity index (Figure 5b) of the formulations during storage for 10 days. This confirms that the ultrasonic process employing non-ionic surfactants in small concentrations is capable of yielding fairly stable nanoemulsions. Only treatment 5 presented a small significant difference ( $p \leq 0.05$ ) for the particle size variable (Figure 5a) after 10 days of storage. Possibly due to the fact that the lowest amount of surfactant was used in this formulation, which consequently also favored changes in the PDI for the referred treatment.

However, the stability in all the tests was obtained as a function of the small particle sizes obtained ( $D < 100\text{nm}$ ). The use of surfactants such as Tween 80, are effective in reducing particle size, which may have favored the process.<sup>27</sup> Small particle diameters indicate that Brownian motion forces are in the same order as gravitational forces, ensuring good stability. Decreasing droplet size changes the repulsive or attractive forces of colloids. This increases the stability of nanoemulsions.<sup>48</sup> Similarly, low PDI values ( $\text{PDI} < 1$ ) in general indicate monodisperse nanoemulsions with a high stability, such as those obtained in this study.<sup>31</sup>

The zeta potential also plays an important role in the stabilization of nanoemulsions because it reflects the surface potential of the particles. As the results indicate, Tween 80 improved the physical-chemical stability of the formulation. This is due to the ethylene oxide and long hydrocarbon chain groups, achieving a stronger steric stabilizing effect.<sup>49</sup> The values obtained for this parameter showed no significant difference during storage (Figure 5c), remaining close to 0 mV. Such a value is consistent since the addition of nonionic surfactants does not affect the surface potential. However, the slight negative charge observed is attributed to the lecithin phospholipids, which are also of great importance in the long-term stability of the nanoemulsions, as they confer a negative final zeta potential at the interface, allowing also the electrostatic repulsion between the dispersed particles.<sup>44</sup>

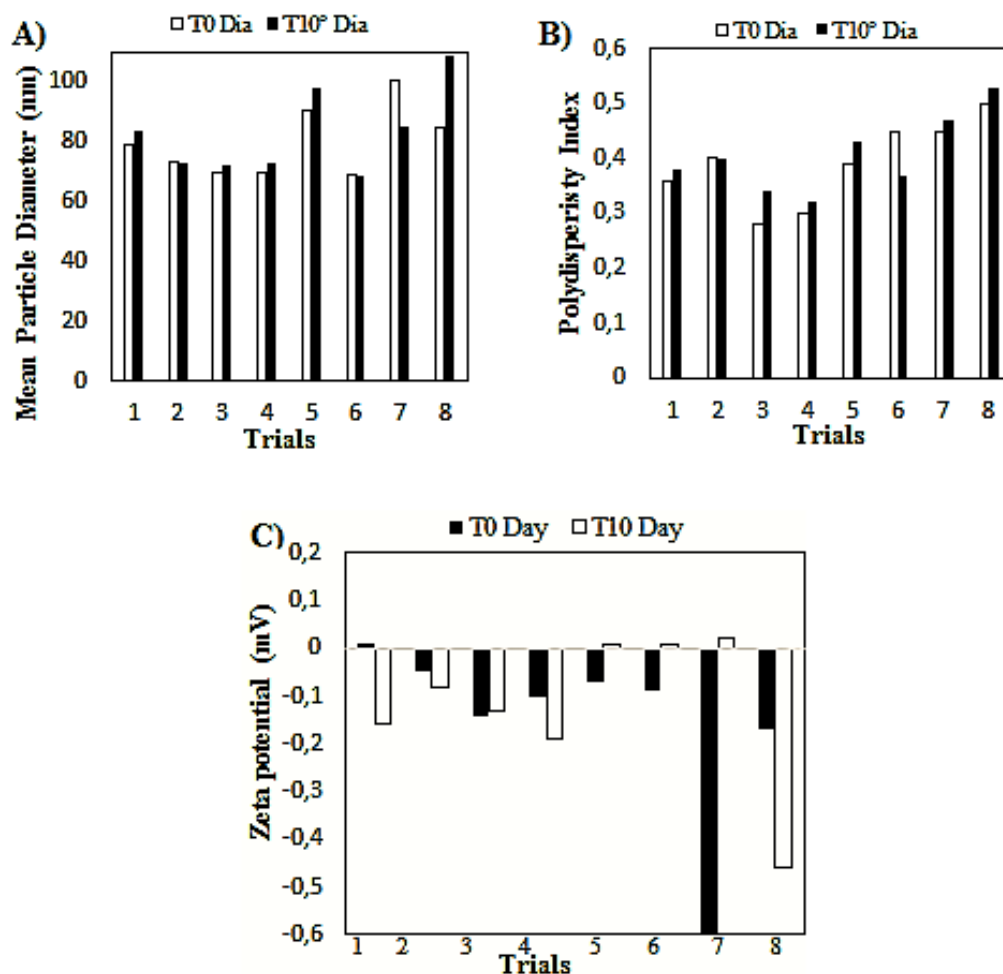


Figure 5 – Influence of storage time on particle size (A), polydispersity index (B) and zeta potential (C) of the nanoemulsions produced.

#### 4. CONCLUSION

The produced nanoemulsions proved to be stable by dispersion forces of steric origin, restricting coalescing effects on the system. This occurred even with a small amount of surfactant, confirming good homogeneity of the formulations. The mean particle size remained around  $69 \pm 0.63$  nm and polydispersity index of  $0.3 \pm 0.01$ . However, process parameters: surfactant concentration and sonication time were shown to be influential in mean particle diameter, however, the ratio of Tween to soy lecithin employed was not significant. The results show that the best formulation was achieved by stirring the nanoemulsion for 10 min at a surfactant concentration of 1.5% (w/w) in the Tween / lecithin ratio (100:0 w/w). From an industrial viability perspective, these results open up new opportunities in the attempt to develop nanoemulsions through a homogenization process that is compatible with industrial needs and can contribute significantly to the reduction of total cost, leading to more sustainable treatments.

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**ARTIGO 2 – PRODUCTION AND EFFICACY OF NEEM OIL NANOEMULSION  
AGAINST ASPERGILLUS FLAVUS AND PENICILLIUM CITRINUM  
IN SOYBEAN SEEDS**

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**Abstract**

Intensive agricultural practices contribute to more precise diagnoses of pests and plant diseases. Therefore, the search for alternative methods that are efficient to control phytopathogens is extremely important. To solve this problem, this study aimed at formulating and characterizing Neem (*Azadirachta indica*) oil-based nanoemulsion bioproducts and at evaluating their applicability against *Aspergillus flavus* and *Penicillium citrinum* in soybean seeds. Soybean seeds of cultivar SYN 1395A IPRO were placed with nanoemulsion containing different concentrations of Neem oil (0.5%; 1%; 2%; and 3%) for 30 minutes and 600 minutes. Control samples (positive: infested and untreated; and negative not infested and treated) were also carried out. Prior to the treatments, the formulated nanoemulsions were characterized by dynamic light dispersion, polydispersity, rheology, and stability test. The fungal sensitivity to nanoemulsions of Neem oil was evaluated. Subsequently, the soybean seeds were treated with nanoemulsion and then tested for germination and health. The nanoemulsion droplets had an average diameter of  $59 \pm 0.6$  nm and the rheology study had a viscosity of  $2.542 \pm 0.07$  mPas. The size of the nanoemulsion remained stable for a period of 20 days. The polydispersity of the particle size remained between  $0.209 \pm 0.02$ . Neem oil had an inhibitory effect on the growth of fungal isolates. The highest antifungal activity was observed at the concentration of 3% (w/v) of Neem oil (28 mm inhibition zone for *A. flavus* And 25 mm for *P. citrinum*.). The nanoemulsion based on Neem oil was efficient against the studied fungi and did not present phytotoxic effects to the seeds. Nanoemulsion is easily accessible, economically viable and, in addition, less toxic than common synthetic pesticides. This study showed that Neem oil nanoemulsions have significant potential applications in agriculture.

**Keywords** Biological control, Fungicide, *Azadirachta indica*, Azadirachtin.

## Introduction

The exponential growth of the population brings about the need for a sustainable food production, which is one of the challenges of the agricultural sector (Godfray and Garnett, 2014; Jordan *et al.*, 2016). Thus, the demand for pesticides and fertilizers plays an important role on effectively maximizing agricultural productivity. However, despite promoting a favorable performance in agriculture, pesticides may be harmful for human health and for non-target organisms, depending on toxicity and degree of contamination (Damalas and Koutroubas, 2016). The fungal population presents health risks due to mycotoxin production. Ochratoxin produced by species of genus *Aspergillusflavus* and *Penicillium citrinum* have nephrotoxic, hepatotoxic and carcinogenic effects (Bognanno and Galvano, 2013; Geremew *et al.*, 2016). Therefore, the use of agrochemical products to control these pathogens is an indispensable tool in current agricultural practices. However, the inadequate management of these products is a common problem. The use of doses above or below the recommended levels result in loss of agrochemical efficiency due to resistant biotypes. Furthermore, excessive use causes considerable waste, increasing production costs of crops and adversely affecting the environment and public health (Oliveira *et al.*, 2014).

Safe and efficient methods of pesticide application are essential for the effective control of these fungi, increasing productivity and decreasing risks to the environment and to humans. Thus, nanotechnology becomes promising as an innovative tool for the safe supply of agrochemicals (Kah and Hofmann, 2014; Werdin González *et al.*, 2014). Conceptually, nanoemulsions are colloidal dispersions containing reduced particles (20-100 nm) dispersed in aqueous medium, depending on the production process (Guttoff *et al.*, 2015). Nanoemulsions are efficient due to increased surface area; increase in systemic activity due to smaller particle size and greater mobility; stability and protection against premature degradation; and decreased toxicity due to the elimination of organic solvents from conventionally used pesticides (Kah and Hofmann, 2014; Oliveira *et al.*, 2014).

In this context, the oil of Neem (*Azadirachta indica*) acts as a growth regulator and as an efficient natural pesticide, which has a strong antifungal (Gomes, 2013; Shah and Wani, 2016), larvicidal and antibacterial (Chandrasekaran *et al.*, 2015) action. The biopesticidal applications of Neem oil is associated with its components, specially Azadirachtin, which is a biologically active tetranortriterpenoid (Jerobin *et al.*, 2012). The content of Azadirachtin in Neem oil is 0.068% w/v, obtained by high performance liquid chromatography (HPLC) (Anjali *et al.*, 2012). (Osman *et al.*, 2017) investigated the development of nanoemulsions

based on Neem and citronella oils against two phytopathogenic fungi *Rhizoctonia solani* and *Sclerotium rolfsii*, and potent antifungal activity was observed on the pathogens. However, there is not much information about plant-based oils used as fungicidal agents in agriculture, mainly because of their low solubility in water.

Therefore, this study aimed at contributing to better formulation and characterization of Neem oil-based nanoemulsion bioproducts and evaluate its applicability against *Aspergillus flavus* and *Penicillium citrinum* fungi in soybean seeds.

## **Material and Methods**

### ***Chemical reagents***

Neem oil was obtained from Lazslo Aromatologia (Brazil) and Tween 20 [polyoxyethylene (20) sorbitan monolaurate (hydrophilic-lipophilic balance, HLB = 16.7)] was obtained from Sigma Aldrich, India. Ultrapure Milli<sup>Q</sup> water, 18.2 MΩ.cm at 25° C was used for all analyses. All chemicals were analytical grade.

### ***Microorganisms, storage and standardization of Aspergillus flavus cd 10508 and Penicillium citrinum cd 10564 inocula***

The fungal isolates *Aspergillus flavus* CD 10508 and *Penicillium citrinum* CD 10564 used in this study belong to the collection of cultures from the Mycotoxins and Food Mycology Laboratory of the Department of Food Science of the Federal University of Lavras (Brazil) and were isolated from grapes.

### ***Formulation of the antimicrobial nanoemulsion***

The nanoemulsion was prepared according to the experimental plan presented in Table 1, varying oil concentrations (0.5%; 1%; 2% and 3%) and proportions (1:2 and 1:3 Oil/Surfactant) between the employed surfactants. Such variations followed (Ghotbi *et al.*, 2014) and (Sekar *et al.*, 2015), using Neem oil and Tween 20 as the dispersed phase, and water as the continuous phase. The dispersed phase was added dropwise to the continuous phase at room temperature using a magnetic stirrer (RQ1210, Remi Metals Ltda) at 250 rpm during 30 minutes to create the emulsion. In addition, the coarse emulsion (produced by

magnetic stirring) was subjected to high-energy emulsification using a 20 kHz ultrasonic processor with a maximum power of 450 W for 45 minutes (Branson Digital – Model 450). The sonication processes were performed in cycles in a glass containing ice cubes to minimize the heat effect. Each cycle consisted of 10 minutes, with 1-minute breaks, with an amplitude of 50%. For the evaluation of the antimicrobial efficiency, a nanoemulsion with the ratios 1:3 and 1:2 (oil/surfactant) was also used, as reported in Table 1.

**Table 1** – Parameters (oil concentration, surfactant ratio, sonication time) investigated to evaluate the final formulation of emulsion and nanoemulsion of Neem.

Trials	Oil concentration (w/v)	Ratio (oil:surfactant)	Sonication Time (min)
T1 (Emulsion)	3.0	01:03	5
T2	0.5	01:03	45
T3	1.0	01:03	45
T4	2.0	01:03	45
T5	3.0	01:03	45
T6 (Emulsion)	3.0	01:02	5
T7	0.5	01:02	45
T8	1.0	01:02	45
T9	2.0	01:02	45
T10	3.0	01:02	45

### *Nanoemulsion characterization*

#### *Determination of particle size (DLS) and size distribution (PDI)*

The size and distribution of nanoemulsion particles of Neem oil were determined according to (Jadhav *et al.*, 2015) using a Zetasizer Nano ZS particle sizer, Malvern Instruments, Malvern United Kingdom. This equipment determines particle size from the relative intensity of fluctuations in the sample dispersion using a laser beam (633 nm) at a 90° angle. Each sample was evaluated in an average of five replicates. Prior to the measurements, the samples were diluted (x100) using a buffer solution (pH 3.0) to avoid multiplication of dispersion effects and ensure free Brownian motion of the particles. The mean particle diameter (DLS) and polydispersity index (PDI) were calculated from the particle size distribution. All measurements were performed at room temperature.

### *Viscosity*

The nanoemulsion viscosity was measured using a Rheostress 6000 (Modular Advanced Rheometer System, Thermo Scientific, Germany) with cone-plate sensor. The Rheowin software was used for data analysis. Flow curves were performed with a shear rate of 0 – 100 s<sup>-1</sup> at 25°C. The analyses were made in triplicate (Anjali *et al.*, 2012).

### *Hydrogen ionic potential – PH*

The pH values of the nanoemulsion were measured using a Tecnal pHmeter calibrated with buffer solutions of 4.0 and 7.0. The determination was made directly on the nanosuspensions at 20°C. All measurements were performed in triplicate and the results were reported as mean values.

### *Storage stability of nanoemulsions*

The nanoemulsions (20 ml) were conditioned in sterile test tube and preincubated at 20°C. During designated intervals (0 day, 10 days, and 20 days of production) changes in size and the polydispersity index of the particles were measured using a NanoZS Zetasizer (Malvern Instr., Malvern, UK). A stability study was also performed by centrifugation of the nanoemulsions (Biofuge, Heraeus, Germany) at room temperature at 3500 rpm for 30 min (Shafiq; Shakeel, 2010). Macroscopic alterations were observed and reported. The temperature was 20 ± 2°C

### *Fungicidal activity of nanoemulsion*

#### *Sensitivity of *Aspergillus flavus* cd 10508 and *Penicillium citrinum* cd10564 in *Neem* nanoemulsion in vitro*

For the inhibitory effect of filamentous fungi, the disc diffusion test established as standard by the National Committee for Clinical Laboratory Standards (NCCLS) (AL Barry and Thornsberry, 1991; Ostrosky *et al.*, 2008) was used. To reach that result, an inoculum was used at the concentration of 10<sup>6</sup> spores mL<sup>-1</sup>, counting in Neubauer chamber. Afterwards, the inoculum was transferred to a Petri dish with malt extract agar medium (MEA) using the

surface scattering technique. Filter paper discs with diameter of 6 mM soaked with 10  $\mu$ L of the nanoemulsions at concentrations of 0.5%; 1%; 2% and 3% (w/v) were placed in the culture medium. In addition, the effect of the mean particle size on the fungitoxic activity of the emulsion and of the pure oil was also evaluated. The negative control was performed using discs added with 10 $\mu$ L of DMSO (dimethylsulfoxide), which is characterized as cryoprotectant. The fungicide Maxim at 10  $\mu$ L/5 ml was used for the positive control. The dishes were incubated in BOD (Biochemical Oxygen Demand) at 25°C for 72 hours. The evaluation was comparative with respect to a reference biological standard (positive control) and the zone or halo of growth inhibition was measured starting from the circumference of the disk until the growth of microorganisms (AL Barry and Thornsberry, 1991). The minimum inhibitory concentration (MIC) is defined as the lowest concentration of nanoemulsion, in which the presence of inhibition halo was identified.

