Universidade Estadual Paulista
"Júlio de Mesquita Filho"

Faculdade de Ciências Farmacêuticas

Antifungal activity of extracts and essential oil of ginger in nanostructured carnauba wax coatings to postharvest conservation of tangerine and papaya

Marcela Miranda

Tese apresentada ao Programa de Pós-graduação em Alimentos e Nutrição para obtenção do título de Doutor em Alimentos e Nutrição.

Área de Concentração: Ciência de Alimentos.

Orientador: Prof. Dr. Marcos David Ferreira
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Araraquara
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Atividade Antifúngica de Extratos e Óleo Essencial de Gengibre em Revestimentos Nanoestruturados de Cera de Carnaúba na Conservação Pós-colheita de Tangerina e Mamão

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Atividade Antifúngica de Extratos e Óleo Essencial de Gengibre em Revestimentos Nanoestruturados de Cera de Carnaúba na Conservação Pós-colheita de Tangerina e Mamão

Marcela Miranda

unesp UNIVERSIDADE ESTADUAL PAULISTA
CÂMPUS DE ARARAQUARA

FACULDADE DE CIÊNCIAS FARMACÊUTICAS

ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE MARCELA MIRANDA, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM ALIMENTOS E NUTRIÇÃO, DA FACULDADE DE CIÊNCIAS FARMACÊUTICAS DO CÂMPUS DE ARARAQUARA-UNESP.

Aos trinta e um dias do mês de julho de 2020, às 14 horas, reuniu-se, virtualmente, a Comissão Examinadora da Defesa Pública, composta pelos seguintes Professores Doutores: Marcos David Ferreira (Orientador) da Embrapa-Instrumentação, Lucimeire Pilon da Embrapa-Hortaliças, Poliana Cristina Spricigo do Escola Superior de Agricultura Luiz de Queiroz da Universidade de São Paulo, Marilene de Mori Morselli Ribeiro do Tanquimica - QGP Quimica Ltda. e Milena Martelli Tosi do Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, sob a presidência do primeiro, a fim de proceder a arguição pública da TESE DE DOUTORADO DE Marcela Miranda, intitulada "Avaliação da Atividade Antifúngica de Extratos de Gengibre e Efeito de Revestimentos Nanoestruturados na Conservação Pós-colheita de Tangerina e Mamão". Os membros participaram da defesa de tese por meio de vídeo-conferência, atendendo o comunicado PROPG de 23 de março de 2020 e o Comunicado 05 - Comitê Unesp Covid-19 de 22 de março de 2020. Após a exposição, a discente foi arguida virtualmente pelos membros da Comissão Examinadora, tendo a candidata recebido o conceito final: _ _ **APROVADA** _ _. Registre-se, que nesta ata, não constarão as assinaturas dos membros da Comissão Examinadora participantes por meio de vídeo-conferência. Serão anexados a esta ata, pareceres circunstanciados dos membros participantes por vídeo-conferência, enviados por e-mail. Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo presidente da Comissão.

Marcos David Ferreira (Orientador)

Lucimeire Pilon(parecer anexo)
Membro participante por vídeo-conferência

Poliana Cristina Spricigo(parecer anexo)
Membro participante por vídeo-conferência

Marilene De Mori Morselli Ribeiro(parecer anexo)
Membro participante por vídeo-conferência

Milena Martelli Tosi (parecer anexo)
Membro participante por vídeo-conferência

Dedico à minha família e aos benfeitores que cruzaram meu caminho durante o doutoramento.

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Resumo

Objetivo: O objetivo deste estudo foi avaliar a atividade antifúngica de extratos alcoólicos de gengibre e de óleos essenciais, contra fitopatógenos pós-colheita, de forma isolada ou associada à nanoemulsão de cera de carnaúba na conservação da qualidade e redução da deterioração natural ou induzida em tangerinas e mamões. Formulações com nanoemulsão de cera de carnaúba e suas associações com hidroxipropil metilcelulose (HPMC), como agente adjuvante na formação de filme, e com a incorporação de óleo essencial de gengibre (GEO), foram aplicadas como revestimento protetor e avaliadas sob diversas condições de armazenamento. **Metodologia**: Extratos alcoólicos (GEs) e óleos essenciais (GEOs) foram extraídos de rizomas de gengibre. A atividade antifúngica desses compostos foi avaliada contra *P. digitatum, P. expansum, F. solani* e *A. alternata* in condições *in vitro.* Dois experimentos foram realizados com tangerinas (‘Nova’ mandarins). Estas frutas foram revestidas com microemulsões de goma laca (*shellac*) e cera carnaúba, e também com formulações nanoparticuladas (nanoemulsões) e comparadas com frutos não revestidos após 7 dias de armazenamento a 20 °C. Os revestimentos também foram testados em tangerinas 'Unique' armazenadas por 14 dias a 10 °C, seguido por um período de simulação de comercialização (7 dias a 20 ºC). A avaliação da qualidade dos frutos incluiu perda de peso, brilho, sólidos solúveis, acidez titulável, pH, ratio, CO_2 e O_2 interno, etanol, teste sensorial e de brilho após 7 dias a 20 °C. Para ensaios pós-colheita com mamões, as formulações filmogênicas foram preparadas nas concentrações de 9% e 18% de nanoemulsão de cera de carnaúba. HPMC foi usado como revestimento controle inerte, a GEO (a 3% v/v), testado como composto ativo contra infestações por fungos. Os frutos foram armazenados e avaliados em dois experimentos separados. O primeiro, realizado após 6 dias a 22 °C e 9 dias a 13 °C, seguidos por 5 dias à temperatura ambiente para simular as condições de comercialização. No segundo ensaio, mamões foram armazenados por 5 dias a 22 °C e 10 dias a 16 °C antes da condição de comercialização simulada, que foi de 3 dias a 22 °C. Análises pós-colheita e ação protetora na redução da severidade de doenças naturais e disseminação de fungos em amostras inoculadas com *C. gloeosporioides* e análise sensorial foram conduzidas. **Resultados:** Os GEOs apresentaram uma maior efetividade que os GEs e em concentrações inibitórias mínimas (MIC), menores para os óleos que as medidas para os extratos alcoólicos. Os GEs não apresentaram ação fungicida contra os fungos avaliados (MCF) na maior concentração testada (6%) em meio líquido. Na condição *in vitro*, os GEOs apresentaram uma melhor atividade antifúngica que os GEs, sendo mais apropriado para incorporação em revestimentos no controle de doenças pós-colheita. GEOs nos ensaios em *in vitro* apresentaram uma melhor atividade que os testes conduzidos em *in vivo.* O fungo *P. digitatum* apresentou maior sensibilidade aos compostos de gengibre (com menor concentração inibitória mínima), seguido por *A. alternata, F. solani* e *P. expansum* (para os quais foram necessários maiores concentração das amostras para que ocorra a inibição do crescimento micelial). Para tangerinas, revestimentos com microemulsão e nanoemulsão de cera de carnaúba resultaram na menor perda de peso em comparação ao controle e *shellac*. Não houve diferenças nas medidas de brilho para a 'Nova', no entanto,

os frutos revestidos com *shellac* tiveram a maior classificação de brilho no teste visual. Todavia, para 'Unique', inicialmente, a cobertura de *shellac* resultou no maior brilho, entretanto no final do armazenamento, a nanoemulsão reteve o maior brilho comparativamente, embora não diferente da microemulsão. Os teores de CO_2 e etanol aumentaram e de O_2 diminuiu internamente durante o armazenamento para todos os tratamentos. Os maiores níveis de CO_2 e etanol foram verificados para *shellac* juntamente com menor O_2, indicando fermentação, sem diferenças entre os demais tratamentos. *Shellac* e a microemulsão também alteraram o perfil dos compostos voláteis, porém mais que os frutos do controle ou a nanoemulsão, especialmente para 'Unique'. Em relação aos ensaios com mamões, todos os revestimentos forneceram alguma proteção, com variações nos efeitos decorrentes das condições de armazenamento e das composições das formulações. Sob armazenamento refrigerado, a maioria das análises não resultou em diferenças significativas entre as amostras. Sob temperatura ambiente e após condições simuladas de mercado, as diferenças tornam-se mais evidentes. As coberturas com nanoemulsão foram capazes de manter melhor a qualidade do mamão sobre as condições de armazenamento e mercado. Foram registradas maiores reduções na perda de firmeza, alterações de cor e taxa de respiração, atuando positivamente no atraso da maturação. Não foram relatadas alterações nos atributos como doçura, acidez, *papaya flavour* ou presença de sabores estranhos relacionados à fermentação interna, devido à cobertura, nos testes sensoriais. O GEO apresentou efeito na redução de doenças na superfície do mamão, principalmente quando associado à carnaúba, embora nenhuma ação tenha sido observada na inibição do crescimento de *C. gloeosporioides* após a inoculação. **Conclusão**: Os GEOs mostraram ser os agentes mais indicados para serem empregados no controle pós-colheita de fungos, por exibirem uma maior atividade antifúngica que as encontradas nos extratos alcoólicos. A combinação de nanoemulsão e GEO em revestimentos foram mais eficazes nos experimentos *in vitro* do que *in vivo*. Para os ensaios de tangerinas, as emulsões de carnaúba resultaram em uma menor perda de água, conferiram brilho e causaram menor produção de etanol do que o *shellac* com a nanoemulsão exibindo maior brilho após refrigeração. Menores também foram as modificações da atmosfera interna e do perfil de voláteis e, consequentemente, preservou o sabor comparado a microemulsão. As coberturas com nanoemulsões de cera de carnaúba foram mais adequadas na proteção e na manutenção da qualidade dos mamões em condições de armazenamento e de mercado. A incorporação de GEO nos revestimentos promoveu a redução de doenças nos mamões, principalmente quando combinado com a nanoemulsão de cera de carnaúba.

Palavras-chave: óleo essencial; nanoemulsão; cera de carnaúba; qualidade da fruta; sensorial; voláteis de aroma.

Abstract

Objective: The aim of the present study was the evaluation of the antifungal activity of ginger alcoholic extracts and essential oils against common post-harvest phytopathogens, as isolated compounds or in association to carnauba wax nanoemulsion. In coating format these composites are able to preserve quality and slow down natural or induced decay on tangerines and papayas. Formulation based in carnauba wax nanoemulsions and their association with hydroxypropyl methylcellulose (HPMC), as a neutral film forming adjuvant, and ginger essential oil (GEO) incorporation, were applied as protective coatings and evaluated under several conditions of storage. **Methodology:** Alcoholic extracts (GEs) and essential oils (GEOs) were extracted from ginger rhizomes. Antifungal activities of GEs and GEOs were evaluated *in vitro* against *P. digitatum, P. expansum, F. solani* and *A. alternata*. Two experiments were performed with citrus (Nova' mandarins). These fruits were coated with shellac and carnauba microemulsions, as well as nanoparticulated carnauba (nanoemulsions) and compared with uncoated control fruits after 7 days storage at 20 °C. Coatings were also tested on 'Unique' tangors stored for 14 days at 10 °C followed by a simulated marketing period of 7 days at 20 ºC. Fruit quality evaluation included weight loss, gloss, soluble solids (SS), titratable acidity (TA), pH, SS/TA ratio, internal CO_2 and O_2, ethanol, and a sensory shine rank test after storage at 20°C for 7 days. For papaya postharvest assays, the formulation was prepared in a concentration of 9% and 18% (w/v) carnauba wax nanoemulsion. HPMC was used as inert coating control and GEO (at 3% v/v) tested as active compounds against fungal infestation. The fruits were stored and evaluated in two separate experiments. The first conducted after 6 days at 22 °C and 9 days at 13 °C followed by 5 days at room temperature to simulate marketing conditions. In the second assay papayas were stored for 5 days at 22 °C, and 10 days at 16 °C before simulated marketing condition of 3 days at 22 °C. Post-harvest analyzes and protective action towards reducing natural diseases severity and inhibit of fungal spread on samples inoculated with *C. gloeosporioides*, were performed along with sensory evaluations. **Results:** GEOs showed a more effectiveness than GEs and at minimum inhibitory concentration (MIC) lower for oils than those measured to alcoholic extracts. The GEs did not show minimum fungicidal concentration (MCF) against the tested fungi even at the highest concentration tested (6%) in broth medium. GEOs on *in vitro* conditions showed a better antifungal activity than GEs, indicating that they are more appropriate for incorporation into edible coatings, aiming phytopathogenic postharvest microorganism's control. When *in vitro* assessment, the GEO showed a better antifungal activity than the *in vivo* conducted tests. The *P. digitatum* fungus was the most sensible to ginger compounds (with lower MIC), followed by *A. alternata*, *F. solani* and *P. expansum*, in this order. These required higher concentrations to attain mycelial inhibition. For tangerines, conventional and nanoemulsion carnauba wax resulted in inferior weight loss compared to control and shellac. There were no differences for gloss measurements for 'Nova' mandarins, however, shellac-coated fruit ranked highest for shine in visual sensory assessment. For 'Unique' tangors, initially, the shellac coating promoted the highest gloss (shine), but at the end of storage, the nanoemulsion exhibited the highest gloss measurement,

although not different from the microemulsion. CO_2 and ethanol generally increased and O_2 decreased internally during storage for all treatments. The highest levels of CO_2 and ethanol were measured in shellac treated samples along with the lowest O_2, indicating internal fermentation, with no differences among the other treatments. Shellac and the carnauba microemulsion also altered the volatile profile more intensely than control or nanoemulsion coating, especially for 'Unique' tangors. For papaya, all coatings provided some protection, with variations on the effects due to storage conditions and between formulations compositions. Under cold storage, most of analyzes did not resulted in any statistically significant differences among samples. At room temperature and after simulated market conditions the differences become more evident. The nanoemulsions were able to a better maintaining of papaya quality over storage and market conditions. Higher reductions on loss of firmness, color alterations and respiration rate were recorded, with positive action in delaying maturity. No changes in attributes as sweetness, sourness, papaya flavor or the presence of off-flavors related to internal fermentation were reported in the sensory test. The GEO presented some effect in reducing natural diseases on papaya skin, particularly when associated with carnauba, although no action was observed in inhibiting *C. gloeosporioides* fungal growth after inoculation. **Conclusion:** GEOs proved to be the most indicated for use as active agents for application in post-harvest fungal control since, in general, they exhibited higher antifungal activity than the alcoholic extracts. The combination of nanoemulsion and GEO into a coating was more effective *in vitro* experiment than *in vivo*. For tangerine assays among the coatings tested, the carnauba emulsions resulted in lesser water loss, imparted more sustainable shine, and caused less ethanol production than did shellac. The carnauba nanoemulsion coatings also exhibited higher shine and fewer modifications of the atmosphere and volatiles profile, and consequently assuring a better flavor compared to the microemulsion. Papayas coated with carnauba wax nanoemulsions had the quality preserved over storage and market conditions. The incorporations of GEO into coatings, promoted a reduction of natural diseases on papaya skin, mainly when combined with carnauba.

Key-words: essential oil; nanoemulsion; carnauba wax; fruit quality; sensory; aroma volatile.

Sumário

Introdução expandida

A produção de alimentos no mundo em 2018 gerou cerca de 4.4 bilhões de toneladas somando grãos e cerais; 1 bilhão de toneladas de hortaliças; 867,8 milhões de toneladas de frutas e 152,4 milhões de toneladas de citros (1). O Brasil, entre 2018 e 2019, produziu cerca de 240 milhões de toneladas de grãos, 41,5 milhões de toneladas de frutas e o setor agropecuário contribui com 21,1% do PIB e 20% da força de trabalho (2).

Em 2018 o valor bruto da produção brasileira foi da ordem da ordem de US$ 164,23 bilhões e a exportação do agronegócio brasileiro de US$ 101,6 bilhões, sendo assim um dos principais produtores e exportadores agrícolas mundiais (o maior exportador global de soja, café, suco de laranja, etanol de cana-de-açúcar, carne bovina e de frango). Terceiro maior produtor de frutas, depois da China e Índia, o Brasil consome internamente aproximadamente 97% de sua produção frutícola e apenas 3% é destinada ao mercado externo, envolvendo cerca de 6 milhões de trabalhadores de modo direto e indireto (2).

Estes dados evidenciam a grande produção mundial e brasileira de alimentos. O avanço das tecnologias propiciou o aumento da produtividade, todavia, as perdas e desperdício de frutas e hortaliças se acumulam no mundo todo nas etapas de produção, pós-colheita, processamento, distribuição e consumo (3).

Em escala mundial, cerca de um terço dos alimentos são inutilizados. Se essa perda fosse reduzida pela metade poder-se-ia alimentar mais de 1 bilhão de pessoas (4).

Ruviaro et al. (4) analisaram em artigo de revisão sistemática, com 431 artigos das bases científicas Google Acadêmico, Scielo e Capes periódicos, a temática de perdas e desperdício de alimentos no Brasil. Os autores concluiram que há poucos estudos que quantificam o desperdício e as perdas. As pesquisas em sua maioria se concentram nas fases de distribuição e consumo e nas fases anteriores (produção, pós-colheita e armazenamento, processamento), e os estudos na área ainda são incipientes. As perdas pós-colheita brasileiras em processamento e transporte são fortemente dependentes do produto, tamanho da cadeia e logística. Os pesquisadores ressaltam que as estatísticas de perdas em termos de produção de alimentos ainda são incipientes e que as estimativas internacionais, como as realizadas pela FAO (3) não consideram publicações em português, idioma no qual a grande parte das publicações sobre o assunto ainda são redigidas no Brasil (4).

Dal´Magro e Talamini (5), em estudo brasileiro de estimativa da magnitude das perdas e desperdício de alimentos, identificou uma média anual de 82,2 mil toneladas entre os anos de 2007 e 2013, representando cerca de 427 kg/habitante/ano. O volume perdido e/ou desperdiçado representou cerca de 42% da oferta média de alimentos do país para o período analisado.

As causas para tais perdas são diversas, e entre elas as principais são inexistência ou quebra da cadeia do frio, manuseio intensivo, embalagens inadequadas, longos períodos de exposição no varejo, falta de treinamento de pessoal, necessidade de melhoria de técnicas de colheita,

educação e conscientização dos consumidores finais (6, 7, 3). Prevenir estas perdas deve ser prioridade, bem como incluir intervenções sobre os sistemas de produção e consumo de alimentos, para que tais sejam mais eficientes (5)

Desta forma, a aplicação de revestimentos comestíveis e/ou protetores destaca-se como uma tecnologia alternativa na conservação pós-colheita que pode auxiliar na redução das perdas de frutas e hortaliças (8, 7). Os revestimentos comestíveis atuam como barreira entre o produto e seu entorno além de apresentar, em alguns casos, atividade antimicrobiana, favorável à manutenção da qualidade do fruto (9, 7), beneficiando toda a cadeia produtiva, desde o produtor até o consumidor final.

Revestimentos a base de lipídeos fornecem uma boa barreira à perda de água, mas em geral requerem solventes agressivos para formação de um filme. Por sua vez, polissacarídeos apresentam uma melhor característica para formação de coberturas, porém apresentam baixa eficiência de barreira à umidade. Desta forma, a combinação de dois grupos – lipídeos e polissacarídeos – gerando revestimentos compósitos, podem trazer benefícios importantes para as propriedades de uma cobertura (10, 7).

Revestimentos comestíveis e protetivos têm sido objeto de interesse de diversos estudos focados em aumentar a estabilidade de alimentos, diminuir perdas e alimentos e a quantidade de resíduos gerada pelo descarte de embalagens plásticas (9, 11, 12). De modo geral, as vantagens do uso de revestimentos formados na superfície de frutas e hortaliças é que estes podem gerar películas muito finas que auxiliam na manutenção das

propriedades sensoriais. Adicionalmente podem agregar partículas e compostos com propriedades bactericida, fungicida, além de melhorar a estabilidade mecânica da superfície (13).

De maneira geral, duas classes de revestimentos podem ser observadas: hidrofóbicas ou hidrofílicas. As coberturas hidrofóbicas são geralmente à base de proteínas ou lipídeos e são mais eficientes como barreiras a gases e a umidade (14). Lipídeos possuem baixa solubilidade em água, sendo, portanto, solúveis em solventes não-polares (15).

Por sua vez os revestimentos hidrofílicos são caracterizados por conter predominantemente grupos polares como as hidroxilas e grupos amino em sua estrutura, os quais as são favoráveis ao rearranjo de moléculas de água ao seu entorno (16). Apresentam boa solubilidade em meio aquoso, auxiliando a dispersão do soluto, favorecendo a de filmes mais homogêneos. Alguns exemplos são os polissacarídeos, como a celulose, hidroxipropil metilcelulose, quitina, goma xantana, pectina, carboximetilcelulose, quitosana, alginato, aloe vera (8, 17).

A cera de carnaúba, na forma de emulsão de tamanho micrométrico, tem sido bastante utilizada na formação de coberturas hidrofóbicas. As emulsões de cera de carnaúba, podem ser comercialmente encontradas sob diversas denominações e nomes fantasia e estão disponíveis em diferentes concentrações (9, 15). Estas emulsões tem sido utilizadas puras ou misturadas com outras ceras e resinas para melhorar o brilho e atingir a permeabilidade desejada (9, 18). Todavia, a formação de revestimentos a base deste composto, na forma de nanoemulsões, não foi ainda

exaustivamente estudada se comparado a outros revestimentos a base de polissacarídeos (19, 20, 17). Em estudo de Miranda (19), foi demonstrado o potencial da aplicação de nanoemulsão de cera de carnaúba na formação de coberturas na conservação pós-colheita de tomates de mesa.

Copernifera Cerifera é a palmeira da qual se extrai a cera de carnaúba de suas folhas, e esta tem sido aplicada em frutas desde 1930 com o objetivo de diminuir a perda de água, reduzir a abrasão entre a superfície dos frutos, proteger em relação a incidência de doenças pós-colheita e controlar a composição gasosa interna dos frutos (21). Esta cera tem composição complexa tendo como principais componentes ésteres, diésteres, álcoois e combinações de hidrocarbonetos. Os conteúdos de ésteres destacam-se como compostos majoritários e pode chegar a 85% sendo ácidos graxos de cadeia longa ligados a álcoois graxos de cadeia longa (22).

A cera de carnaúba é uma cera dura, quebradiça, com alto ponto de fusão (entre 83 e 86ºC) e é classificada de acordo a sua cor e qualidade pela instrução normativa SARC Nº 10 (11 de dezembro de 2002). Reconhecida como substância GRAS do inglês *Generally Recognized as Safe (*geralmente reconhecida como segura) pelo *Food and Drug Administration* (23) e pela Agência Nacional de Vigilância Sanitária (ANVISA) na Resolução da Diretoria Colegiada (RDC) de 8 de junho de 2013 (24) esta cera tem sido avaliada em diversas estudos na conservação de frutas e hortaliças, como tomate (19, 25), mangas (26); maçãs (27); tangerinas (28); mamões (29, 30); pepinos (31) entre outras.

Silva et al. (26) em estudo avaliando a conservação pós-colheita de diferentes recobrimentos em manga ‘Tommy Atkins’, observaram vantagem na utilização de recobrimentos a base de carnaúba na manutenção da qualidade dos frutos durante armazenamento. Os revestimentos com ou sem adição 1% de triclosano ou 0,1% de ácido sórbico mostraram atividade antimicrobiana contra bactérias mesófilas, bolores e leveduras comparado a mangas revestidas somente com cera.

O hidroxipropil metilcelulose (HPMC), é um polissacarídeo de caráter hidrofílico derivado da celulose e utilizado principalmente na indústria farmacêutica (32, 33), com potencial de uso associado ou não a outros compostos para aplicações como revestimento comestível. O HPMC é um hidrocolóide, solúvel em água, com boas características filmogênicas e tem sido utilizado em uma variedade de alimentos como barreira ao oxigênio (10).

Por sua vez, Navarro-Tarazaga et al. (34) destacam que o HPMC proporciona a formação de filmes flexíveis, sem odor, sem adição de sabor, solúveis em água, com baixa permeação para oxigênio e aromas diversos. Todavia, por serem hidrofílicos, não são tão efetivos como barreira à umidade.

A utilização de cera de abelha em associação com HPMC aplicados como revestimento comestível em ameixas, em diferentes concentrações, reduziu a resistência mecânica e barreira ao oxigênio e melhorou a barreira a perda de água (34). Resultados semelhantes foram obtidos com a associação de HPMC, com outros compostos (cera de abelha e goma-laca),

juntamente com a aplicação de antifúngicos em laranjas ‘Valencia’ (35) e tangerinas (36), demonstrando o potencial de utilização dessas combinações como revestimentos protetores em frutas.

Perez- Gago et al. (37) avaliaram a conservação pós-colheita de tangerinas com a utilização de revestimentos comestíveis de base lipídica, cera de abelha, carnaúba e goma-laca em diferentes proporções associadas ao polissacarídeo HPMC. Foi observado que frutos revestidos com baixos teores de lipídeos (20%) proporcionaram menores concentrações de O_2 e elevados teores de etanol, quando comparados aqueles com altos teores de lipídeos (60%), indicando a possibilidade de uma relação com a baixa permeabilidade do HPMC e espessura da camada de cobertura devido à elevada viscosidade das emulsões.

Adicionalmente, os óleos essências produzidos por plantas são complexas misturas de compostos voláteis provenientes do metabolismo secundário das plantas, conhecidos pelo seu controle de fungos fitopatógenos (38), e tem ganhado grande visibilidade na conservação de alimentos por suas propriedades antimicrobianas.

Estudos relatam propriedades antimicrobianas de óleos essenciais (OE) aplicados a alimentos diversos, como leite, carne, frutas, hortaliças, etc. OE e vários dos seus componentes individuais, apresentam atividade contra patógenos de alimentos (39, 40).

Sridhar et al. (41), relatam propriedades antifúngicas do óleo essencial de gengibre frente aos fitopatógenos *Alternaria alternata*, *Aspergilus flavus* e *Penicillium sp*. em testes *in vitro*. Kouame et al. (42),

verificaram inibição do crescimento micelial de *Colletotrichum gloeosporioides* inoculado em mangas tratadas com óleo essencial de gengibre.

Oliveira et al. (43) em experimento *in vitro* e *in vivo* com óleos essenciais de *Eucalyptus staigeriana, Lippia sidoides* e *Pimenta pseudocaryophyllus* contra *Rhizopus stolonife relataram boa atividade antifúngica destes óleos na pós-colheita de morangos*. Revestimentos com carboximetilcelulose associados ao óleo de *L. sidoides* demonstraram ação preventiva e curativa quanto a infeção por *R. stolonife.* Redução significativa na severidade da doença, em especial quando tratados de modo curativo também foi observado.

Camilo et al. (40) reportaram em ensaio, com óleos essenciais de gengibre e açafrão, eficácia destes contra o crescimento de *S. aureus* e *E. coli*, com valores de concentração inibitória mínima (CIM) ≥ 213,3 µg/mL e 85,3 µg/mL, respectivamente para óleo de gengibre e MIC ≥ 1024 µg/mL para ambas as bactérias testada com óleo de açafrão. Os autores encontraram neral (22,9%), zingibereno (15,5%) e geranial (14,9%) como compostos majoritários do óleo de gengibre.

Cutrim et al. (44) avaliaram a atividade antimicrobiana de óleos essenciais de gengibre e alecrim e seus extratos hidroalcóolicos (etanol 70%) e reportaram que o óleo essencial de gengibre apresentou melhor atividade antimicrobiana comparado com o de alecrim. As concentrações inibitórias mínimas (MIC) para *Escherichia coli* (ATCC 25922) e *Staphylococcus aureus* (ATCC 25923), foram de 1000 e 200 µg. mL^{-1} para o

óleo de gengibre, já para o extrato hidroalcóolico as concentrações foram de 80,000 µg. mL^{-1}.

O gengibre (*Zingiber officinale)* tem sido estudado, principalmente nos países asiáticos, para diversas aplicações médicas em função de seus compostos fenólicos ativos (44, 45) e compostos antioxidantes (46). Vários estudos indicam o potencial de uso de extratos desse rizoma na redução de infestações por micro-organismos em alimentos (47,48) e, consequentemente, com potencial emprego em revestimentos comestíveis (49, 50).

Sa-Nguanpuag et al. (48) relatam a capacidade de compostos químicos extraídos do óleo de gengibre inibir o crescimento microbiano em estudos *in vitro* e *in vivo.* Foi observada a inibição de crescimento para *Bacillus subtilis, Bacillus nutto, Pseudomonas aerugenosa, Rhodoturola sp*, *Samonella newport, Samonella enteritidis* e *Fusarium sp*. Todavia, não foi evidenciado efeito inibitório em *Escherichia coli, Campylobactor coli* e *Campylobacteor jejuni*. Os autores concluíram que o óleo essencial de gengibre pode ser utilizado na redução do crescimento microbiano em mamão verde ralado, com potencial uso para outros produtos.

Nikolić et al. (47) em estudo referente a ação antibacteriana do extrato etanólico de gengibre em estudos *in vitro*, confirmaram a eficiência no controle de bactérias gram-positiva, em especial *Staphylococcus aureus*. Entre os extratos e óleos essenciais, o de gengibre tem sido largamente utilizado na área de saúde humana (45), e apesar de apresentar grande potencial de uso em recobrimentos comestíveis, tem sido pouco estudado.