*Efficacy of Neem nanoemulsion against Aspergillus flavus cd 10508 and Penicillium citrinum cd10564 in infected soybean seeds*

The soybean, cultivar SYN 1359S IPRO, without chemical treatment were supplied by Syngenta Seeds Ltda, Uberlândia – MG, Brazil. The seeds were inoculated and germinated according to the methodology adapted from (Abd-Elsalam and Khokhlov, 2015; Galletti *et al.*, 2015).

Contamination of seeds by the physiological conditioning method in the presence of fungi

Following the methodology described by (Costa *et al.*, 2003), the fungi culture was placed in BDA medium at 25°C  $\pm$  2 to obtain colonies that were later used for the production of a suspension with 10<sup>6</sup> conidia/mL. From this suspension, aliquots of 1mL were spread on BDA solid medium containing mannitol with a water potential of -1.0 Mpa in 15-cm Petri dish. The dish were maintained at 25°C with a photoperiod of 12 hours/day. After incubating the fungi for seven days, 300 seeds were placed on each dish on a single layer for incubation at 25° C. The seeds remained on the fungal colony for 48 hours. Regarding period of time, t<sub>2</sub> = 30 min and t<sub>1</sub> = 600 min, 4 replications (4 plates of 15 cm) with 300 soybean seeds each were used at the respective concentrations (0.5%; 1%; 2%; 3%). Therefore, 72 dishes were used, being 36 for seeds exposed to *Aspergillus flavus* After the contact period, the seeds were removed and dried for 48 hours in laboratory environment until water content of 12%, then in



storage (10°C and 50% RH) for later use. For the period of 30 and 600 minutes, four replicates (4.15 cm plates) containing 300 soybean seeds were used (0.5%; 1%, 2%, 3%). A total of 72 plates were used, being 36 for seeds exposed to the isolate of *Aspergillus flavus* and 36 for seeds exposed to the isolate *Penicillium citrinum*. After the contact period, the seeds were removed and dried for 48 hours in a study environment up to 12% and then in a storage chamber (10°C and 50% RH) until there was a use of the people in the later trials.

#### Treatment of infected seeds

After contamination and drying, the seeds were immersed in 50 ml of nanoemulsion at concentrations of 0.5%; 1%; 2% and 3% (w/v) during 600min (Time 1) or 30min (Time 2). Untreated and fungi-contaminated seeds were used as positive controls of phytotoxicity, whereas treated and uncontaminated seeds were used as negative controls.

#### Germination test in lab

For the germination test, eight replicates of 25 seeds were used. Paper sheets (germitest) were moistened with distilled water up to 2.5 times its dry weight to form rolls. The rolls were kept in a BOD chamber at 25°C for 7 days, having photoperiods of eight hours a day. Thus, the seeds were uniformly distributed on the sheets to decrease competition and avoid contamination among seeds and seedlings. The results were expressed as percentage of normal seedlings (having all essential structures that are crucial for development).

#### *Health test (modified blotter)*

A total of 200 seeds were used for each treatment, divided into eight replicates of 25 ones, which were arranged individually on a layer of filter paper (3 overlapped discs) in a solution of 2,4-D (sodium dichlorophenoxyacetate), which is an inhibitor of germination, at the concentration of 10 ppm. Seeds were spaced 1-2 cm apart in 25-cm petri plates. The plates were kept at  $20 \pm 2^\circ\text{C}$  for 10 days. Then, the seeds were examined individually using a stereoscopic microscope (resolution of 30-80X) and an optical microscope. Results were expressed as percentage of infected seeds.

### ***Statistical analysis***

The experiments were set up in a 4 x 2 factorial scheme, with four nanoemulsions at different concentrations of Neem oil (0.5%, 1%, 2%, and 3%) and left in contact with the seeds for 2 different periods (600 and 30 min). Two additional treatments/controls (positive: contaminated untreated with nanoemulsion (3) and negative: uncontaminated treated with nanoemulsion (3)) were also included. A completely randomized design with 3, 5, and 8 replications was used, according to the recommendation of each protocol. The data were submitted to individual analysis of variance for each fungus. The treatments were compared with the controls by means of Scheffé test ( $p \leq 0.05$ ). The comparison of the means of the germination and blotter tests were done by the Scott Knott test at 5% significant level. The percentage data of contaminated seeds obtained through the blotter were transformed using  $(x+0.5)^{0.5}$ . Regression analyses were performed to describe the effects of nanoemulsion concentrations. These analyses were performed using the SISVAR® software (Ferreira, 2014). The other analyses were performed using the Statistica Analysis Systems 8.0 software and the means were compared using the Tukey test with a significance level of 5% ( $p \leq 0.05$ ).

## **Results and discussion**

### ***Nanoemulsion characterization***

#### ***Determination of particle size (DLS) and size distribution (PDI)***

The produced emulsion had diameters above the nanometer scale ( $397.8 \pm 26.9$  nm) whereas nanoemulsions had diameters below 60 nm (Table 2). Nanoemulsions particles corresponding to the concentration of Neem oil employed were reduced as the concentration of surfactant increased. This benefited the development of small diameters (up to  $59 \pm 0.61$ nm) and reduced turbidity. Emulsion turbidity is a characteristic of concentration and particle size. These nanoemulsions were stable at a ratio of 1:3 due to the surfactant, which restricted the interfacial free energy and provided a mechanical barrier to coalescence (Anjali *et al.*, 2012). Likewise, as reported by (Moghimi *et al.*, 2016), the addition of surfactants to nanoemulsion systems caused the interfacial film to condense and stabilize, resulting in small diameters.

**Table 2** – Effects on particle size, polydispersity, viscosity and pH, applying different ratios of oil and surfactant to formulate nanoemulsions (T2, T3, T4, T5, T7, T8, T9 and T10) and emulsions (T1 and T6).

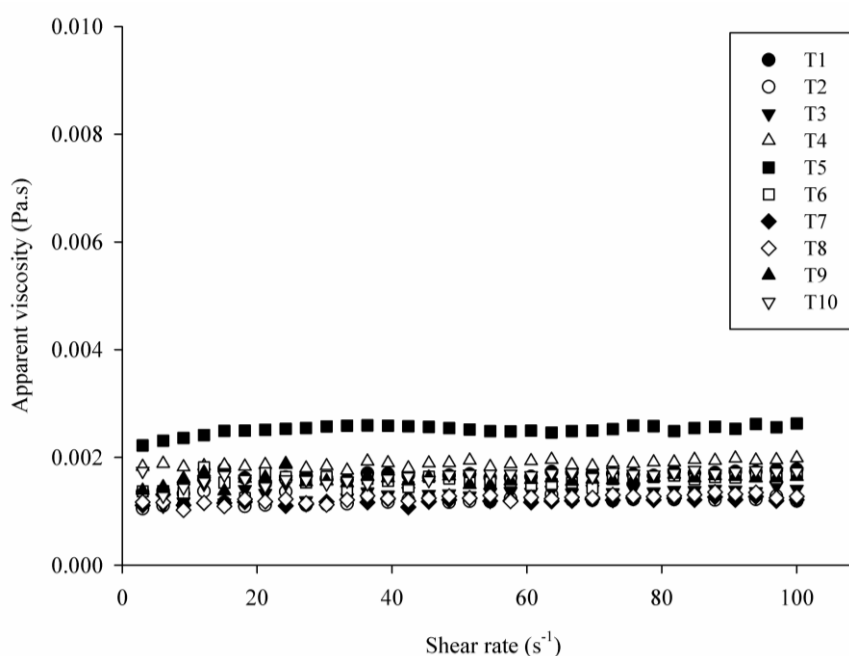
Trials	Oil (w/v)	Ratio (oil:surfactant)	Droplet size (nm) <sup>a</sup>	Polydispersity	Viscosity (mPa s) <sup>a</sup>	pH
T1	3.0	01:03	397.8 ± 26.9c	0.528 ± 0.21b	1.998 ± 0,05b	5.26
T2	0.5	01:03	56.2 ± 1.32a	0.313 ± 0.08a	1.231 ± 0,01a	5.56
T3	1.0	01:03	56.6 ± 0.88a	0.308 ± 0.01a	1.386 ± 0,04d	5.57
T4	2.0	01:03	51.0 ± 0.12a	0.264 ± 0.00a	1.922 ± 0,08g	5.60
T5	3.0	01:03	59.9 ± 0.61a	0.209 ± 0.02a	2.542 ± 0,07h	5.62
T6	3.0	01:02	260.1 ± 1.27c	0.510 ± 0.03b	1.800 ± 0,01e	4.72
T7	0.5	01:02	81.1 ± 1.51b	0.368 ± 0.04a	1.215 ± 0,05a	4.55
T8	1.0	01:02	69.0 ± 1.54b	0.368 ± 0.05a	1.279 ± 0,04c	4.62
T9	2.0	01:02	69.0 ± 0.26b	0.303 ± 0.01a	1.613 ± 0,05f	4.77
T10	3.0	01:02	70.5 ± 1.9b	0.361 ± 0.06a	1.702 ± 0,02b	4.80

The results obtained in this study corroborate with the literature, in which they reported the formation of NE particles of Neem oil (ratio 1:3) of size in the 30-70 nm range and spherical in shape (Ghotbi *et al.*, 2014). As for the size distribution of these particles, the polydispersity was  $0.361 \pm 0.06$  and  $0.209 \pm 0.02$  for nanoemulsion formulations with oil and surfactant ratios of 1:2 and 1:3, respectively. These values differed statistically from those found for the polydispersity of the emulsion ( $0.528 \pm 0.21$ ), which was higher. The polydispersity index is a measure of the particle size distribution, thus measures the homogeneity of the nanoemulsion particles, which is an important factor used to evaluate the nanoemulsion stability (Tan *et al.*, 2016). The particle size in the nanoscale range may be due to low polydispersity values (Shinoda and Saito, 1969). High polydispersity results in low uniformity of droplet size.

### Viscosity

The rheological behavior of the emulsions has been of great interest for practical industrial applications. The viscosity of the selected formulations is shown in Figure 1. The emulsions (T1 and T6) had a similar viscosity to the ones found in nanometric scale (T5 and T10). The 13 nanoemulsion formulations had the highest viscosity,  $2.542 \pm 0.07$  mPa.s (Table 2). Similarly, the slight increase in particle diameter also provided an increase in the dispersed

phase viscosity and, consequently, an increase in flow resistance and restricted rate of droplet disruption (McClements and Rao, 2011). The main factors that influence emulsion viscosity are the dispersed phase volume fraction, particle size and charge as well as colloidal interactions (Guerra-Rosas *et al.*, 2016). However, the increase in viscosity may still be due to the increase in the concentration of non-ionic surfactants. According to (Florence, 1969), this phenomenon occurs because water molecules are trapped in the non-ionic surfactant cross-linking chains. This can be attributed to increased hydration by water molecules around the hydrophilic portion of the surfactants. The same behavior found in this study were reported by other studies (Anjali *et al.*, 2012; Dias *et al.*, 2014).



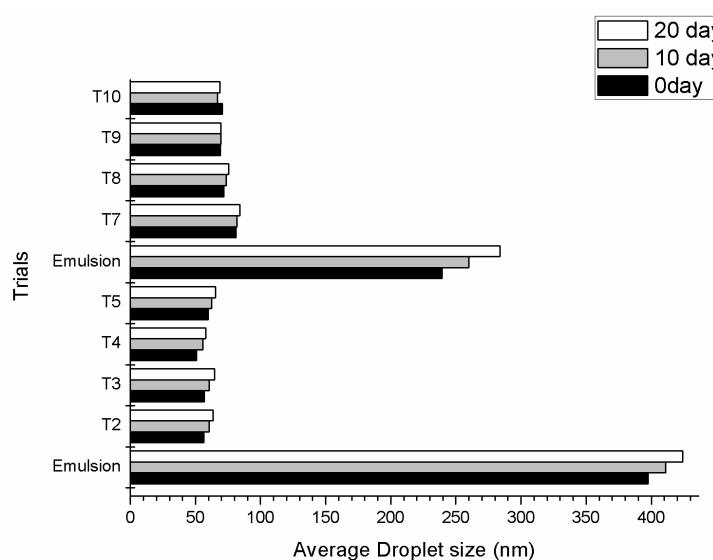
**Figure I** – Changes in viscosity values of Neem oil nanoemulsions (T1 and T6) and nanoemulsions (T2, T3, T4, T5, T7, T8, T9 and T10) of Neem oil.

### *pH*

The increased surfactant and oil concentrations also provided an increase in the nanoemulsion pH values. The nanoemulsion formulations with 1:2 and 1:3 ratios had pH values of 4.80 and 5.65, respectively (Table 2). Similar behaviors were reported by (Anjali *et al.*, 2012). The nanoemulsion is more effective at physiological pH (4.5 and 6), which is considered another good feature in its potential application as an anti-fungal (Myc *et al.*, 2002).

### Nanoemulsion storage stability

The nanoemulsion stability is distinguished from emulsions due to its greater kinetic stability and less phase separation (Donsì and Ferrari, 2016). In this context, the results obtained in this study confirmed that the nanoemulsions resisted the stability tests in a centrifuge, as opposed to the emulsions. No statistical difference was observed among the diameters of nanoemulsion particle for 20 days. They preserved their size, which proves good stability and prevents the occurrence of coalescence (phase separation) (Figure 2).



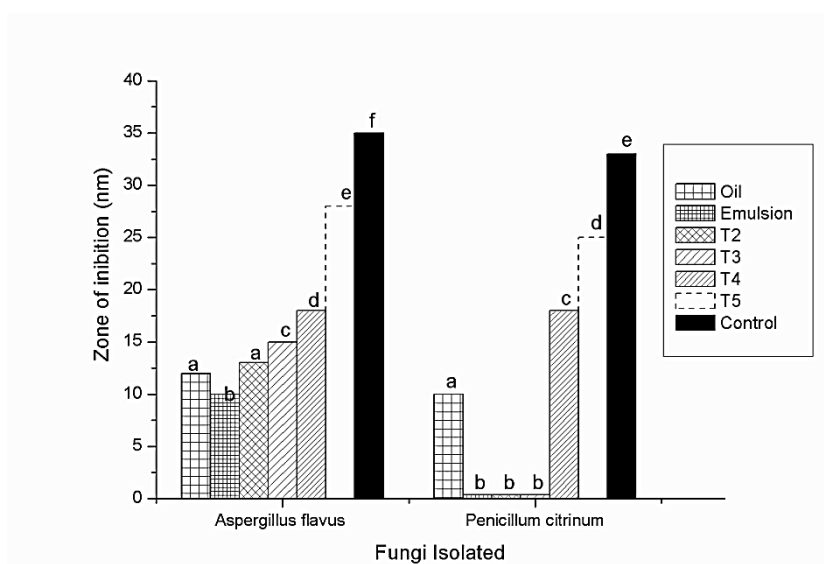
**Figure II** – Particle size (nm) of emulsions and nanoemulsions during storage period.

Nanoemulsions were physically stable at room temperature with no phase separation while emulsions were not stable. This finding confirmed the fact that nanoemulsions generally have a high kinetic stability (Calligaris *et al.*, 2016). To ensure the long-term kinetic stability of a nanoemulsion, the control of particle size distribution and the selection of a suitable emulsifier are extremely important (McClements, 2012). Tween 20 has been a suitable surfactant for the NE formulation; because of its non-ionic nature, it is not affected by pH, besides being considered a non-toxic and biocompatible compound (Ostertag *et al.*, 2012; Chandrasekaran *et al.*, 2015). In this context, the steric effect plays an important role in the nanoemulsion stabilization (Gupta *et al.*, 2016). Nevertheless, (Niu *et al.*, 2015) reported that the stability of nanoemulsions can improve as surface charge increases, due to forces produced between the particles against flocculation and coalescence.