Atarés et al. (49, 50), realizaram estudo para avaliação da preparação de emulsões formuladas pela combinação de isolado de proteína de soja com dois óleos essenciais (canela e gengibre). Os filmes com óleo de gengibre foram menos resistentes e menos propensos a elongação do que os de canela. Essa interação proporcionou uma melhor integração do óleo na matriz proteica na formação dos filmes, gerando superfícies menos rugosas, à medida que o teor de óleo aumentou.

Revestimentos comestíveis podem afetar o sabor e o perfil de compostos voláteis em frutas não climatéricas (51), como frutas cítricas e climatéricas, como em maçãs e mangas (52, 53).

A produção mundial de tangerinas, mandarinas, clementinas e satsuma em 2018-2019 foi de 32,0 milhões de toneladas segundo USDA-FAS (54). China é o maior produtor de tangerinas (22,0 milhões de toneladas) e também o segundo produtor de laranja de mesa do mundo, com 7,2 milhões de toneladas. O maior produtor de laranjas é Brasil, com 19, 4 milhões de toneladas (1).

Para frutas não climatéricas, como laranjas e tangerinas, os revestimentos podem reduzir a atividade respiratória e a perda de água. Entretanto, uma permeabilidade inadequada à gases atmosféricos de revestimentos para citros pode criar sabores indesejáveis (55), sendo necessário o uso de revestimentos com barreiras efetivas, uma vez que grande parte da produção de citros é revestida com ceras para proporcionar brilho e reduzir a perda de água e encolhimento de frutas (18, 28, 56).

As tangerinas são um grupo diverso de citros, como satsumas e clementinas, atraentes para os consumidores, porque são fáceis de descascar e consumir em comparação com as laranjas comuns (57). As tangerinas têm um tempo de armazenamento menor que as laranjas. A qualidade do sabor da tangerina geralmente diminui rapidamente após a colheita e pode estar associado a um acúmulo interno de metabólitos anaeróbicos devido à redução da disponibilidade de oxigênio no tecido da fruta com altos valores de CO_2 interno, etanol, acetaldeído (55, 57, 58, 52). A propensão a um maior acúmulo de metabólitos anaeróbicos em comparação às laranjas pode estar associada à uma menor permeabilidade da casca às trocas gasosas e à uma maior atividade da enzima álcool-desidrogenase no suco das tangerinas (55,59, 57).

Estudos indicam que tangerinas respondem produzindo etileno quando o suporte de oxigênio é insuficiente (59) e este etileno pode elevar a produção de aromas e sabores desagradáveis (60), diminuindo a qualidade de sabor.

Tietel et al. (28) atribuíram a deterioração do sabor ao revestimento de tangerinas e uma ligeira diminuição da acidez e sugeriram que o enceramento reduziu o sabor típico de tangerinas e aumentou a sensação de sabores indesejáveis para tangerinas da cultivar 'Mor'. Os autores realizaram este estudo com cera comercial Tag e Zivdar da Safepack Products Ltd., empregadas para revestir citros (28).

Alguns aromas voláteis estão associados a uma percepção de sabores desagradáveis que podem aumentar, especialmente os

relacionados ao etanol e ao acetato de etila. Portanto, autores recomendam o uso de revestimentos com alta permeabilidade à oxigênio, mesmo que às custas da redução do brilho (61,62). Consequentemente, os revestimentos comestíveis devem ser utilizados adequadamente, uma vez que o principal objetivo da qualidade pós-colheita é contribuir para manter a qualidade e reduzir as perdas.

Laranjas e tangerinas contêm cerca de 90% de água e nutrientes, sendo facilmente expostas a doenças pós-colheita. Para citros, *Penicillium digitatum* e *Penicillium italicum* causadores do mofo verde e azul, respectivamente, podem resultar em perda pós-colheita. Vários métodos ao longo dos anos têm sido empregados para o controle da doença, de modo a minimizar a contaminação de frutas no campo ou na embalagem e aplicação de tratamentos físicos e químicos, como fungicidas artificiais, para prevenir ou reduzir a infecção por fitopatógenos (63, 64, 65). No entanto, alguns fatores, como resistência ao uso de fungicidas, regulamentação restritiva e conscientização dos consumidores (65), levaram à pesquisas de estratégias mais seguras e amigáveis para o controle de podridões e doenças (64).

Nesse sentido, estudos empregando estratégias alternativas têm sido conduzidos, como a exposição a radiação UV para reduzir a colonização por *Penicillium digitatum* em tangerinas 'Fallglo' (64). Outra estratégia é a introdução de óleos essenciais na matriz polimérica dos revestimentos, para diminuir a taxa de difusão e a exposição de compostos antimicrobianos na superfície da fruta, onde a contaminação geralmente ocorre por um período mais longo (66).

Won e Min (67) estudaram revestimentos de cera de carnaúba combinados com extrato de sementes de toranjas (1:1) e óleo de orégano (2:1) em tangerinas *Citrus unshiu Marc*. e avaliaram a qualidade pós-colheita. Os dois revestimentos reduziram em 23% e 25%, respectivamente, o índice de incidência de *P. italicum* na superfície das tangerinas, quando comparado com frutos não revestidos. Complementarmente, os revestimentos foram capazes de reduzir a perda de água e diminuir a taxa respiratória dos frutos.

Os óleos essenciais (OE) também podem reduzir a difusão de O_2 através do filme, gerando uma maior concentração interna de CO_2 principalmente devido à baixa difusão de gases decorrente do revestimento, como resultado da natureza lipofílica dos OEs. A combinação de revestimentos comestíveis a óleos essenciais, pode ser mais eficaz na criação de condições favoráveis ao prolongamento da vida pós-colheita dos produtos hortícolas, mantendo a qualidade geral das frutas, os compostos nutricionais e a aceitação do produto pelos consumidores (68)

Adicionalmente as tangerinas, entre os produtos de maiores perdas pós-colheita, temos o mamão (*Carica papaya* L.) que é uma das frutas tropicais de maior consumo e economicamente importante em países tropicais e subtropicais, sendo consumida mundialmente como fruta fresca ou processada. As características desejáveis de qualidade estão relacionadas com o amadurecimento que envolve vários processos bioquímicos que convertem o fruto em comestível, doce, macio e aromático (69). Todavia, o mamão consiste em um fruto climatérico altamente

perecível, com altas porcentagens de perdas pós-colheita, principalmente devido a contaminações por micro-organismos, desordens fisiológicas, danos físicos e manuseio inadequado (70, 71).

O Brasil ocupa a segunda posição entre os cinco maiores produtores de mamão, sendo eles Índia, Brasil, México, Indonésia e Republica Dominicana (1). A produção brasileira no ano de 2018 foi em torno de 1,1 milhão de toneladas (1) e a exportação deste fruto tem aumentado anualmente, atingindo 43,3 mil toneladas em 2019 (72). O mamão é cultivado em todo o território brasileiro, sendo os maiores produtores em 2018 os estados do Espírito Santo, Bahia, Ceará, Rio Grande do Norte e Minas Gerais, responsáveis por cerca de 90% da produção nacional (73).

De modo mais geral, as perdas pós-colheita de mamão são estimadas entre 30 a 60% em países desenvolvidos e em desenvolvimento (74), sendo que sendo que a incidência de fitopatógenos podem causar perdas pós-colheita, de até 75% dos mamões na fase de comercialização (38, 75). Nestes contextos, o desenvolvimento de tecnologias que minimizem perdas e promovam a manutenção da qualidade, podem representar significativos lucros em todos os segmentos da cadeia produtiva.

De acordo com Ventura et al. (76), de modo geral, as perdas por ação microbiana da produção são majoritariamente atribuídas aos fungos *Phytophthora spp., Phoma caricae-papayae, Asperisporium caricae, Oidium caricae* e *Colletotrichum spp.*

No Brasil, a antracnose em mamões tem sido associada com *Colletotrichum*. acutatum (77), *Colletotrichum brevisporum* (78) e

Colletotrichum karstii (79). Adicionalmente, *Colletotrichum truncatum* foi reportado por Dos Santos Vieira et al. (80) pela primeira vez no Brasil, em mamões oriundos de Porto Seguro, Bahia, espécie esta que já tem sido reportada incidência de antracnose no México e em Trindade e Tobago.

Dantas et al. (81) avaliaram doenças fúngicas de ocorrência pós-colheita em mamões comercializados na central de abastecimento de Recife e verificaram grande diversidade de doenças em mamões. Neste estudo, a podridão peduncular causada por *Colletotrichum gloeosporioides* obteve frequência média de 39,7% de ocorrência dentre os patógenos, seguidos de antracnose (20,32%); mancha chocolate (10,5%) e podridão por *Fusarium spp* com 5,6% das incidências. Outros autores apontam *Fusarium spp* sendo o gênero de maior ocorrência no Brasil (82).

Com o objetivo de minimizar as perdas vários tratamentos como com água quente, embalagem em atmosfera modificada, irradiação gama tem sido utilizados na condição pós-colheita para manutenção da qualidade do mamão, incluindo a aplicação de revestimentos protetores (83, 30, 84).

A aplicação de revestimento a base de goma arábica (10%), combinada com óleo de gengibre (2%) e extrato (1,5%) foram avaliados para manutenção da qualidade e controle de antracnose em mamão 'Eksotika II' armazenados a 12 °C e 80% UR. Revestimentos combinado com óleo de gengibre mostraram inibir significativamente a germinação de esporos (93%). Adicionalmente o revestimento atrasou o amadurecimento e teve efeito fungicida para mamões (83).

Ohashi et al. (30) avaliaram mamões ‘Golden’ revestidos com emulsão de cera de carnaúba em baixas concentrações (2,4 e 4,8%) e verificaram retardo no amadurecimento, redução de perda de peso e diminuição de incidência de podridões para a menor concentração testada.

Bosquez-Molina et al. (85) estudaram o efeito antifúngico *in vitro e in vivo* de óleos essenciais de tomilho e de limão mexicano contra *Colletotrichum gloeosporioides* e *Rhizopus stolonifer* e incorporação destes óleos em revestimentos a base de goma de mesquite (*Prosopis spp*) para conservação pós-colheita de mamões. Os autores reportaram redução entre 40 e 50% de podridões causadas pelos fungos quando os mamões foram imersos nos óleos. Revestimentos de goma mesquite combinados com os óleos a de tomilho e limão mexicano a 0,1 e 0,5 %, respectivamente, foram capazes de reduzir em 100% a incidência de C. *gloeosporioides*. Óleo de tomilho mostrou-se extremamente eficiente, com CIM de 0.060% para ambos os fungos. No estudo, os autores concluíram que o óleo de tomilho se mostrou mais eficiente, além de aumentar a vida pós-colheita dos frutos.

A combinação de extratos e óleos essenciais com outras matrizes lipídicas e/ou poliméricas, como já comentado, pode ser uma alternativa para adequar e melhorar as propriedades finais de um filme. Permite aliar características e propriedades que podem proporcionar um acréscimo na ação do revestimento, melhorando propriedades físicas, químicas, mecânicas e antimicrobiana.

Neste contexto, o objetivo do presente trabalho foi de avaliar a atividade de extratos e óleos essenciais de rizoma de gengibre *(Zingiber*

officinale) visando aplicações como potenciais antifúngicos naturais, e associa-los ao desenvolvimento de revestimentos protetores nanoestruturados, tendo por base formulações de nanoemulsão de cera de carnaúba e do polissacarídeo HPMC. As avaliações da capacidade protetora dessas coberturas foram conduzidas na conservação pós-colheita de tangerinas e mamões, sob diversas condições de armazenamento. Na Figura 1 está apresentado o esquema geral da organização dos capítulos.

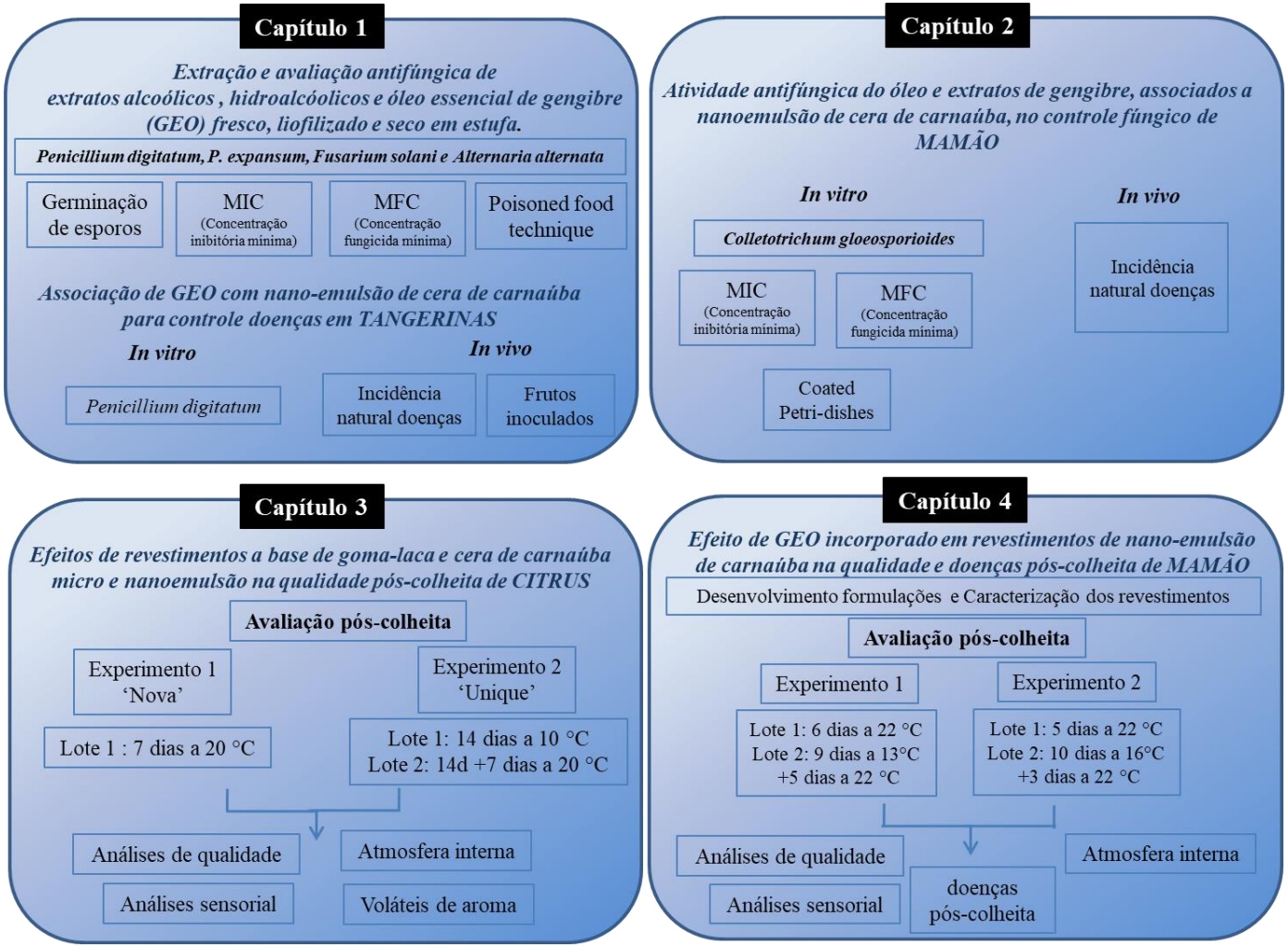

Figura 1. Esquema geral da composição dos capítulos.

Capítulo 1.

Antifungal evaluation of and extracts and essential oil from ginger (*Zingiber officinale Roscoe*), in association to carnauba nanoemulsions, as protective postharvest coatings on tangerines.

Artigo a ser submetido.

Antifungal evaluation of essential oils and extracts from ginger (*Zingiber officinale Roscoe*), in association to carnauba nanoemulsions, as protective postharvest coatings on tangerines.

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ABSTRACT

Phytopathogens can cause considerable economic losses in several crops, being fungi the group with the highest occurrence of postharvest diseases. Against the proliferation of microorganisms in food, plant extracts and their compounds may be an alternative antifungal agent considering their low toxicity and biodegradability. The extracts and oils from the *Zingiber officinale Roscoe* have shown potential to be used in the control of plant pathogens, though more studies have to be done in order to evaluate the actual activity of this rhizome against fungi that causes postharvest diseases. This is the aim of the present study, evaluating ginger extracts (GEs) and essential oil (GEO) as antifungal activity against phytopathogens (*Penicillium digitatum, Penicillium expansum*, *Fusarium solani* and *Alternaria alternate)* and GEO action for natural and inoculated *P. digitatum* in association to protective coatings applied on 'Unique' tangerines fruits. *In vitro* antimicrobial activity of alcoholic ginger extracts (GEs) and GEOs was evaluated by the spore germinated test (SGT), minimum inhibitory concentration (MIC), minimum fungicide concentration (MFC) and poisoned food (PF) against *Penicillium digitatum, Penicillium expansum*, *Fusarium solani* and *Alternaria alternata* phytopathogens. Additionaly, the effect on natural diseases incidence on tangerines inoculated with *P. digitatum* stored at 20°C, was also evaluated up to 31 days and after 14 and 21 days at 20°C. The MIC analyzes

pointed GEOs as more effective than GEs, requiring an inferior concentration than the alcoholic extracts in inhibiting *P. digitatum* spores germination. Comparing the tested fungi, *P. expansum* was the least sensitive by displaying a higher amount of germinated spores. In general, *P. digitatum, P. expansum, A. alternata* and *F.solani* resulted in an *in vitro* reduction of spores germinated when the proportion of GEs and GEOs in broth medium increased from 1 to 3%. Conversely, GEE (ginger ethanolic extracts) and GHEs (ginger hydroethanolic extracts) did not showed fungicidal activity against *P. digitatum, P. expansum, F. solani* and *A. alternata,* even at the highest concentration tested concentration (6%). The *P. digitatum*, was the fungus with higher sensitivity to ginger compounds followed by *A. alternata, F. solani* and *P. expansum*, being that *F. solani* and *P. expansum* at the highest tested GEO concentration (1.6%) did not resulted in growth inhibition. The MFC measured for *P*. *digitatum,* and *A*. *alternata* was 0.8 and 1.6, respectively, in broth test. On *in vivo* trials, GEO at 0.8% added into nanoemulsion carnauba wax coatings, was not enough to provide significantly reduce natural diseases on 'Unique' tangerines along 31 days of storage. On the other hand, the combination of nanoemulsion + GEO showed to be, *in vitro,* significantly more effective than the nanoemulsion or the GEO alone, but not when applied on fruits. Nano-emulsion showed antifungal ability by its own, with or without GEO incorporation, performing better than neat GEO, indicating possible use of its antimicrobial effect. GEOs showed a potential to applyed as coating for postharvest fungal control, with better results than alcoholic extracts. The use of essential oils for antifungal treatments is more wholesome and environmentally friendly than using chemical preservatives and fungicides.
Key-words: ginger essential oil, phytopathogens, antifungal activity, alcoholic extract, ethanolic extract.

1. Introduction

Essential oils and several of their individual components have broad activities against food pathogens as confirmed by *in vitro* experiments (Singh, 2010, Bakkali et al., 2008; Burt et al., 2004). Particularly, ginger extracts have the potential to be used as a natural antimicrobial agent, in replacing artificial preservatives and synthetic fungicides. Ginger has been studied in several applications, due to its composition rich in active phenolic compounds and the relative ease way of obtaining extracts from its rhizome extracts (Nikolić et al., 2014; Rahmani et al., 2014; Sa-Nguanpuag et al., 2011), with potential to be used as active agent in edible coatings (Atarés et al., 2010a, 2010b).

Extracts and oleoresins are produced from fresh or dry plant material, and usually from non-peeled ginger, considering that when peeled most of its essential oil could be lost (Reverchon, 1997; Leung, 1980). The oleoresins are practically solid at room temperature after the elimination of the solvent. In their composition is found a range of lipophilic constitutes of the vegetal material, such as essential oil, fatty acids, fatty acid methyl esters, pigments (carotenoids), coumarin, steroids and flavonoids (Reverchon, 1997; Dogeneski, 2013). Studies have demonstrated that oleoresin, which is present in crude extracts, has antimicrobial and antioxidant properties (Sing et al., 2008; Dogenski, 2013).

The essential oil contains volatile compounds responsible for the aroma, while oleoresin also has non-volatiles pungent properties (Singh, 2010; Govindarajan, 1982). The fungicidal nature of essential oils and their extracts are attributed to the monoterpenes, sesquiterpenes and their derivatives (Singh, 2010).

The antifungal properties of ginger essential oil against phytopathogens such as *Alternaria alternata, A. flavus* and *Penicillium sp*. has been reported by Sridhar et al. (2003), in *in vitro* assays. In the study of Gaston et al. (2015) reported mycelial growth inhibition of *Colletotrichum gloeosporioides,* in inoculated mangoes, when treated with ginger essential oil. Sa-Nguanpuag et al. (2011) also reported the ability of chemical compounds extracted from ginger oil in inhibiting the growth of human and

phytophatogen, such as *Bacillus subtilis, Bacillus nutto, Pseudomonas aerugenosa, Rhodoturolasp, Samonella newport, Samonella enteritidis* and *Fusarium sp* both *in vitro* and *in vivo* conditions.

Citrus fruits as oranges and mandarins contain about 90% of water and nutrients, which makes them easily susceptible to postharvest diseases and consequently decay losses. In citrus, *Penicillium digitatum* and *Penicillium italicum* cause green and blue mould, respectively, with severe important economic losses during commercialization (Chen et al. 2019). To control these diseases, the application of physical and chemical treatments, as artificial fungicides, is the most used method. However, some factors, such as fungicide resistance, restrictive regulations and consumer awareness (Smilanick et al., 2006) have pressured researches to look for safety and friendly environmental alternatives for increasing decay control (Alferez et al., 2012).

Most of the studies about GEO antimicrobial activity have been performed *in vitro* against several bacteria and fungi, with a lack of experiments concerning the addition of GEO for active coatings preparation. The association of ginger essential oil to biopolymeric matrices has been reported in tests in the formation of films based on fish skin-gelatin (Tongnuanchan et al., 2013); HPMC (Atarés et al., 2011), sodium caseinate (Noori et al., 2018; Atarés et al., 2010a) and in a soy protein matrices (Atarés et al., 2010b). These studies, however, are not focused on the GEO incorporation in formulations suitable for preparing edible coatings for fruit quality preservation.

In this context, the main aim of the present study was to evaluate the antifungal activities of ginger extracts (GE) and essential oils (GEO) in controlling postharvest phytopathogens, especially in some of those fungi with most incidences on citrus crops. The citrus decay – natural and fungus inoculated - after application of nanoemulsion coatings containing GEO was also assessed.

2. Material and Methods

2.1 Ginger extract and essential oil extraction and evaluation of the antifungal activity

2.1.1 Plant material and processing

Ginger ‘Gigante’ (*Zingiber officinale*) from Piedade city in São Paulo State, Brazil, was purchased at Ceasa - São Paulo, in physical integrity conditions (absence of injuries or damages). The ginger rhizomes were washed, rinsed, sanitized and processed in small pieces (around 0.5 cm) using a domestic blender. Subsequently, the material was grounded and separated into three parts. One part was frozen (named here as fresh sample), and the other submitted to oven at 40 °C with air circulation – oven-dried. The remaining part was freeze-dried until 3 to 9% of final moisture – freeze-dried. After drying, the samples were stored in a dry environment at room temperature.

2.1.2 Extraction of alcoholic ginger extracts (GE)

GE was extracted by mixing 50 g of each sample (fresh or dried) with 300 mL of absolute ethanol (ethanolic extract - GEE) or 70% ethanol (hydroethanolic extract - GHE), followed by agitation at 200 rpm in a shaker at 25 °C during 24 h. Then, the extracts were filtered, and the resulted material added to 300 mL of respective ethanol and the extraction processes repeated. The residues were washed with 300 mL of ethanol, and the filtered extracts mixed and evaporated using a rotary evaporator at 40 °C, resulting in crude extracts (Breda et al., 2016).

The yield of the crude extracts was calculated according to Equation 1.

$$\mathrm{R}\% = \frac{Mext \times 100\%}{M}, \qquad (1)$$

Where:

R % is the percentage of yield of the crude extract;

M_{ext}, the of crude extract obtained (g)

M, the mass of initial material used for the extraction (g)

2.1.3 Extraction of ginger essential oils (GEO)

GEOs were extracted by hydro-distillation using a Clevenger's type apparatus, according to Natta et al. (2008), with modifications. Fresh ginger (600 g), oven-dried ginger (50 g) or freeze-dried (50 g) was combined with distilled water (1 L). The flask containing the material and water was heated under soft boil during 24 h. The oil collected and dried over anhydrous sodium sulfate to remove moisture. Each extraction was conducted in triplicate and then mixed. The oil and the extracts obtained were stored at low temperature (5 °C) in a dark container for further use. Commercial ginger oil (C GEO), food-grade, CAS Number: 8007-08-7 was purchased from Sigma-Aldrich (W252204-250G-K, China) and used as GEO control.

2.1.4 Dilution of GE in polyvinylpyrrolidone (PVP) and GEO in dimethyl sulfoxide (DMSO)

Crude GEs were solubilized in 0.016 $g \times mL^{-1}$ ethanolic solutions of PVP at the proportion of 2:1 (v/v), according to the procedure described by El-Arini and Leuenberger (1998), with some modifications. Crude GEOs were dissolved in DMSO 10% at a proportion of 2:1 (v/v). PVP and DMSO were used in order to facilitate the ginger compounds perfusion into the culture mediums, and guarantee contact to microorganism and subsequent activity.

2.1.5 Microorganisms and Culture Maintenance

Antifungal activities were evaluated against cultures of *Penicillium expansum* - CMIIAA PEN 001*, Fusarium solani* – CCT 2876*, Alternaria alternata* CCT 1250, which were provided by Embrapa Instrumentação, São Carlos-SP. P*enicillium digitatum* isolated from citrus fruit as described by Narciso (2009). The fruits were from the Horticultural Research Laboratory

(USHRL) in Ft. Pierce, FL, US- Citrus and Other Subtropical Products Unit, Agricultural Research Service (USDA-ARS). The strains of *P. digitatum* and *P. expansum* were maintained and grown in potato dextrose agar - PDA, *F. solani* in oat medium, and *A. alternata* in malt extract agar - MEA. After inoculation, the cultures were incubated at 25 °C until sporulation (between 7 to 10 days).

2.1.6 Standardized inoculum preparation

Petri dishes containing the sporulating fungal cultures were filled with 1 mL of sterilized solution of Tween 20 at 0.5 % (v/v) and gently homogenized with Drigalski spatel. The supernatant (spores suspension) was removed with a sterilized Pasteur pipette, filtered in cheesecloth to retain hyphae fragments and transferred to a sterilized glass tube. An aliquot of spore suspension was analyzed in Neubauer chamber and adjusted with 0.85 % sodium chloride solution for a density corresponding to approximately 0.4×10^6 CFU·mL^{-1} (spore germination, minimum inhibitory concentration-MIC) or 0.4×10^5 CFU·mL^{-1} (poisoned food technique).

2.1.7 Spore germination test

P*enicillium digitatum, Penicillium expansum, Fusarium solani* and *Alternaria alternata* spores germination tests, in liquid medium - potato-dextrose – PD, were performed following Mendes et al. (2016) with slight modification. Spore suspensions and broth with samples were prepared at twice the final concentration desirable, taking into account the dilution when inoculum and PD were combined. Incubation was carried out at 25 ° C for 24 h under 120 RPM orbital shaking. The final GEs and GEOs concentrations were 1% and 3% (v/v). An aliquot of 10 µL of the suspension was transferred and observed in a Neubauer camera, using optical microscope and images recorded. The germinated and non-germinated spores counting was performed and expressed as percentage. Cycloheximide (1 g×mL^{-1}) and the broth without sample (GEOs or GEs) were used as positive and negative controls respectively. DMSO and PVP control were included in the test in

order to investigate any antifungal activity of these compounds. Results were obtained in triplicate and each measurement performed at 5 different sites of each slide.

2.1.8 Minimum inhibitory concentration (MIC) and Minimal Fungicidal Concentration (MFC)

The MIC test was performed using a sterile test tube containing 500 µL of potato-dextrose (PD) broth with the samples, and 500 µL of a standardized inoculum of each microorganism was added to all test tubes and incubated at 25 °C for 7 days under agitation of 125 rpm. The observations were carried out after 24, 48, 72, 96 and 168 h. The concentration ranges evaluated for GEs activities were 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 and 6.0 % (v/v) and 1.6, 0.8, 0.4, 0.2, 0.1, 0.5, 0.025, 0.0125, 0.0062, 0.0031 and 0.0015% (v/v) for GEOs. From the diluted sample the desirable concentration was prepared by adding to PD broth. Cycloheximide and broth without samples (GEOs or GEs) were used as positive and negative controls, respectively. The MIC was defined as the lowest concentration of GEs or GEOs in which the inhibit microorganism growth is visible (PUJOL et al., 2016 with slight modifications). The results were expressed as the average of three repetitions.