### ***Fungicidal activity of the nanoemulsion***

#### *Sensitivity of Aspergillus flavus cd 10508 and Penicillium citrinum cd10564 in neem nanoemulsion in vitro*

The strength and antifungal activity of nanoemulsions against examined microorganisms were quantitatively assessed by the presence or absence of inhibition zones and MIC values (Figure 3). Nanoemulsions based on oil and surfactant at a ratio of 1:3 were selected for this study. Nanoemulsion droplet sizes were considered an important parameter for the antifungal study. The results showed that Neem oil has an inhibitory effect on the growth of fungal isolates. The highest antifungal activity was observed at the concentration of 3% (w/v) of Neem oil, T5 (28 mm inhibition zone for *Aspergillus Flavus* and 25 mm for *Penicillium Citrinum*). The fungicidal effect at this concentration was equivalent to the positive control used in this study. However, the antifungal action persisted even at lower concentrations (minimum inhibitory concentrations MIC), even in smaller inhibition zones (Figure 3).



**Figure III** – Effect of pure oil, emulsion and nanoemulsion concentrations (T2, T3, T4, T5) on the inhibition zone of *Aspergillus flavus* and *Penicillium citrinum* isolates.

The effect of the mean size of the particles on the fungitoxic activity of the emulsion was also studied. The nanoemulsion ability to inhibit mycelial growth was compared with that of coarse emulsions and pure oil. Thinner and more homogeneous particle size distributions

increased the antifungal activity of NEs significantly ( $p \leq 0.05$ ) and improved the physical stability of nanoemulsions. This behavior is clearly visible in T5 samples. Coarse emulsions formulated with 3% oil exhibited a 9mm halo inhibition for *Aspergillus flavus* and absence of inhibition for *Penicillium citrinum*, whereas nanoemulsions exhibited inhibition with halos larger than 25mm.

Likewise, growth inhibition for T5 samples showed a significant increase ( $p \leq 0.05$ ) in inhibition when using pure oil [from 10mm to 25mm (Figure 3)]. Because of the sub-micron size of the particles, O/W nanoemulsions can penetrate more easily through the fungi membrane when compared to micrometer particles of the emulsion. This leads to an evident increase in antifungal activity (Ribes *et al.*, 2016). This inhibitory effect is due to the fact that nanoemulsions affect fungal hyphae and spore, reducing their viability. This activity is associated with the fact that nanoemulsions fuse with lipid-containing organisms and destabilize the lipid membrane of the pathogen, resulting in cell lysis and death (Joe *et al.*, 2012).

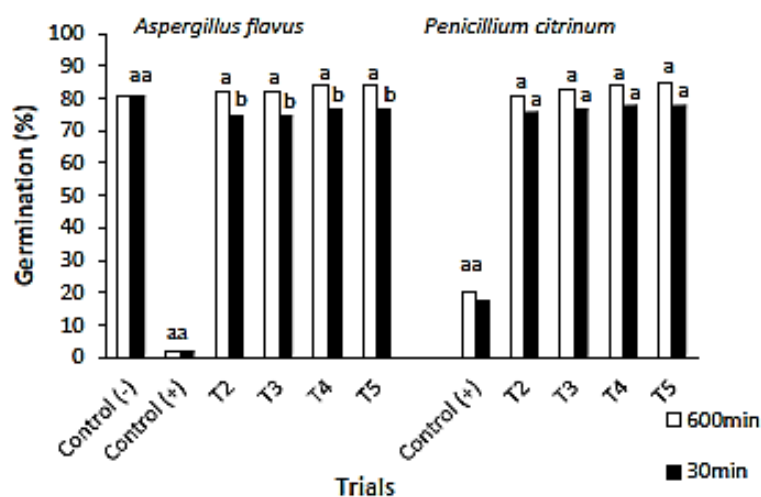
On one hand, Neem oil has good insecticidal action on a variety of pathogens (Ghosh *et al.*, 2014). On the other hand, little attention has been paid to the antifungal activity of Neem nanoemulsions with different levels of azadirachtin. However, due to rapid action, small concentrations of nanoemulsions are unlikely to result in the development of resistant strains (Myc *et al.*, 2002). Antifungal properties of *Azadirachta indica* in inhibition of the production of AFB1 by *Aspergillus flavus* was also established by Sudha *et al.*, (2013). Several studies also reported the significant improvement of the antimicrobial activity when encapsulated in submicron emulsions of different NEs, such as limonene (Donsi *et al.*, 2011), vegetable oil (Joe *et al.*, 2012), among others.

### ***Effects of nanoemulsion on the quality of soybean seeds***

#### ***Germination test***

When comparing treatments T2, T3, T4, T5 with the positive control, the nanoemulsion (T5) immersed for 600min provided 78% more germination for the *Aspergillus flavus* fungus and 60% more germination for the *Penicillium citrinum* fungus (Figure 4). Therefore, the nanoemulsion was very efficient in controlling the effect of these fungi on the germination of soybean seeds. As for the negative control, there were no significant differences ( $p \geq 0.05$ ) when compared to other treatments in seed germination, which suggests

that the nanoemulsion does not have any phytotoxic effect. A germination test was carried out to determine the initial physiological potential of the seeds, and they presented an average of 99% germination.



**Figure IV** – Percentages of germination of seeds contaminated with *Aspergillus flavus* and *Penicillium citrinum* treated with NEs at different concentrations (T2, T3, T4, T5) for two durations (t1 and t2).

**Table 3** – Means of percentage of germination of soybean seeds after contamination with *Aspergillus flavus* e *Penicillium citrinum*.

Time (min)	<i>Aspergillus flavus</i> (%G)	<i>Penicillium citrinum</i> (G%)
600	83 a	82 a
30	75 b	78 a
Treatments	80 a	80 a
Positive Control	2 b	20 b
Negative Control	81 a	81 a

\*Means followed by the same letter in the column do not differ statistically at the 5% probability level by the Scott Knott test. \*\*Comparative mean of germination of seeds contaminated with fungi and that were in touch with nanoemulsions (treatments), germination of seeds contaminated with fungi (positive control) and germination of uncontaminated seeds (negative control).

Within each inoculum, the nanoemulsion concentration was not significant for the percentage of germination. On the other hand, duration influenced the final stand of normal plants when the inoculation was carried out with the fungus of the genus *Aspergillus flavus*. The duration of 600 minutes (t1) was favorable to seed germination when compared to 30 min (t2) (Table 3). Seeds infected by *Aspergillus flavus* and in contact with the nanoemulsion for a longer duration (t1) had a higher germination percentage. Thus, it is necessary to consider that



the nanoemulsion also provides some advantage to germination, in addition to not presenting phytotoxicity (negative control) to the seeds. Because this is an innovative topic, more research is necessary to better understand why it happened, especially to the physiological quality of the seeds.

Some treatment methods such as high concentrations of chlorine and decontamination by pasteurization impact the germination rate of treated seeds and results in lower yields of germination (Landry *et al.*, 2014). The Neem nanoemulsion was able to inactivate the pathogens without affecting the seed yield. Landry *et al.* (2014), working with carvacrol nanoemulsion on alfalfa seeds also observed a similar behavior in which the nanoemulsion did not affect seed germination. However, (Berahmand and Panahi, 2012), while using a combination of nanoparticles of SiO<sub>2</sub> and TiO<sub>2</sub>, concluded that nanoparticles could increase the nitrate reductase enzyme in soybean (*Glycine max*), improve its water absorption capacity, promote its antioxidant system and, in fact, accelerate and increase its germination.

#### *Health evaluation of the seeds contaminated by fungi*

Using the blotter test, the concentration and time factors were significant for the isolation ( $p \leq 0.05$ ) of both inocula (Table 4).

**Table 4** – Percentage of infested soybean seeds obtained in the sanity test after inoculation with fungi *Aspergillus flavus* and *Penicillium citrinum*.

Time	<i>Aspergillus flavus</i> (%)	<i>Penicillium citrinum</i> (%)
1	93 a	90 a
2	50 b	80 b
Treatments	75 b	86 b
Positive Control	100 a	100 a
Negative Control	3 c	9 c

\*Means followed by the same letter in the column do not differ statistically at the 5% probability level by the Scott Knott test. \*\*Duration of immersion in nanoemulsion in t1 = 600 min and in t2 = 30 min.

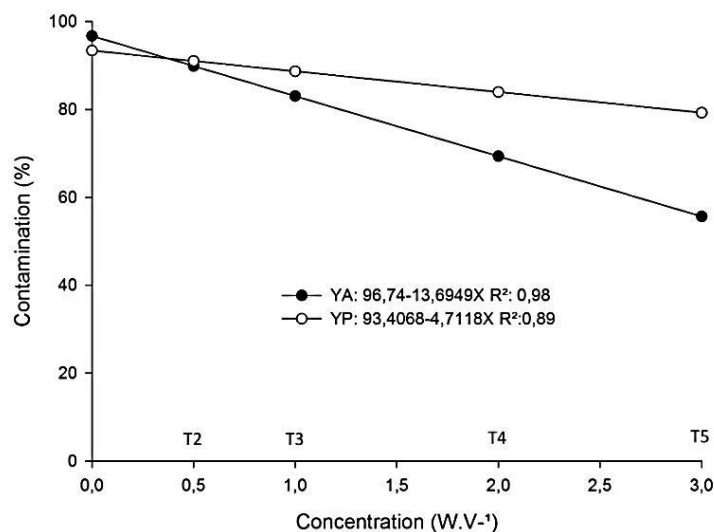
\*\*\*Comparative mean of germination of seeds contaminated with fungi and that were in touch with nanoemulsions (treatments), germination of seeds contaminated with fungi (positive control) and germination of uncontaminated seeds (negative control).

The concentration of fungi was 10<sup>6</sup> conidia / mL in soybean seeds. The contamination operation was efficient, since the inoculated seeds (positive control) showed 100% contamination for both fungi. Nanoemulsions based on oil and surfactant were selected in a

ratio of 1: 3. Nanoemulsion droplet sizes were considered an important parameter for the antifungal study. It has been widely accepted that smaller NPs would have higher surface energy and, therefore, proved to be more toxic to fungal cell (Tang *et al.*, 2012).

Regarding the times, treatments of the seeds contaminated with different concentrations of nanoemulsion for 30 or 600 minutes produced similar behavior for both fungi. For both inocula, at time 2 (30min) the percentage of infested seeds was lower than in the time 1 (600 min). The treatments performed with immersion of 30 min were sufficient to promote a reduction of on average 20% of the seeds contaminated with *Penicillium citrinum* in relation to the positive control. As for the seeds contaminated with *Aspergillus flavus*, this reduction reached an average of 50%. A higher time of seed exposure with nanoemulsion favored germination (Figure 3), but by providing a high moisture content in the seed, it probably benefited the emergence of new fungi, which explains the inefficiency of time 1 (Dannemiller *et al.*, 2016). The percentage of fungi that appear in the negative control comes from spores that were already carried by the seed. That means that, if the seeds are not treated, these fungi will proliferate, under favorable conditions, and cause damages to the seeds.

Regarding concentration, a reduction in the percentage of infested seeds was observed in proportion to the increase of the nanoemulsion concentration, for both fungi tested (Figure 6).



**Figure V** – Percentage of contaminated soybean seeds after inoculation with the *Aspergillus flavus* and *Penicillium citrinum* fungi in the blotter test.

When a microorganism is exposed to external stress at different concentrations of bioactive compounds, there are effects on the genomes that regulates the proteins (Abd-

Elsalam and Khokhlov, 2015). (Pandey *et al.*, 2014) also observed that increasing the concentration of botanical oils nanoemulsions in fungal-contaminated pea seeds provided greater elimination of these pathogens. There are several possibilities for the antifungal action of oils, as reported by researchers (Chouhan *et al.*, 2017; Valdés *et al.*, 2017), but the exact mechanism has not been determined yet. For bioactivity, the oil penetrates through the cell wall and the cytoplasmic membrane (Chouhan *et al.*, 2017). These results have allowed us to hypothesize the action of azadirachtin, such as its proportional increase, to inactivate the essential enzymes, to react with the cell membrane or to disturb the genetic material functionality. Nevertheless, the hydrophobic fraction in the phenolic oils are dissolved in the hydrophobic domain of the cytoplasmic membrane between the acyl lipid chains. This disintegrates the outer membrane and increases the cytoplasm membrane permeability to the adenosine triphosphate of fungal cells, eventually leading to its death (Pandey *et al.*, 2014). In addition, the susceptibility of the microorganisms to the oils has been shown to be dependent on the treatment pH. A lower pH not only increases stress for cells, but also increases the transfer of fungal membrane oils, increasing their observed efficacy (Landry *et al.*, 2014). The Neem nanoemulsion treatment presented in this study has a pH of 4.8 (Table 2) which utilizes the synergistic effects of both pH stress and an increase in the affinity of azadirachtin to the fungal membrane. Lower pH values not only increase cell stress, but also increases the transfer of oils from fungal membranes and increases efficacy (Landry *et al.*, 2014).

## **Conclusion**

The nanoemulsion formulation containing Neem oil, Tween 20, and deionized water was successfully optimized by the high-energy method. A drop size of less than 60 nm and stability over a long period was obtained. The Neem oil nanoemulsion with the smallest particle size (T5) was found to be more effective in the antifungal control of the genera *Aspergillus flavus* and *Penicillium citrinum*. The reduced size and uniform spread of these fine particles increased antifungal efficacy both in *in vitro* tests and contaminated seeds. In this study, the Neem oil-based nanoemulsions were efficient in the control of fungi and did not present phytotoxic effects to the seeds. Moreover, they may also favor the increase of contaminated seeds germination. Nanoemulsions are accessible, economically viable and less toxic than the usual synthetic pesticides. This study shows that Neem oil nanoemulsions may contain considerable potential applications in agriculture as they can selectively inhibit harmful fungi in plant fields and release essential elements for plant growth.

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**ARTIGO 3 – DEVELOPMENT OF PECTIN BIONANOCOMPOSITES AND NANOEMULSIONS OF CARNAUBA WAX AND NEEM OIL FOR SEED COATING**

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**Abstract**

The growing demand for biodegradable packaging materials motivated the development of new materials as an alternative to replace conventional packaging. This study aimed to develop nanocomposite films with Neem oil (Noil) nanoemulsions and Carnauba wax (Nwax) using a polymer matrix of HDM pectin; and to evaluate the nanoemulsions effects on the mechanical, vapor barrier, thermal and biodegradability properties of the films. These properties were monitored and they were related to the nanocomposites structural characteristics. The nanoemulsions were characterized by the polydispersity index and mean particle diameter. The nanocomposites thickness, appearance, mechanical, thermal and barrier properties were also analyzed. Carnauba wax and Neem oil nanoemulsions presented average diameters of 69 nm and 59 nm respectively and they were stable over time. The nanoemulsions addition in the films, mainly with Noil30, improved the water vapor permeability regarding the pectin films. Mechanical properties were optimized, being the elastic modulus the most affected mechanical attribute (it reduced from 1990.29 to 668.47 MPa for pectin; and from 1990.29 to 493.19 MPa for Nwax30 and Noil30 respectively). It was also noted a variation on the nanocomposites biodegradability. There was a mass loss after 45 days of testing. Regarding the synthetic polymer, it remained practically intact after 90 days. Thus, the nanocomposites presented promising results in the development of new materials containing green compounds with biocompatibility in the packaging application.

**KEYWORDS** packing materials, biopolymers, property of materials.

## 1 INTRODUCTION

The fossil energy depletion and the substantial increase in the use of petroleum-based plastics have raised environmental concerns. These aspects leveraged researches searching for innovations for the application of environmentally friendly materials. Minimizing the environmental impact of synthetic polymers is the main drivers for this trend. These new materials must have (1) physical properties equal or greater than conventional packages, (2) they must be obtained from renewable sources and be biodegradable, and (3) they must preserve the freshness of food (Haq, Hasnain, & Azam, 2014). Currently, the traditional agricultural commodities appear as interesting sources of film-forming material. Thus, the commercialization of biopolymers films such as polysaccharides, proteins and lipids gained importance (Tavassoli-kafrani, Shekarchizadeh, & Masoudpour-behabadi, 2016). They denote viable alternatives in complementing or replacing the traditional materials.

Pectins are composed of natural complex heteropolysaccharides composed of residues of  $\alpha$ -D-galacturonic acid (1, 4) and a variety of neutral sugars such as rhamnose, galactose and arabinose. It is the second most abundant component of the cell wall of terrestrial plants (Noreen, Nazli, Akram, Rasul, & Mansha, 2017); and it is used to produce edible films for applications in food packaging (Oliveira et al., 2016). Gezgin, Lee, & Huang, (2017) reported that pectin properties vary with the degree of methyl esterification (DM), being classified as high pectin (HDM) or low methyl esterification (LDM). Thus, it has several applications due to its gelling agent capacity, besides to be non-toxic, biocompatible and biodegradable. However, pectin-based packaging generally does not correspond to the physical properties of conventional packages (Naqash, Masoodi, Rather, Wani, & Gani, 2017).

Considering this aspect, the nanostructures addition in polysaccharide matrices may allow the preparation of nanocomposite films with improved physical properties. The improved packaging concentrates on nanomaterials that improve barrier properties, strength, flexibility and stability (Dufresne, 2017). Bora & Mishra (2016) reported improvements in antimicrobial properties and an increase in the light barrier property of multifunctional films (pectin-sodium alginate/casein) containing silver-casein nanoparticles. Otoni et al. (2014) noted an improvement in physical, mechanical, barrier and antimicrobial properties in pectin/papaya purée based films incorporated with essential oil nanoemulsions. Pectin films with chitosan nanoparticles also showed an improvement in their mechanical and thermal properties (Lorevice, Otoni, Moura, & Mattoso, 2016).

Therefore, this work aimed to formulate and characterize nanoemulsions, in addition to improving the physical properties of hydrophilic pectin films by adopting a nanocomposite strategy to be used in seed coating. Carnauba wax and Neem oil were selected as the nanoemulsion layer due to their good adhesion and inherent hydrophobicity. The influence of the incorporation of the different concentrations of these hydrophobic materials on the mechanical, thermal and biodegradation properties of the films was investigated. Moreover, the nanocomposites properties were compared in relation to the commercial polymer used used in the seed industry.

## **2 MATERIAL AND METHODS**

### **2.1 Materials**

The materials used in the study were: Neem oil (obtained from Laszlo Aromatologia); Carnauba Was-type 1 (Megh Wax ECF-124, obtained from Megh Indústria e Comércio Ltda, BRAZIL); Tween 20 (polyoxyethylene (20) sorbitan monolaurate (HLB = 16.7), obtained from Sigma Aldrich, IND); pectin HDM (DM > 50% and  $M_w = 130,000 \text{ g mol}^{-1}$ ) (provided by CP Kelco Brazil S/A, BRAZIL) and Ultrapure Water of a Milli Q,  $18.2 \text{ M}\Omega\cdot\text{cm}$  at  $25^\circ \text{C}$ .

### **2.2 Nanoparticles formulation and characterization**

#### **2.2.1 Formulation**

Nanoemulsions were prepared by the casting technique with adaptations proposed by Jadhav, Holkar, Karekar, Pinjari, & Pandit (2015) and Sekar, Sivakumar, Mukherjee, & Chandrasekaran, (2015). Carnauba wax (5% w/v), Tween 20, 1.5% (w/v) and water were used as fase lipid, surfactant and as the aqueous phase respectively. The emulsion was stirred for 10 minutes using a sonicator (Branson Digital Model-Model 450, Danbury-USA) at 450 W.

In the Neem oil nanoemulsion (3% m/v), the Tween 20 was used in the ratio of 3:1 (oil: surfactant) and the water was used as the continuous phase. The emulsion was subjected to stirring using a 20 kHz ultrasound processor - 450 W for 45 minutes (Branson Digital-Model 450). The sonication procedures were applied with an amplitude of 50%.

#### **2.3 Particle size, size distribution, and short-term stability**

The mean size and particle size distributions of the nanoemulsion were determined by dynamic scattering of light in a Zanosizer Nano Series (Malvern Instruments Inc., Worcestershire, U.K.) 1h and 48h after the emulsification. Ultrapure water was used as a

dispersant to avoid multiple dispersion effects and interactions between the particles. The cumulative mean (z-diameter) and the polydispersity index (PdI) were used to describe the size and particle size distribution respectively.

## 2.4 Film preparation

The solutions (3% w/v) of HMP pectin were prepared by dissolving the powdered pectin in ultrapure water, homogenized at 1500 rpm for 24 hours until the complete solubilization. Then, the solutions were incorporated with 3% (m/v) Neem oil or 5% (m/v) carnauba wax, producing Neem oil nanocomposites: NOil10, NOil20 and NOil30; and carnauba wax nanocomposites: NWax10, NWax20 and NWax30. These contents were based in pre-testes.

The composition of the film-forming solutions are shown in Table 1. The solutions were rested for 4 h for bubble elimination before applying the casting method at  $25 \pm 2^\circ \text{C}$  for 48 h to make the films. The dried films were cut and stored hermetically under refrigeration until the analyzes moment. A commercial L232 low permeability polymer from Incontec was used for comparison.

**TABLE 1** – Compositions of aqueous film-forming solutions

<b>Film</b>	<b>HMP (% wt)</b>	<b>Neem Oil (%wt)</b>	<b>Carnauba Wax (%wt)</b>	<b>Ratio HMP/Lipid</b>
HMP - Control (-)	3	-	-	100/0
L232 - Control (+)	-	-	-	-
NOil10	3	3	-	90/10
NOil20	3	3	-	80/20
NOil30	3	3	-	70/30
NWax10	3	-	5	90/10
NWax20	3	-	5	80/20
NWax30	3	-	5	70/30

*Note.* HMP: high methylester pectin. L232: Polymer for industrial seed coating. Noil: Nanocomposite of pectin and Neem oil. Nwax: Nanocomposite of pectin and Carnauba wax.

## 2.5 Nanocomposites characterization

### 2.5.1 Film thickness

The film thickness was measured using a Micrometer 250 from Lorentzen & Wettre (L & W), Sweden. Measurements were made in at least eight random points of the same sample and the mean value was used to calculate the tensile strength of the film.

### 2.5.2 FT-IR characterization

The characterization was performed according to Li, Nie, Chen, Xiang, & Li, (2016) with modifications. The samples were prepared on KBr discs (1 mg sample in 100 mg KBr) with hydrostatic pressure at a force of 5 cm<sup>2</sup>. Measurements were recorded by a Shimadzu-IRAffinity (Shimadzu Co., Kyoto, Japan) spectrophotometer equipped with an Attenuated Total Reflectance device for spectral region analysis (4000-500 cm<sup>-1</sup>) with 64 scans recorded at a resolution of 4 cm<sup>-1</sup>. Analyzes were performed in duplicate.

### 2.5.3 Mechanical properties

The tensile tests were determined using a texture analyzer model TA-XT2i (Stable Micro Systems, UK) according to Núñez-Flores et al., (2013). The specimens were dimensioned in 20 mm x 100 mm. Assays were run using a 1 kN load cell at a crosshead speed of 125 mm.sec<sup>-1</sup>. The tensile strength and the elasticity modulus were determined by the mean values of ten measurements.

The films were cut into 9 cm<sup>2</sup> squares and they were fixed to a support with central aperture for puncture testing. Then, a 5.0 mm diameter spherical probe was perpendicular displaced to the film surface at a constant velocity of 0.8 mm/s until the tube passed through the film. The puncture resistance was calculated by dividing the load at the break point by the film thickness. The results were calculated by averaging 10 measurements (Zivanovic S., Chi S., Draughon, 2005).

### 2.5.4 Water vapor permeability (WVP)

The water vapor transmission rate (WVTR) of the films was determined by the standard gravimetric method proposed by the ASTM E 96-00. The specimens were cut to a 5.25 mm radius and placed in amber glass vial holes with  $\frac{3}{4}$  of their volume containing silica previously dried for 24 h at 150 ° C.

The glass had a diameter, length and base diameter of 10 mm, 58 mm and 26 mm respectively. The caps (height = 18 mm, diameter = 20 mm) were drilled in their upper portion with the diameter of the glass permeation area. The glass flasks were placed in airtight desiccators at 18.5 ± 2 ° C. This system defines a water activity equal to 0 (zero) in the atmosphere in contact with the sample face.

Weight gain measurements were performed by weighing the test vial daily, using an electronic scale of 0.001 g. thus, the water uptake in the vial was measured every 24 h during 168 h (7 days).

Weight gain versus time was used to determine the WVTR. The slope of the graph linear portion represented the steady state amount of water vapor that diffused through the sample per time unit (g / h). The WVTR was expressed in gram per square meter per day units. The steady state over time (slope) yielded a regression coefficient of 0.93 or greater.

Three replicates were performed for each nanocomposite, yielding a total of 21 replicates, including the pure pectin film. Sample WVPs were calculated by multiplying the WVTR constant by the sample thickness (mm) and dividing this result by the water vapor pressure difference ( $\Delta p$  in KPa) through the sample (Thawien Bourtoom & Chinnan, 2008).  $\Delta p$  is the ambient vapor pressure difference containing silica gel (zero) and pure water (2.11297 KPa at 18.5 °C). Calculated using the Tetens equation (VO, 1930).

### **2.5.5 Thermal analysis**

The thermal stability was investigated using a thermogravimetric analyzer DTG-60AH, TGA (Shimadzu Co., Kyoto, Japan). The samples were placed in platinum crucibles and then they were subjected to heating at  $10^\circ \text{C} \cdot \text{min}^{-1}$ , from 25 to  $600^\circ \text{C}$  under a nitrogen atmosphere with a flow rate of  $50 \text{ mL} \cdot \text{min}^{-1}$ . The differential scanning calorimetry (DSC) was determined using a calorimeter DSC-60 (Shimadzu Co., Kyoto, Japan). Each sample (3-5 mg) was heated in a crucible at a heating rate of  $5^\circ \text{C} \cdot \text{min}^{-1}$  from 25 to  $200^\circ \text{C}$ , under a nitrogen atmosphere with a flow rate of  $40 \text{ mL} \cdot \text{min}^{-1}$ . The determinations were performed in triplicate (Mishra, Majeed, & Banthia, 2016).

### **2.5.6 Biodegradation Test**

#### **2.5.6.1 Soil preparation**

The soil was prepared with three equal parts of fertile (low clay) soil, manure and sand (42 mesh), yielding a total dry land weight of 15 kg as described by ASTM G160-98. After mixing, the soil was sieved using a 4 mesh sieve and it was aged for 3 months, being its pH (6.5-7.5) and humidity (20-30%) monitored twice monthly. The desired pH was reached by the calcium carbonate (increase pH) or sulfur (decrease pH) addition. After 3 months, a cotton cloth ( $400\text{-}475 \text{ gm}^{-2}$ ) was buried for the viability control and the tensile strength was measured after 5 days. When a decrease of more than 50% this property was detected, the land was considered suitable for use. Then it was placed in deep containers with a height of 17 cm.

### 2.5.6.2 Biodegradation test

The pectin films (5 x 5 cm) with different carnauba wax (Nwax10, Nwax20 and Nwax30) and Neem oil (Noil10, Noil20 and Noil30) contents, and the commercial polymer L232 were buried in the above-mentioned containers in a soil of 17 cm depth for 90 days. Soil moisture was maintained between 20 and 30%, based on their dry weight. The water lost due to the evaporation was replaced without soil deformation. The containers were placed in a room with controlled temperature ( $30 \pm 2^\circ \text{C}$ ) and humidity (between 85 and 95%). Samples were analyzed every 15 days by visual morphology and by optical microscopy before and after biodegradation.

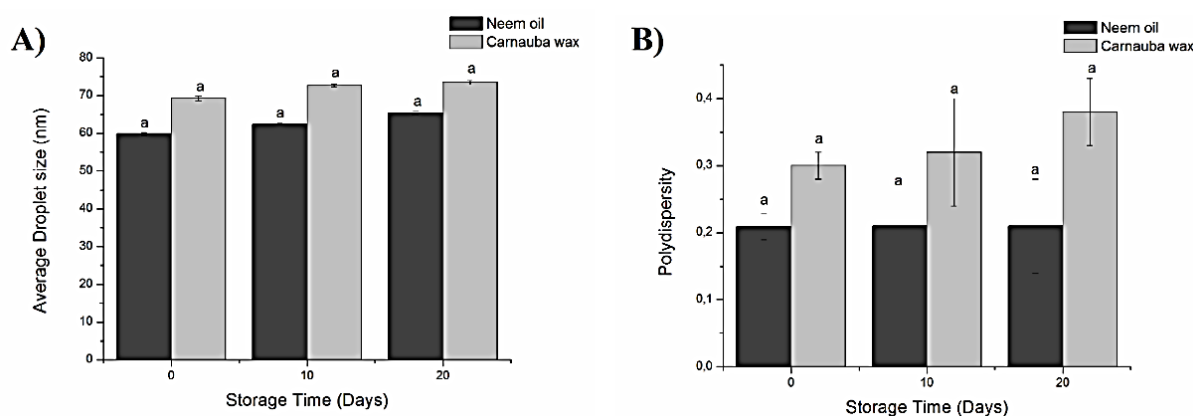
## 2.6 Statistical analysis

The results were evaluated by ANOVA and Tukey's test, using the software "Statistica Analysis Systems" 8.0.

## 3 RESULTS AND DISCUSSION

### 3.1 Particle size, size distribution and stability

Figure 1 shows the values obtained for the particle size, polydispersity index (PdI) and stability of the two nanoemulsion systems studied.



**FIGURE 1** – Particle size (DLS) (A), polydispersity index (PDI) (B) and stability of carnauba wax and neem oil nanoemulsions

The average particle diameter of both systems measured by dynamic light scattering differed statically ( $p \leq 0.05$ ), with values of 69.28 nm and 59.87 nm for carnauba wax and Neem nanoemulsions respectively. According to (Dammak, Carvalho, Sílvia, Trindade, &



José, 2017) and (Kheradmandnia & Farahani, 2010), the nanoemulsions particle size reduction may present improvements in the physical properties of polysaccharide matrix films. Moreover, it was noted that the nanoemulsions particle size remained unchanged after 20 days of emulsification (Figure 1A). This indicates that the solution stability would not be lost by the particles coalescence during the drying period of the films preparation (Otoni et al., 2014). In addition, being the Brownian motion lower than the gravity-induced cracking rate, the nanoparticles sedimentation process probably would not occur (Yi, Li, Zhong, & Yokoyama, 2014).

The polydispersity index indicates how the particle size distribution is dispersed. The particle size distributions of carnauba wax and Neem oil presented values smaller than 0.30 (Figure 1 B) and they did not differ statistically from each other ( $P \geq 0.05$ ). According to (Polychniatou & Tzia, 2016), small PdI values between 0.2 and 0.3 are related to narrow-size distributions. The low PdI values observed in this study may have contributed to a greater nanoemulsions stability (Tan et al., 2016).

## 3.2 Nanocomposites characterization

### 3.2.1 Thickness

The thickness values obtained from pectin nanocomposite films are shown in Table 2. The nanocomposites added by the nanoparticles of both wax and Neem oil showed an increase in their thicknesses ( $p \leq 0.05$ ) regarding the pectin control film. This increase occurred due to the incorporation of the solid nanoemulsion contents, associated with the changes in the casting technique applied in the preparation of the filmogenic solutions.