In order to determine the MFC, an aliquot of 10 µL of each incubated test tube of MIC and the higher concentrations were sub-cultured on PDA Petri dishes and incubated at 25 °C for 5 days (Donlan and Costerton, 2002). The MFC was defined as the lowest concentration that allowed no visible growth. The results are expressed as the average of three repetitions.

2.1.9 Poisoned food technique

The direct contact method in solid culture medium against fungi and inverted Petri dish test was undertaken using Ramdas et al. (1998) and Sing et al. (2008) adapted procedures. The calculated amount of each diluted GE and GEO (to obtain a final concentration of 1% and 3% v/v) were mixed with 20 mL of the sterilized culture medium (45 °C) and poured in sterilized Petri plates, followed by addition of Tween 80 (100 µL to 100mL of medium) to

disperse sample in the medium. After medium solidification, plates were inoculated with 10 µL of spores suspension of each microorganism in a concentration of 10^5 and incubated at 25 °C. Negative control (without sample) was similarly prepared. The positive control was cycloheximide. DMSO, PVP and tween 80 controls were included in the tests to investigate possible antifungal activity. Petri dishes were sealed by parafilm to avoid the release of volatile compounds. The observations were carried out after 24, 48, 72, 96 and 168 h. The diameter of radial growths (mm) recorded on the 7th day and used for calculating the percent of mycelial zone inhibition (% MZI):

$$\% \text{ MZI} = \frac{dc - dt}{dc} \times 100, \qquad (2)$$

Where:

dc is the average diameters of mycelial colonies of the negative control

dt is the average diameters of mycelia colony as measured for each treatment

2.1.10 Inverted Petri dish test

Petri dishes inoculated with 10 µL of *Penicillium digitatum* spores suspension 10^5 on PDA medium were inverted upside down and sterilized filter paper disc (Whatman 6 mm) placed on the center of its inverted lid. The required doses (5, 10 and 30 µL) of crude samples (samples without dilution in DMSO or PVP) were soaked on filter paper discs. Petri dishes were then incubated at 25 °C during 5 days in inverted position. Each test was performed in triplicate, and fungal sensitivity calculated in terms of mycelial percentage of zone inhibition compared to the negative control. The negative and positive controls were similarly prepared by replacing samples to sterile distilled water and ammonium hydroxide 30%, respectively. The diameters of radial growths (mm) as recorded on the 5th day, were used for calculating the percent of mycelial zone inhibition and compared to the negative control.

2.2. Natural diseases and fungal inoculation on citrus and coating protection

2.2.1 Coatings preparation

Carnauba wax nanoemulsion (CWN) coating, was prepared with the oil phase (O) comprising carnauba wax type 1 (8 to 18% wt/v), and oleic acid (2.6 to 6% wt/v), from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The aqueous phase (W) was composed of ammonium hydroxide (1 to 3% wt/v), and dimethylpolysiloxane (0.02 to 0.1% v/v) (from Sigma-Aldrich) and deionized water (71 to 89% wt/v). The nanoemulsion was prepared following Hagenmaier and Baker (1997) with modifications. The resulted carnauba wax nanoemulsion is characterized by having an average diameter size of 44 nm, with a narrow polydispersion index (0.28) and zeta potential -43.8 mV, according to measurements previously carried out by Zetasizer Nano ZS (Malvern Instruments Inc., Westborough, MA, USA), (Miranda, 2015).

Oil-in-water (O/W) GEO emulsions were prepared by adding 0.8% (v/v) of ginger oil; 0.6% (v/v) of Tween 80 in ultrapure water, followed by mixing in an Ultra-Turrax at 16,000 rpm for 4 min to obtain ginger essential oil nanoemulsion according Otoni et al. (2014).

Carnauba wax nanoemulsion containing GEO was obtained by a continuous and gradually additions of Tween 80 at 0.6% (v/v) and GO 0.8% (v/v), into previous prepared CWN, followed by mixing using an Ultra-Turrax at 16,000 rpm for 4 min to get the final coating formulation.

Considering the low amount of oil generated from the steam distillation extraction in laboratorial scale, commercial ginger oil (CGEO) was also used to guarantee the necessary volume for all experiments.

The extract ginger oil chosen was the GEO-oven-dried ginger, which attained the highest yield among the extraction. The concentration of 0.8% was chosen due to *in vitro* experiments for *P. digitatum.*

2.2.2 Analyzes of the coating formulations in inoculated Petri dishes

An aliquot of 10µL *Penicillium digitatum* spore suspension at 10^6 CFU·mL-1 was placed on PDA for 24h incubation at 25°C. Then, 1 mL of each coating (previously filtered through a sterile 0.2 µm pore size membrane) was poured on the Petri dishes and spread with Drigalski spatel on the agar medium surface. The plates were allowed to dry until complete solvent evaporation and formation of a superficial film. In sequence, Petri dishes were incubated at 25 °C for 7 days. Control was similarly prepared by replacing coatings with sterile distilled water. Each assay was performed in triplicate and the readings based on the diameter (mm) of mycelial zone.

2.2.3 Fruit processing and coating

'Unique' tangor was obtained at Al´s Family Farms Citrus-Fort Pierce, a commercial citrus plantation in Florida, US. Fruits were selected, washed and sanitized by immersion in a 200 mg·L^{-1} peroxyacetic acid during 3 minutes and dried at room temperature. Experiments were conducted in completely randomized factorial design.

The sanitized tangerines were hand coated by pouring separately 1 mL of each formulation on the fruit and spread with latex gloves. Treatments were: a) carnauba nanoemulsion; b) carnauba nanoemulsion with 0.8% of extracted GEO; c) carnauba nanoemulsion with 0.8% of commercial oil (CGEO); d); 0.8% of neat GEO; e) 0.8% of neat commercial CGEO and f) control (sterile water instead of coatings). For each treatment 30 fruits were used.

2.2.3.1 Natural diseases incidence

All fruits were stored at 20°C for 31 days. Fruit which shows soft rot at the stem scar and/or green/blue mould occurrences on skin surface were observed, was counted as decayed fruit and put apart from the lot. Diseases incidence scored as the percentage of decayed samples of each

treatment during storage. (Fagundes et al., 2014). The evaluations were carried on days 14, 21 and 31. The assay finished when uncoated fruits lot achieved decay of 100%.

2.2.3.2 Fungal inoculation on citrus

Penicillium digitatum is a major postharvest pathogen of citrus diseases. Since, in this study we tested the extracts against *Penicillium digitatum,* this fungus was chosen to be assayed *in vivo* experiments, due to economic importance in citrus losses and compatible interaction (*P. digitatum*-oranges) compared to the incompatible interaction (*P. expansum*-oranges), according (Vilanova, et. al 2013).

After sanitation, samples of 'Unique' tangerines had the barks superficially wounded, twice on the equator (on opposites sides), with a probe tip 1 mm x 2 mm in length. Inoculation in the wounds were carried out by dropping 10 µL of a *Penicillium digitatum* spore suspension (10^6 CFU·mL^{-1}). After incubation at 20 °C for 24 h, the samples had the whole surface coated by manual spreading of 1 mL of each formulation. Inoculated fruits with sterile water spreading were taken as control. The coatings were allowed to dry at room temperature and the fruits stored at 20 °C. For each treatment 10 fruits were considered. Samples showing any green mould incidence was counted as a decayed fruit. The disease incidence on inoculated samples was estimated as the percentage of decayed fruit (Fagundes et al., 2014), after 14 and 21 days. The assay was finished when treatment reached 90% of green mould incidence.

3. Statistical analyzes

Statistical analyzes were performed using the parametric one-way variance (ANOVA). Spores germination data were analyses by multiple comparison Tukey test and the poisoned food technique and inverted Petri dishes by multiple comparison Duncan. The significance level for all analyzes was set as 5%. Significance differences in the percentage of diseases

incidence were analyzed by a 95% confidence interval. The software used was the IBB SPSS Statistics Inc. (Chicago, IL).

4. Results and discussion

4.1 Ginger alcoholic (GE) extracts and essential oil (GEO) yield

Yields of crude GE and GEOs obtained from fresh (% wet basis, w/w) and freeze-dried and oven-dried ginger (% dry basis, w/w) are shown in Table 1. The amount of the extracts and essential oils obtained in this study varied according to the methodology used (by solvent or steam-distillation) and can be influenced by substrate chemical composition (Dapkevicius et al., 1998).

There was a variation in the oil yielding according to fresh or dried samples used in hydro-distillation, the highest quantity was observed for the dried ginger (freeze-dried or oven-dried) and the lowest for fresh ginger (Table 1). For fresh ginger oil, a similar yield values were recorded by Philippe et al. (2012) as 0.23 ± 0.7%. Singh et al., 2005, attained 1.2% of oil from fresh ginger. Higher values were reported by Chidozie-Onyenekwe and Hashimoto, 1999, with 2.4%, and by Magalhães et al. (1997), which come up a range of 1.0 to 2.25%.

The differences found between ethanolic (around 160%) and hydroethanolic (approximately 220%) extracts (Table 1) are assumed as due to the nature of the solvent, however, the obtained in this study was notably higher than those reported in the literature for solvent extraction. Generally, plant extracts resulted in crude extracts of around 3 and 11% and can reach up to 20% (Magalhães et al., 1997). Breda et al. (2016) reported yields between 7 and 64% from residues of Brazilian savanna fruits (including leaves, peels and seeds). The extractions of GEE and GHE (Table 1) in our study were higher than the values found in the literature. The solvent time evaporation could be a determinant factor in achieving this high percentage of the extracts.

Essential oils have a higher number of components than extracts. Singh et al. (2005) identified 69 components for ginger oil and just 34 for the ethanolic oleoresin, therefore, in microbial assays, the ginger oil results in better antifungal activity than the oleoresins. Radu et al. 2017 also found a higher number of ginger compounds for bioproducts obtained by ginger rhizomes from hydro-distillation method compared to pressing fresh rhizome (aqueous extract). The aqueous extract showed to have inferior antioxidant properties and fungicidal activities.

Nevertheless, it is clear that hydro-distillation - the oil extraction method employed, despite low ratio of production compared to plant material, presents superior number of compounds compared to ethanolic extraction. On the other hand, the extraction by solvent, which has a higher yield production generates extracts thermally sensitive (Mukhopadhyay, 2000).

Table 1. *Zingiber officinale Roscoe* (ginger) extracts and essential oil yields from fresh, freeze-dried and oven-dried ginger rhizomes.

Class	Samples	Yield (% w/w)
Ginger extracts (GE)	GEE-fresh ginger	163.1 ± 2.5
	GEE-freeze-dried ginger	162.0 ± 4.2
	GEE-oven-dried ginger	160.4 ± 3.5
	GHE-fresh ginger	217.3 ± 5.6
	GHE-freeze-dried ginger	218.6 ± 4.9
	GHE-oven-dried ginger	222.4 ± 7.9
Ginger essential oils (GEO)	GEO-fresh ginger	0.2 ± 0.0
	GEO-freeze-dried ginger	3.2 ± 0.1
	GEO-oven-dried ginger	3.5 ± 0.1

GEE = ginger ethanolic extracts; GHE = ginger hydroethanolic extract.
Mean of 3 extractions ± standard deviation

4.2 Antifungal activity of alcoholic ginger extracts (GEs) and essential oils (GEOs)

4.2.1 Spore germination test

The evaluation of *Penicillium digitatum, Penicillium expansum, Fusarium solani* and *Alternaria alternata in vitro* spore germination suppress on samples treated with GEs or GEOs are summarized in Table 2. From the data presented, is clear that alcoholic ginger extracts and essential oils have different levels of activity.

The ginger ethanolic extracts (GEE) from fresh, freeze-dried ginger, and oven-dried rhizomes at 3% (v/v) and GEO at 1 and 3%, acts better than GHE, resulting in the lowest percentage of *P. digitatum* germinated spores (0 to 5.3 %).

At a concentration of 1%, GE a high percentage of spores germination is observed for *P. digitatum.* At this concentration, the ginger hydroethanolic extracts (GHE) showed to be more effective than GEE. However, when the concentration was increased to 3% (v/v), the GHE maintains the number of spores germination while GEE reduce them. Among the hydroethanolic extracts, the samples obtained from oven-dried ginger stands out among this class, with a lower amount of germinated spores. At a large, considering the *P. digitatum,* the GEOs have a more accentuated antifungal response among the samples.

Meanwhile, the *P. expansum* showed to be less sensible, generating a higher percentage of germinated spores when compared to *P. digitatum,* after 24h of incubation. Generally, ginger exhibited a potential to inhibit the germinated spores, as compared to negative control of *P. expansum,* especially at a concentration 3%, however, not statically different. A reduction in germination is also observed when the concentration of GEs and GEOs increased from 1 to 3%, though no significant statistical differences among different treatments were evidenced. *Alternaria alternata* exhibited the same behavior as *P. expansum* related to spores germination after 24 hours of incubation at 1 and 3 % of GEs and GEOs samples.

Overall, *Fusarium solani,* germination of spores attained 60.7 % for the negative control not statically different from those measured to GEs and GEOs samples, excluding GEE-oven-dried sample, which resulted in the lowest percentage of germinated spores, 20.6 and 14.4% at 1 and 3%, respectively. GEOs at 3% showed reductions of around 25 to 50% compared to the negative control.

Othman et al. (2020), in testing aqueous and ethanol extracts from different plant origin (camphor, peppermint, basil, rosemary, ginger, fenugreek, cinnamon, dill and Japanese green tea) against *Aspergillus flavus*, *Aspergillus versicolor*, *Penicillium sp*. and *Penicillium purpurogenum* reported that ginger water extract was the one which had the broadest spectrum of antifungal activity, inhibiting in different levels of fungal growth. Water ginger extract showed an inhibition ratio of 10% against *Penicillium sp*. and *Penicillium purpurogenum*.

It is well known that GEO and oleoresin have different types and amount of phenolic compounds in their compositions, which give rise different responses against fungi strains (Reverchon 1997).

Ginger extracts or oleoresins contain phenolic alkenones such as gingerols, shogaols, zingerones, paradols, gingerdiols and other pungent principles like diarylheptanoids, gingerenones, dehydroshogaol and cyclic diarylheptanoids (Kikuzaki and Nakatani, 1996; Connell and Jordan, 1971). In addition, Singh et al. (2008, 2005) reported that the compounds in oleoresins samples (crude extracts from several solvents) could be different and dependent on the variety, solvent and extraction method, environment, among other factors (Singh et al., 2008; 2005).

Phenolic compounds are assumed as the chemicals responsible for the antimicrobial activity found in essential oils. Ginger oil contains sesquiterpene hydrocarbons such as zingiberene, ar-curcumene, β-bisabolene and β-sesquiphellandrene and monoterpenes such as geranial, neral and camphene in different amount and it depends on the factors cited before (Reverchom, 1997; Wohlmuth et al. 2006; Singh et al. 2008).

The better effectiveness of GEOs compared to GEs might be understand in terms of essential oils contain volatile compounds that are prone to interact and inhibiting spore germination and spore tube elongation (Sivakumar, Baustista-Banos, 2014; da Cruz et al., 2013).

Essential oil reduces the growth of postharvest pathogens by acting directly on the mycelial and spore germination, which affects the pathogen cellular metabolism (Regnier et al., 2010; Serrano et al., 2005). Essential oils contain phenolics and when it interacts with the microorganism envelope, it could result in deformation of the glycoprotein structure, deregulating internal functions. Basically, the essential oil molecules can cross over the fungal lipid cell membrane, causing disruption which results in imbalances of hydrogen and potassium ions exchanges, leading the microorganism to death (Beckman, 2000; Fung et al., 1977).

The levels of compounds may explain the different antifungal activities found between GEOs and GEs samples.

Table 2. Percentage of *in vitro* germinated spores of *Penicillium digitatum (PD)*, *Penicillium expansum* (PE), *Fusarium solani* (FS) and *Alternaria alternata* (AA) after 24 h incubation in GEs and GEOs.

Class	Samples	PD		PE		FS		AA	
		****1% v/v**	****3% v/v**	**^{ns}1% v/v**	**^{ns}3% v/v**	****1% v/v**	****3% v/v**	**^{ns}1% v/v**	**^{ns}3% v/v**
Ginger Extracts (GE)	GEE-fresh ginger	18.1 ± 1.0^{bc}	2.9 ±1.0^{a}	49.7 ± 6.7^{a}	38.2 ± 6.4^{a}	62.4 ± 2.0^{b}	43.6 ± 11.9^{ab}	50.6 ± 4.7^{a}	44.0 ± 10.1^{a}
	GEE-freeze-dried ginger	35.7 ± 4.4^{d}	1.2 ± 1.0^{a}	50.8 ± 11.3^{a}	38.0 ± 10.4^{a}	63.4 ± 2.0^{b}	52.8 ± 4.8^{b}	56.0 ± 7.4^{a}	38.1 ± 13.3^{a}
	GEE- oven-dried ginger	32.2 ± 4.4^{d}	0.6 ± 1.0^{a}	53.8 ± 10.2^{a}	45.0 ± 4.4^{a}	20.6 ± 5.1^{a}	14.4 ± 8.3^{a}	49.2 ± 11.3^{a}	41.1 ± 8.4^{a}
	GHE-fresh ginger	24.6 ± 1.8^{c}	25.1 ± 1.0^{c}	45.4 ± 9.4^{a}	41.6 ± 7.3^{a}	63.4 ± 17.5^{b}	37.4 ± 10.8^{ab}	49.4 ± 14.1^{a}	43.4 ± 13.4^{a}
	GHE-freeze-dried ginger	23.4 ± 1.0^{c}	19.9 ± 2.7^{b}	46.3 ± 13.4^{a}	33.3 ± 5.8^{a}	61.5 ± 16.7^{b}	53.3 ± 5.8^{b}	43.0 ± 1.0^{a}	39.2 ± 3.3^{a}
	GHE-oven- dried ginger	14.6 ± 1.0^{b}	2.3 ± 1.0^{a}	54.2 ± 3.8^{a}	31.5 ± 12.4^{a}	41.3 ± 13.7^{ab}	34.3 ± 12.5^{ab}	52.1 ± 3.6^{a}	46.0 ± 9.4^{a}
Ginger Essential Oils (GEO)	GEO-commercial	5.3 ± 1.8^{a}	0.6 ± 1.0^{a}	40.3 ± 8.7^{a}	37.4 ± 14.1^{a}	38.3 ± 10.3^{ab}	30.6 ± 12.8^{ab}	51.0 ± 4.9^{a}	36.7 ± 9.1^{a}
	GEO-fresh ginger	0.0 ± 0.0^{a}	0.0 ± 0. 0^{a}	41.4 ± 16.2^{a}	38.6 ± 8.7^{a}	57.0 ± 15.7^{b}	41.8 ± 9.2^{ab}	52.2 ± 12.7^{a}	37.8 ± 6.7^{a}
	GEO-freeze- dried ginger	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	52.2 ± 13.5^{a}	50.3 ± 10.7^{a}	47.8 ± 19.4^{ab}	45.8 ± 11.7^{ab}	49.1 ± 5.9^{a}	33.5 ± 2.8^{a}
	GEO-oven-dried ginger	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	39.3 ± 5.6^{a}	37.3 ± 11.3^{a}	53.6 ± 2.1^{ab}	32.3 ± 18.7^{ab}	50.0 ± 14.3^{a}	51.2 ± 6.6^{a}
	Negative control	37.4 ± 3.7^{d}	37.4 ± 3.7^{d}	53.7 ± 3.6^{a}	53.7 ± 3.6^{a}	60.7 ± 6.6^{b}	60.7 ± 6.6^{b}	55.3 ± 1.9^{a}	55.3 ± 1.9^{a}

GEE = ginger ethanolic extracts; GHE = ginger hydroethanolic extract. Mean ± std. deviation. Columns with different letters are significantly different by Tukey test ($p<0.05$) applied after ANOVA (^{ns} = non significantly ** = $p < 0.001$).

4.2.2 Minimum Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

The results of MIC and MFC for ginger samples are displayed in Table 3. Generally, GEO had inferior values of MIC and MFC, compared to ginger extracts (GEs) indicating that the oils are effective at lowers concentrations.

Among the tested fungi, *Penicillium digitatum* was found to be more sensible than *Penicillium expansum, Altenaria alternata* and *Fusarium solani* (lower MIC for GEs and GEO) (Table 3).

The assays with GEOs were more efficient in inhibiting *Penicillium digitatum* visible growth, with capacity to inhibit growth at low concentrations as 0.4 to 0.8 % (v/v). On the other hand, MICs measured for GEEs and GHEs (2.5 to 3.0%) are higher. *Penicillium expansum* showed to be more resistant than *Penicillium digitatum*, requiring greater concentrations (higher MIC) for a visual identification of growth inhibiting (Table 3). The same response was recorded to *Fusarium solani* and *Alternaria alternata* in relation to the measured MICs, i.e., high MIC for GE and low for GEO.

Alcoholic extracts, GEEs and GHE, did not exhibit fungicidal activity against *Penicillium digitatum, Penicillium expansum, Fusarium solani* and *Alternaria alternata.* However, the GEOs were also able to confer antifungal activity against *Penicillium digitatum* and *Alternaria alternata* at concentrations ranging from 0.8 to 1.6%.

GEO extracted from fresh and freeze-dried ginger, were more fungicidal than the oven-dried extract and commercial ginger oil. GEO was quite effective in controlling *Penicillium digitatum,* and *Alternaria alternata*. At the highest concentration tested (1.6%), no fungicidal activity was observed against *Penicillium expansum* and *Fusarium solani.*

Despite GEOs did not confirmed an MFC at 1.6 % against *Penicillium expansum and Fusarium solani* standard of sporulation and the fungal growth were reduced at this concentration. *Penicillium expansum* was less sensitive when compared to *Penicillium digitattum*, with observable fungal inhibition response only when interacting to freeze-dried GEO.

Concerning *Fusarium solani*, the GEO-freeze-dried and GEO-fresh samples showed better performance than the GEOs obtained from air oven-dried and the commercial ginger oil. GHEs presented a better antifungal activity than GEEs. For *Alternaria alternata,* the GEO from fresh ginger was most effective (the lower MIC, 1.6%).

Against the fungi evaluated (*Penicillium digitatum, Penicillium expansum, Fusarium solani and Alternaria alternata*), GEEs and GHEs a complete inhibition was not observed, (did not resulted in MFC), even at the highest used concentration (6%).

Ficker et al. (2003), in evaluating the minimum lethal concentration of purified ginger ethanolic extract antifungal compounds (6-gingeral, 8-gingerol, 10-gingerol and gingerdiol), found values ranging from 0.1% to 0.0075% and a MIC inferior to 0.1% in assaying several filamentous fungi (*Alternaria, Aspergillus, Rhizopus and Fusarium* genus). The levels of minimum concentrations in our study were even expected to be higher than those presented in the literature since our samples were diluted at the proportion of 2:1 (v/v). Additionally, several factors could explain the differences between the results, such as genetic variety and climatic factors. Dabague et al. (2011) reported in a study, about ginger oil composition, that the levels of several compounds are influenced by drying time/temperature. The authors demonstrated that geranial and neral present in the ginger essential oil increased with rhizome ginger drying time; on the other hand, geraniol and geranyl acetate content decreased. Consequently, the levels of fungi inhibition in our work could be explained by the ginger extraction processes (dry or fresh) and the solvent used (polar or non-polar), which according to the literature, are able to extract varied quantity and components.

Table 3. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *Penicillium digitatum (PD), Penicillium expansum* (PE), *Fusarium solani (FS)* and *Alternaria alternata* (AA) for GEs and GEOs in broth.

Class	Samples	MIC (% v/v)				MFC (% v/v)			
		PD	PE	FS	AA	PD	PE	FS	AA
Ginger Extracts (GE)	GEE-fresh ginger	2.0 < MIC ≤ **2.5**[1]	5.0 < MIC ≤ **6.0**	5.0 < MIC ≤ **6.0**	5.0 < MIC ≤ **4.0**	**	**	**	**
	GEE-freeze-dried ginger	2.0 < MIC ≤ **2.5**	1.5 < MIC ≤ **2.0**	4.0 < MIC ≤ **5.0**	3.0 < MIC ≤ **4.0**	**	**	**	**
	GEE-air oven ginger	2.0 < MIC ≤ **2.5**	1.5 < MIC ≤ **2.0**	4.0 < MIC ≤ **5.0**	2.5 < MIC ≤ **3.0**	**	**	**	**
	Ethanol 96% - control	2.5 < MIC ≤ **3.0**	4.0 < MIC ≤ **5.0**	*	3.0 < MIC ≤ **4.0**	**	**	**	**
	GHE-fresh ginger	2.5 < MIC ≤ **3.0**	*	3.0 < MIC ≤ **4.0**	*	**	**	**	**
	GHE-freeze- dried ginger	2.5 < MIC ≤ **3.0**	4.0 < MIC ≤ **5.0**	3.0 < MIC ≤ **4.0**	2.5 < MIC ≤ **3.0**	**	**	**	**
	GHE-air oven ginger	2.0 < MIC ≤ **2.5**	*	3.0 < MIC ≤ **4.0**	2.5 < MIC ≤ **3.0**	**	**	**	**
	Ethanol 70%- control	3.0 < MIC ≤ **4.0**	*	*	3.0 < MIC ≤ **4.0**	**	**	**	**
Ginger Essential Oils (GEO)	GEO-fresh ginger	0.2 < MIC ≤ **0.4**	*	0.8 < MIC ≤ **1.6**	0.4 < MIC ≤ **0.8**	**0.8**	**	**	**1.6**
	GEO-freeze- dried ginger	0.2 < MIC ≤ **0.4**	0.8 < MIC ≤ **1.6**	0.4 < MIC ≤ **0.8**	0.8 < MIC ≤ **1.6**	**0.8**	**	**	**1.6**
	GEO-air oven ginger	0.2 < MIC ≤ **0.4**	*	*	*	**0.8**	**	**	**
	GEO-commercial	0.4 < MIC ≤ **0.8**	*	*	0.8 < MIC ≤ **1.6**	**	**	**	**1.6**

GEE = ginger ethanolic extracts; GHE = ginger hydroethanolic extract. n = 3 for each sample tested. Ginger extracts concentrations tested: 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0 % (v/v) and ginger oil: 0.0015, 0.0031, 0.0062, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 % (v/v). * no inhibition of visible microorganism growth was observed at the highest concentration tested; ** = total inhibition not observed at the highest concentration tested. [1] Lowest concentration of GEE, GHE or GO that inhibited microorganism visible growth in broth medium.

4.2.3 Poisoned food technique

The percentage of mycelial zone inhibition of the fungi *Penicillium digitatum*, *Penicillium expansum*, *Fusarium solani* and *Alternaria alternata* in solid medium containing different concentrations (1 and 3%) of GEs or GEOs from ginger rhizome are shown in table 4.

The GEO performed better than GE, by exhibiting a higher reduction in the tested fungi.

GHEs and GEEs incorporated in solid medium did not reduce significantly mycelial growth of *P. digitatum* at a concentration of 1% and 3%. At 3% was expected that GEs could have some action against mycelial growth, considering that this extract presented a reduction on spore germination and a MIC at 3%. However, it is important to mention that in both techniques (spore germination and MIC) PD broth – liquid medium - were used, which allow a better contact between ginger components and the fungi spores/hyphae. On the other hand, poisoned food technique solid medium diffusion was used to evaluate the percentage of mycelial zone inhibition. Previous research has showed that the solid medium method has a limitation for fast-growing microorganisms, aerobic or facultative aerobic (Ostrosky et al. 2008). Many authors have pointed to the uneven lipophilic components distribution in EO composition results in an unequal concentration of EO in agar, causing regions with a distinct antimicrobial activity, which can lead to misinterpretations (Da Silva et al., 2012; Suhr, Nielsen, 2003; Lambert et al., 2001).

The tween 80 was used to disperse the sample in the medium, however it did not provide any improvement of GEs in reducing compared to control samples. Although, it is known that in the diffusion method, the concentration and origin of the medium, the pH, the oxygen viability and the amount of inoculum and concentration, and incubation conditions, might influence results (Ostrosky et al., 2008).

When applying GEOs, significant inhibition of mycelial zone was observed for both tested concentrations (1 and 3%) against *P. digitatum*.

GEO-freeze-dried and GEO-oven-dried samples showed the best activity. The commercial GEO exhibited the lower inhibition for both concentrations tested and was the less effective among the oils, however reducing of the mycelial zone was significant different compared to that measured in the control (Table 4).

P. expansum showed more sensibility than *P. digitatum* to GEO, achieving higher percentage of inhibition. In general, the GEO was more effective in reducing mycelial zone than GEs against *P. expansum*. By comparing GHEs and GEEs results, the last group has a better action against *P. expasum* growth, after commercial GEO, which showed the highest mycelial zone inhibition.

The *Penicillium digitatum* exhibited higher susceptibility to the alcoholic extracts than the *Penicillium expansum* in broth liquid medium. However, when in solid medium - *P. expansum* had a higher percentage of mycelial zone inhibiting than *P. digitatum* for GEs and GEOs.

For the strain *F. solani*, GEO-commercial displayed the greatest inhibition, at both concentrations tested, 23 and 41% of inhibition. respectively. The others extract samples at 1% did not reduce significantly fungus growth compared to control. The most significant difference with respect to control plates was observed to the 3% GEE-freeze-dried and air oven ginger samples.

GEO-commercial was the most efficient against *A. alternata* mycelial growth, resulting in the best inhibiting among tested fungi (inhibition of 62 and 82% at 1 and 3% of GEO, respectively). At 3% all the samples, GEs and GEO, were able to reduced significantly mycelial growth of *A. alternata* compared to control.

Summarizing, on solid medium, the GEO stands out as the most effective agent against the 4 fungi tested. *P. expasum* and *A. altenata* were more sensitively to ginger compounds. Ginger extracts against *P. digitatum* and *F. solani* resulting generally in lower percentage of inhibition than *P. expansum* and *A. alternata.*

Investigation of antifungal activity of ginger essential oil (from fresh ginger) and oleoresin (from dried ginger) using the food poison assay, against filamentous fungi (*Curvulariapallescens, Aspergillus terreus and Aspergillus nige*r) showed that essential oil had a higher effective inhibitory action than oleoresin (Singh et al., 2005), what is in agreement with our results. Sasidharan and Menon (2010), indicated a 4 mm of zone inhibition attained by ginger oil (from dried ginger) and an absence of growth for fresh ginger oil against *Penicillium spp,* in tests by disc diffusion with paper discs impregnated with 10µL of each sample. In opposition to these results, we found that dried essential oil was more potent fungi inhibitor than fresh oil (test on *P. digitatum*).

In relation to the literature, many variations of concentrations and percentage inhibition zones for ginger oil can be found. Sridhar et al., (2003) reported a 9.31% of zone inhibition to *Penicillium sp* at 0.1% of ginger essential oil, while Seasou et al., (2012) registered a complete inhibition for *Penicillium griseofulvum* and 79.6% for *Penicillium citrinum* growth at a concentration of 1000 mg/L, which corresponded to 0.1% dose.

Radu et al. 2017 reported doses of GEO around 0.3% as efficient for several fungus strains (*Aspergilius, Fusarium graminearum, F. oxysporum, Botritys allii*), though higher doses were necessary for *A. terreus* (1.2%) fungal inhibition in solid medium.

The extent of the inhibitory effects of the ginger essential oil can be attributed to the presence of an aromatic nucleus containing polar groups (Singh et al., 2005). Different concentrations of compounds, found in dry ginger compared to fresh ginger such as: zingiberene (28.6 and 30.3%, respectively), curcume (5.6 and 11%), β-bisabolene (5.8 and 7.2%) and β-sesquiphellandrene (2.5 and 6.6%). However, some compounds in inferior amount in dried ginger compared to fresh, as geranial (8.5 and 4.4%, respectively), oxygenated compounds (29.2% versus 14.4%, respectively), as measured by Sasidharan and Menon (2010), also interferes on the levels of mycelial inhibition.

Agarwal et al. (2001) in a study testing ginger essential oil, oleoresin and isolated compounds, reported the oleoresin was more polar than the essential oil and the isolated compounds, although, ginger oleoresin was less active than other samples, what is also in agreement with our study.

It is important to highlight that ginger samples, even when not showing significant differences in the reduction of mycelial growth when tested in solid medium, were able to change sporulation and cause morphological damage to fungal hyphae.

Such effects have been observed by Bordoh et al. (2020) in essaying ginger extract against *Colletotrichum gloeosporioides* (Penz.). The ginger extract was able to distorted, shrunken and swollen hyphae.

Table 4. Percentage mycelial zone inhibition of the fungi *Penicillium digitatum (PD)*, *Penicillium expansum* (PE), *Fusarium solani* (FS) and *Alternaria alternata* (AA) in solid medium containing different concentrations (1 and 3%) of GEs or GEOs from ginger rhizome.

Class	Samples	PD		PE		FS		AA	
		** **1% v/v**	** **3% v/v**	** **1% v/v**	** **3% v/v**	* **1% v/v**	** **3% v/v**	** **1% v/v**	** **3% v/v**
Ginger Extracts (GE)	GEE-fresh ginger	3.8 ± 1.3^{d}	6.7± 3.2^{cde}	24.4 ± 1.1^{bc}	36.2 ± 1.8^{b}	0.2 ± 12.1^{b}	11.2 ± 6.7^{bcd}	9.5 ± 0.7^{bc}	**22.2 ± 16.2^{d}**
	GEE-freeze-dried ginger	3.9 ± 2.2^{d}	6.5 ±1.9^{cde}	**31.3 ± 2.4^{b}**	**60.8 ± 1.7^{a}**	**12.2 ± 4.8^{ab}**	**20.0 ± 5.0^{b}**	**21.2 ± 6.2^{b}**	**49.4 ± 3.9^{b}**
	GEE-oven-dried ginger	4.4 ± 4.2^{d}	10.7 ± 2.6^{cd}	25.5 ± 0.6^{bc}	35.6 ± 4.4^{b}	0.5 ± 5.3^{b}	**17.1 ± 4.7^{bc}**	**20.1 ± 16.1^{b}**	**43.2 ± 3.7^{bc}**
	GHE-fresh ginger	1.5 ± 3.1^{d}	3.1 ± 1.8^{de}	12.9 ± 0.3^{d}	22.5 ± 1.3^{c}	6.3 ± 3.2^{b}	12.6 ± 13.7^{bcd}	10.4 ± 2.0^{bc}	**29.7 ± 6.5^{cd}**
	GHE-freeze- dried ginger	0.3 ± 2.5^{d}	3.5 ± 5.6^{de}	21.7 ± 3.8^{c}	37.7 ± 1.1^{b}	**12.3 ± 12.8^{ab}**	12.0 ± 8.5^{bcd}	**13.9 ± 5.9^{b}**	**40.0 ± 10.2^{bc}**
	GHE-oven-dried ginger	0.2 ± 0.4^{d}	0.4 ± 0.1^{e}	24.0 ± 1.4^{bc}	33.0 ± 3.2^{b}	1.1 ± 5.4^{b}	6.0 ± 5.4^{cd}	12.1 ± 5.3^{bc}	**24.3 ± 3.4^{d}**
Ginger Essential Oils (GEO)	GEO-fresh ginger	**20.2 ± 3.9^{b}**	**20.7 ± 3.1^{b}**	NT	NT	NT	NT	NT	NT
	GEO-freeze- dried ginger	**31.2 ± 2.2^{a}**	**34.6 ± 2.0^{a}**	NT	NT	NT	NT	NT	NT
	GEO-oven-dried ginger	**18.8 ± 3.8^{b}**	**29.5 ± 2.2^{a}**	NT	NT	NT	NT	NT	NT
	GEO-commercial	**11.8 ± 8.5^{c}**	**12.5 ± 1.3^{bc}**	**43.7 ± 11.6^{a}**	**62.2 ± 7.9^{a}**	**23.0 ±2 .0^{a}**	**41.4 ± 1.8^{a}**	**62.7 ± 6.0^{a}**	**82.0 ± 3.0^{a}**
	Negative control	0^{d}	0^{e}	0^{e}	0^{d}	0^{b}	0^{d}	0^{c}	0^{e}

GEE = ginger ethanolic extracts; GHE = ginger hydroethanolic extract. Data expressed in % of mycelial zone inhibition of fungi. Columns with different letters are significantly different by Duncan test ($p<0.05$) applied after ANOVA (** = $p < 0.001$; * = $p < 0.01$). NT= not tested

4.2.4 Inverted Petri dish test

Additionally, mycelial zone inhibitions (MZI) were also tested for *Penicillium digitatum* against ginger oil volatile vapor using the inverted Petri dishes method. The results are summarized in Table 5.

Low doses of crude ginger oil (5µL and 10 µL) were ineffective to reduce mycelial growth. When the concentration was increased to 30 µL of and placed on filter paper discs, the fungal inhibition is observed.

GEO from freeze-dried rhizomes was the most effective oil against *P. digitatum* growth while commercial ginger showed the inferior activity, 52.6 and 11.6% MZI, respectively. Fresh and oven-dried ginger oil have intermediate activities (34.1 and 36.1, respectively). According to Sharma and Srivastava (2013), in similar experiment (volatile activity assay- inverted Petri plate), a complete control of *Penicillium expansum* growth was attained at a concentration of 0.5%, while our result shows that 30µL was necessary to attain 52.6% of growth inhibition against *Penicillium digitatum.* The strain severity and/or oil quality might be a factor for this discrepancy.

In the GEs testing, the percentage mycelial zone inhibition was very low (2 to 0% - data not showed), similar to results reported by Singh et al. (2005), in testing ginger essential oil and oleoresins vapor against filamentous fungus. The authors pointed out that oleoresin could be less or ineffective, s it has no vapor action, compared with essential oil.

Table 5. Percentage of mycelial zone inhibition of the fungus *Penicillium digitatum* measured in the solid medium, by indirect contact method (inverted Petri dishes) of ginger essential oils.

Class	Samples	Doses on filter paper discs		
		5 µL	10 µL	*30 µL
Ginger Essential Oils (GEO)	GEO-fresh ginger	8.8 ± 3.2[a]	9.4 ± 3.1[a]	34.1± 5.7[b]
	GEO-freeze-dried ginger	7.9 ± 2.8[a]	7.3 ± 2.2[a]	52.6±1.6[a]
	GEO-oven-dried ginger	0.0 ± 3.4[a]	1.3 ± 7.2[a]	36.1± 8.5[b]
	GEO-commercial	0.0 ±3.6[a]	5.3 ± 1.1[a]	11.6± 1.7[c]
	Negative control	0[a]	0[a]	0[d]

Data expressed in % MZI. Columns with different letters are significantly different by Duncan test ($p<0.05$) applied after ANOVA (* = $p < 0.001$)

4.3 Natural diseases and fungal inoculation on citrus under coating protection

4.3.1 Coating formulations in inoculated Petri dishes

Figure 1 shows mycelial zone growth of *P. digitatum* inoculated on PDA Petri dishes and coated with different coatings.

For this *in vitro* assay, the nanoemulsion coating improved the antifungal activity of the GEO. Nano-emulsion formulation in association with GEO and commercial GEO, showed comparatively reduced mycelial growth in 68.3 and 67.0% for each oil, respectively. Neat carnauba nanoemulsion also presented some activity, with 29.2 % of inhibition. Commercial GEO (C GEO) had similar activity to extracted GEO in this assay (Figure 1), inhibiting 20.4 and 19% the growth, respectively.

Carnauba wax is basically an inert coating, however, the experimental tests of carnauba wax in nano-sized emulsions provides protection against fungal colonization. The processing of nanoemulsion using ammonium hydroxide at 30% as solvent, could be explanation for some effect against mycelial growth, as confirmed in Petri dishes coated with neat nanoemulsion compared to the control (with sterile water).

Another possible explanation for the antifungal activity of carnauba wax lies in its hydrophobic nature. This wax creates a water repellent surface which makes difficult to microorganism attach and the fungal hyphae penetrate into the fruit. This reduces the supply of nutrients, thereby affecting the survival of the fungus. Additionally, Cruz et al. (2002) suggested that defense active proteins present in carnauba wax act against the fungal structure, such as chitinase and a β-1,3-glucanase, as confirmed by *in vitro* analyzes of the inhibiting of *Colletotrichum lindemuthianum*, *Colletotrichum musae*, and *Fusarium oxysporum* growth in their early stage. Gonçalves et al. (2010) also observed antifungal activity of carnauba wax, under *in vitro* and *in vivo* conditions against the development of *Monilinia fructicola* and *Rhizopus stolonifera* infection on coated nectarines and plums.

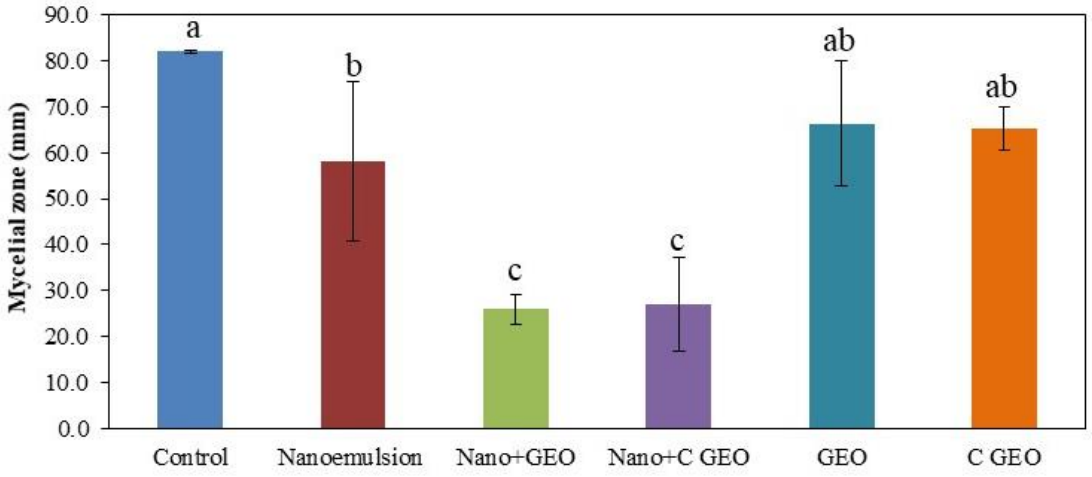

Figure 1. Mycelial zone growth (mm) of *Penicillium digitatum* inoculated on PDA Petri dishes and coated with different formulations, incubated at 25 °C for 7 days. Columns with different letters are significantly different by Games Howell ($p<0.05$) applied after ANOVA. Nano: nanoemulsion; GEO: extracted ginger oil; C GEO: commercial ginger oil.

4.5.1 Natural diseases and *P. digitatum* inoculated fruit disease incidence

Diseases incidence naturally increases during storage time. When assessed directly on the treated fruits, significant differences among treatments are observed after 31 days of storage. As expected, the uncoated samples underwent higher levels of diseases incidence when compared to nanoemulsion and GEO treated fruits (Figure 2).

The same trend was observed for *in vitro* inoculation in Petri dishes, where the nanoemulsion formulations, with/without GEO incorporation, showed inferior diseases incidence (73 to 77%). When coated with neat GEOs, despite a little reduction in the average values, these were not statistical different from the uncoated control. (Figure 2).

For tangerines with *P. digitatum* inoculated, there was a slight trend in a reduction over the fungal infection, when nanoemulsions (with or without GEO) were used as coatings (Figure 3).

In the present study, it was observed that 0.8% is the Minimal Fungicidal Concentration (MFC) for GEO, in the *in vitro* assay. However, this concentration was not enough to decrease diseases incidence when tested *in vivo* on tangerines, requiring higher concentration for an *in vivo* effective action.

Noori et al. (2018) have reported a significant decrease of total aerobic psychrophilic bacteria, moulds and yeasts on food matrix when using GEO at 6% as coating. A higher concentration of GEO demanded to present any significant antifungal activity against inoculated *P. digitatum* on tangerines could be attributed to the complexity of the food matrix and other factors as the availability of the compounds to interact with the fungi hyphae. As pointed out by Sanchez-Gonzalez et al. (2011), the incorporation of essential oil into a polymeric matrix can reduce the release and diffusion of the agent. In this sense, the combination of proper coatings, that does not hinder the migrations of active oils, might be useful in creating the right conditions for a joint action in prolonging the postharvest life of fruits and vegetables.

Concerning concentration on *in vivo* tests, Bordoh et al. (2020) evaluated antimicrobial effect of rhizome of ginger, turmeric rhizome and “dukumg anak” against *C. gloesoporioides* (Penz.) *in vitro* and on dragon fruit - *in vivo*. For the *in vivo* analyzes, the authors highlighted that all crude extracts at a concentration of 15.0 gL^{-1} boost disease incidence and severity due to their phytotoxicity. For ginger extract concentrations below 5.0 gL^{-1} were sufficient to inhibit the fungal growth and above that, the diseases are intensified. The other two extract at 5.0 or 10.0 gL^{-1} have a good

performance in reducing anthracnose and its severity on dragon fruits, absent of phytotoxic effect. Based on these data, it is significant to note that there is a limit to used extract dose and exceeding it the contrary effect from the desired one may occur.

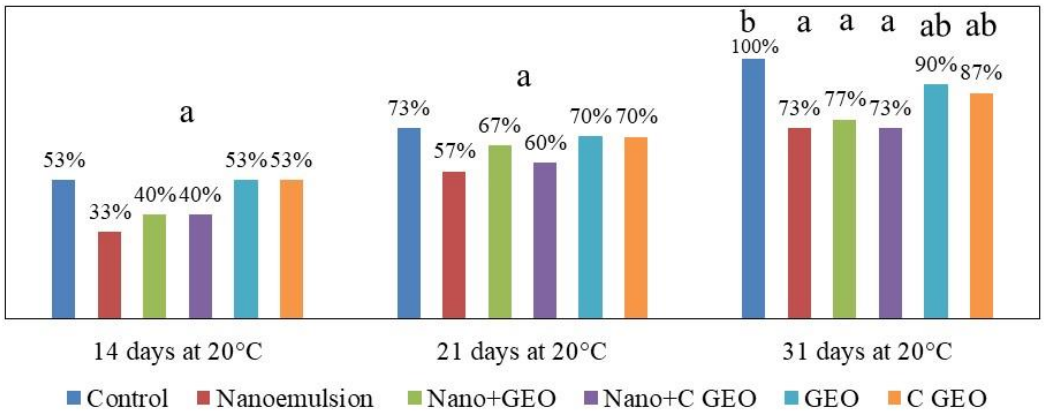

Figure 2. Natural diseases incidence on coated fruits with different formulations and storage at 20 °C for 31 days. Columns with different letters are significantly different at p<0.05. Nano: nanoemulsion; GEO: extracted ginger oil; C GEO: commercial ginger oil.

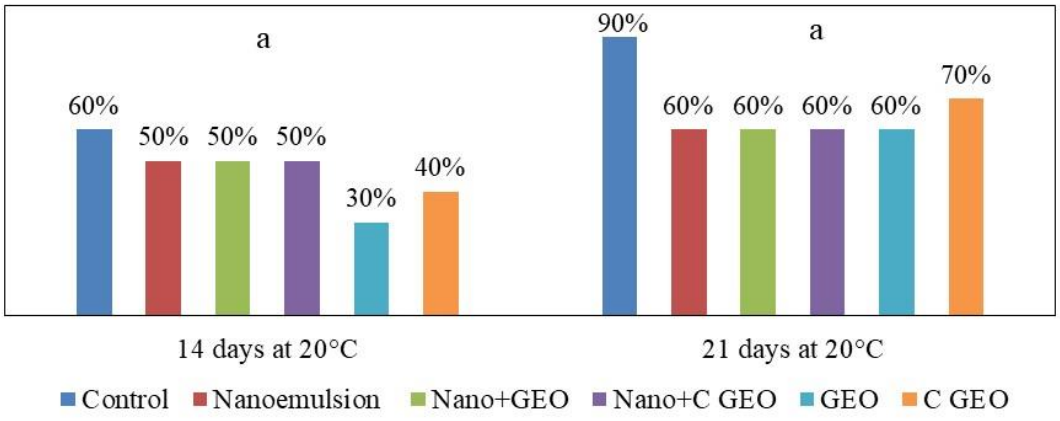

Figure 3. Disease incidence of *P. digitatum* inoculated fruit coated with different formulations and storage for 21 days at 20 °C. No differences between treatments at p<0.05, where observed along storage period. Nano: nanoemulsion; GEO: extracted ginger oil; C GEO: commercial ginger oil.

5. Conclusions

The general conclusions of this study are that the GEOs present a better effectiveness than GEs in reducing spores germination, when analyzed in *in vitro* conditions.

The GEOs reduced significantly the *P. digitatum* spores germination growth, among the samples and fungi tested. The *P. expansum* and *A. alternata* strains showed to be less sensitively to ginger derivatives.

No fungicidal activity was observed to GEO against *F. solani* and *P. expansum,* even at the highest concentration tested (1.6%). The MFC measured for *P. digitatum* and *F. solani* were between 0.8 to 1.6%. The extracts GEE and GHEs, also have no fungicidal activity against the tested fungi.

The *P. digitatum,* is the most sensible to ginger compounds (with lower MIC), followed by *A. alternata, F. solani* and *P. expansum,* in this order.

The MIC related to the addition of GEO at a concentration of 0.8%, in the carnauba nanoemulsion wax coating, as determined *in vitro* for *P. digitatum* added in, was not enough to reduce significantly the diseases on ‘Unique’ tangerines along 31 days of storage at 20 °C.

The combination of nanoemulsion containing GEO was more effective against *P. digita*tum *in vitro* assay (coated Petri dishes) than the neat nanoemulsion or the GEO alone. On the fruits the neat nanoemulsion coatings showed to be sufficient for a satisfactory fungal protection.

Despite of ginger oil showed a higher antifungal activity on *in vitro* condition, it is a more indicated to be incorporated into an edible coating, for the control against phytopathogenic postharvest diseases, since in general, the oil antifungal activity was greater than the GEs.

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Capítulo 2.

Antifungal activity of *Zingiber officinale Roscoe* (ginger) oil and extracts, associated with carnauba wax nanoemulsions, on fungal control of harvest papaya.

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Antifungal activity of *Zingiber officinale Roscoe* (ginger) oil and extracts, associated with carnauba wax nanoemulsions, on fungal control of harvest papaya

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Abstract

Essential oils and plant extracts can be safe alternatives for reducing post-harvest decay in foods compared to synthetic preservatives. Ginger oil (GO) and ethanolic extracts (GE) has been *in vitro* studied. Antifungal activity associated with fruit coatings on papaya has not been exhaustively investigated until now. In this study, the antifungal activity of GOs and GEs to control *Colletotrichum gloeosporioides* was investigated. *In vitro* results showed that GO has higher activity compared to GE, significantly reducing mycelial growth. The measured minimum inhibitory concentration (MIC) of GOs and GE were 0.1% to 0.8% (v/v) and 2.5% to 5%, respectively. Petri dishes inoculated with *C. gloeosporioides* were coated with carnauba wax nanoemulsion (CWN), GO nanoemulsion (at 3 and 6%), and their combination. Results showed that after 24h plates treated with 3% or 6% of GO, and CWN exhibited significant inhibition of the mycelial zone (MZI). The combination of CWN coating and GO was more effective than GO alone. CWN coatings resulted in significantly higher MZI alone or when associated with GO, compared to GO itself or control-water. After 7 days, plates treated with GO resulted in the same MZI as control, and CWN demonstrated inhibition against *C. gloeosporioides. In vivo* tests on 'Redland' papayas coated with CWN showed effective control upon fungal growth. The lowest values of fungal disease severity occurred when CWN was associated with GO (at 3 and 6% v/v). By comparing the CWN and GO alone activities, CWN resulted in higher decay inhibition. At a concentration of 6% GO, the diseases severity was superior than that found in uncoated fruits, suggesting GO phytotoxicity at that level.

Keywords: *Colletotrichum gloeosporioides,* papaya decay, ethanolic extracts, edible coatings, carnauba wax nanoemulsion

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INTRODUCTION

Considerable losses of fruits and vegetables are caused by plant pathogens, mainly by fungal-induced post-harvest spoilage and deterioration (Palou et al., 2016). Papaya (*Carica papaya* L.), a native fruit of tropical America, is particularly susceptible to postharvest decay as a consequence of fungal and bacterial infestation that reduce shelf-life, and consequently fruit marketability (Singh, 2010). To tackle this problem, essential oils and plant extracts were explored as an eco-friendly alternative for reducing microorganisms compared to commercial, chemical fungicides.

Potential control of phytopathogens using essential oils (EOs) has been intensified in the last years and showed the promising application in the post-harvest field (Palou et al. 2016, Singh, 2010). The development of low cost, low tech postharvest techniques can represent a significant advance for post-harvest product conservation.

Several essential oils have been tested against post-harvest diseases of papaya fruit, including ginger oil (*Zingiber officinale*), which has active phenolic compounds (Singh et al., 2008;) and the capacity for reducing common microorganisms in food (Sa-Guanpuag et al., 2011) with potential use associated with edible coatings (Atarés et al., 2010). Ginger extracts have potential to be used as a natural antimicrobial agent, replacing artificial preservatives and chemical fungicides. Additionally, combining essential oils (EO) and plant extracts with coatings can be a suitable alternative to overcome problems related to EO *in vivo* application, such as undesirable flavor and odor, phytotoxicity, and efficacy *in vivo*. By combining EO in a coating matrix, volatile release of antimicrobial volatiles could be controlled and phytotoxicity reduced (Oliveira, 2019).

The aim of the present study was to evaluate the antifungal activity of ginger oil (GO) and ginger extracts (GE) for control of fungal decay on papaya fruit, and also to explore the potential of their incorporation into a postharvest coating consisting of carnauba wax nanoemulsion for reduction of postharvest decay.

MATERIALS AND METHODS

Extraction of ginger extracts (GE) and essential oils (GO)

Ginger rhizome (*Zingiber officinale*), 'Gigante' was purchased from Ceasa-SP (Brazil). Samples were washed, rinsed, sanitized and cut in small pieces (0.5 cm). Then, the material was ground and divided into three parts: One portion was frozen; another submitted to oven air at 40 °C and the other freeze-dried until 3 to 9% final moisture. After drying, the material was packed in low-density polyethylene packages and stored in a dry environment at room temperature (~ 25 °C).

GE was obtained by mixing each plant (fresh or dried) with absolute ethanol (ethanolic extract -GEE) or 70% ethanol (hydroethanolic extract-GHE), according to Breda et al., 2016). Each extraction was carried out in triplicate and then combined.

GOs were obtained by hydro-distillation (steam distillation) using a Clevenger's type apparatus according to Natta et al. (2008) procedure with slight modifications. In short, fresh ginger (600 g), freeze-dried (50 g) and oven air dried ginger (50 g) were mixed with distilled water (1 L), and heated under soft boil over 24 h. The oil was collected and dried over anhydrous sodium sulfate. Extractions were conducted in triplicate and stored at 4 ± 2 °C in a dark container for further use. Commercial ginger oil (GOCom), food grade, CAS Number: 8007-08-7, was purchased from Sigma-Aldrich (China) and used as a reference.

In vitro antifungal activity evaluation

Microorganism maintenance and inoculum preparation

The plant extracts and essential oils were evaluated for activity against *Colletotrichum gloeosporioides* provided by Horticultural Research Laboratory (USHRL) in Ft. Pierce, FL, US-Agricultural Research Service (USDA-ARS). The microorganisms were grown in potato-dextrose-agar (PDA). After inoculation, the cultures were incubated at 25 °C until sporulation (between 7 to 10 days).

Petri dishes containing the sporulating fungal cultures were covered with 1 mL of a steril solution of Tween 20 at 0.5 % (v/v) and gently homogenized with a Drigalski spatel. An aliquot of spore suspension was analyzed in Neubauer chamber and adjusted with 0.85 % sodium chloride solution to approximately 0.4×10^6 CFU·mL^{-1} to determine MIC (Minimum Inhibitory Concentration), MFC (Minimum Fungal Concentration), and to inoculate Petri dishes with postharvest coatings.

Dilution of GEs and EOs for the direct contact method

Crude extracts were dissolved in 0.016 g×mL^{-1} ethanolic solution of polyvinylpyrrolidone at 2:1 (v/v), according to El-Arini and Leuenberger (1998). Essential oils were dissolved in dimethyl sulfoxide 10% at a proportion of 2:1 (v/v).

The direct contact method in broth

The MIC test was performed using a sterile test tube containing 500 µL of potato-dextrose (PD) broth with the sample, and 500 µL of a standardized inoculum of *C. gloeosporioides* was added to all test tubes, which were incubated at 25 °C for 7 days at 125 rpm. The observations were carried out after 24, 48, 72, 96 and 168 h. The concentration ranges for extracts were 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 and 6.0 % (v/v) and for essential oil were 1.6, 0.8, 0.4, 0.2, 0.1, 0.5, 0.025, 0.0125, 0.0062, 0.0031 and 0.0015% (v/v). The diluted sample was added to broth in an amount calculated to obtain the final desirable concentration. Cycloheximide and broth were used as positive and negative controls, respectively. The MIC was defined as the lowest concentration of extract or ginger essential oil that inhibited visible microorganism growth. In order to determine the MFC, an aliquot of 10 µL of each incubated test tube (7d) of MIC and the higher concentrations were sub-cultured on PDA Petri dishes and incubated at 25 °C for 5 days (Donlan and Costerton, 2002). The MFC was defined as the lowest concentration of extract that prevented visible growth. The results are expressed as the average of three repetitions.