**TABLE 2** – Mechanical Properties: tensile strength (TS), modulus of elasticity (EM) and elongation at rupture (EB), of pectin films incorporated with carnauba wax and neem oil nanoemulsions

Film	Thickness ( $\mu\text{m}$ )	TS (MPa)	EM (MPa)	EB (%)	PS (N/mm)
HDM pectin	76.67 $\pm$ 0.12 <sup>a</sup>	28.0 $\pm$ 0.22 <sup>a</sup>	1990.29 $\pm$ 18.17 <sup>a</sup>	1.08 $\pm$ 0.05 <sup>a</sup>	359.93 $\pm$ 37.48 <sup>a</sup>
Nwax10	104.23 $\pm$ 0.23 <sup>b</sup>	28.99 $\pm$ 0.44 <sup>a</sup>	885.53 $\pm$ 6.14 <sup>b</sup>	3.23 $\pm$ 0.22 <sup>b</sup>	232.28 $\pm$ 9.70 <sup>c</sup>
Nwax20	112.7 $\pm$ 0.56 <sup>b</sup>	29.02 $\pm$ 0.20 <sup>a</sup>	721.86 $\pm$ 6.14 <sup>c</sup>	3.17 $\pm$ 0.19 <sup>b</sup>	200.42 $\pm$ 6.86 <sup>d</sup>
Nwax30	96.98 $\pm$ 0.45 <sup>ab</sup>	29.49 $\pm$ 0.34 <sup>a</sup>	668.47 $\pm$ 51.36 <sup>c</sup>	3.84 $\pm$ 0.11 <sup>bc</sup>	157.78 $\pm$ 7.49 <sup>cd</sup>
Noil10	112.14 $\pm$ 0.68 <sup>b</sup>	29.86 $\pm$ 0.92 <sup>a</sup>	525.99 $\pm$ 13.75 <sup>d</sup>	3.38 $\pm$ 0.31 <sup>bc</sup>	280.39 $\pm$ 9.46 <sup>b</sup>
Noil20	101,34 $\pm$ 0,32 <sup>b</sup>	30,50 $\pm$ 0,46 <sup>a</sup>	497,74 $\pm$ 21,16 <sup>d</sup>	3,80 $\pm$ 0,62 <sup>bc</sup>	234,95 $\pm$ 17,10 <sup>c</sup>
Noil 30	123,28 $\pm$ 0,76 <sup>b</sup>	30,28 $\pm$ 0,83 <sup>a</sup>	493,19 $\pm$ 16,50 <sup>d</sup>	4,28 $\pm$ 0,36 <sup>d</sup>	93,26 $\pm$ 2,56 <sup>e</sup>

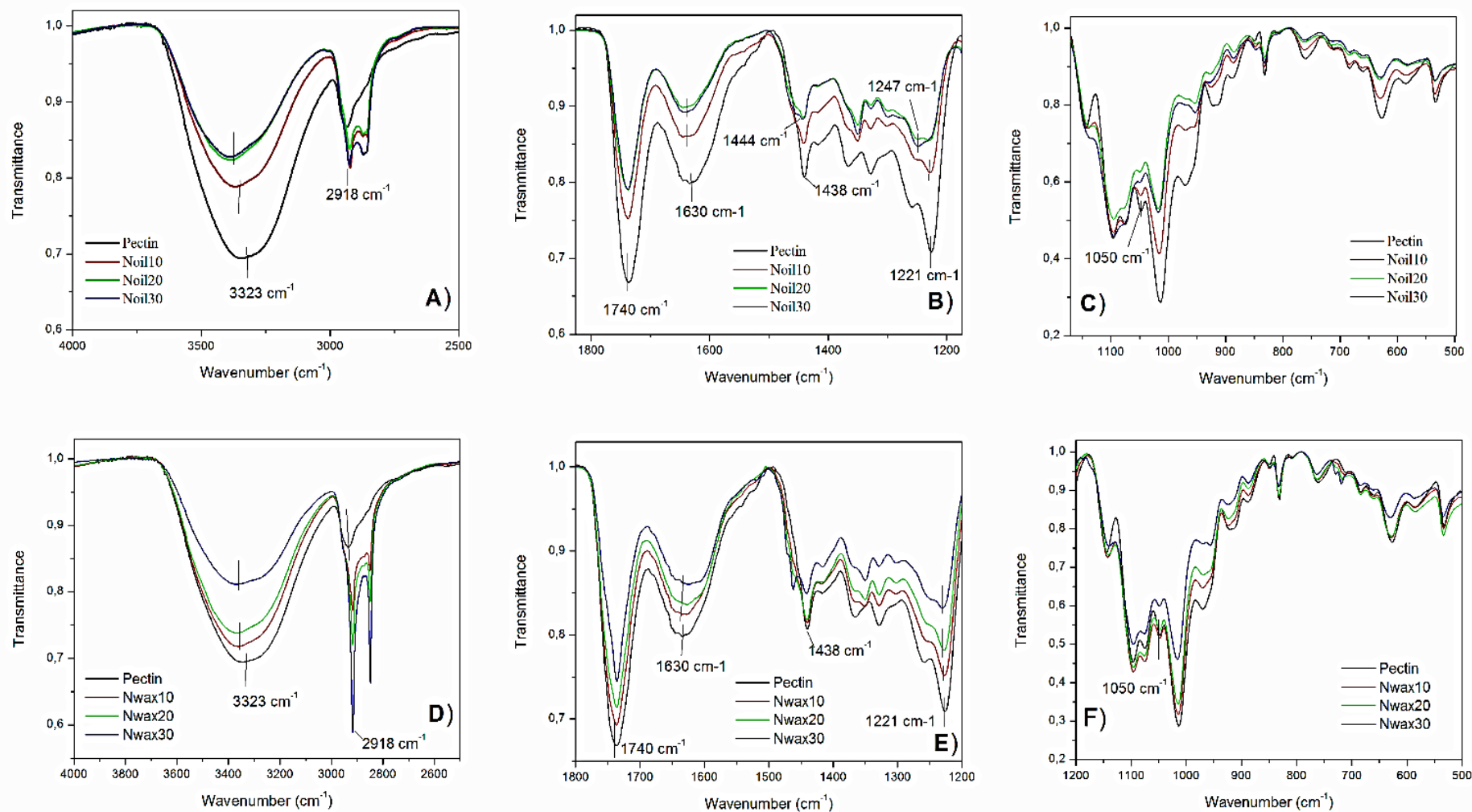
*Note.* Means in the same column with the same letter did not differ statistically ( $p > 0.05$ ).

### 3.2.2 Infrared Spectroscopy

FT-IR spectroscopy provides information on the biomolecules conformational and structural dynamics (Figure 2). The pectin spectrum showed absorption bands of  $1050\text{ cm}^{-1}$ , axial strain absorption characteristics of  $\text{-CH-OH}$ , and  $1622\text{ cm}^{-1}$  correspond to the  $\text{COOH}$  deformation. The band in the  $1740\text{ cm}^{-1}$  region corresponds to the  $\text{C}=\text{O}$  axial methylcarboxylate (methyl ester) group deformation (Abbas et al., 2015; Kumar, Sharma, Pathania, & Kothiyal, 2015). The bands associated to  $2934\text{ cm}^{-1}$  correspond to the elongation of the  $\text{-O-CH}_3$  groups present in galacturonic acid (Lorevice et al., 2016). In addition, the bands between  $3400$  and  $2500\text{ cm}^{-1}$  represent the elongation of the hydroxyl groups due to intermolecular hydrogen bonding between pectin monomers (Singthong, Cui, Ningsanond, & Goff, 2004).

Noil and Nwax nanocomposites were analyzed by FT-IR to find interactions between the nanoemulsions and the pectin matrix. One way of identifying interactions between the nanoemulsions and the pectin matrix is by analyzing changes in particular bands of each compound. Such interactions provide explanations for the mechanical, thermal and barrier properties of polymer films (Lorevice et al., 2016). Changes in stretching vibrations of the Noil and Nwax spectra were observed at  $3323\text{ cm}^{-1}$ . This suggests the existence of interactions between the alcohol of the lipophilic material and the pectin hydroxyl ( $\text{-OH}$ ), resulting in stronger interactions between the polymer matrix and nanoemulsions (Volli & Singh, 2012). Stretching vibrations at  $2918\text{ cm}^{-1}$  suggest the methyl ester groups stretches of galaturonic acid (Lim, Yoo, Ko, & Lee, 2015). These small changes are directly related to the increased lipophilic material concentration and demonstrate that the binding among the functional pectin groups and the Azadirachtin Neem Oil reactive group (Sanuja, Agalya, & Umopathy, 2015). An elongation value of  $1630\text{ cm}^{-1}$  can be related to the  $\text{COOH}$  of the pectin structure. The displacement and intensity of  $\text{C-O}$  ester bonds at  $1438\text{ cm}^{-1}$  to  $1444\text{ cm}^{-1}$  of pectin nanocomposite films showed the presence of different interactions as polar bonds between the nanoemulsions and the pectin (De, Gupta, Mandal, & Karak, 2015b).

In addition, displacement of the  $\text{C}=\text{O}$  vibrational elongation in the  $1221\text{ cm}^{-1}$  region by acids, esters, ethers and alcohols presence can be observed in Neem oil nanocomposites (Volli & Singh, 2012). These interactions, together with the acetylated pectates of polymer matrix, are more intense regarding to the band identified in nanocomposites with higher neem oil concentrations. As the neem oil and the pectin have similar groups in their molecules, these interactions can not be conclusive. They only suggest one sign of the Noil interactions. No other changes could be found in the pectin/nanoemulsion spectra that would be useful to indicate interactions between the nanoemulsions and the pectin matrix.



**FIGURE 2** – FTIR spectra of pectin, carnauba wax nanocomposite (D, E and F) and neem oil nanocomposite (A, B and C), in different proportions

### 3.2.3 Mechanical Property

The films mechanical properties (tensile strength (TS), modulus of elasticity (EM) and elongation at rupture (EB)) evaluate their integrity and performance during the use, storage and handling. They may be associated with chemical conformations and interactions (Mchught & Krochta, 1994). Table 2 shows that the nanoemulsions incorporation did not promote an effect on the tensile strength regarding to the control film ( $p \geq 0.05$ ). Li et al. (2016) and Otoni et al (2014) reported a reduction in tensile strength with the lipid phase incorporation in pectin films. However, it was not observed in this study. In general, oil phases additions lead to the heterogeneous films formation with discontinuity in the polymer matrix (Zúniga, Skurtys, Osorio, Aguilera, & Pedreschi, 2012). Thus, it is hypothesized that both nanoemulsions decreased the number of free hydroxyl groups by interactions with pectin acids groups due to the lipids incorporation. This possibly explains the no differences detected in this parameter. This result is in agreement with the water vapor permeability reduction obtained.

There was a significant difference ( $p \leq 0.05$ ) between the films evaluated in the modulus of elasticity parameter. A reduction in the elasticity modulus of the films was observed with the nanoemulsions incorporation to the polymer matrix. The highest ME reduction was observed in the incorporation of Neem oil nanoemulsion. Thus, the films presented a reduction in their rigidity and a greater material malleability. It is assumed that the lipid phase acted as a plasticizing agent, generating a greater mobility between the adjacent polymer chains, and conferring extensibility to the nanocomposite films (Aguirre, Borneo, & Leo, 2013; Sharma, Khatkar, Kaushik, Sharma, & Sharma, 2017). The increase in malleability due to the reduction of EM possibility to the coating to adapt to the irregularity of the application surface of this material. Associated to this, the elongation at break (EB%) parameter presented a significant difference ( $p \leq 0.05$ ) among the evaluated films. The results presented in Table 2 corroborated the discussion regarding to the nanoemulsions participation as plasticizing agents due to the films elongation increase.

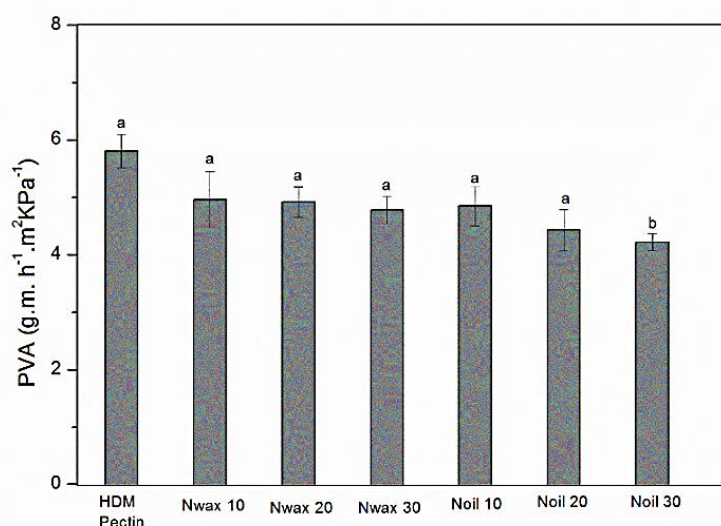
According to (Kokoszka, Debeaufort, Hambleton, Lenart, & Voilley, 2010), the plasticizers weaken the intermolecular interactions among the polymer chains in order to increase the free volume and mobility, leading to a decrease in stiffness and the film extensibility and flexibility improvement. High E% values suggest good flexibility, extensibility and resistance due to the cohesion among the polymer chains (Lorevice et al., 2016). According to (Bonnaillie, Zhang, Akkurt, Yam, & Tomasula, 2014) the pectin can

present different molecular arrangements depending on the composition of the film, favoring its mechanical properties.

The nanoemulsions incorporation to the polymer matrix differed to the control sample ( $p \leq 0.05$ ) in the puncture tests, promoting a reduction in the force applied to the puncture (Table 2). TS is a measure related to the hardness of a film under stress applied at right angles to its surface, generating multidirectional forces. PS is a measure of the elasticity under a vertically charged tension (PARK & ZHAO, 2004). This reduction occurred due to the plasticizing effect of nanoemulsions incorporated into the matrix, which promoted mobility to the polymer chains (Maran, Sivakumar, Sridhar, & Immanuel, 2013).

### 3.2.4 Water vapor transmission rate (WVTR)

The water vapor permeability (PVA) predicts the rate of water vapor transmission per unit area through the film of known thickness, induced by a pressure gradient between two specific surfaces, and of relative humidity and specified temperature. It implies moisture barrier properties of the materials (Otoni et al., 2014). The incorporation of the different nanoemulsions types and concentrations to the pectin matrix promoted a significant reduction ( $p \leq 0.05$ ) of the PVA in the Noil30 films (Figure 3). The pectin films presented WVTR of  $5,81 \pm 0,89 \text{ g.m.h}^{-1}.\text{m}^2\text{kPa}^{-1}$ , whereas in the Nwax30 and Noil30 films, this value decreased to  $4,78 \pm 0,08 \text{ g.m.h}^{-1}.\text{m}^2\text{kPa}^{-1}$  and  $4,22 \pm 0,15 \text{ g.m.h}^{-1}.\text{m}^2\text{kPa}^{-1}$  respectively. In addition to the feasibility of the polysaccharides application for the packages elaboration, nanoemulsions were added to polysaccharide matrices with the aim of improving water barrier properties in this study.



**FIGURE 3** – Permeability to water vapor of pure pectin films and nanocomposites

The main reason for the WVTRs reduction in Noil films compared to the pectin is possibly related to the amount of polar bonds and the hydrophilic interaction of the lipid material with acids groups in the polymer matrix. It was noted that Noil increased the water vapor barrier by obstructing the direct path of water vapor transposition. According to (Antoniou, Liu, Majeed, & Zhong, 2015), the arrangement of nanoemulsions can clog the films pores causing more tortuous pathways for the diffusion of the water vapor molecules.

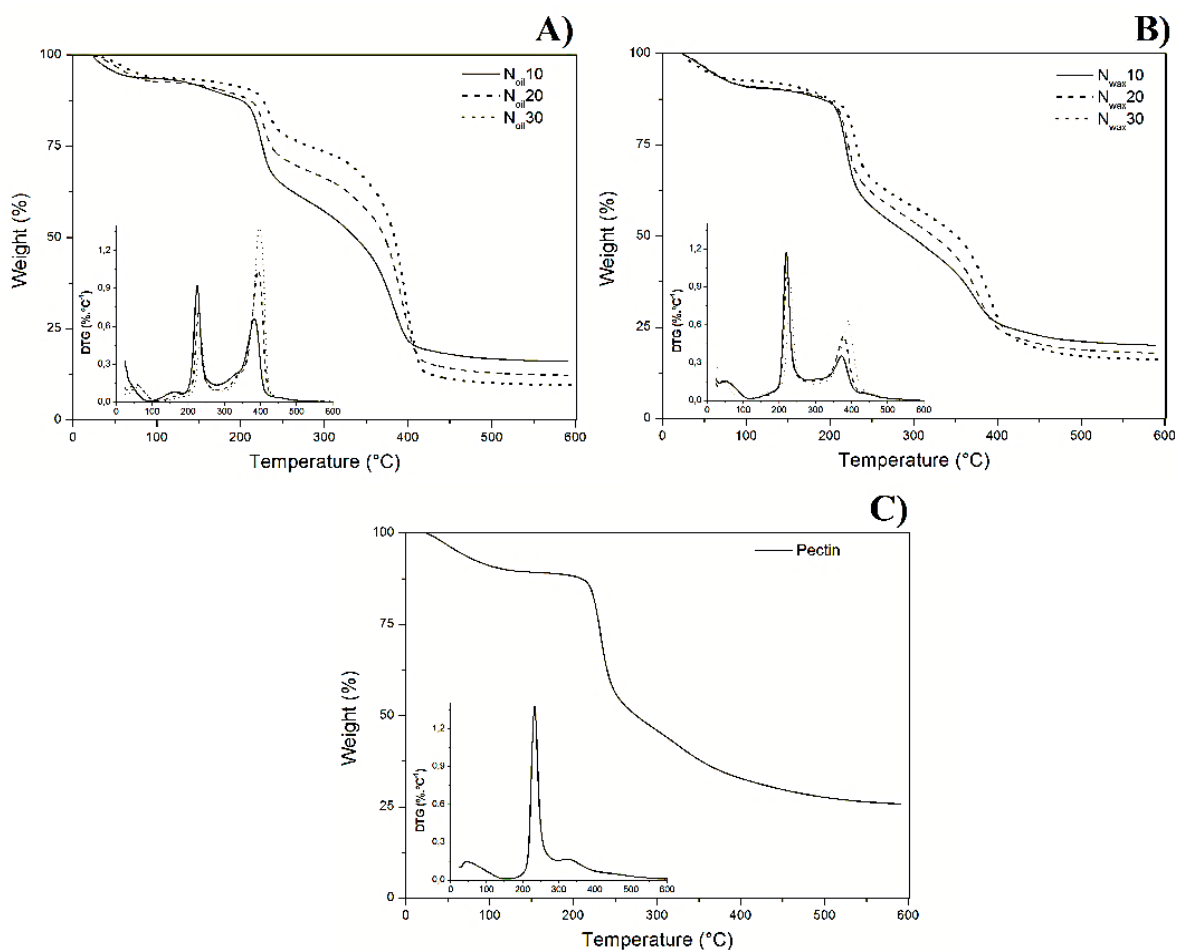
When the nanoemulsions were added to the pectin matrix, their surfaces interacted with the pectin hydroxyl groups, promoting a reduction of the band elongation around  $3323\text{ cm}^{-1}$  in FT-IR spectra (Figure 2). Moreover, the TS values increase (Table 2) after the Noil and Nwax addition indicates the presence of interactions between the nanoemulsions and the pectin. This also corroborates the hypothesis that both nanoemulsions decreased the number of free hydroxyl groups. (Antoniou et al., 2015).