Coatings preparations

Carnauba wax nanoemulsion (CWN) coating, was prepared with the oil phase (O) composed for carnauba wax type 1 (8 to 18% wt/v), and oleic acid (2.6 to 6% wt/v), from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The aqueous phase (W) was composed of ammonium hydroxide (1 to 3% wt/v), and dimethylpolysiloxane (0.02 to 0.1% v/v) (from Sigma-Aldrich) and deionized water (71 to 89% wt/v). The nanoemulsion was accomplished by the inversion phase of W/O to O/W system, using the morpholine-free method as proposed by Hagenmaier and Baker (1997) with modifications. The carnauba wax nanoemulsion with average diameter size of 44.1 ± 7.6 nm was generated, with a narrow polydispersion index (0.28) and zeta potential -43.8 mV, according to measurements previously carried out by Zetasizer Nano ZS (Malvern Instruments Inc., Westborough, MA, USA), (Miranda, 2015). Oil-in-water

(O/W) GO nanoemulsions (with 3 and 6% v/v) were prepared following Otoni et al. (2014). Commercial ginger oil was used considering the low yield attained by steam distillation extraction in laboratorial scale. Higher concentrations of GO (3 and 6%) were chosen due to preliminary *in vivo* experiments which showed interactions among the EO components and the fruit matrix, what reduces the antimicrobial EO efficacy. Carnauba wax nanoemulsion containing GO was prepared with continuous and gradually additions of Tween 80 at 0.6% (v/v) and GO 3 or 6% (v/v), into previous prepared CWN, followed by mixing using an Ultra-Turrax at 16,000 rpm for 4 min to obtain the final coating.

Inoculated Petri dishes coated with postharvest coatings

The amount of 10 µL of *C. gloeosporioides* spore suspension 10^6 CFU·mL^{-1} was placed on solid PDA. After 24 h incubation, aliquots of 1 mL of each coating formulation (filtered through sterile 0.2 µm pore size membrane) were cast on the inoculated Petri dishes and gently spread with Drigalski spatel and incubated at 25 °C for 7 days. A control was similarly prepared by replacing coating with sterile deionized water. Three Petri dishes per treatment were assayed. The coatings treatments were: CWN at 18%; CWN at 18% with commercial GO at 3% and 6%, and GO at 3% and 6%. Percentage of mycelial inhibition (%MZI) was estimated after 2 and 7 days and expressed as the mean value of three repetitions per treatment. Radial growth reduction was calculated in relation to fungal growth as measured in the control, as follows: (C-T/C) ×100= % MZI. Where C is the radial growth measurement in control and T is radial growth of the pathogen in the presence coating formulations (Sivakumar et al., 2002).

Coated papaya fruits natural decay

'Redland' papaya (*Carica papaya L.)* were harvested at the first maturity stage, with 10% yellow peel color, from a commercial farm in Homestead-FL. The fruit was selected, washed and sanitized by immersion in 200 mg L^{-1} peroxyacetic acid during 3 min, air-dried and stored at room temperature with 80% relative humidity (RH). When fruits achieved 40 % yellow peel color, they were coated with 2 mL of the different formulations. The coatings compositions were the same six that were applied to the Petri dishes. Coated fruit was air-dried at room temperature and stored for 6 days at 20 °C. Experiments were conducted in a completely randomized factorial design. The severity of natural diseases was determined based on the correlation between fruit surface and the percentage of affected area. For samples from each treatment during the storage period were assessed. Each fruit was scored using a 6-point category scale. The scores were: 1 (0% of affected area), 2 (1% to 20% of affected area), 3 (21% to 40%), 4 (41% to 60%), 5 (61% to 80%) and 6 - 81% to 100% of affection (Romanazzi et al. 2013).

Statistical analysis

Statistical analysis for percentage of mycelial zone inhibition for *C. gloeosporioides*- inoculated Petri dishes coated with postharvest coatings was performed using univariate parametric analysis of variance (ANOVA) and multiple comparisons Tukey test. The comparison between disease severity scores was performed using non-parametric ANOVA and Kruskal-Wallis multiple comparisons due to the ordinal level of the variable and independent samples assumed in each experiment. The significance level for all analyzes was 5% ($p < 0.05$). Statistical software R (Core Team, 2016) was used.

RESULTS AND DISCUSSION

Table 1 shows the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *C. gloeosporioides* after exposure by direct contact in broth medium for ginger extracts (GE) and ginger oils (GO).

Oven air and freeze-dried ginger extracts resulted in lower MIC compared to fresh ginger extracts, indicating that the drying process may influence the activity of the extracts. GEs from fresh ginger showed a MIC equal to the alcoholic controls (ethanol 96% and 70%), showing that the ethanolic portion might takes place in the antifungal activity of these extracts. Additionally, alcoholic controls showed MFC at 5 and 6% (v/v) concentrations, corroborating the participation of ethanol in the extract activity. GEs exhibited MFC against *C. gloeosporioides* between 4 and 6% (v/v), except GHE-fresh ginger which did not show any fungicidal activity at the highest concentration tested.

GOs performed better for MIC and MFC assays compared to GEs. GO from freeze-dried ginger was the most effective at low concentration in inhibiting growth and fungal activity, with 0.1 and 0.2 % (v/v) for MIC and MFC, respectively. Commercial ginger oil required a higher concentration (1.6%) when compared to extracted GOs. However, because the low yield of oil extraction processing, the use of commercial ginger oils for postharvest decay control was also adopted. Isolation of antifungal compounds from ginger could be another alternative to reduce concentration, for example [6], [8] and [10]-gingerols and [6]-gingerdiol metabolites. The antifungal mechanism of these compounds has been associated with the fungal cell wall and intracellular organelles, provoking morphological alterations in pathogen´s hyphae, according to a study presented by Oliveira et al. (2019).

Table 1. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) for *Colletotrichum gloeosporioides* after exposure by contact to different concentrations of ginger extracts and essential oils incorporated into the medium.

Class	**Samples**	***MIC (% v/v)***	***MFC (% v/v)***
Ginger Extracts (GE)	GEE-fresh ginger	3.0 < MIC ≤ **4.0**[1]	6.0
	GEE-freeze-dried ginger	2.0 < MIC ≤ **2.5**[1]	5.0
	GEE-air oven ginger	2.0 < MIC ≤ **2.5**[1]	5.0
	Ethanol 96% - control	3.0 < MIC ≤ **4.0**[1]	5.0
	GHE-fresh ginger	4.0 < MIC ≤ **5.0**[1]	> 6.0*
	GHE-freeze- dried ginger	2.5 < MIC ≤ **3.0**[1]	4.0
	GHE-air oven ginger	2.0 < MIC ≤ **2.5**[1]	4.0
	Ethanol 70%- control	4.0 < MIC ≤ **5.0**[1]	6.0
Ginger Essential Oils (GO)	GO-fresh ginger	0.2 < MIC ≤ **0.4**[1]	0.4
	GO-freeze- dried ginger	0.05 < MIC ≤ **0.1**[1]	0.2
	GO-air oven ginger	0.4 < MIC ≤ **0.8**[1]	1.6
	GO-commercial	0.8 < MIC ≤ **1.6**[1]	>1.6*

Note: GEE = ethanolic ginger extract, GHE = hydroethanolic extract, GO = ginger oil. n = 3 for each sample tested. Ginger extracts concentrations tested: 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0 % (v/v) and ginger oil: 0.0015, 0.0031, 0.0062, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 % (v/v). * = total inhibition not observed at the highest concentration tested. [1] Lowest concentration of GEE, GHE or GO that inhibited microorganism visible growth in broth medium.

Table 2 shows the percentage of mycelial zone inhibition (% MZI) of *C. gloeosporioides* after exposure by contact with the coatings in Petri dishes. After 2 days of inoculation, CWN coating showed a significantly higher percentage of fungal inhibition, alone or with addition of GO, as compared to GO itself and the control. Similar MZI (21.0 and 22.5 %) were attained by GO nanoemulsion for both evaluated concentrations: 3 and 6% respectively. However, after 7h no significant mycelial inhibition was observed in Petri dishes treated with GO nanoemulsion compared to control plates and to treatments containing CWN, which resulted in satisfactory inhibition of *C. gloeosporioides.*

Antimicrobial activity of essential oils (EOs) and extracts from spices and herbs are attributed to phenolic compounds and there are considerable differences in chemical structures and amount of phenolics present in GO and oleoresin extracts (Singh et al., 2008). The hypothesis for EO activity is based in the hydrophilic character of its components. Oil compounds can cross the lipid layers of fungal cell membranes causing detachment and disruption, impairing membrane integrity and structure. There is an imbalance of hydrogenic and potassium cation exchange, which may lead to cell death (Beckman, 2000). GO also can induce high levels of cytoplasmic vacuolation, vacuole fusion and detachment of the plasma membrane from the cell wall, making it thinner causing loss of integrity (Da Cruz et al., 2013).

After 7 days (Table 2), % MZI for Petri dishes treated with GO nanoemulsion coating at both concentrations were not significantly different compared to the untreated control. Coatings containing CWN resulted in the highest percentage of mycelial growth inhibition (Table 2). It is known that carnauba wax is an inert substance; however, for the experimental conditions adopted in this work, the carnauba wax nanoemulsion coatings have showed protection against fungal colonization and invasive infection. The potential antimicrobial activity of carnauba can be attributed mainly to its hydrophobic nature. Carnauba wax provides a water-repellent coating which difficulties microorganisms attachment and fruit penetration, reducing feed on living cells what have strong influence on pathogen survival. Additionally, other compounds found in wax composition have been suggested as having antifungal activity, as reported by Cruz et al. (2002), that showed that defense active proteins, such as chitinase and a β-1,3-glucanase, in carnauba wax presents *in vitro* action of inhibiting *Colletotrichum lindemuthianum*, *Colletotrichum musae*, and *Fusarium oxysporum* growth in their early stage. Gonçalves et al. (2010) also reported antifungal activity of carnauba wax, under *in vitro* and *in vivo* conditions (as coating on nectarines and plums), against the development of *Monilinia fructicola* and *Rhizopus stolonifera* infection.

Table 2. Percentage of mycelial zone inhibition (% MZI) of *Colletotrichum gloeosporioides* after contact with coated Petri-dishes with different coatings (mean values ± SD, n = 3).

Coatings	% MZI	
	After 2 days	After 7 days
Water - control	00.0 ± 0.0 [c]	00.0 ± 0.0 [b]
GO nanoemulsion at 3%	21.9 ± 5.5 [b]	0.2 ± 0.7 [b]
GO nanoemulsion at 6%	22.5 ± 2.6 [b]	2.5 ± 1.6 [b]
CWN coating	50.6 ± 3.0 [a]	45.0 ± 2.6 [a]
CWN containing GO at 3%	42.0 ± 7.6 [a]	44.4 ± 1.9 [a]
CWN containing GO at 6%	48.2 ± 8.0 [a]	47.8 ± 2.8 [a]

Note: CWN = carnauba wax nanoemulsion, GO = ginger oil nanoemulsion made from commercial oil. Means followed by a different letter in the same column are significantly different by Tukey test ($p < 0.05$).

Figure 1 shows the performance of GO, CWN coating and their combination when *in vivo* experiment with papaya. CWN coatings containing GO (3 or 6%) exhibited the smallest decay severity, however statistically not different from CWN neat formulation (without GO). The nanoemulsion coating containing 3% GO resulted similarly to the control, however, not different if compared to 6% GO, which have shown some peel phytotoxicity. By considering that, the results attained by CWN formulation was not different from that of 3% GO addition and of untreated control. Although it is not possible to confirm the joint effect of both compounds, a probable synergic action between coating and GO was reported by Ali. et al. (2016). In evaluating coating combinations of 2 % GO and 10 % gum Arabic (GA) applied on 'Eksotika-II' papaya fruits along 28 days storage at 12 ± 1 °C it was shown that the mixture of GO and GA provided to be most effective in reducing anthracnose development than the individual application of GO or GA.

It is worth noticing that carnauba wax nanoemulsion itself showed some antifungal control, despite being an inert coating. In addition to the previously commented features of carnauba active compounds, there is the possibility that some ammonia residues may remain in coating. An ammonium is used as emusificate during the preparation of wax nanoemulsions. When the coating is drying, is expected that both ammonium and water evaporate and a thin carnauba film is formed. However, some ammonia residues may remain, what can provide certain action towards reducing fungal severity, when compared to uncoated fruits. Nevertheless, Gonçalves et al. (2010), have pointed out three possible antifungal mechanisms acting, either conjunctly or separately, in neat carnauba wax coatings: i) the formation of a steady physical barrier that prevents pathogen passage through the film; ii) alteration of the fruit internal atmosphere and, iii) a presumed direct action of the wax against the pathogens.

The incorporation of essential oils can interfere in the water vapor diffusion of the coatings but allow the delivery of active compounds to fruit's surface, where contamination occurs (Sanchez-Gonzalez et al., 2011). For an effective inhibition of fungal growth, a high concentration of phenolic compounds could be required, particularly when the attack takes place in a microorganism favorable environment, such as the food matrix (Ali et al., 2016). However, the use of high concentrations of GO should be carefully considered due its phytotoxicity, as here observed when coating fruits with a 6% GO nanoemulsion. Normally for *in vivo* analyzes of the

antifungal activity, the concentration of active oil required is higher than the amount tested in *in vitro* conditions. This is because several interactions occur between oil phenolic compounds and the food matrix, decreasing overall activity (Feng and Zheng, 2007). These complex interactions may justify why less inhibition of *C. gloeosporioides* fungus is observed on the surface of the fruits. (Alia et al., 2016).

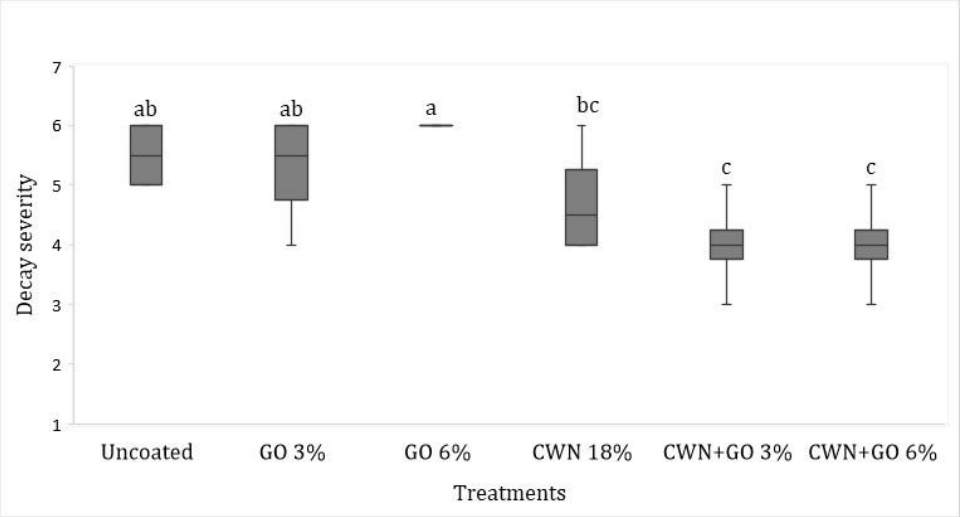

Figure 1. A) Median of the score for natural decay severity of 'Redland' papaya fruit coated with different coatings. Fruits were storage for 6 days at 80% RH. CWN = carnauba wax nanoemulsion and GO = ginger oil nanoemulsion made up with commercial oil. Bars with different letter are significantly different by Kruskal-Wallis test ($p < 0.05$).

CONCLUSIONS

GOs and GEs showed activity against *C. gloeosporioides,* and GOs required lower concentrations compared to alcoholic GE. Extracts from fresh material displayed less activity than oven air and freeze-dried ginger roots. Petri dishes inoculated with CWN coatings showed mycelial inhibition (after 48 hours and 7 days), in neat condition or associated with commercial GO. On the other hand, GO at 3, or 6% reduced fungal growth after 48 hours compared to uncoated plates. However, after 7 days, only GO was not efficient to further reduction of fungus spreading. The *in vivo* assays pointed to the complexity of the processes involving the volatile compound release, interactions with fruit matrix, and action on fungal development, when EOs are incorporated. To control disease severity in papayas, CWN containing GO showed the highest activity, with superior performance than GO alone, followed by the CWN neat coating. GO at 6% exhibited some peel phytotoxicity and consequent elevated fungal severity (probably due to peel injuries resulting from the GO toxicity). In summary, CWN coating alone or associated with EOs might be a potential alternative to chemical fungicides as a natural and safe product to reduce papaya fungal decay.

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Capítulo 3.

Effect of shellac and carnauba wax micro- and nanoemulsion coatings on post-harvest quality of citrus.

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Effect of Shellac and Carnauba Wax Micro- and Nanoemulsion Coatings on Post-harvest Quality of Citrus

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Abstract. Coatings are generally applied to fruits as microemulsions, whereas nanoemulsions are still experimental. 'Nova' mandarins were coated with shellac and carnauba microemulsions, and an experimental carnauba nanoemulsion for comparison with uncoated control during storage for 7 d at 20 °C. Coatings were also tested on 'Unique' tangors stored for 14 d at 10 °C followed by a simulated marketing period of seven d at 20 ºC. Fruit quality evaluation included weight loss, gloss, soluble solids (SS), titratable acidity (TA), pH, SS/TA ratio, internal CO_2, and O_2 as well as fruit juice ethanol and other aroma volatile content. Sensory visual shine and tangerine flavor rank tests after storage were conducted followed by an off-flavor rating. The carnauba waxes resulted in less weight loss compared to the uncoated control and shellac coating in both experiments. There were no differences for gloss measurements for 'Nova' mandarins, however, shellac-coated fruit ranked highest for shine in a sensory test. For 'Unique' tangors, initially shellac showed the highest gloss (shine), but at the end of storage the nanoemulsion exhibited the highest gloss measurement, although not different from the microemulsion. Similarly, after storage the nanoemulsion ranked highest for shine, although not different from the microemulsion. There were only minor differences for SS, TA, pH and SS/TA among treatments. CO_2 and ethanol generally increased and O_2 decreased during storage, with the highest levels of CO_2 and ethanol found for shellac treatment along with the lowest O_2, indicating anaerobic respiration. There were only minor differences among the other coating treatments, although sometimes different from control, which generally had the highest O_2, lowest CO_2 and lowest ethanol. Shellac and the carnauba microemulsion also altered the volatile profile more than did control or the nanoemulsion, especially for 'Unique' tangors. For 'Unique' tangors, the control and the nanoemulsion ranked highest for tangerine flavor and had the least off-flavor at the end of storage. Among the coatings tested, the carnauba emulsions demonstrated less water loss, imparted more sustainable shine and caused less ethanol production than did shellac with the nanoemulsion exhibiting more shine, less modification of the atmosphere and volatile profile and consequently better flavor compared to the microemulsion.

Keywords*:* Aroma volatiles, ethanol, fermentation, fruit quality, mandarin, modified atmosphere, tangor.

Introduction

Tangerine/mandarin, clementine and satsuma world production in 2018-19 was 32.0 million tons fresh fruit (USDA-FAS, 2020). China is the largest producer of mandarins (22.0 million tons) and is also the second largest producer of fresh oranges in the world at 7.2 million tons. The largest orange producer is Brazil with a yield of 19.4 million tons (FAOSTAT, 2020). The development and application of protective coatings is recognized as an alternative environmentally-friendly approach to reduce losses and improve post-harvest conservation (Nayak et al., 2019).

Coatings provide a barrier between fruit and the external environment, induce a modified atmosphere within fruit (decreased O_2 and increased CO_2), and reduce water vapor diffusion, influencing respiration and transpiration rates, respectively. Coatings also can influence the aroma volatile profile (Baldwin et al., 1995; El Hadi et al., 2013). For citrus, the main criteria for coatings are the ability to impart shine, to improve sales, and to retard water loss to reduce shrinkage, while maintaining fresh flavor (Hall, 2012). Resins and waxes are generally used (Bai and Plotto, 2012), as they are hydrophobic to varying degrees, and impart shine.

Certain coating formulations can cause anaerobic respiration due to modification of the fruit internal atmosphere, causing fruits to produce high levels of ethanol and acetaldehyde leading to off-flavor and trapping off-flavors within the fruit (Baldwin et al., 1999). In general, fruit produce a collection of volatile compounds that constitute their characteristic aroma, which is important for acceptability by consumers (El Hadi et al., 2013). Nisperos-Carriedo et al. (1991) reported that citrus, stored for 13 d at 21 °C, showed an increase in some aroma

volatiles, including alcohols, which were higher in commercial wax-coated oranges than in uncoated, thus altering the aroma profile. This highlights the need to tailor functional and permeability proprieties of coatings to suit the unique requirements of every fruit and vegetable, under specific storage regimes, to achieve the best quality.

Coating fruit surfaces serves to replace the natural layer of wax removed by cleaning and handling processes in packing houses. Coatings generally used for citrus include microemulsions containing resins, waxes or blends of shellac, candelilla wax, carnauba wax, beeswax, polyethylene, or petroleum waxes (Palou et al. 2015; Hall, 2012). Among commercial citrus coatings, shellac resin has been widely used alone or as a major component of "waxes" (Hagenmaier and Shaw, 1991), due to its ability to impart high gloss or shine. Shellac does help to maintain moisture and reduce shrinkage, but not to the extent of true waxes. Carnauba wax also has been largely used to form conventional microemulsion coatings, alone or mixed with other waxes and resins to optimize shine (De Freitas, et al 2019, Luangtana-Anan and Limmatvapirat, 2019; Palou et al. 2015; Bai and Plotto, 2012).

There are few studies on carnauba nanostructure-based coatings, as compared to polysaccharide-based nano-coatings such as nanochitosan (Pilon et al., 2014; González-Saucedo et al. 2019; Nguyen and Nguyen, 2020). On the other hand, Miranda (2015) demonstrated that an experimental carnauba wax nanoemulsion coating (without other resins and/or gloss additives) allowed gas exchange while reducing water loss. This coating exhibited small (nano-sized) lipid micelles, forming stable and shiny coatings. Nanoemulsions may impart more shine than microemulsions, as generally the smaller the lipid micelles, the greater the gloss or

shine and stability of the emulsion. These coatings, however, may also have different gas permeability properties than conventional microemulsions.

In this context, edible coatings can contribute to fruit quality and shelf life by improving visual quality and reducing postharvest losses due to desiccation, provided the formulation does not cause off-flavor due to modification of the internal fruit atmosphere. In this study, a carnauba nanoemulsion coating was evaluated and compared to typical shellac and carnauba microemulsion coatings on two citrus cultivars.

Materials and Methods

Fruit. Approximately 200 'Nova' mandarins (*Citrus reticulata*) and 300 'Unique' tangors (*C. reticulata x C. sinensis*) were used in two experiments. 'Unique' or 'Ortanique' is believed to be a spontaneous hybrid of a sweet orange and a mandarin, that was first found in Jamaica (Nugent et al., 1967). Fruit were obtained at Al´s Family Farms Citrus, Fort Pierce, Florida. The fruit were selected for uniformity and lack of defects, washed with JBT® 395 fruit cleaner (JBT® FoodTech, Lakeland, FL), rinsed and sanitized with 100 $mg \cdot L^{-1}$ peroxyacetic acid (PAA) (Jet-Oxide, Jet Harvest Solutions, Longwood, FL) for 3 min in a then air dried at room temperature.

Coating preparations. A conventional carnauba wax microemulsion was formulated according to Hagenmaier and Baker (1997), with a slight modification. Carnauba wax emulsion was prepared in an open reactor by heating 45 g of carnauba wax type I (Strahl & Pitsch, Inc., West Babylon, NY), 5 g oleic acid and 5 g myristic

acid, 28 g ammonium hydroxide 8% (all Sigma-Aldrich Chemical Co. St. Louis, MO) and deionized water (175 g), at 105 °C with constant mechanical stirring (800 rpm) for 3 min. Then, under mechanical stirring the emulsion was cooled to 50 °C. Carnauba wax nanoemulsion was prepared with an oil phase composed of carnauba wax type 1 (18% w/v), and oleic acid (6% w/v), from Sigma-Aldrich Chemical Co. (St. Louis, MO,). The water phase was composed of ammonium hydroxide (3% w/v), dimethylpolysiloxane (0.01% v/v, Sigma-Aldrich), and deionized water (89% w/v). Formulation of the nanoemulsion was accomplished by the inversion phase of W/O to O/W system. The diameter size of the lipid mycelles obtained was 44 ± 8 nm, with a narrow polydispersion index (0.28) and zeta potential of -43.8 mV (Miranda, 2015), measured by Zetasizer Nano ZS (Malvern Instruments Inc., Westborough, MA).

Fruit processing and coating. This research was carried out in two experiments. The first experiment tested coatings on 189 'Nova' mandarin fruits with the following treatments: lab-made, simulated commercial carnauba wax microemulsion, a commercial shellac microemulsion (Stay Fresh 590HS High Gloss, JBT, Lakeland, FL), an experimental carnauba nanoemulsion wax and uncoated fruits. The second experiment was conducted with the same treatments, but applied to 289 'Unique' tangor fruits. Fruit were coated by spreading one mL of coating per fruit by hand, using latex gloves. Coated fruits were then dried with a heat gun (50 °C) for 60 seconds prior to analyses and storage.

For the first experiment, 'Nova' mandarins were stored for 7 d at 20 °C and quality analyses were done initially (0 d) and at the end of storage (7 d). For the second experiment, 'Unique' fruits were stored at 10 °C for 14 d followed by a

simulated marketing period of 7 d at 20 °C, more typical for the industry. Quality analyses were performed initially (0 d), after 14 d cold storage (10 °C) and again after the simulated marketing period (7 d at 20 ºC).

***Fruit quality*.** Fruit were gently juiced so as not to allow peel oil into the juice (Baldwin et al., 2012). Soluble solids content (SS) of the juice was determined by refractive index with a digital refractometer (ATAGO PR-101, Tokyo, Japan). Titratable acidity (TA) and pH were calculated from titration of 10 mL of juice with 0.1 mol·L^{-1} NaOH to a pH 8.1 end point using an autotitrator (Mettler Toledo DL50, Columbus, OH) and expressed as percent of juice. The ratio (SS/TA) was the proportion between sugar and acid (Baldwin et al., 2012). For SS, TA, pH, SS/TA, fifteen fruits were juiced per treatment (five composite replicates of three fruits each).

From this same juice, ethanol and other volatile compounds were quantified by gas chromatography (GC) using a standard curve with authentic standards (Baldwin et al., 2012). Temperatures of oven, injector, and detector were 70, 250, and 250 °C, respectively. For quantification of ethanol and other aroma volatiles, 3 mL of fruit juice were transferred to a 10 mL crimp-capped vial, rapidly frozen in liquid nitrogen and then stored at −80 °C. Frozen samples were later thawed under running tap water and inserted into a Gerstel multipurpose auto sampler for head space injection (3 mL) onto a gas chromatograph (GC, Agilent 6890, Santa Clara, CA) equipped with a Stabilwax column (Restek Corporation, Bellefonte, PA), a HP-5 low bleed column (Agilent) and a flame ionization detector. The gas flow rate for He, H_2, and air were 10, 35, and 350 mL·min^{-1}, respectively. Temperatures of oven, injector, and detector were 90, 200, and 250 °C, respectively.

In addition to ethanol, common citrus volatiles were analyzed including the alcohols hexanol, *cis*-3-hexenol, *trans*-2-hexenol, methanol, 2-methylpropanol, α-terpineol and linalool; aldehydes acetaldehyde, decanal and hexanal; esters methybutanoate, ethylbutanoate, ethylacetate, ethylhexanoate and ethyl-3-hydroxyhexanoate; and terpenoids valencene, limonene, myrcene, α-pinene, sabinene and γ-terpinene. Volatile compound identification was confirmed using solid phase micro extraction (SPME) fibers with mass spectroscopy (MS). Confirmation by MS was accomplished by solid phase microextraction (SPME, 50/30 um DVB/CAR/PDMS, Supelco, Bellefonte, PA), as reported by Wang et al. (2015). The instrument and settings for SPME injection: GC-MS (model 6890 GC + 5973NMS; Agilent) with a non-polar column (0.25 mm × 60 m, 0.50 μ film thickness, DB-5, Agilent).

Internal gases, CO_2 and O_2, from ten fruit samples per treatment were evaluated by withdrawing a 10 mL internal fruit gas sample by syringe from the fruit columella while the fruit was submerged in water. These samples were analyzed by a GC (Hewlett Packard HP 5890A; Hewlett Packard, Avondale, PA) fitted with a CTR column (Cole-Parmer, Vernon Hills, IL) and a thermal conductivity detector. The gas flow rate for helium and air were 80 and 350 $mL \cdot min^{-1}$, respectively. Temperatures of oven, injector, and detector were 70, 250, and 250 °C, respectively. Weight loss of 10 fruit per treatment was measured during storage and results were expressed as a percentage of initial weight.

Gloss measurements. A reflectometer (micro-TRI-gloss; BYK-Gardner, Silver Spring, MD) was used to evaluate gloss (shine) of coating formulations on test paper and on the fruit. For test papers, an aliquot of 0.5 mL of each coating was spread on a 0.05 mm-thick polished paper (Leneta Co., Mahwah, NJ) with a 4-mil

castor (BYK-Gardner, Columbia, MD) with a speed of 1 cm s^{-1}. The control consisted of uncoated paper. Five papers per treatment were used and measurements were repeated five times per paper at different positions. The reflectance was measured at an angle of 20° for test papers (Bai et. al, 2003a) and 60° for fruit (Bai et. al, 2003b). For coated fruit, a case with a circular 19 mm diameter orifice was attached to the equipment to accommodate the round fruit shape. Ten fruits per treatment were used and measurements were made twice at opposite points of the equatorial region of the fruit.

Sensory visual shine analyses. Twenty-five and nineteen panelists visually evaluated fruit shine of coated 'Nova' and 'Unique' fruit, respectively. Panelists consisted of laboratory staff familiar with assessment of citrus products. For 'Nova', two groups of four fruits were presented, each group containing all four treatments, and panelists were asked to pair fruits by appearance (Tetrad test, Ennis, 2012). Then panelists were asked to rank the pairs for shine in decreasing order of gloss intensity (most shine to least shine). For 'Unique' tangors, panelists were also instructed to squeeze fruit between middle finger and thumb to rate firmness/hardness of the fruit on 10-point scale (0 soft to 10 hard). In both tests, fruits were presented with a three-digit randomized code each on a tray under daylight illumination.

Sensory flavor evaluation. 'Unique' Fruit were juiced as described above, and frozen at 20 ºC. On day of panel the juice was thawed overnight at 5 ºC, and 40 mL of juice was poured into 120 mL cups with lids (Solo, Urbana, IL, USA). Coded juice samples were presented to panelists at 14 ± 1 ºC, following a William's design. An additional cup of control juice was presented and labelled as "warm up".

Panelists were instructed to first taste the "warm up" sample to familiarize their taste buds to the juice (O'Mahony et al., 1988). Then they were instructed to take a sip from each coded cup, and rank the samples for overall flavor, from best to worst. They were allowed to taste as many times as necessary. Finally, a question was asked about any off-flavor perceived in each sample. Panelists were asked to rate off-flavor from 1 (no off-flavor) to 10 (extreme off-flavor) and to describe the off-flavor if perceived. Tasting took place in isolated booths under red lighting and data were recorded using Compusense® Cloud (Compusense, Guelph, ON, Canada). Twenty-four panelists participated in flavor evaluation of 'Unique' juice.

***Statistical analyses*.** Physical and chemical parameters were analyzed using univariate parametric analysis of variance (ANOVA) at each storage time, and multiple comparison Duncan test or Games-Howell (according to homogeneity of variance assumed or not) and a t-test for paired samples. The significance level for all analyzes was 5%. The software programs used in the analysis were IMB SPSS Statistics Inc. (Chicago IL), Statistical/Statsoft, version 7 (SAS, 1989; STATSOFT version 7, 2011) and R Core Team (2016). Ranked sensory data were analyzed using a critical absolute rank sum differences table at $p < 0.05$ (Newell and MacFarlane, 1987). Tetrad test data were analyzed using approximation equation for tetrad (Z-test) at $p=0.05$. The comparison between sensory firmness scores was performed using non-parametric ANOVA and multiple comparison of Kruskal-Wallis, due to the ordinal level of the variables and four independent samples in the experiment ($p < 0.05$). Off-flavor ratings were analyzed by ANOVA in a mixed model with random panelists using Senpaq v. 5.01 (Qi Statistics, Reading, UK). Principal component analysis (PCA) were performed using JMP, Ver. 13 (SAS, Cary, NC) to

test the separation among coating treatments and storage times based on the volatile compounds.

Results and Discussion

Weight loss. A major function of coatings on fruits is to retard water loss, measured as weight loss. Microemulsion (conventional) and nanoemulsion (experimental) carnauba wax coatings resulted in less weight loss compared to control and shellac coated fruit, which were not different from each other, in the first experiment with 'Nova' mandarins (Fig. 1A). The microemulsion fared slightly better than the nanoemulsion for retarding weight (water) loss. For the second experiment with 'Unique' tangors, fruit weight loss was minimal after 14 d at 10 °C (less than 2%) (Fig. 1B), with control showing the most weight loss, not different from shellac. At the end of storage (14 d at 10 °C + 7 d at 21 °C), the microemulsion followed by the nanoemulsion coating exhibited less weight loss when compared to uncoated fruits, with the shellac coating being intermediate between control and the waxes.

Carnauba wax nano- and microemulsions are less permeable to water vapor than shellac, due to the lipophilic nature of this plant wax. Solid lipid structure is dense and restricts water diffusion (Morillon et al., 2002), thus the results are not surprising. Several studies have reported that carnauba-based coatings decrease water loss (Kim et al., 2014; Jo et al., 2014).

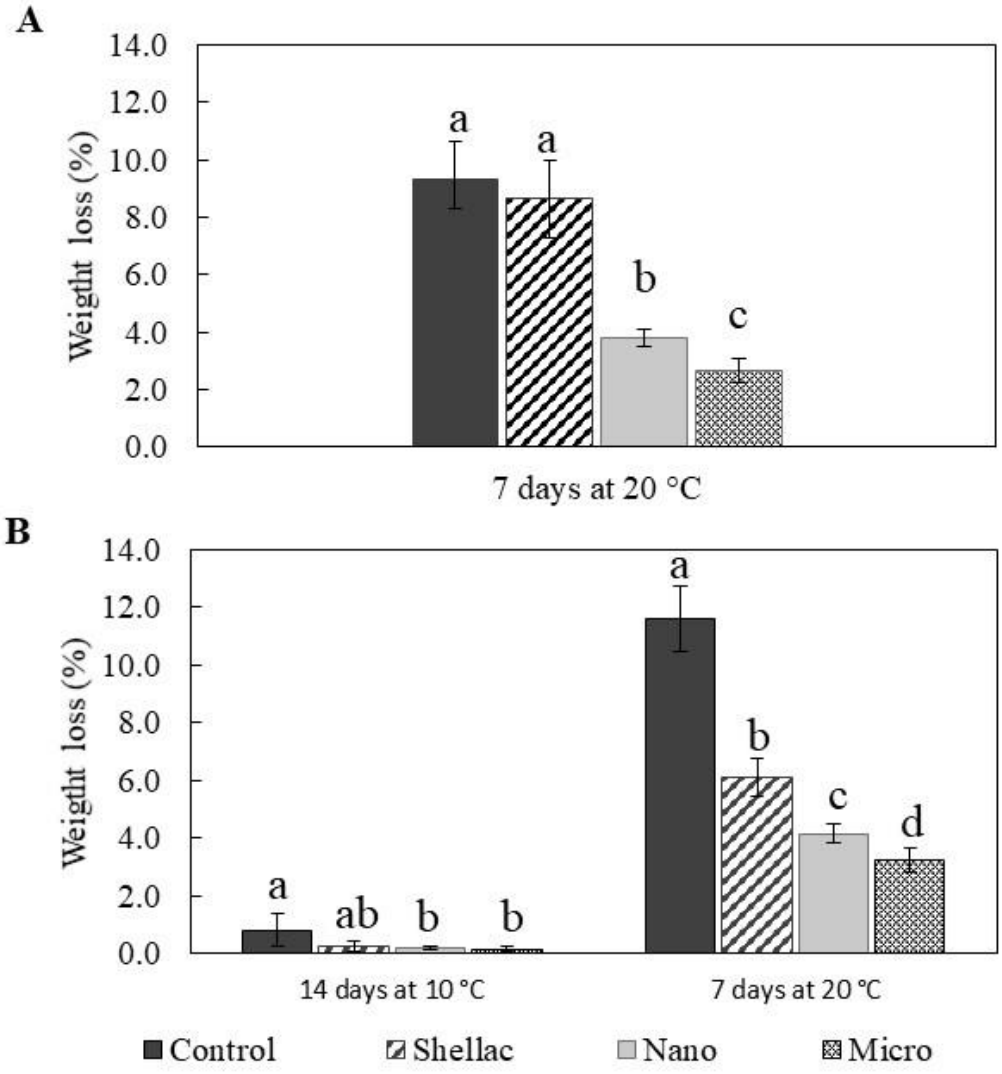

Figure 1. Weight loss of **A)** 'Nova' mandarins and **B)** 'Unique' tangors coated and uncoated (10 fruit/treatment). For each storage period, columns with different letters are significantly different by Duncan test ($p<0.05$).

Sugars and acids. Sweet and sour tastes, attributed to sugars and acids, are important to fruit flavor quality. There were minor statistical but no practical differences for SS, TA, pH and ratio among treatments (Fig. 2). For 'Nova' mandarin coated fruits (Fig. 2A, C, E and G), there were no differences in SS between coatings (Fig. 2A), however, shellac had the highest TA and lowest pH (not different from the microemulsion), which resulted in the lowest SS/TA (not different from control) (Fig. 2C, E and G). Generally, although not tested statistically, there was a slight increase in SS, pH and SS/TA and decrease in TA (except shellac for TA and pH and microemulsion for pH) after 7 d at 20 °C. Similar results were reported by Obenland et al. (2011). The authors associated the higher SS/TA with slightly superior flavor for mandarin fruit stored at 8 °C for up to 6 weeks, with one week at 20°C.

For 'Unique' tangors, there were also no substantial differences for SS, TA, pH or ratio among treatments (Figure 2B, D, F and H). However, the pH and ratio for uncoated fruit was statistically the lowest among the treatments after 14 d at 10°C (not different from shellac or nanoemulsion for SS/TA) (Fig. 2F and H, respectively), and there were no differences after an added 7 d at 20°C. Similarly, no significant differences for SS or TA were detected for different storage temperatures or over the storage period in 'Valencia' oranges coated with shellac, a cellulose-based coating or uncoated fruit (Baldwin et al., 1995).

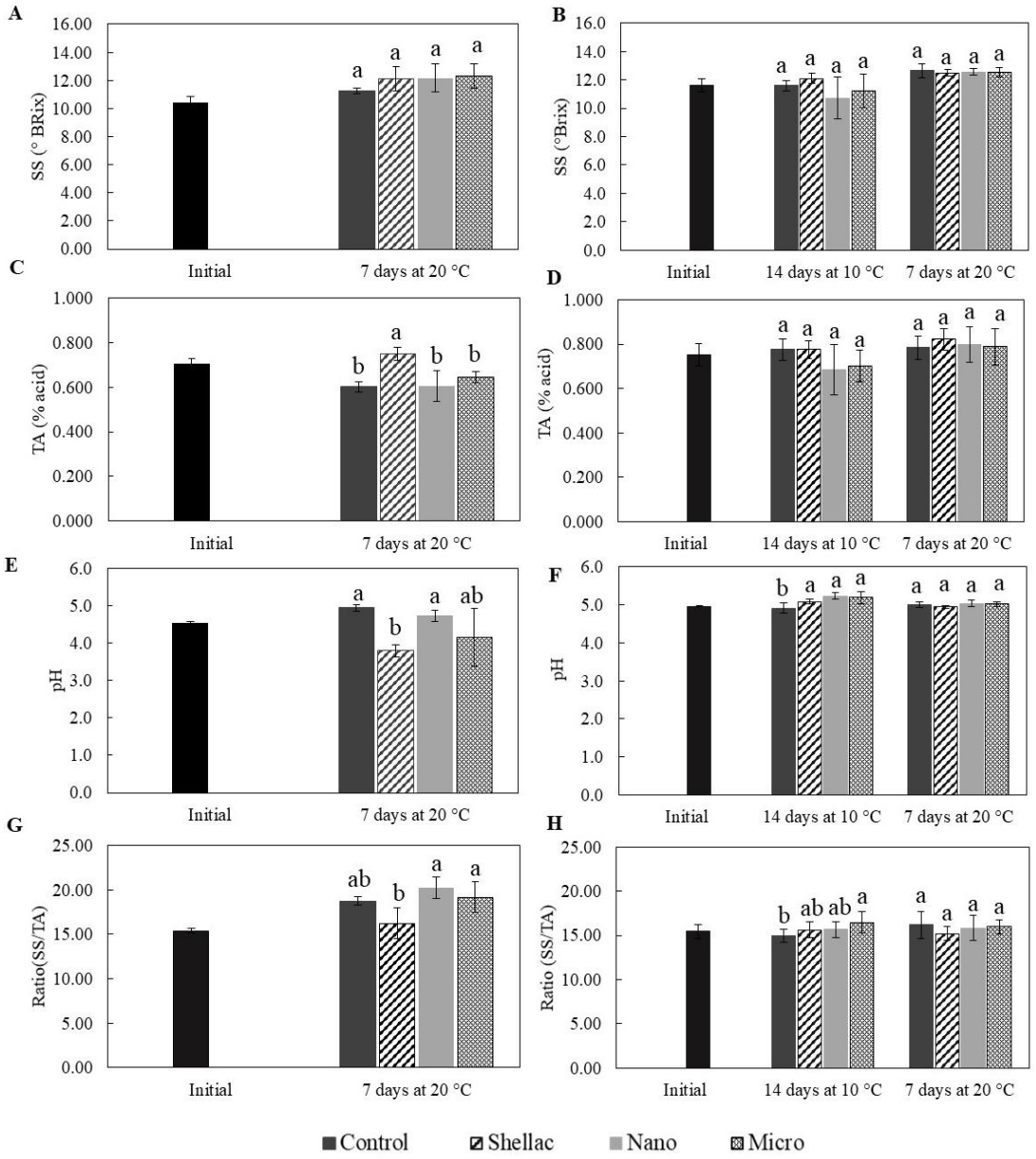

Figure 2. **A** and **B**) SS – soluble solids; **C** and **D**) TA - titratable acidity; **E** and **F**) pH; **G** and **H**) SS/TA ratio values for 'Nova' mandarin and Unique' tangors (respectively) uncoated and coated with different coatings (five composite reps of three fruit each). Columns with different letters are significantly diferent within each storage period by Duncan ($p<0.05$).

Internal gases. Fruit coatings can result in a modified fruit internal atmosphere that can affect flavor. Ethanol is discussed here as it is affected by O_2 levels and is an indicator of anaerobic respiration and resulting off-flavor. For fruit in these two experiments, internal CO_2 and ethanol generally increased and O_2 decreased during storage (Fig. 3). For 'Nova' mandarins, the highest level of CO_2 and ethanol were found in the shellac treatment along with the lowest O_2, suggesting anaerobic respiration. The lowest level of CO_2 and ethanol were found in uncoated control fruits along with the highest O_2. The nano- and microemulsion carnauba wax coatings were intermediate, and not different from each other (Fig. 3A, B and C). High ethanol has been directly linked to off- and altered flavor (Baldwin et al., 1995, Hagenmaier, 2000, 2002; Hagenmaier and Goodner, 2002; Ke and Kader, 1990). Hagenmaier (2002) coated several mandarin hybrids (eight varieties) with wax (polyethylene and candelilla) and resin (shellac), and reported that a sensory taste panel found that fruit coated with the low gas-permeability coating (shellac) had less fresh flavor compared with those coated with higher gas-permeability coatings (polyethylene and candelilla waxes). The author demonstrated that mandarin flavor may be affected when internal CO_2 is higher than 14%, internal O_2 is lower than 4% and juice ethanol content is greater than 1500 $uL \cdot L^{-1}$ after 7 d storage at 21 °C (see dashed lines at Fig. 3A to F).

'Unique' tangors showed the same trend as 'Nova' mandarins in that fruits coated with shellac showed the lowest O_2 along with highest CO_2 and ethanol, followed by the carnauba microemulsion, then the nanoemulsion (Fig. 3D to F), especially after seven d at 20 °C. Meanwhile, uncoated fruit had the lowest ethanol and CO_2 levels, along with the highest O_2. There were no differences between the

nanoemulsion and control for ethanol levels or between the nanoemulsion and the microemulsion for O_2 or ethanol after 14 d cold storage, and after the simulated marketing condition for ethanol. Lower ethanol levels in the nanoemulsion coating could indicate better flavor, similar to uncoated fruit. Thus, both carnauba coatings in this study were shown to be suitable for mandarins and tangors. Similar results were reported by Navarro et al. (2007). These authors reported that internal CO_2 and ethanol were highest for fruit coated with shellac.

Shellac coatings can reduce gas exchange, modifying the internal atmosphere and creating an anaerobic/fermentative environment (Baldwin et al., 1995; Hagenmaier, 2000; Hagenmaier and Baker, 1994). Usually resin coatings have low oxygen permeability properties and generally shellac and wood resin are less permeable than waxes such as polyethylene, candelilla (Hagenmaier, 2002) and carnauba (Assis et al., 2008; Lin and Zhao, 2007). Therefore, lipid-based coatings, such as carnauba wax, present a more effective moisture barrier, and are relatively permeable to gases (Assis et al., 2008; Lin and Zhao, 2007), resulting in less off-flavor.

Low oxygen atmospheres have been shown to result in increased production of ethanol, methanol, and acetaldehyde in citrus fruit (Davis, 1970; Shaw et al., 1990). Summarizing this study, carnauba wax emulsions exhibited intermediate modification of the fruit internal atmosphere between uncoated controls and shellac, presented a better moisture barrier than shellac, and resulted in less ethanol, with nanoemulsion ethanol levels not being different from uncoated fruit (Fig. 3 C and F).

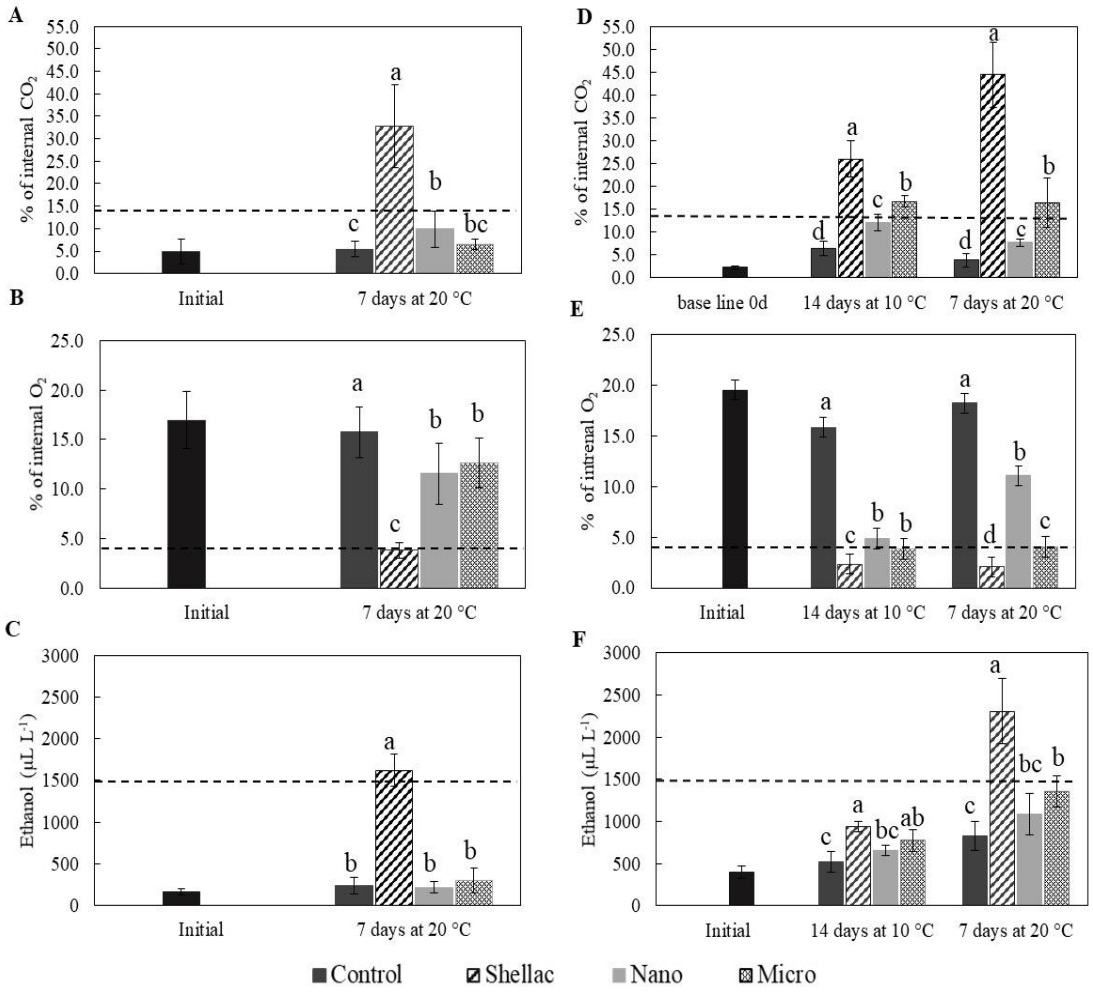

Figure 3. **A** and **D**) internal CO_2; **B** and **E**) internal O_2 (10 fruit/treatment); **C** and **F**) juice ethanol values for 'Nova' mandarins and Unique' tangors, respectively (five replicates of three fruit each). Columns with different letters are significantly different for each storage period by Duncan or Games Howell ($p<0.05$). Dashed line represents the critical level according to Hagenmaier (2002) that mandarin flavor may be affected (internal CO_2 and juice ethanol higher than 14% and 1500 uL L^{-1}, respectively, and, O_2 lower than 4%).

Aroma volatile analysis. The PCA plots of aroma volatiles from 'Nova' mandarins and 'Unique' tangors are shown in Fig. 4A and B, respectively. The PCA for 'Nova' mandarins (Fig. 4A) explained 59.5% of the data variation (33.2% and 26.3% for components 1 and 2, respectively) and 62.4 % of the variation (mostly in component 1) for 'Unique' tangors (Fig. 4B) offering more evidence of the altered internal atmosphere due to coatings. For 'Nova' mandarins, shellac coatings were associated with ethanol, methanol and acetaldehyde, indicating fermentation, among other volatiles. The carnauba wax emulsions were either clustered with control and 0 d samples or further down to the right on component 1, especially one nanoemulsion outlier, that was associated with common citrus terpenes, esters and an aldehyde (Fig. 4A, limonene, myrcene, α-pinene, γ-terpinene, ethyl hexanoate and decanal among others). For 'Unique' tangors, there were not a lot of volatile differences after 14 d at 10 °C for any of the treatments, which were similar to 0 d juice, as metabolism at this temperature is relatively slow (Fig. 4B). However, after cold storage followed by the simulated marketing period at 21 °C there were more differences between treatments. The aroma volatiles in the juice from 0 d fruit, controls and the carnauba nanoemulsion treatment were associated with less volatiles, being on the left side of component 1, although associated with methanol and α-terpineol. The juice from fruit coated with the microemulsion or shellac were associated with more volatiles on the right side of component 1, including acetaldehyde, ethylacetate, ethanol and acetone (Fig. 4), which are associated with anaerobic respiration. One control outlier was also associated with acetaldehyde as well as valencene. While the other volatiles shown in Fig. 4 are mostly desirable and make up citrus aroma, the coatings may have trapped them and/or affected their

synthesis, resulting in an altered flavor profile compared to normal (as evidenced for 0 d and control uncoated fruit juice) resulting in possible off-flavor.

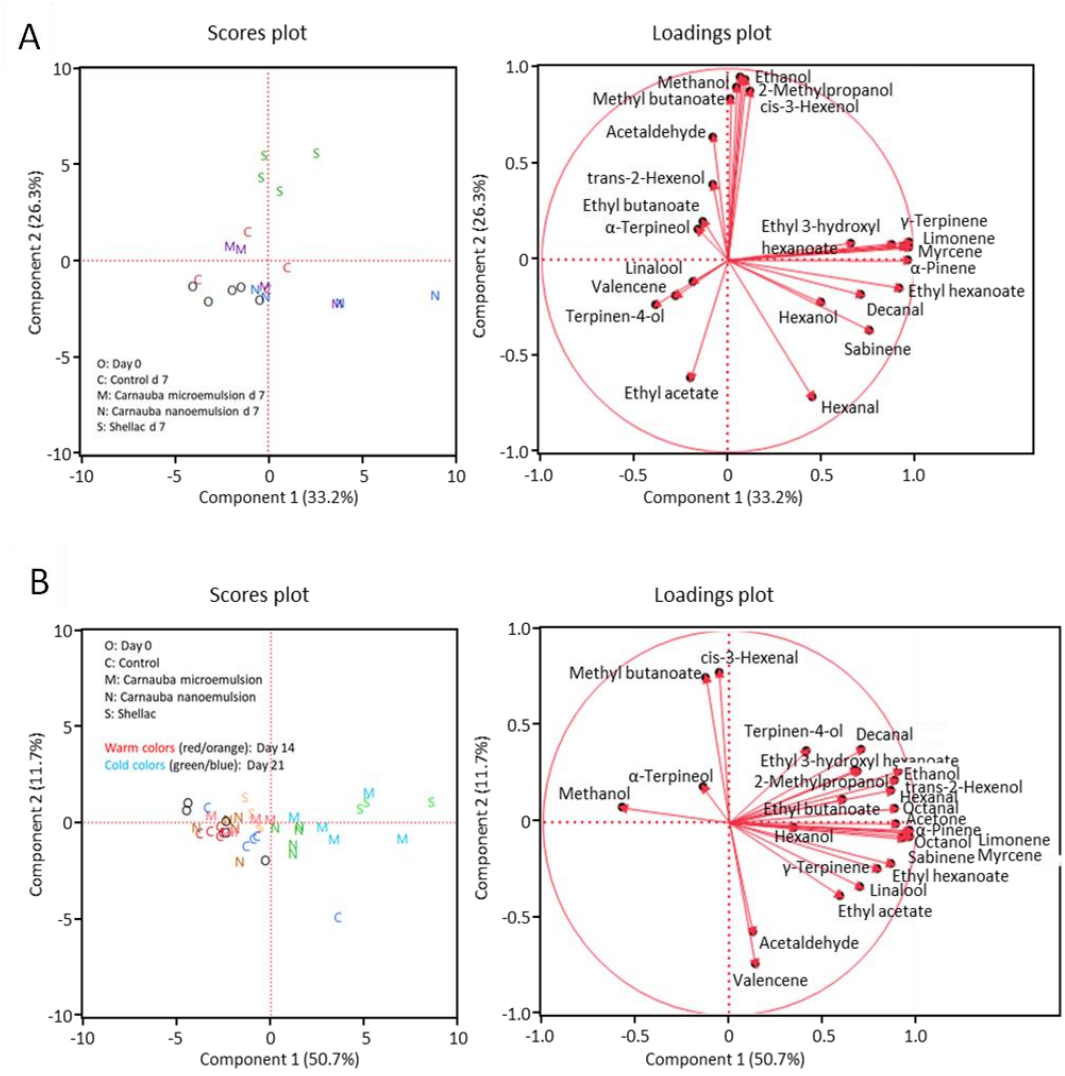

Figure 4. Principle Components Analysis of 26 aroma volatile compounds measured in the juice of 15 fruits (five composite replicates of three fruit each) for untreated fruit (Control - C) or fruit coated with three different coatings and stored at room temperature (Day 0 - O), **A)** 7 d at 21 °C (C, S, N and M) and **B)** 14 d at 10 °C (red/orange C, S, N and M) or 14 d at 10 °C + 7 d at 21 °C (green/blue C, S, N and M). S = Shellac; N = nanoemulsion and M = micromeulsion.

Fruit flavor evaluation. In the second experiment with 'Unique' tangors, the juice used to measure aroma volatiles was also evaluated by a sensory panel for flavor (Fig. 5A, B and C). After 14 d cold storage, control fruit juice was ranked as having the best mandarin flavor (lowest value), although not different from the carnauba microemulsion, while the carnauba nanoemulsion and shellac-coated fruit ranked as having the worst mandarin flavor (highest value), although not different from the carnauba microemulsion (Fig. 5A). For off-flavor at 14 d, there were no differences between treatments (Fig. 5C). This reflects the volatile data that showed little difference between treatments after cold storage (Fig. 4). After 14 d cold storage followed by 7 d at 20 °C, control fruit juice and the carnauba nanoemulsion-coated fruit juice were ranked highest for mandarin flavor, and the shellac and microemulsion were ranked lowest (Fig 5B). This also reflects the increased volatile changes after the simulated marketing period at 21 °C (Fig. 4). Similarly, for off-flavor the control and carnauba nanoemulsion-treated fruit juice were rated lowest for off-flavor, and shellac and microemulsion rated highest (Fig 5C). This is consistent with the ethanol and other volatile levels, especially for shellac. The CO2, O2 and ethanol levels for the carnauba microemulsion were at or near levels that would predict flavor problems, but it is surprising that there were no flavor differences from shellac-coated fruit, which induced much more extreme modification of the atmosphere than did the carnauba microemulsion, resulting in higher ethanol. Off-flavor descriptors for the shellac and microemulsion-coated fruit were "rancid, "fermented", "chemical", "rotten fruit" and "soapy", which might be explained by higher amounts of ethyl acetate, ethyl hexanoate, ethyl-3-hydroxy hexanoate, and some aldehydes (Plotto et al., 2008).