The reaction among the pectin functional groups and the OH group of the active neem compound (Azadirachtin) interacted more effectively, promoting the reduction of the number of polar groups responsible for the hydrophilic nature present in the water vapor (Sanuja et al., 2015). T Bourtoom & Chinnan (2008) have suggested that lipids with lower chains are more efficient in reducing the transfer of moisture through films due to their greater homogeneity. This can be explained by the better dispersion of the Noil in composite films. These results corroborated the discussion about the water vapor barrier increase in lipid-embedded polysaccharide films (Cano, Cháfer, Chiralt, & González-martínez, 2016; Chiumarelli & Hubinger, 2014; Santos, Ribeiro, Caceres, Ito, & Azeredo, 2014; Sanuja et al., 2015).

### 3.2.5 Thermogravimetric analysis

Figure 4 shows the thermograms of the pectin, Noil and Nwax films. They show the mass loss and the maximum decomposition temperature ( $T_{max}$ ) through the peaks of the TGA derivatives (DTGA).

The pure pectin thermogram (Figure 4C) showed a thermal degradation profile during three moments: (I) at  $80^{\circ}\text{C}$ , which corresponds to the water loss (reduction of 10% the initial mass); (II) at  $232^{\circ}\text{C}$  -  $326^{\circ}\text{C}$ . In this temperature range, the degradation due to 42% the mass loss, occurs mainly due to the material pyrolytic decomposition, occurring processes of primary and secondary decarboxylation (Einhorn-stoll & Kunzek, 2009); (III) A final mass loss at  $350^{\circ}\text{C}$  -  $600^{\circ}\text{C}$  was observed in TG graphs, with a loss of 21% corresponding to the oxidation region. The three moments of the pectin thermogram were observed in the nanocomposite films.



**FIGURE 4** – TGA and DTGA curves of Noil (A), Nwax (B) and pure pectin (C) films

It is observed that the moment I is slightly anticipated and reduced for nanocomposites (Table 3) regarding to the pure pectin. It is inferred that samples incorporating nanocomposites have a smaller amount of water absorbed than pure pectin. This corroborates the results obtained in the permeability test.

**TABLE 3** – TGA data of nanocomposites and pectin film

Treatments	T <sub>onset</sub> (°C)	Peak 1 (°C)	Peak 2 (°C)	Peak 3 (°C)	Peak 4 (°C)	Volatiles (%)
<b>HDM Pectina</b>	221.15	45.95	232.55	326.51	-	10.85
<b>N<sub>oil</sub>10</b>	215.81	169.02	231.89	337.10	390.03	6.51
<b>N<sub>oil</sub>20</b>	215.77	55.26	231.66	340.55	396.14	7.51
<b>N<sub>oil</sub>30</b>	225.03	59.23	231.09	342.25	425.87	6.43
<b>N<sub>wax</sub>10</b>	214.30	55.26	226.55	378.55	-	9.56
<b>N<sub>wax</sub>20</b>	214.65	59.80	227.69	384.23	-	9.65
<b>N<sub>wax</sub>30</b>	222.64	55.87	239.16	399.30	-	7.48

The degradation steps appear delayed in all the nanocomposites at time II, at 230 °C (Figures 4A and B). In Figure 4 (A and B) it is observed that the incorporation of the nanoemulsions promoted an increase of the degradation temperature relative to the pure pectin (326° C), Nwax (399° C) and Noil (342° C); and a mass loss of a 42% and 62% for the Nwax and Noil respectively. According to Gorrasi, Bugatti, & Vittoria (2012), the Tmax increase is directly related to the increase of the nanoemulsion concentration and to the higher chemical interactions of the lipid phase with the polymer matrix.

The same can be observed in the DTG peaks. Figure 4 shows pectin degradation at 232° C and the oxidation of unsaturated oleic and linoleic acids present in the lipid phases incorporated in the polymer matrix (Figures 4 A and B). At 425 °C, the degradation is related to the saturated fatty acids oxidation (Almeida et al., 2017). The nanoemulsions presence helps degrade the pectin at higher temperatures. This improvement may be due to the nanoemulsions layers, which create a more tortuous path, preventing the volatile material permeation. Other possibility would be the existence of a protective effect of the lipid material, which increases the thermal stability of the nanocomposite. This increase is a confirmation of a strong interaction between the pectin and the lipid phase (Gorrasi et al., 2012). The importance of this increase in degradation temperature is to confirm that a coating will be capable of transporting and storing even at higher temperatures without any damage to its structure.

### **3.2.6 Soil biodegradation**

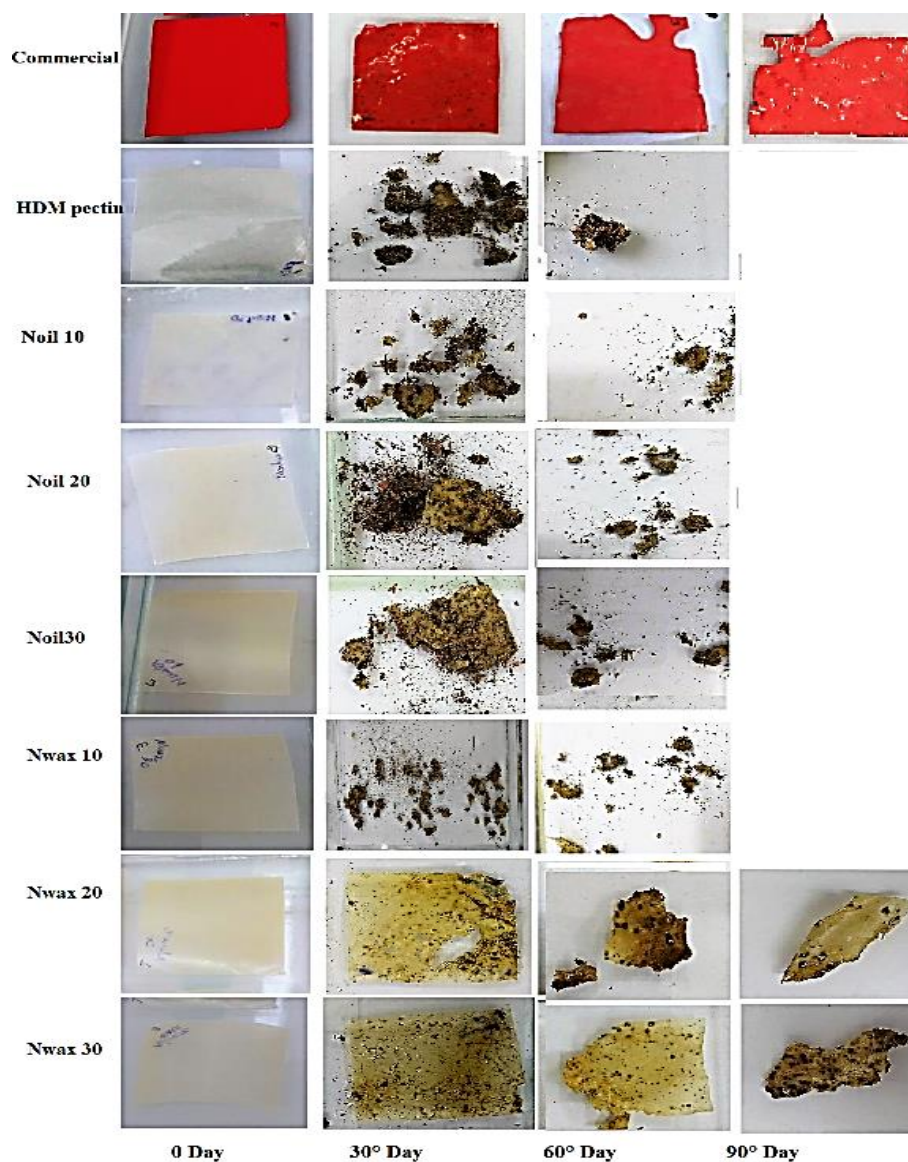
The nanocomposites biodegradability was evaluated in an active wet soil during 3 months. The samples were evaluated visually (Figure 5) and by light microscopy (Figure 6) before and after the soil degradation. All samples were compared with a commercial synthetic polymer applied to the seed coat (Figure 7).

Figure 5 shows a darkening and a degradation increase of the nanocomposites during the decomposition time in the soil. These results differed from those observed for the synthetic polymer.

The pectin biodegradation process occurs by the hydrolytic oxidation processes by natural biological systems. Pectin can be readily used as a carbon source in the biodegradation process, once it is a natural polymer. A large number of microorganisms are involved in the pectin digestion. Thus, several fungal agents produce polygalacturonases (PGs), which depolymerize the pectin HG domain and cause the cell wall decomposition. Other enzymes, that degrade the wall cellulose and hemicellulose (cellulase and xylanase respectively) are



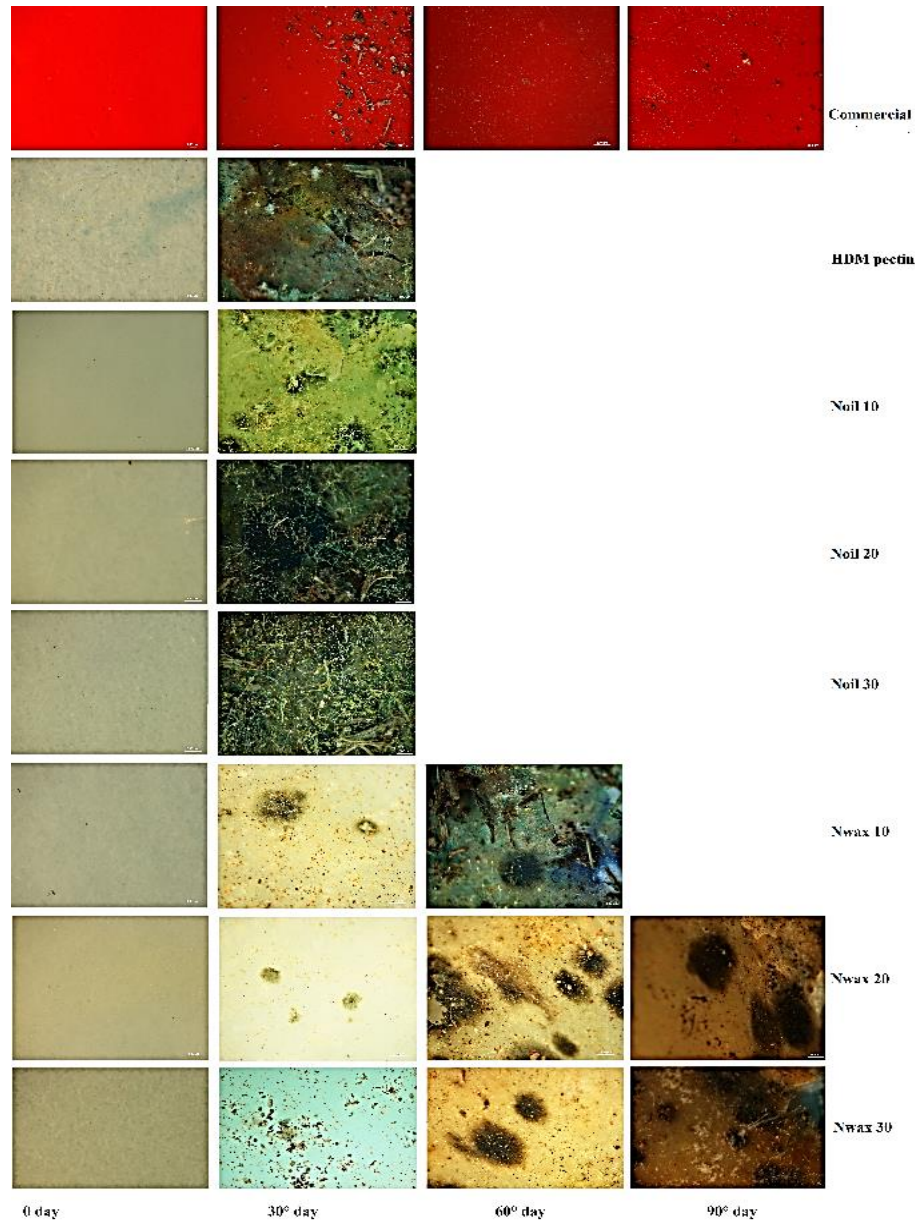
also produced by the pathogens to promote their degradation (Wang, Ren, Ding, Xu, & Chen, 2018). On the other hand, synthetic polymers generally have small repeating units in their structure and this regularity in their chain increases their crystallization, causing hydrolysable groups of the polymer to be inaccessible to the enzymes. Therefore, many synthetic polymers are considered resistant to the microorganisms attack (Chandra & Rustgi, 1997).



**FIGURE 5** – Pectin films, commercial polymer and bionacomposites visual analysis after different days of contact with the soil

Figure 6 shows the appearance of whitish spots throughout the material during the biodegradation qualitative evaluation period. According to the literature, this can occur due to the microorganisms adhesion to the material surface (Araújo, Damasceno, Carvalho, Alves, & Barbosa, 2015). It is possible to note that the films have undergone changes in their surfaces.

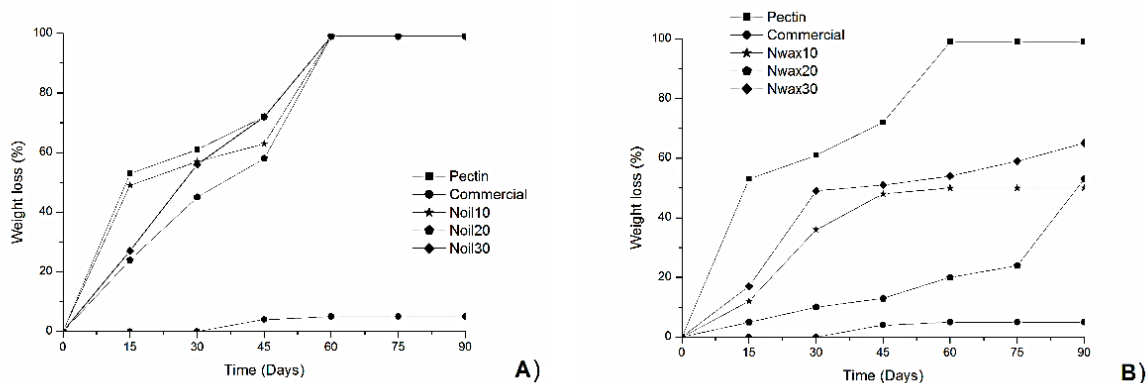
This occurred due to the degradation of the biopolymer surface by microorganisms, promoting modifications through the deposition of released extracellular material. Associated with the accumulation of water, this generates the fungal colonies proliferation with the microbial filaments invagination to the material interfaces (Flemming, 1998).



**FIGURE 6** – Reflective optical micrograph (20 ×) of the pectin films, synthetic polymer and biocomposites exposed to soil biodegradation for 0-3 months

It can be verified that the nanocomposite materials, mainly the Nwax, presented lower indices of mass loss in the initial phase (15 days) (Figure 7), acting as a protective barrier to biodegradation. However, Noil nanocomposites presented advanced degradation

stage, with a mass loss between 65 and 70% from the 45th day. These results are similar to those observed in pectin control films. After 60 days, fragments of these samples could not be found.