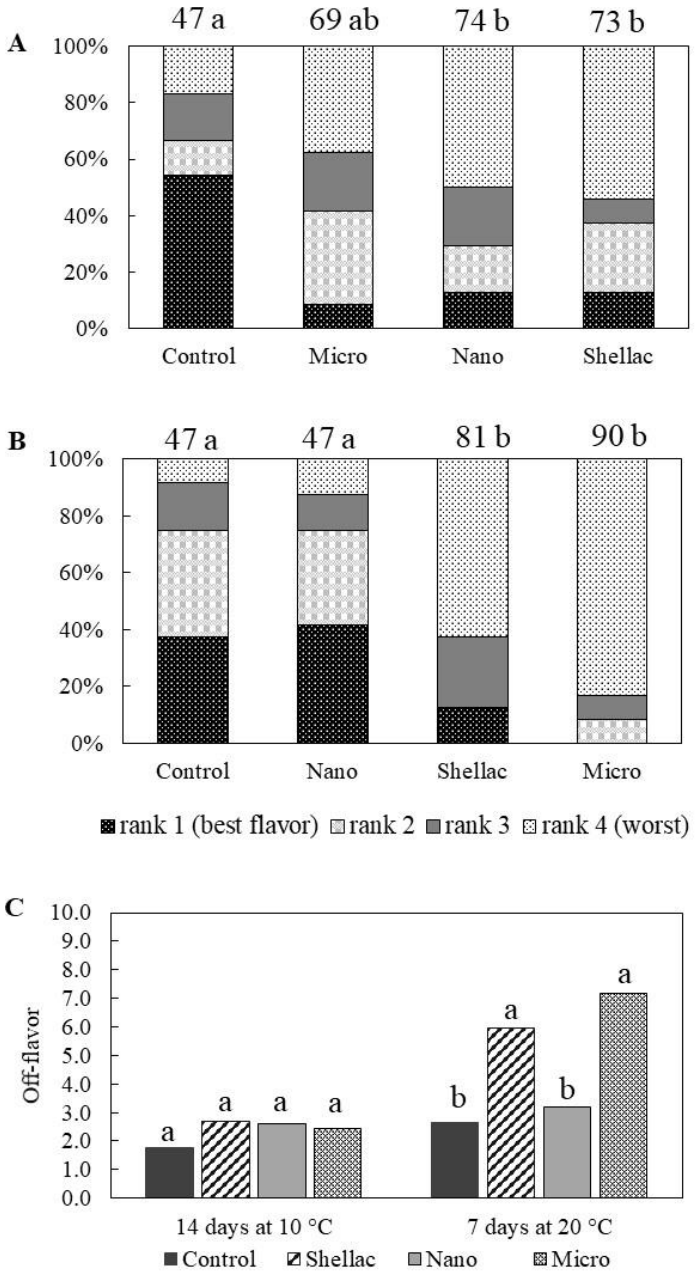

Figure 5. Fruit juice was ranked for tangerine flavor of 'Unique' ('Ortanique') tangors after 14 d at 10 °C (A) and after an additional 7 d at 21 °C (B). Columns with different letters are significantly different by critical absolute rank sum differences (value on top of figure) table at $p < 0.05$ (Newell and MacFarlane, 1987, data shown in order of ranking) for the four samples as determined by 24 panelists. Color shading depicts % of panelists' selection of treatment. (C) Juice rated for off-flavor on a scale of 1-10, where 1 = none and 10 = extreme. Numbers (mean rating, n = 24) followed with the same letter are not statistically different using the Fisher's LSD test ($p < 0.05$).

***Gloss analyses*.** Fruit shine boosts sales, therefore, coatings that impart shine are sought after by the coating industry, especially for citrus. In preliminary tests with paper sheets, gloss on coated paper sheets increased when hot air was used for coating surface drying, being generally higher for coatings dried at 50 °C compared to 20 ºC (Fig. 6A), significant for the two carnauba emulsions. This indicates that application of heated air helps coatings to dry and tends to impart more shine to the fruit. In fact, heated air-drying tunnels are often used for citrus by the industry (Hall, 2012). Shellac as expected had the highest gloss, significantly different when compared to carnauba wax nano- and microemulsions and the uncoated paper (Fig. 6A).

For gloss on 'Nova' mandarin fruit (Fig. 6B), shellac coating showed higher gloss than the uncoated control initially, with carnauba wax coatings being not different from shellac or control. However, after 7 d of storage, all coatings showed more gloss than control and with no differences among coating treatments. For 'Unique' tangors (Fig. 6C), initially shellac showed the highest gloss, significantly different from the other treatments, which were not different from each other. After 14 days of storage at 10 °C, shellac and the carnauba wax nanoemulsion showed the highest gloss readings with no difference between them and no difference between the carnauba microemulsion and control. However, after 7 d at 20 °C, the nanoemulsion showed the highest gloss with the microemulsion being not different from the nanoemulsion or shellac. Citrus fruit loss of shine was observed for all treatments over time in storage, however, this was greater for shellac-coated fruit. Similar results were reported by Navarro et al. (2007) for 'Valencia' oranges and 'Marisol' tangerines, treated with different coatings, with fruit gloss being highest for

the shellac coating. In another study, after storage shellac gloss/shine decreased but was still shinier than uncoated fruit. For that study, the shellac coating provided fruits with more shine and exhibited better weight loss control when compared to commercial cellulose-based coatings (Baldwin et al., 1995).

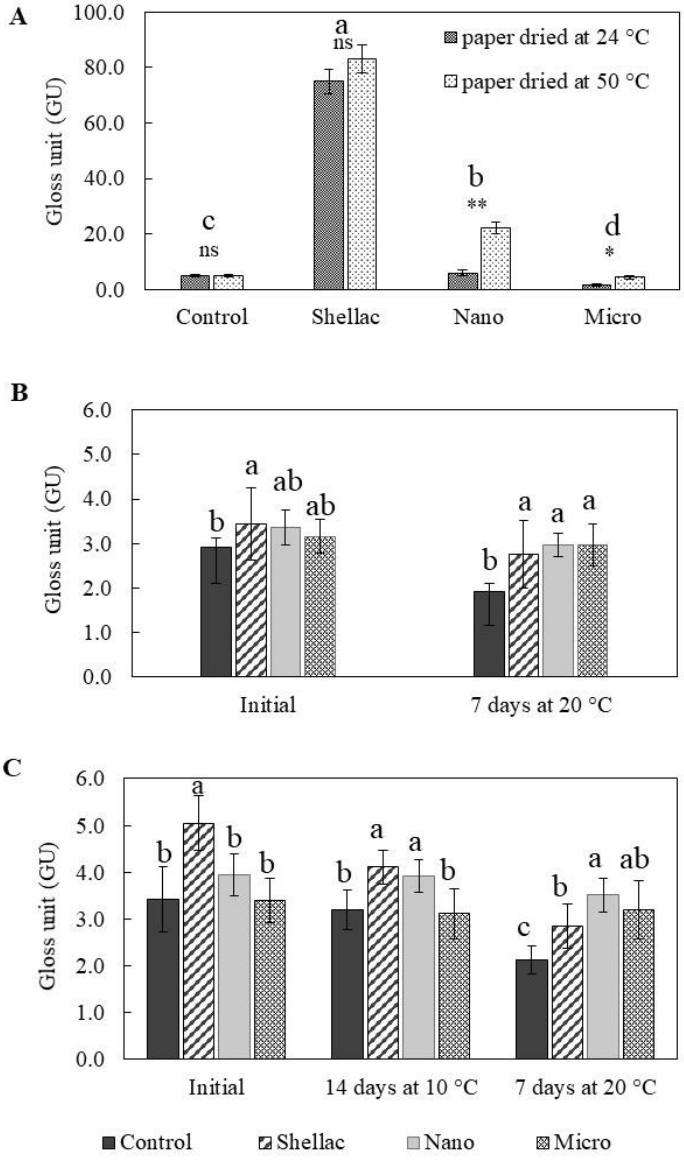

Figure 6. A) Gloss on black paper (five test papers with five measurements each), differences determined by t-test (*, $p<0.05$; ** $p<0.1$) **B)** Gloss on 'Nova' mandarins; **C)** Gloss on 'Unique' tangors (10 fruits/treatment with two measurements/fruit). For each storage period, columns with different letters are significantly different by Duncan test or Games Howell at $p<0.05$.

Sensory visual shine analyses. For ‘Nova’ mandarins, shellac-coated fruit were ranked highest for shine by panelists after 7 d at 20 °C, followed by carnauba nanoemulsion, carnauba microemulsion and finally the uncoated control (Fig. 7A). For ‘Unique’ tangors, the carnauba nanoemulsion ranked highest, although not different from the microemulsion, but higher than the shellac, and all were higher than the uncoated fruit (Figure 7B) after 14 d 10 °C and 7 d at 20 °C. There was no difference between the carnauba microemulsion and shellac for shine. For the sensory pairing test (Tetrad) with ‘Nova’ mandarins, panelists recognized shellac-coated and uncoated fruit, but could not differentiate between carnauba wax microemulsion and nanoemulsion in appearance for coated fruit (Fig. 7C).

The results for ranking based on shine are in agreement with Navarro et al. (2007), who reported that shellac coated fruit ranked third for appearance (out of five treatments) and was rated as too shiny. At the end of the cold storage (14 d at 10 °C) and simulated marketing condition (7 d at 20 °C), uncoated tangors were more firm (hard, dried out) followed by the microemulsion, with the nanoemulsion being softest and shellac not different from either of the carnauba emulsions (Fig. 5D).

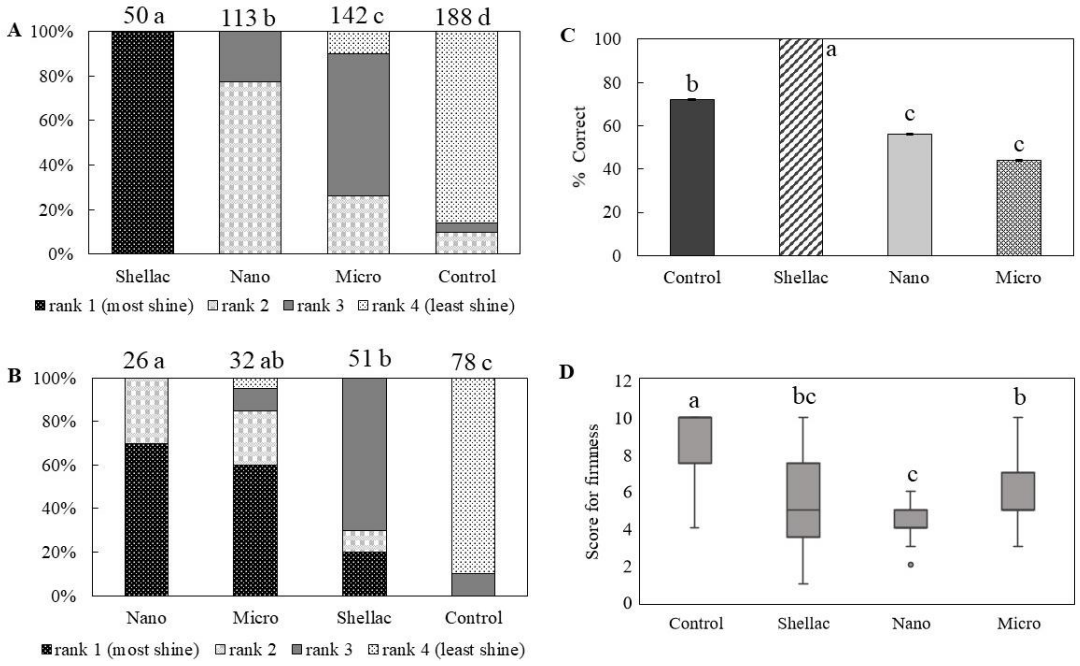

Figure 7. Sensory shine ranking for 'Nova' mandarins **(A)** and 'Unique' tangors **(B)** at end of the storage period. Columns with different letters are significantly different by critical absolute rank sum differences table at $p<0.05$ (Newell and MacFarlane, 1987, value on top of figure, shown in order of ranking). Color shading depicts % panelists' selection of treatment. **C)** Sensory tetrad test for 'Nova' mandarins at end of the storage period. Columns with different letters are significantly different by approximation equation for tetrad (Z-test) at $p \leq 0.05$. **D)** Sensory firmness perception for 'Unique' tangerine after 21 days of storage. Columns with different letters are different by Kruskal-Wallis multiple comparisons test ($p< 0.05$).

In conclusion, carnauba wax emulsion coatings allowed more gas exchange resulting in less fruit internal atmosphere modification, added more sustainable shine and reduced water loss more effectively than did a commercial shellac microemulsion on citrus fruit. Carnauba coatings did not affect fruit sugar and acid levels, while shellac effects were minimal. The shellac-coating induced the lowest O_2 along with highest CO_2 and ethanol levels in fruit, which indicated anaerobic respiration and would predict undesirable flavor changes, confirmed by a sensory panel. The panel found the uncoated controls and the nanoemulsion-coated fruit to have the most tangerine flavor and least off-flavor. Aroma volatile analysis showed

that the shellac and microemulsion resulted in increased volatile levels, indicating alteration of the normal volatile profile. Comparing the two carnauba coatings, the microemulsion showed slightly better water loss control, while the nanoemulsion generally exhibited higher gloss/shine, less modification of the fruit internal atmosphere, less alteration of the aroma volatile profile, and lower ethanol levels. This resulted in better flavor quality for nanoemulsion-coated fruit juice that was not different from the uncoated control. Use of edible coatings is an environmentally friendly technology to reduce post-harvest losses and maintain fruit quality when used appropriately, and the carnauba nanoemulsion was as good as or better for shine and flavor impacts compared to the carnauba microemulsion. Future work will look at decay control for carnauba and shellac microemulsion compared to the carnauba nanoemulsion-coated citrus fruits.

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Capítulo 4.

Effect of incorporation ginger essential oil in carnauba nanoemulsion coatings on papaya: evaluation of quality and post-harvest fruit decay.

Artigo a ser submetido.

Effect of incorporation ginger essential oil in carnauba nanoemulsion coatings on papaya: evaluation of quality and post-harvest fruit decay.

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Abstract

Formulations with particulate emulsions of carnauba, and its association with hydroxypropyl methylcellulose (HPMC) and ginger essential oils (GEO), were applied as protective coatings on papayas and evaluated under several conditions of storage. Uncoated fruits were taken as control. Carnauba micro and nano emulsions were prepared from carnauba wax type I in a closed reactor, and the formulations prepared in a concentration of 9% and 18% (w/v) of particulate fraction in suspension. HPMC (1% w/v) was used as inert coating control and as adjuvant in film formation. GEO (3% v/v) was tested as active compounds against fungal infestation. The fruits were stored and evaluated in two separately experiments. Experiment 1: 6 days at 22 °C (Lot 1) and 9 days at 13 °C followed by a 5 days at room temperature to simulate marketing conditions (Lot 2), and Experiment 2: 5 days at 22 °C (Lot 1), and 10 days at 16 °C, before simulated marketing condition for 3 days at 22 °C (Lot 2). Post-harvest analyzes comprised measurement of weight loss, soluble solids (SS), titratable acidity (TA), pH and ratio (SS/TA), firmness, skin color determinations. The protective action in reducing the incidence of natural diseases and the inhibition of fungal spread were conducted on samples inoculated with *Colletotrichum gloeosporioides.* Sensory evaluation as also carried out. Although all coatings provide some protection, the effects are dependent on the storage conditions, which differ between formulations. Under cold storage, most of the analysis did not resulted in statistically significant differences among samples. At room temperature, and after simulated market conditions, the differences become more evident. Nanoemulsions with 18% of particulate carnauba were particularly able to a better maintaining of papaya quality over storage and market conditions. Higher reductions on loss of firmness, color alterations and respiration rate were recorded, meaning a positive action in delaying maturity. No changes in attributes as sweetness, sourness, papaya flavor or the presence of off-flavors related to internal fermentation were reported by panelist in tasting samples from all treatments after 5- and 13-days storage. GEO presented some effect in reducing natural diseases on papaya skin, particularly when associated to carnauba, although no activity was observed in inhibiting *C. gloeosporioides* fungal growth after inoculation.
Key-words: papaya, carnauba emulsions, edible coatings, post-harvest quality, diseases control.

1. Introduction

Papaya (*Carica papaya* L.) is a native fruit of tropical America and disseminated throughout the tropics. Brazil is the second-largest global producer of papaya (1.1 million tons) after India, with a production up to 5.9 million of tones (FAO, 2020). According to Secex (Brazilian's Secretariat of Foreign Trade), the Brazilian exportation of papaya fruit increases annually, reaching 43.3 thousand tons by 2019 (CONAB, 2020).

Papaya, however, is a fragile, highly perishable fruit with post-harvest life not superior to four weeks (Pérez-Carrillo & Yahia, 2004). Due to its thin skin, the papaya is very susceptible to mechanical damages and post-harvest injuries. Additionally, the great volume of water in the mesocarp renders a considerable fruit susceptibility to microorganisms attack and other physiological disorders (Singh & Rao, 2011).

Estimations of papaya post-harvest losses are approximately 30 to 60 percent of the whole production in both developed and developing countries (FAO, 2019). The application of coatings, edible or merely protective, has been considered a valuable strategy in providing additional integrity to fruit skin by forming a semipermeable barrier that lowers water vapor permeability and inhibits microbial adherence and growth (Bai & Plotto, 2011). Several types of materials have been proposed as suitable to coat fruits, each having advantages and disadvantages in their applications (Dhall, 2013).

Particularly hydrophilic polysaccharides such as chitosan, starch and cellulose salt derivatives, and hydrophobic compounds as carnauba wax, proteins and lipid-based formulations, or their combinations (composites),

have been extensively evaluated as protective edible films and coatings (Santos et al., 2017; Formiga et al., 2019; Freitas et al. 2019; Zambrano-Zaragoza et al. 2020, Arroyo et al. 2020). Recently, particulated systems as chitosan nanoparticles and nanoemulsions of carnauba wax, instead of conventional continuous coating, have been tested on apples (Pilon et al., 2015), grape berry (Kim et al., 2014) and papayas (Ohashi et al., 2015), showing improved effectiveness concerning reductions in losses of weight, firmness and preserving post-harvest attributes.

In addition to the effect of reducing gas exchange and retarding water vapor loss and maturity, one attractive advantage of coating application lies in the ease of incorporating active compounds in the formulation, enhancing the microbial spoilage prevention when topically applied (Campos et al., 2011). The incorporation of active agents in nanoparticulate form seems, in principle, to be more friendly and efficient than the additions of continuous phases in coating formulations.

Therefore, the aim of the present study was twofold: First, the development and evaluation of carnauba nano and micro-sized emulsions suitable for coating application. Second the incorporation of ginger essential oil in the coating formulations, as an active agent in inhibiting fungal proliferation on papayas. The ginger essential oil has known antimicrobial activity against a series of foodborne pathogens (Hasan et al., 2012; López et al., 2017), with activity either in liquid or in solid coating medium (Noori et al., 2018). Neutral coatings consisting of hydroxypropyl methylcellulose

(HPMC) were also assayed, with and without ginger oil, as comparative formulations.

2. Material and methods

2.1 Preparation of coatings formulations

2.1.1 Carnauba wax nanoemulsion coatings (Nano).

Emulsion of carnauba in nanosize dimensions was prepared following the methodology proposed by Hagenmaier and Baker (1997) with adaptations. A closed reactor was used to heat a mixture of 180 g of carnauba wax type I (Strahl & Pitsch, Inc. - West Babylon, NY), 30 g of oleic acid, 20 g of ammonium hydroxide 8% (Sigma-Aldrich Chemical Co. - St. Louis, MO, USA), and deionized water (775 g), at 105 °C under constant mechanical stirring (800 rpm) for 10 min. Then, the emulsion was cooled to 90 °C at a rate of 1°C/min approximately, under agitation and subject to high-pressure homogenization at 400 bar and rapidly cooled down to room temperature. The final emulsion was formed by nanosized oil drops.

2.1.2 Carnauba wax microemulsion coating (Micro).

Wax-in-water conventional carnauba emulsion, in micro-sized particles, was also prepared as described by Hagenmaier and Baker (1997). In an open cylindrical reactor, 45 g of carnauba wax SP200 (Strahl & Pitsch, Inc. - West Babylon, NY) was melted with 5 g of oleic acid, 5 g of myristic acid and 15 mL of deionized water at 105 °C. 28 g ammonium hydroxide 8% (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was added under

constant mechanic stirring (800 rpm) for 10 min. Thereafter, 160 mL of water was heated and added. Then, the solution remained under mechanical stirring for 10 min at 95 °C and cooled down to 50 °C at a rate of 5 °C/min. The final emulsion was formed by micro-sized oil drops (0.2 to 1.7 μm) dispersed in the water phase previously characterized (Miranda, 2015).

2.1.3 Hydroxypropyl methylcellulose coating (HPMC 1%).

1 g of HPMC, from Sigma Aldrich, (molar weight ~90,000 da) was slowly dispersed in 100 mL of hot water at 80 °C and homogenized under magnetic agitation for 5 min. Then, the suspension was left to cool to room temperature and remaining overnight under stirring. No plasticizer was used in this formulation.

2.1.4 Ginger essential oil nanoemulsion preparation (GEO).

Commercial food grade ginger oil (from Sigma-Aldrich, purity 97% - CAS Number: 8007-08-7) was used as active agent. GEO emulsion was prepared by adding gradually and continuously 3% (v/v) of ginger oil and 0.6% (v/v) of Tween 80 in distilled water when mixing in an Ultra-Turrax at 16,000 rpm for 4 min for complete homogenization.

2.1.5 Coatings containing GEO.

3% (v/v) of ginger oil and 0.6% (v/v) of Tween 80 were gradually and continuously adding into previously prepared coatings (nanoemulsion,

microemulsion, or HPMC), followed by mixing in an Ultra-Turrax at 16,000 rpm for 4 min to assure homogenized coating.

2.1.6 Coatings formulation combining wax emulsion (Nano or Micro) with HPMC.

1 g of HPMC powder was slowly added into 100 mL of each emulsion (Nano or Micro), under magnetic stirring, and left to homogenize overnight at room temperature. These formulations were named as Nano + HPMC and Micro + HPMC.

2.1.7 Surface wettability

Surface wettability of papaya skin (15 x 4mm) and on papaya coated surfaces were analyzed by contact angle measurements using the sessile deionized water drop method (volume ~ 3 µL). Strips of skin were cut with a scalpel blade and carefully mounted on glass slides and contact angles automatically registered in a CAN101 Optical Contact Angle Meter (KSV Instruments, Finland). The recorded angles were the average of tree measurements on each sample, determined using an adaptation of the ASTM D5725-99. The times 1.0; 20.0; 40.0 and 60.0 s were selected as check points. All measurements were repeated five times.

2.1.8 Particle size and zeta potential

Particle size distribution and zeta potential were obtained using a suspension (1:100) with concentrated coating dispersed in deionized water at

room temperature using a Zetasizer Nano ZS (Malvern Instruments Inc., Westborough, MA, USA). The data was acquired from 10 measurements with five runs each and 1s delay between the runs. All samples were analyzed in four replicates.

2.1.9 Scanning Electron Microscopy

Papaya skins coated and uncoated were characterized using field emission gun scanning electron microscopy (MEV-SEM JEOL JSM-6701F). For surface micrographs, stripes of papaya´s skins (coated by dipping method) were allowed to dry for 48 h, mounted on carbon slides and gold-coated. For fracture micrographs, samples were frozen in liquid nitrogen and fractured. Considering the interest in the coating characterization, spontaneous air drying was allowed for preserve biologic tissue integrity. After the fracture, the samples were dried for 24 h in a desiccator and then gold-coated using an SCD 050 sputter coater (Leica Microsystems, Germany). The microscopy operated at an acceleration voltage of 10 kV.

2.2 Coatings on papayas

‘Redland’ papayas (*Carica papaya L.)* were provided by at a commercial papaya plantation located in Homestead, Florida, USA. The fruits were harvested at the first maturity stage, sorted, washed and sanitized by immersion in a 200 mg L^{-1} of peroxide acetic acid (PAA), during 3 min and air-dried at room temperature. Previously sanitized cold room and utensils were used.

Filmogenic formulations comprising carnauba emulsions (micro and nano) with 9% and 18% of solid phase in suspension were prepared. Isolated coatings with HPMC and carnauba emulsions with 1% (w/v) HPMC addition were also applied. The incorporation of ginger essential oil (GEO), as an active agent, was considered and also evaluated. The prepared formulations are summarized in Table 1.

Table 1. Coating formulations applied on papayas fruits.

Coatings Identification	**Formulations**
Control	Rinsed with distilled water.
HPMC	Aqueous solution at 1% of HPMC.
Nano 9%	Carnauba wax nanoemulsion with 9% of solid phase in suspension.
Nano 18 %	Carnauba wax with 18% of solid phase in suspension.
Nano 9 % + HPMC	1% of HPMC incorporated in Nano 9%
Micro 9 %	Carnauba wax microemulsion with 9% of solid phase in suspension.
Micro 9 % + HPMC	1% of HPMC incorporated in Micro 9 % coating
HPMC + GEO	HPMC at 1% (w/v) with addition 3% (v/v) of ginger essential oil (GEO).
Nano 9% + GEO	Carnauba wax nanoemulsion with 9% of solid phase in suspension plus 3% (v/v) of GEO.
Nano 9% + HPMC + GEO	Carnauba wax nanoemulsion with 9% plus 1% HPMC with 3% of GEO addition.
GEO	Ginger essential oil nanoemulsion prepared at 3% (v/v).

The coatings were carried out manually by pouring 2 mL of filmogenic solution on latex-gloved hands and manually spread on sanitized papayas. The coated samples were spontaneously dried at room temperature. The hand-coating procedure assures the use of a minimal amount of solution per sample with no contamination of the filmogenic formulation (Sun et al., 2015).

Two post-harvest papaya experiments were carried. The first comprising seven treatments, control, neat HPMC, Nano 9%, Nano 18 %, Nano 9 % + HPMC, Micro 9 % and Micro 9 % + HPMC, (Table 1), distributed in two lots and stored. Lot 1 with 70 samples, was kept at 22 °C for 6 days and, Lot 2 with 98 samples, was stored along 9 days at 13 °C and transferred to simulated marketing condition, remaining 5 days at room temperature. A total of 168 fruits were essayed, counting destructive non-destructive analyses (termed Experiment 1).

The second experiment consisted in evaluating the papayas coatings with the addition of ginger essential oil as an active agent. The applied formulations were: Control, neat HPMC, Nano 9%, Nano 18 %, Nano 9 % + HPMC, HPMC + GEO, Nano 9% + GEO, Nano 9% + HPMC + GEO and neat GEO (Table 1). For these, the samples were also divided into two lots as follows: Lot 1 with 90 samples stored at 22 °C for 5 days and Lot 2 composed by 126 samples stored at 16 °C for 10 days before simulated marketing condition (3 days at 22 °C). A total of 216 fruits were essayed, counting destructive non-destructive analyses. That was termed as Experiment 2.

2.3 Post-harvest analyzes

2.3.1 Soluble solids, titratable acidity, pH, and ratio.

Before analyzes, papaya juice was prepared. The fruits were peeled, seeds discarded and the pulp cut in cubic pieces of about 2 cm^3. 50 g of the pulp was blended in a Walita Mixer (LiqFaz, Walita, São Paulo, Brazil) with

50 mL of deionized water for 30 s. Four papayas per treatment were essayed. Soluble solids content (SS) was determined by a refractive index with a digital refractometer (ATAGO PR-101, Tokyo, Japan). Titratable acidity (TA) and pH were calculated using a titration of 10 mL of juice with 0.1 mol/ L of NaOH to pH 8.1 endpoint in an auto titrator (Mettler Toledo DL50, Columbus, USA). The titratable acidity (TA) was expressed as grams of citric acid per 100 mL of juice. The ratio (SS/TA) was estimated using SS (%) and TA (%) and expressed in absolute values (Baldwin et al., 2012).

2.3.2 Weight loss percentage.

Variations in loss percentage were determined to the nearest 0.01 g using a digital scale (AS 2000C, Marte Balanças Ltda, SP, Brazil). The weight loss percentage for each interval was calculated in relation to the initial weight. At the end of each stored condition (after 6, 9, and 14 days for Experiment 1 and, 5, 10, and 13 days for Experiment 2). Six fruits per treatment were individually weighted.

2.3.3 Flesh firmness evaluation.

Fruit flesh firmness was measured using a TA. XT Plus Texture Analyzer (Stable Micro Systems, London, UK), equipped with a 50N load cell and stainless-steel probe with 6 mm diameter. The penetration speed was 1 mm/s to a depth of 5 mm in the equatorial region perpendicular to probe into peeled papaya fruit. Four fruits per treatment and three penetrations in each

sample were measured, for each stored sampling time. The firmness results were expressed in Newtons (N).

2.3.4 Skin color determination.

Color parameters were determined with a colorimeter Minolta® CR-400 Chroma Meter (Minolta Camera Co., Osaka, Japan), using the CIELAB L*a*b* system. Lightness (L*) ranges from completely opaque/black (0) to completely transparent/white (100). Chroma (C*) and hue angle (H*) were computed from colorimetric unities as $C^* = (a^{*2} + b^{*2})^{1/2}$ and $H^* = \text{arctg}\ (b^*/a^*)$, respectively. The instrument was calibrated using a standard white reflector plate. Values were obtained at three different positions in each fruit, in a total of six fruits per treatment at each sampling time.