**FIGURE 7** – Mass loss percentage of the pectin, synthetic polymer and nanocomposite films (Nem - A) and carnauba wax - B) as a function of the biodegradation time.

The results indicated that the kinetic biodegradation of the pure pectin and Noil films in the soil were faster than the Nwax film. Nwax showed a low degradation after 75 days, with 55% its initial mass. However, this degradation was superior regarding to the synthetic polymer degradation, which presented only 7% its biodegraded mass after 90 days. Therefore, the nanoemulsion content visibly altered the biodegradability.

Jarerat & Tokiwa (2003), reported that the microorganisms colonization on the films surface occurs without the distinction of the amorphous or crystalline region. However, microorganisms secrete enzymes that promote degradation preferentially in the amorphous region. The soluble products obtained from the degradation are then absorbed through the cell wall and metabolized. As a result of the enzymatic action, surface erosion occurs, forming spherical or tubular holes. Schneider et al. (2010) noted that the degree increase and the crystallinity modulus decreased the degrading bacteria colonization on the films surface and consequently on their degradation rate. The polymers degradation rate depends on the environment around the sample (moisture, nutrient availability, pH, temperature), as well as the crystallinity degree, polymer molar mass and the monomeric units incorporation in its chain (Wei, Liang, & McDonald, 2015).

The biocomposite materials presented a rapid biodegradation, presenting application potential as a coating for agricultural seeds, in which a rapid and effective degradation time is necessary for the delay in the radicles formation.

## 4 CONCLUSION

This study demonstrated that it was possible to obtain nanobiocomposites with monodisperse particles, smaller than 70 nm in diameter. HDM pectin films with different nanoemulsions concentrations were successfully produced, presenting characteristics of homogeneous, compact and continuous materials. The increased nanoemulsion concentration provided materials with good thermal stability and intermolecular interactions between the nanoemulsions and the polymer matrix, resulting in increased water vapor barrier and elongation. The Neem oil incorporation in the pectin films promoted an increase in the tensile strength and a reduction in the elastic modulus regarding to the carnauba wax. The nanocomposite materials, mainly with Neem oil, presented excellent results in relation to the non-formation of macro-residue in the soil during the biodegradation process. These new materials present potential application in the films and coatings preparation for the industrial and agricultural area of seeds.

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**ARTIGO 4 – ANTIMICROBIAL PROPERTIES OF PECTIN NANCOMPOSITES/  
NEEM OIL NANOEMULSION FOR SEED COATING**

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**Abstract:** Petroleum based packaging materials have caused serious environmental problems due to their inappropriate disposal. Biodegradable polymers reinforced with nanostructures are used to produce new and environmentally friendly packaging, being considered interesting alternatives to replace the conventional packages. In this context, biopesticides are also considered safe, biodegradable and environmentally friendly. They, especially the *Azadirachta indica* A. Juss. (Neem), are used in emulsified concentrates. There is a growing interest in nanoemulsions based on phytochemical mixtures. This occurs due to their better effectiveness compared to the synthetic biopesticides. This study aimed to aggregate Neem oil nanoemulsions and pectin matrices to produce nanocomposite films. In addition, the study aimed to evaluate the nanoemulsions effect on the films properties for coating soybean seeds. Nanoemulsions were characterized assessing their medium diameter and stability, while the nanocomposites antimicrobial, morphology, mechanical and barrier properties were analyzed. The nanoemulsions had an average diameter close to  $59 \pm 0.61\text{nm}$  and they showed a good stability. The nanoemulsions addition improved the films mechanical properties compared to the pure pectin film. They reduced the films stiffness, resistance, and water vapor permeability, and increased their extensibility. In addition, Neem oil provided antimicrobial properties against *Aspergillus Flavus* and *Penicillium Citrinum*. Moreover, the seed coating process did not change their moisture content. The seeds coating promoted a positive effect on the germination process of soybean seeds. In summary, antimicrobial nanocomposite films from renewable sources were successfully produced. The fungicidal inhibition of Neem oil as nanoemulsions makes these new materials promising for the production of seed coatings.

**Keywords:** Active packaging, Nanotechnology, Biopolymer, *Azadirachta*, Nanoemulsions, Antimicrobial properties.

## Introduction

Soybean stands out as an important crop in agriculture. A basic requirement in the soybeans production is the use of high quality seeds. Thus, it is important to use technologies to maintain their quality (Zhang et al., 2016). However, the agricultural community faces a range of losses challenges such as the yield stagnation, the crops destruction due to the fungal attacks, the negative influence on vigor, the exposure to an inadequate humidity and to fluctuating climatic conditions (Conceição et al., 2017). A progress in the development of biodegradable coatings has been pointed out to sustainably solve these difficulties (Baker et al., 2017). Fossil fuels are the main source of commercial polymers. However, there is a growing environmental concern about their use. Therefore, materials based on biopolymers have been extensively investigated to replace petroleum-based polymers.

Among the renewable sources for the biopolymers production, is the pectin. It is a natural hydrophilic polysaccharide composed of  $\alpha$ -D-galacturonic acid residues (1.4). It is present in the plant cell walls; it is non-toxic, biocompatible and biodegradable and has good film forming properties, (Oliveira et al., 2016). However, films made from natural polymers, such as the pure pectin, have poor thermomechanical properties. They also presents poor moisture barriers due to their hydrophilic nature (Shankar et al., 2016). One approach to enhancing the functionality of these films is to combine hydrophobic materials with polysaccharides to form composite films.

Due to its cost-effectiveness, nanocomposites have been applied to improve the characteristics of biodegradable polymers (Tornuk et al., 2017). Nanocomposites are a wide range of materials consisting of two or more components, wherein at least one of these has nm scale dimensions (i.e., between 1 and 100 nm). The dispersion in the form of nanoparticles causes a very wide interfacial area. This modifies the molecular motility and, consequently, the substance mechanical and thermal properties (Chaichi et al., 2017). The use of

nanoemulsions gained specific attention among the nanocomposites. The lipid phase can act as a solvent for various additives and the coating is used as an active packaging. This is of interest once the active films are biodegradable and can provide a range of benefits, such as the antimicrobial activity (Dammak et al., 2017).

Following this line, recent studies have showed the advantages of seeding lipid material in the nanoemulsions form to be applied in films based on biopolymers. Otoni et al. (2014) worked with cinnamaldehyde pectin / papaya / nanoemulsion edible composite films and found positive results for their mechanical and moisture barrier properties. Moreover, they noted that the antimicrobial activity provided by cinnamaldehyde against foodborne pathogens was remarkable. Nanobiocomposites based on pectin and cinnamon nanoemulsion developed by (Moura et al., 2015) also presented mechanical properties and moisture barrier even more satisfactory for the application as active packaging.

Neem oil (*Azadirachta indica*) is already used as an effective natural pesticide. It also has strong antifungal action and acts as a growth regulator, being interesting for the nanobiocomposites preparation (Shah; Wani, 2016). Azadirachtin is a biologically active tetranortriterpenoid that exhibits biopesticidal applications present in the Neem (Jerobin et al., 2012).

There is no report of the Neem oil nanoemulsion incorporation into pectin based films. In addition, its antimicrobial properties evaluation is still incipient. Thus, the present study aimed to produce Neem oil nanoemulsions to develop new environmentally correct nanocomposites to be used as active coatings; to evaluate its antimicrobial effect and the pectin films; and evaluate the nanocomposite application in soybean seeds through the evaluation of the physiological quality of the treated seeds.

## **Material and Methods**

### **Materials**

The materials used in the study were: Neem oil (obtained from Laszlo Aromatologia); Tween 20 (polyoxyethylene (20) sorbitan monolaurate, HLB = 16.7), obtained from Sigma Aldrich, IND); pectin HDM (DM > 50% and Mw = 130000 g mol<sup>-1</sup>) (provided by CP Kelco Brazil S/A, BRAZIL) and Ultrapure Water of a Milli Q, 18.2 MΩ.cm at 25° C.

### **Nanoparticle formulation and characterization**

Nanoemulsions were prepared by the casting technique with adaptations proposed by Sekar et al. (2015). Neem oil, 3% w / v and Tween 20, 1: 3 ratio (oil: surfactant) as the lipid phase and water as the continuous phase were used for the nanoemulsion production. The emulsion was then blended using a 20 kHz ultrasonic processor (Branson Digital-Model 450) (450 W) for 45 minutes. The sonication processes were performed in cycles, using cups containing ice cubes to minimize the heat effect. Each cycle lasted 10 min with a pause of 1min, using the 50% amplitude in the ultrasound.

### **Particle size, size distribution, and short-term stability**

The mean size and particle size distributions of the nanoemulsion were determined according to (Jadhav et al., 2015), using a dynamic scattering of light in a Zanosizer Nano Series (Malvern Instruments Inc., Worcestershire, U.K.). This equipment determines the particle size from the relative intensity of the sample dispersion fluctuations with a laser beam (633 nm) at a 90° angle. Each sample was evaluated from an average of five replicates. The samples were diluted (x 100) using a buffer solution (pH 3.0) to avoid the dispersion effects multiplication and to ensure the particles free Brownian motion before the measurements. The

cumulative mean (z-diameter) and the polydispersity index (PDI) were calculated from the particle size distribution. All measurements were performed at room temperature.

### Film preparation

The solutions (3% w/v) of HMP pectin were prepared by dissolving the powdered pectin in ultrapure water, homogenized at 1500 rpm for 24 hours until the complete solubilization (Lorevice *et al.*, 2016). This solution was used as a control film (-). Then, the solutions were incorporated with 3% (m/v) Neem oil nanoemulsion, producing Neem oil nanocomposites: NOil10, NOil20 and NOil30. The low permeability commercial polymer L232 (Incontec) was used as control (+) against the nanocomposites produced.

The composition of the film-forming solutions are shown in Table 1. The solutions were rested for 4 h for bubble elimination before applying the casting method at  $25 \pm 2$  °C for 48 h to make the films. The dried films were cut and stored hermetically under refrigeration until the analysis moment.

Four films were produced for each treatment. The Neem oil concentration (3% m/v) against *Aspergillus flavus* fungi and *Penicillium citrinum* was chosen from pre-tests.

**Table 1 – Compositions of aqueous film-forming solutions.**

Film	HMP (% wt)	Neem Oil (% wt)	Ratio HMP/Oil
HMP <sup>a</sup> - Control (-)	3	-	100/0
L232 <sup>b</sup> - Control (+)	-	-	-
NOil10	3	3	90/10
NOil20	3	3	80/20
NOil30	3	3	70/30

<sup>a</sup> HMP: high methylester pectin

<sup>b</sup> L232: Polymer for industrial seed coating

Noil: Nanocomposite of pectin and Neem oil

## **Nanocomposites characterization**

### *Scanning Electron Microscopy (SEM)*

The fracture surface was analyzed on a LEO EVO 40 XVP scanning electron microscope (Carl Zeiss, Jena, Germany) equipped with a secondary electron detector. The cross-sectional images were taken from cryogenic fractionated samples previously immersed in liquid nitrogen during 2 min. Samples were mounted on stubs, using a double-sided carbon tape and metallized with gold (Balzer, SCD 050). The images were taken at 15-20 kV with the magnifications of 1000 and 5000X. Pore size was measured using the Image J 1.50i program (Wayne Rasband, National Institutes of Health, USA).

### *Antimicrobial properties*

The *Aspergillus flavus* CD 10508 and *Penicillium citrinum* CD10564 used belong to the Mycotoxins and Mycology Foods Laboratory collection of the Department of Food Science of UFLA - Brazil. They were isolated from grapes. The disc diffusion test established as a standard by the National Committee for Clinical Laboratory Standards (NCCLS) (AL Barry and Thornsberry, 1991; Ostrosky *et al.*, 2008). Was used to evaluate the inhibitory effect of filamentous fungi. An inoculum at the concentration of  $10^6$  spores  $\text{mL}^{-1}$ , counted in Neubauer's chamber was used. Subsequently, this inoculum was transferred to a petri dish containing medium malt extract agar (MEA) using the surface scattering technique. Filter paper disks of 6 mm diameter, soaked with 10  $\mu\text{L}$  of the nanoemulsions at 0.5%; 1%; 2% and 3% (w / v) concentrations were placed on the culture medium. In addition, the average particle size effect on the fungitoxic activity of the emulsion and still pure oil was studied.

Disks impregnated with 10 $\mu\text{L}$  of DMSO (dimethylsulfoxide) - characterized as cell cryoprotectant – were considered as the negative control. The Maxim fungicide 10  $\mu\text{l}$  / 5 ml was used as the positive control. The plates were incubated in a Biochemical Oxygen Demand



BOD incubator at 25 ° C during 72 hours. The evaluation was comparative with a reference biological standard (positive control). The zone or growth inhibition halo was measured starting from the circumference of the disc to the margin where there was growth of microorganisms (AL Barry and Thornsberry, 1991). The minimum inhibitory concentration (MIC) was defined as the lowest nanoemulsion concentration in which the presence of inhibition halo was identified.

#### *Coated soybean seed moisture*

The moisture content of soybean seeds was determined according to the Rules of Analysis of Seeds (RAS) (BRASIL, 2009), using the oven method 105 ° C +/- 2 ° C. Four replicates of 50 grams of seeds were dried for 24 hours.

#### *Germination of coated seeds*

Eight replicates of 25 seeds for each coated treatment were assessed for the germination test. Paper towel (germitest) moistened with distilled water (2.5 times its dry weight) and made in the rolls form were used as substrate. The rolls were kept in a BOD chamber at 25o C during 8 days. The results were expressed as the percentage of normal seedlings (BRASIL, 2009). The first germination count was made 5 days after sowing (DAS). Seeds with radicles longer than 2 mm were considered as germinated seeds. The percentage of normal seedlings were counted after 8 days. Afterwards, root and shoot lengths were evaluated using a 30 cm graduated ruler.

#### **Statistical analysis**

All analyzes were performed in triplicate and the results were presented by the means and standard deviations of these measurements. Statistical analysis was performed using the

Statistica Analysis Systems 8.0 software. ANOVA and Tukey test were used to determine statistically significant differences among the means ( $p \leq 0.05$ ).

## Results and Discussion

### Particle size, size distribution, and stability

The mechanical ultrasonic process successfully formulated the Neem nanoemulsion. The nanoemulsion (O/W) was prepared using Neem oil, Tween 20 and water. The 1: 3 ratio of NE was evaluated as stable, with a particle size of  $59 \pm 0.61$ nm and polydispersity index of  $0.209 \pm 0.02$  (Figure 1).

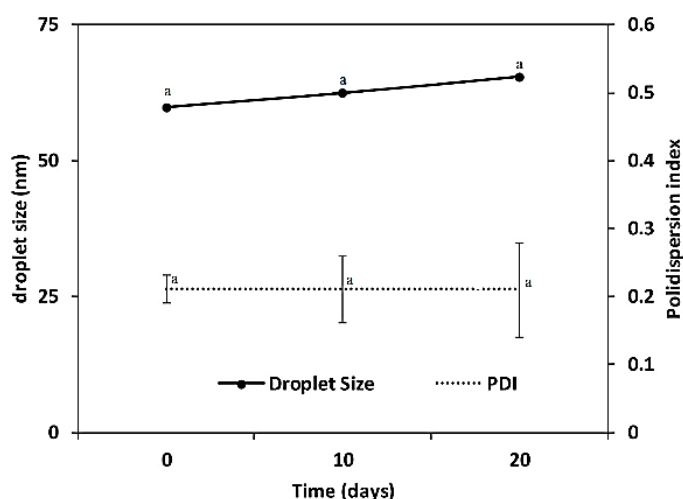


Figure 1 – Neem oil nanoemulsions particle size (DLS), polydispersity index (PDI) and stability.

The results are in agreement with other studies that reported the formation of spherical NE size drops of Neem oil (ratio 1: 3) in the range of 30-70 nm (Ghotbi *et al.*, 2014; Osman *et al.*, 2017). Thus, Tween 20 was considered as a suitable surfactant for the NE formulation, since Tween is less affected by the pH and is considered non-toxic and biocompatible. (Azeem *et al.*, 2009). In addition, it is important that nanoemulsions remain physically stable during storage, transport, and use for commercial applications, for commercial applications

(Polychniatou and Tzia, 2016). The particle size distribution control and the suitable emulsifier selection is of utmost importance to ensure the long-term nanoemulsion stability (McClements, 2012). The Neem nanoemulsion stability may persist for 20 days due to the presence of the stabilizing surfactant, which inhibited the coalescence of the nanoparticles (Ozturk, 2017).

## Nanocomposites characterization

### *Film Microstructure*

Scanning electron microscopy (SEM) was used to determine the morphology of the cross sections of the pectin, nanocomposite and commercial polymer films (Figure 2). It was possible to illustrate the relationship among the nanocomposites surface characteristics and their moisture barrier properties.

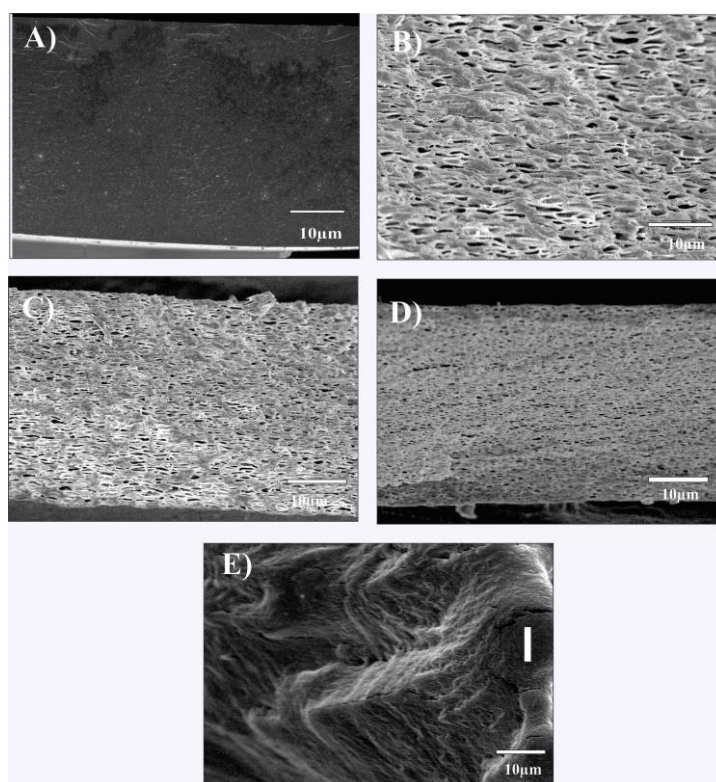


Figure 2 – Scanning electron microscopy (SEM) images of commercial polymer films (A) and pectin (E), added with Neem oil nanoemulsions: Noil10, Noil20 and Noil30 (B, C and D, respectively).

The SEM images showed a condensed, homogeneous, fracture-free and pore-free structure. The film A (commercial polymer) has better mechanical, thermal and barrier properties. The film E (HDM pectin) exhibited some imperfections, such as pores and fractures (Fig, 2). They are possibly derived from the film drying process and the solvent evaporation rate. This resulted in weaker mechanical properties. These imperfections also led to lesser interactions among the pectin chains. On the other hand, the nanoemulsions caused some changes in the films, as in the chain density and homogeneity. There were intermolecular interactions between the pectin network and the nanoemulsions surface. Noil addition (samples B, C and D) promoted a compact structure with holes, increasing the tortuosity. This matrix configuration reduced the molecular diffusion and improved the film mechanical properties. The results showed that the oil particles were more intense as the concentration of incorporated lipids increased, resulting in a more tortuous surface due to the holes caused by the oil nanoemulsions. However, Noil did not substantially alter the pectin matrices, indicating to be a great alternative to produce new nanocomposite materials.

#### *Antimicrobial properties*

The disc inhibition test was performed to assess the antifungal properties of the Neem oil nanocomposites against storage fungi (*Aspergillus flavus* and *Penicillium citrinum*) in seeds. The film without oil were used as a negative control. As expected, the Neem oil-free films did not present light zones around the film discs, as well as an inhibitory effect (Figure 3). This indicates that pectin does not have antimicrobial properties against the evaluated fungi.

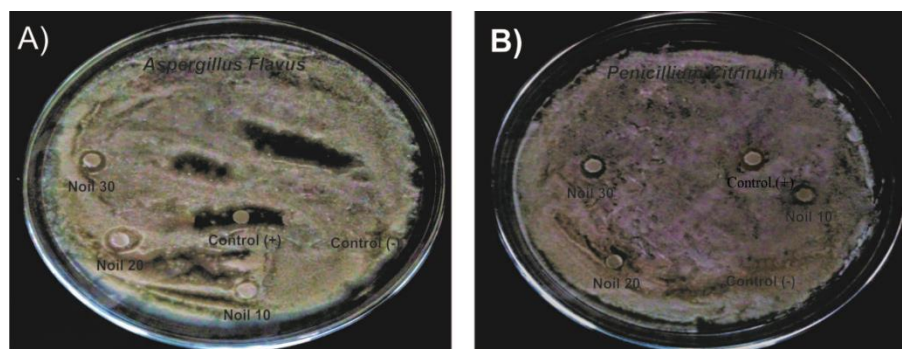


Figure 3 – Typical inhibition zones created around Control (+), Control (-) (pure pectin), Noil 10, Noil 20 and Noil 30 against fungi (A) *Aspergillus Flavus* and (B) *Penicillium Citrinum*.

The Neem microbial films presented inhibition zones around the disks (Table 3), inhibiting the microbial growth. An antifungal action was observed even at the lowest concentrations used (minimum inhibitory MIC concentrations). Maxim synthetic fungicide was used as a positive control. It did not present a significant difference ( $p \leq 0.05$ ) in the inhibition halo against *Penicillium Citrinum*, indicating its high efficiency compared to nanocomposites. A significant difference ( $p \leq 0.05$ ) in the inhibition halo against *Aspergillus flavus* was detected. However, the nanocomposites inhibition zones remained close to those observed in the positive control against both tested fungi (Table 2).

**Table 2 – Antimicrobial effect of Neem pectin/nanoemulsion bionanocomposite films against *Aspergillus Flavus* and *Penicillium Citrinum*.**

Film	<i>Aspergillus Flavus</i>		<i>Penicillium Citrinum</i>	
	IZ (mm <sup>2</sup> ) <sup>a</sup>	IE <sup>b</sup>	IZ (mm <sup>2</sup> ) <sup>a</sup>	IE <sup>b</sup>
Control (-)	0	-	0	-
Control (+)	33 ± 0.80a	+	29 ± 0.54a	+
Noil10	27.0 ± 0.78b	+	27.5 ± 0.87a	+
Noil20	27.9 ± 0.56b	+	28.2 ± 0.54a	+
Noil30	28.6 ± 0.43b	+	28.4 ± 0.21a	+

a-b For Film, IZ values followed by different lowercase letters were significantly different ( $p < 0.05$ ).

<sup>a</sup> IZ: area of inhibitory zone, reported as mean value ± standard deviation.

<sup>b</sup> IE: inhibitory effect underneath film disks, reported as present (+) or absent (-).

The film added with the highest Neem oil concentration (Noil30) presented better antimicrobial properties (Table 3; Fig. 4). This can be attributed to the *Azadiractina* oil active compound that migrated from the film to the microbial cells. (Huang *et al.*, 2010) suggested that nanoemulsions promote the facilitated transport of phytochemicals. This occurs through the cell membranes. As a result, there is an increase in the plasma phytochemical concentrations and therefore an increase in the bioavailability. Inhibition occurs because the nanoemulsions act on the fungi through fungal and sports hyphes, reducing their cellular viability. This activity is imposed on the fact that nanoemulsions merge with organisms that contain lipids. Thus, this fusion destabilizes the pathogen lipid membrane, resulting in the cell lysis and its death (Joe *et al.*, 2012).

Neem oil has an adequate insecticidal action against several pathogens (Ghosh *et al.*, 2014). However, little attention has been given to the antifungal activity of the Neem nanoemulsion. In addition, due to its rapid action, it is unlikely that the small concentrations employed may result in the resistant strains development (Myc *et al.*, 2002). (Sudha *et al.*, 2013) also noted antifungal properties of *Azadirachta* and an AFB1 production inhibition by *Aspergillus flavus*. (Osman *et al.*, 2017) confirmed the Neem nanoemulsion fungitoxic activity, classifying it as a strong biopesticide to control plant pathogens.

#### *Coated soybean seed moisture*

Films were produced with HDM pectin (Fig. 4C - control) and incorporated with different proportions of nanoemulsion, resulting in nanocomposites (Fig. 4D, E and F). The seeds were also coated with the L232 commercial polymer (Fig 4B). These coatings conferred continuity, homogeneity, transparency and gloss to the coated seeds (Fig 4A), and an absence of fractures and interruptions were observed (Fig. 4).

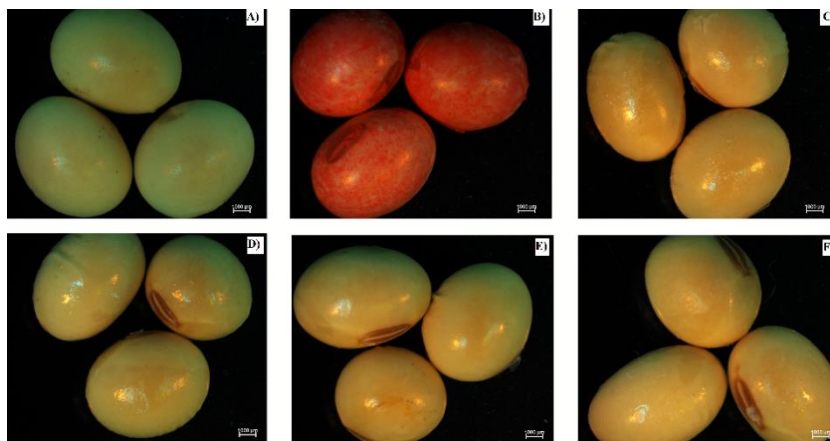


Figure 4 – Seed less micrographs (A), coated with commercial polymer L322 (B), coated with pectin (C), and coated with Noil 10, Noil20 and Noil 30 (D), (E) and (F) nanocomposites.

It was found that the coating using polymers did not significantly affect ( $p > 0.05$ ) the water content of the soybean seeds compared to the uncoated seeds (Figure 5). The water content in both remained equal to 14%. This result indicates the preservation of the seed viability and vigor.

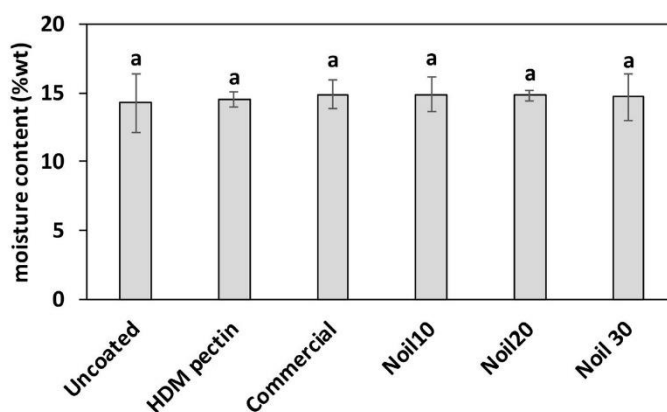


Figure 5 – Moisture content percentages of seeds without coating and coated with different nanocomposites.

The quantitative relationships between the water content and the longevity allow a better prediction of the useful life of the seeds. (Balouchi *et al.*, 2017). Thus, the seed water content is influenced by the relative humidity. According to (Smaniotto *et al.*, 2014), the water content is the most significant factor in preventing grain deterioration during the storage

period. Thus, the microorganisms attack and the respiration effect will be minimized maintaining a low grain water content and temperature. (Liu *et al.*, 2017) suggested that there is an increase in the respiratory rate proportional to the increase in temperature, which is dependent on the seed water content. With a water content of more than 14% (b.u.), the respiration increases rapidly in most cereals, causing their deterioration. Therefore, the conservation of the seed properties is necessary before and after them be coated.

### *Germination test*

Different effects among the treatments in the shoot length; root length and seedling length variables were observed. The coating application showed a positive effect on the soybean seeds germination process (Figure 6). The low performances noted in the uncoated seeds can be attributed to their moisture content and to infections that accelerate their deterioration. The impermeable nature of water vapor nanocomposites prevented the seed moisture increase. However, the polymer did not inhibit the water absorption during the germination. However, the film (although it is hydrophilic) acted as a physical barrier to water entry. Thus, there was a more balanced imbibition, optimizing the germination process (Rathinavel, 2015).

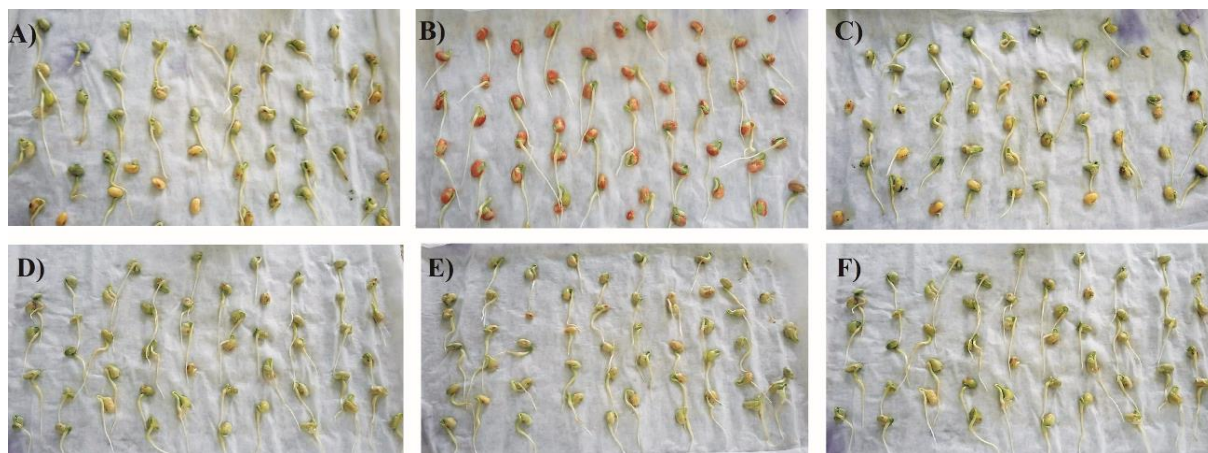


Figure 6 – Germination of soybean seeds after 8 days without coating (A), coated with commercial polymer L232 (B), pectin (C), Noil10 (D), Noil20 (E) and Noil30 (F).



All treatments presented similar results ( $p \geq 0.05$ ) for the first germination count and normal seedlings percentage variables. Films made with pectin and Noil 10 presented the worst performances. Seeds treated with Noil 30 formed seedlings with a longer shoot length and, consequently, showed a longer seedling length. Moreover, they presented the second best performance regarding to the root length (Table 3). These results agreed with the (Jerobin *et al.*, 2012) study. These authors noted that the *Azadirachtin* - a limonoid founded in the Neem - is biologically active and can act as an effective natural pesticide and growth regulator.

Thus, it was observed that the coating favored the seeds vigor, without affect their germination, indicating its great application potential. Similarly, an germination increase and the plant establishment were noted by (Ludwig *et al.*, 2011) in soybean seeds coated with polymer and amino acids.

**Table 3 – Observed means for the variables: first germination count (GPC); percentage of normal seedlings (PN); root length (CPA) root length (CR) and seedling length (CP).**

Treatments	PCG	PN	CPA	CR	CP
Control	96 a	94 a	5,54 c	8,34 a	13,88 b
Comercial	97 a	93 a	5,13 f	7,81 c	12,94 d
Pectina	94 a	88 a	5,23 e	7,68 d	12,91 e
Noil10	93 a	94 a	5,43 d	7,43 e	12,86 f
Noil20	93 a	92 a	5,61 b	7,41 f	13,02 c
Noil30	92 a	91 a	6,42 a	7,95 b	14,37 a

Means followed by the same letter in the column do not differ statistically at the 5% probability level by the Tukey test.

## Conclusion

The nanocomposite pectin films using Neem oil nanoemulsion is feasible. Particle size stability was observed, and it was possible to produce uniform films. Noil addition did not

substantially altered the pectin matrices. Therefore, this seems to be a great alternative to produce new nanocomposite materials. Moreover, the Neem nanocomposites, considering the increase of the employed concentration, presented an excellent fungicidal activity against the tested pathogens (related to the seeds storage); and the seed coating process did not change their moisture content. It is possible to highlight the successfully production of films from renewable sources such as the pectin, promoting the use of agroindustrial residues and their value aggregation. Nanoemulsion films are an ecologically promising antimicrobial material for applications in seed and grain coatings.

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