2.3.5 Internal ethylene, CO_2, and O_2.

10 mL of internal gas were withdrawn with a syringe from four samples of each treatment. Ethylene concentrations were analyzed using a gas chromatograph - GC (Hewlett Packard HP 5890A, Avondale, USA) equipped with a flame ionization detector and a GSQ column (Agilent, Santa Clara, USA). The analyzes followed Sun et al. (2017) procedures. The gas flow rate for He, H_2, and air were 10, 35, and 350 mL/min, respectively. Temperatures of the oven, injector, and detector were 90, 200, and 250 °C, respectively. The CO_2 and O_2 concentrations were quantified with a CTR column (Cole-Parmer, Vernon Hills, USA) and a thermal conductivity detector, for these, the temperatures of the oven, injector, and detector were 70, 250, and 250

°C, respectively. The gas flow rate for helium and air was 80 and 350 mL/min, respectively.

2.3.6 Determination of natural diseases severity.

The occurrence of spontaneous diseases was assessed by visual inspection. Each fruit was rated into a 6-point severity scale: 1 (no visible disease), 2 (1% to 20% of the proportional area affected), 3 (21% to 40%), 4 (41% to 60%), 5 (61% to 80%) and 6 (81% to 100% of area affected), (Nunes et al, 2011; Romanazzi et al. 2013). For Experiment 1, samples of Lot 1 were assessed after 5 and 6-days storage and those from Lot 2 after 9, 11, and 14 days. Analyzes in Experiment 2 were performed after 5 days of storage at room temperature (Lot 1) and for Lot 2, after 5, 10 stored at 16 °C and more 3 days at room temperature. The data were analyzed as nonparametric tests of significance.

2.3.7 Fungal inoculation and coating protection.

Colletotrichum gloeosporioides obtained from USDA-ARS collection was grown on potato dextrose agar in Petri dishes at 25 °C for 7–14 days. The spore suspensions were prepared to give a final concentration of 1×10^{6} spores mL^{-1}. A total of 72 fruits was sanitized, as described before, and divided into two lots of 36 samples each. Fruit from the first lot were superficially wounded twice by piercing on the equator region (1 mm × 2 mm in length) and inoculated by dropping 10 µL of the conidial suspension into the injuries. After incubation at 20 °C for 24 h (Fagundes et al., 2014), they

were hand-coated with the formulations displayed in Table 1 in exception of microemulsion 9 %. The remained 36 non-inoculated samples were also coated by way of comparison. All samples were stored at 20 °C for 11 days and 80% RH. Assays were carried out using four replicates for each treatment. Determination of diseases severity were recorded after 4, 6, 11 days based on 6-point category scale.

2.3.8 Sensory analysis.

Fruits from Experiment 2 were subject to sensory evaluation. The analyzes were carried out at room temperature under fluorescent light in samples stored 5 days at room temperature and after 13 days of simulated marketing conditions. For flavor evaluation, papayas were peeled, seeds removed, and the pulp cut in cubic pieces of approximately 2 cm^3. Samples were presented with a three-digit randomized code each on a plate. A total of 37 panelists ranked attributes as sweetness, sourness, papaya flavor, fermented and off-flavor perception. A 10-point hedonic scale (1 to 10 - extreme) was used. Fifteen and twenty-two panelists took place in the flavor evaluation of the 5^{th} and 13^{th} day, respectively.

2.4 Statistical analyzes

The univariate parametric analyzes of variance (ANOVA) and multiple comparisons Duncan or Tukey tests were carried out by using the IBM SPSS Statistics software (Inc., Chicago, IL). The comparison between disease severity scores was performed using nonparametric ANOVA and adequate

multiple comparisons of Kruskal-Wallis, Friedman, or Wilcoxon. For sensory analyzes, the scores were compared by means of nonparametric ANOVA and Mann-Whitney U Wilcoxon test. All tests considered the significance level of 5%.

4. Results and discussion

4.1 Coating characterization

4.1.1 Surface wettability

According to the wetting analysis, the surface hydrophobicity decreases for coated papaya compared to uncoated, except when coated with GEO or Nano 18% which had preserved similar angles to that measured in the control fruits. HPMC itself possesses a hygroscopic nature due to the high density of polar functional groups, mainly hydroxypropoxy groups ($OCH_2CH(OH)CH_3$), and a higher hydrophilicity (low contact angles) was fully expected (Figure 1).

Amongst carnauba Nano 18%, Nano 9%, and Nano 9%+GEO no statistical differences were found. However, the incorporation of 1% (w/v) HMPC into the carnauba solution resulted in a reduction of nearly 40% in the hydrophobicity of the coatings compared to those without HPMC (Figure 1). Excluding GEO, that slightly increased the hydrophobicity, all the other formulations resulted in more hydrophilic surfaces than the original papaya skin. Anyway, by consisting in an additional barrier, effect in retarding moisture loss is expected over treatments.

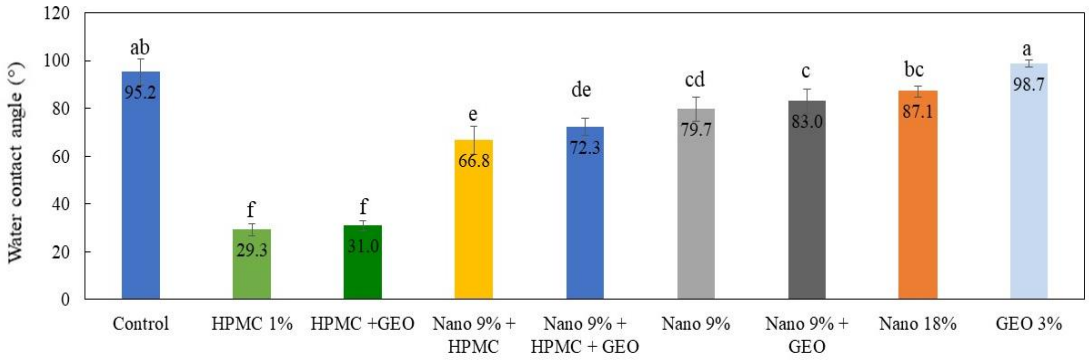

Figure 1. Water contact angle on uncoated skin papaya and coated papaya surfaces, (mean ± SD, n=5).

4.1.2 Particle size and zeta potential

Neat carnauba Nano 18 and 9% formulation are nano-sized in droplet diameters of approximately 40 nm, and when combined with HPM or GEO the diameter size of particles in suspensions increased (Table 2). Geo emulsion coating at 3% resulted in the biggest drop size, around 400 nm. All the coatings presented zeta potential values higher than |30| mV (Table 2), indicating colloidal stability according to Attama et al. (2007).

Table 2. Size and zeta potential of coating formulations.

Coatings Identification	Size (d.nm) Mean ±SD	Zeta Potential (mV) Mean ±SD
Nano 9%	40.6 ± 1.0	- 52.2 ± 4.2
Nano 18 %	39.6 ± 1.0	-62.5 ± 19.2
Nano 9 % + HPMC	122.6 ±13.0	-57.1 ± 13.4
GEO 3%	398.6 ± 39.0	-31.4 ± 2.3
Nano 9% + GEO	140.1 ± 2.1	-85.3 ±3.0
Nano 9% + HPMC + GEO	148.3 ± 40.2	-73.5 ±3.6

4.1.3 Scanning Electron Microscopy

The SEM micrographs of uncoated papaya skin are presented in Figure 2A, B and C. Films formed from carnauba at 18% emulsion (Figure 1E) showed a more compact matrix compared with carnauba 9% (Figure 2H) and Nano 9 + GEO (Figure 3A and B), where cracks can be evidenced on film (Figure 2F and I). Coatings contain HPMC also demonstrated a consolidation of uniform matrix, confirming its film forming capacity (Figure 3E and H), however detachments from papaya skin (Figure 3 D) and coating ruptures (Figure 3C) were observed. Cracks and detachments interfere in the barrier property by allowing the increase of gas exchange (Baldwin, 1995). The closer microstructural observations reveal surface roughness, what is consequence of the velocity of solvent evaporation.

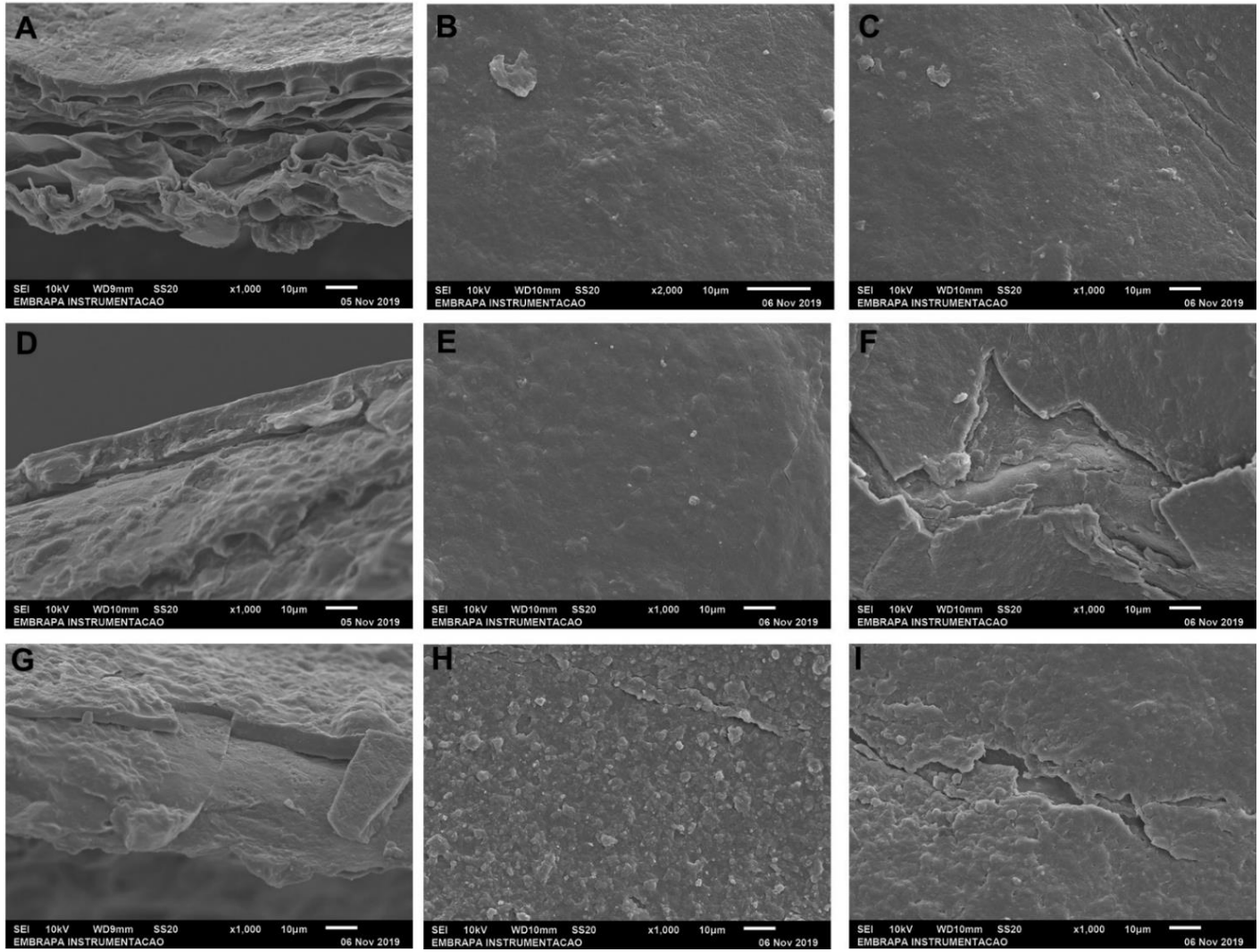

Figure 2. Field emission gun scanning electron microscopy (FEG-SEM) of papaya skin uncoated (A, B and C) and coated with Nano 18% (D, E and F) and Nano 9% (G, H and I).

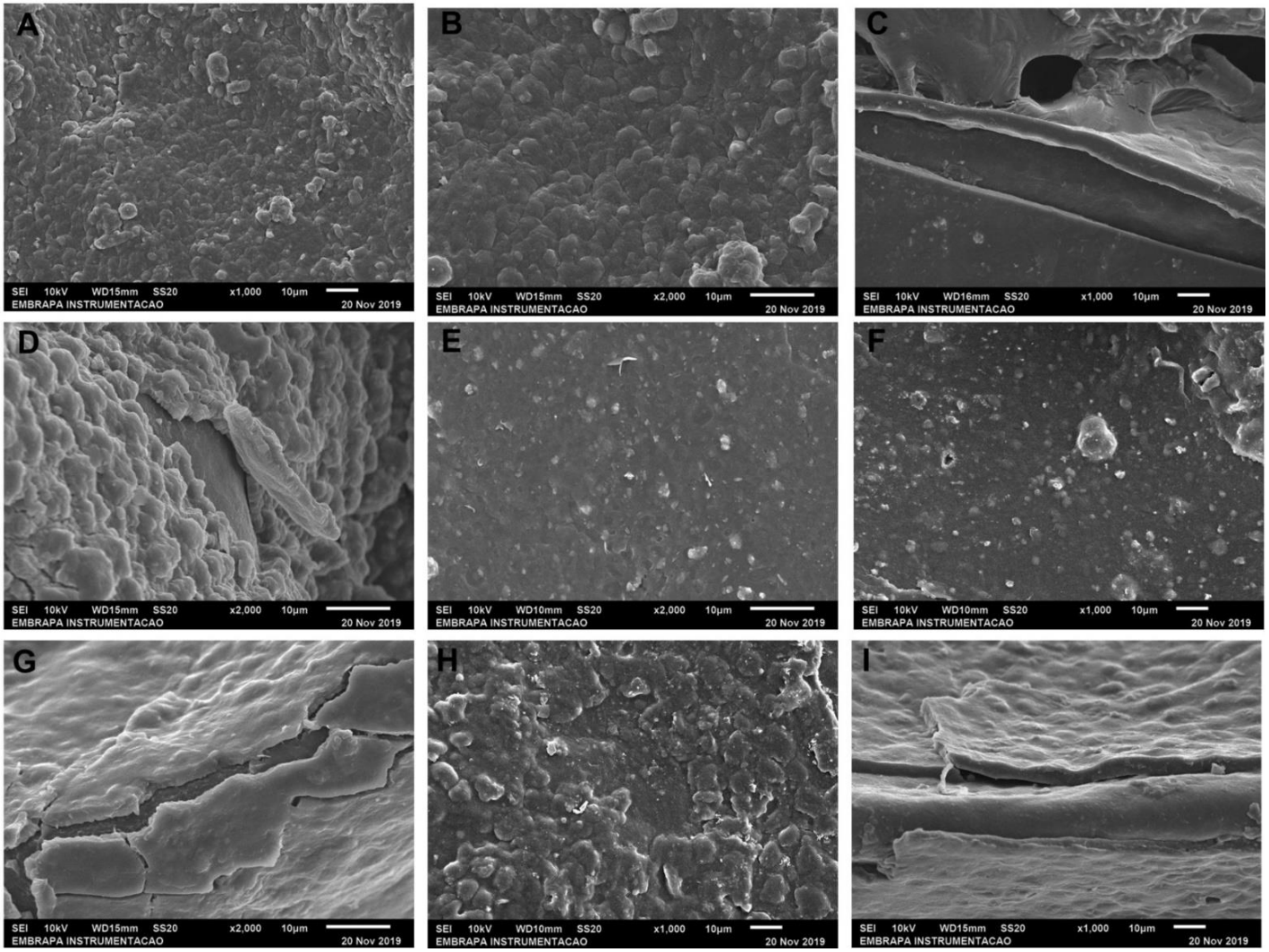

Figure 3. Field emission gun scanning electron microscopy (FEG-SEM) of papaya skin coated with Nano 9% + GEO (A and B), Nano 9% + HPMC (C), HPMC (D and E), Micro 9% (F and G), and Micro 9% + HPMC (H and I).

4.2 Postharvest Evaluation

4.2.1 Weight loss

The measurement of papayas from Experiment 1 revealed that all coating formulation acts positively in reducing the weight loss. The fruits treated with nanoemulsions resulted in better mass conservation along the storage time, particularly under refrigeration. When maintained at room temperature, after 6 days, all samples presented weight loss (Figure 4A). The carnauba-based coatings performed better and similarly by reducing nearly 74% the mass loss compared to the control samples. The neat HPMC resulted in the lowest effect as a barrier to water loss (30%), what was expected considering its hydrophilic nature, as already pointed out by Baldwin et al. (1997).

Under refrigerated storage (at 13 °C), several physiological and biochemical processes are slowed down, reducing by consequence, the transport of moisture through the skin. In such conditions all samples behaves similarly with no statistical significances between carnauba based coatings (mass reductions were all below 1%).

When transferred to room temperature (22 °C), after 5 days of marketing simulation, significant differences were recorded (Experiment 1, Figure 4 A). In the uncoated fruits (control) a reduction of weight superior to 10% was measured. The coated samples behave accordingly: inferior protection for neat HPMC and similar performance for carnauba emulsions-based coatings. The average reductions imparted by the coatings in this condition were around 70% for Lot 1, and 53% for Lot 2, when compared to

respective controls. Overall, coating with 18% of solid carnauba nanoparticles in suspension performs better than 9%.

Concerning Experiment 2 (Figure 4B), a similar tendency was observed. The measurement of weight after 5 days at room temperature, proved the efficiency of carnauba nanoemulsion, the highest for 18%, in reducing water loss. The pattern is repeated in the samples maintained under refrigeration. Proportionally, the highest reductions were found in samples from the lot that alternated between refrigeration and market simulation. For these mass losses were greater than 10%, as measured to uncoated samples, and around 4 % for samples coated with the carnauba-based formulations. It is worth noticing that in all conditions the application of neat GEO did not result in any effective barrier against water migration. Despite no significant statistical differences, it is evident that the carnauba concentration in suspension interferes on the barrier properties, as tendency of reduction of water loss (from 9 to 18% m/v), as visually observed in Figure 4B.

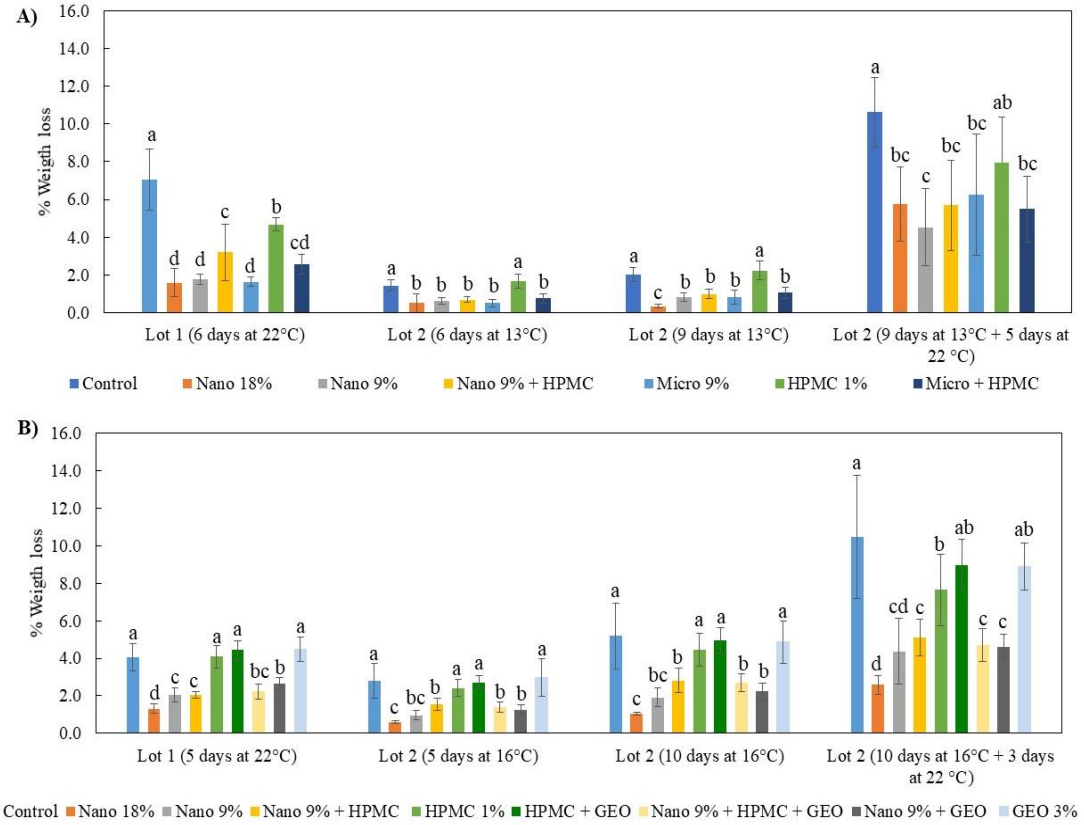

Figure 4. Weight loss (%) of 'Redland' papaya fruit with different coatings related to experiment 1 (A) and experiment 2 (B), for each storage period. Columns with different letters are significantly different by Duncan test ($p<0.05$). Nano: carnauba wax nanoemulsion coating. Micro: carnauba wax microemulsion coating. HPMC: hydroxypropyl methylcellulose coating, and GEO: ginger essential oil nano-emulsion.

4.2.2 Soluble solids (SS), titratable acidity (TA), pH, and ratio SS/TA.

The first experiment resulted in no statistical differences between lots for measured values of SS and pH, regardless of treatments or storage conditions (Figures 5 A and C, respectively). The SS values are the same in all samples indicating that there was no significant variation in the sugar concentration due to the different treatments. Similar results were reported by Azene et al. (2011) in papaya fruits packaged and stored under different conditions. Schweiggert et al. (2011) also reported slight SS increasing, from

10.5 to 10.8° Brix, in red fleshed papaya at different postharvest stage maturation. Sucrose, glucose and fructose are the main sugars in papaya with gradually increased until the fruit is fully ripe (Selvaraj et al.1982). The increasing of solid soluble during ripening is hypothesized as mechanism of cell wall disassembly providing a source of carbon for sugar synthesis (Schweiggert et al. 2011; Fabi et al, 2007; Lazan et al. 1995). It is important noticing that in all treatments samples the content of SS measured was below 11° Brix (Figure 5A), characterizing unripe fruits, according to ideal maturity stage for commercialization as established by the Brazilian Technical Regulation on Quality of Papaya (BRASIL, 2010). Nevertheless, a tendency of increasing the pH values over time (Figure5C), is expected and indicates an ongoing ripening process, in which the citric and malic acid (the most common acids in papaya) is diminished (Schweiggert et al. 2011; Selvaraj et al.1982).

Concerning the amount of titratable acidity (TA), it was observed that after 6 days at 22 °C, the fruits coated with HPMC resulted in the lowest TA, similar to the uncoated samples (Figure 5B). In general, the neat HPMC coating has inferior effectiveness in delaying the rate of TA decreasing in all essayed conditions (Figure 5B). On the other hand, the carnauba Nano 9% performed better as protective coating between treatments, particularly after refrigerated storage and 5 days market simulation. In such condition the carnauba Nano 9% coating preserved around 30% more of TA when compared to the average values as measure to other coatings. A decrease in titratable acidity is related to maturity development, so proportional higher TA

values are interpreted as an action in retarding ripeness. The efficiency presented by the carnauba formulation is related to a better formation of a semi-permeable coating on the fruit's surface, which modify the internal atmosphere by decreasing O_2 and increasing CO_2 production, as pointed out by De Medeiros et al., 2012.

The SS/TA ratio is an important indicator of fruit quality, ordinary used to assess the balance of sweet/sour. In all experimental conditions it is observed a large fluctuation of SS/TA ratio with increase values with time (Figure 5D). According to Schweiggert et al. (2011), the ratio (TSS/TA) found for red fleshed papaya at pre-harvest stage increased from 55 to 85 and 105 during maturation, indicating the attainment of a balance equilibrium.

Concerning Experiment 2 (Figure 6), after 5 days of room temperature storage, fruits coated with ginger essential oil (GEO) resulted in the low values of TA associated to high pH (low acidity), Figure 6 (B and C). The amount of soluble solids (SS) is statistically the same for all samples with exception to those coated with GEO, with resulted in a lower SS after 5 days at 22°C. The fruits coated with Nano 9%, have higher average amount of TA and consequently the lower SS/TA. On the other side, treatments with HPMC showed intermediate values, depending on the treatment combination (Figure 6B and D).

After 10 days at cold storage (16 °C), no differences regarding TA contents can be observed between treatments. However, when undergo market simulation (10 days at 13°C plus 3 days at room temperature), small differences are detected with respect to titratable acids, having the Nano 9%

coating a little higher content and neat GEO with a little low (Figure 6B). In general, the differences between treatments were not sufficient for a reliable estimation of the optimum coating in preventing biochemical activity.

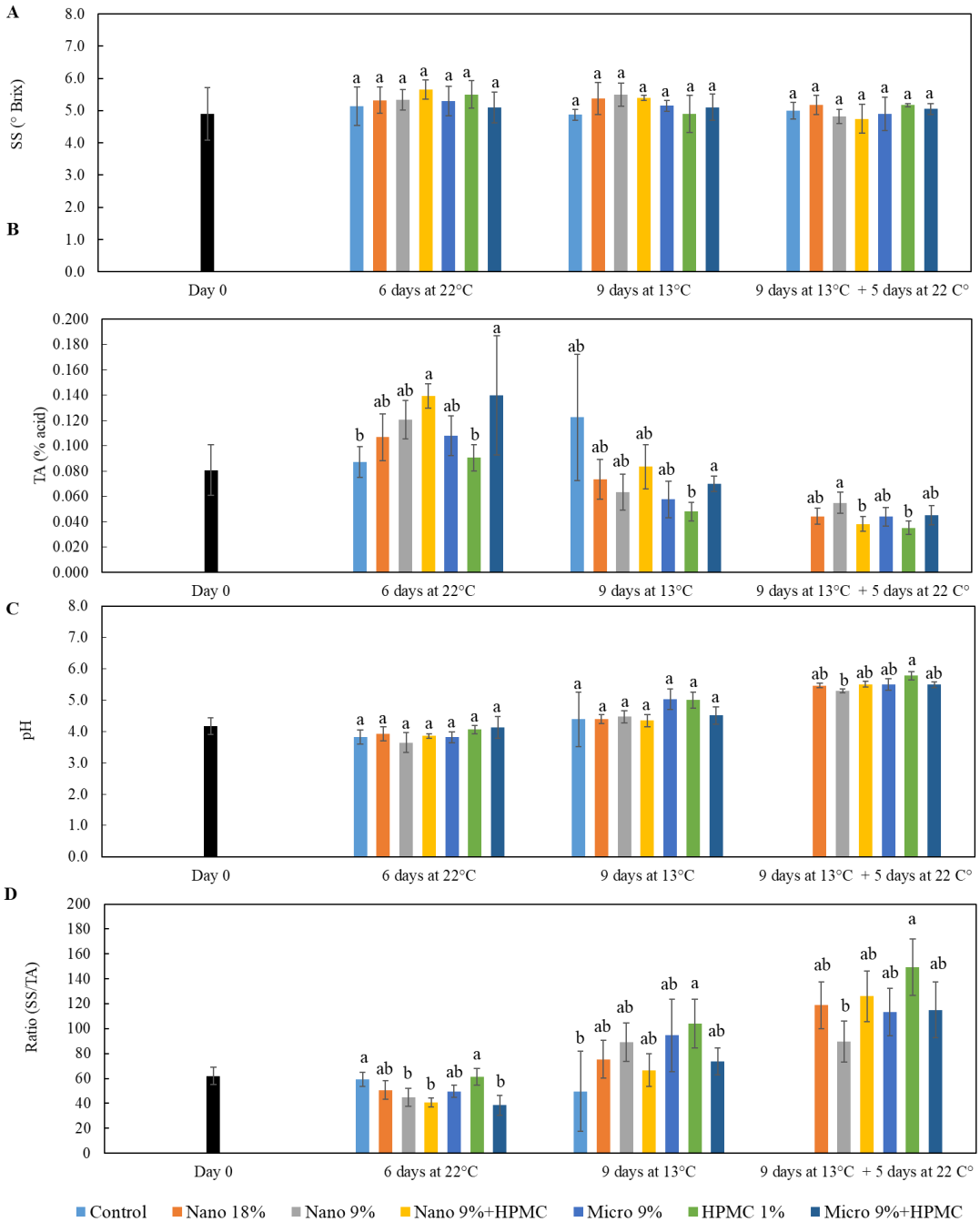

Figure 5. A) SS – solid soluble; **B**) TA – titratable acid; **C**) pH; **D**) ratio – SS/TA values for uncoated papaya and coated with different coatings at experiment 1. Columns with different letters are significantly different by Tukey or Games Howell (according to homogeneity of variances) applied after ANOVA one way ($p<0.05$) within the storage period.

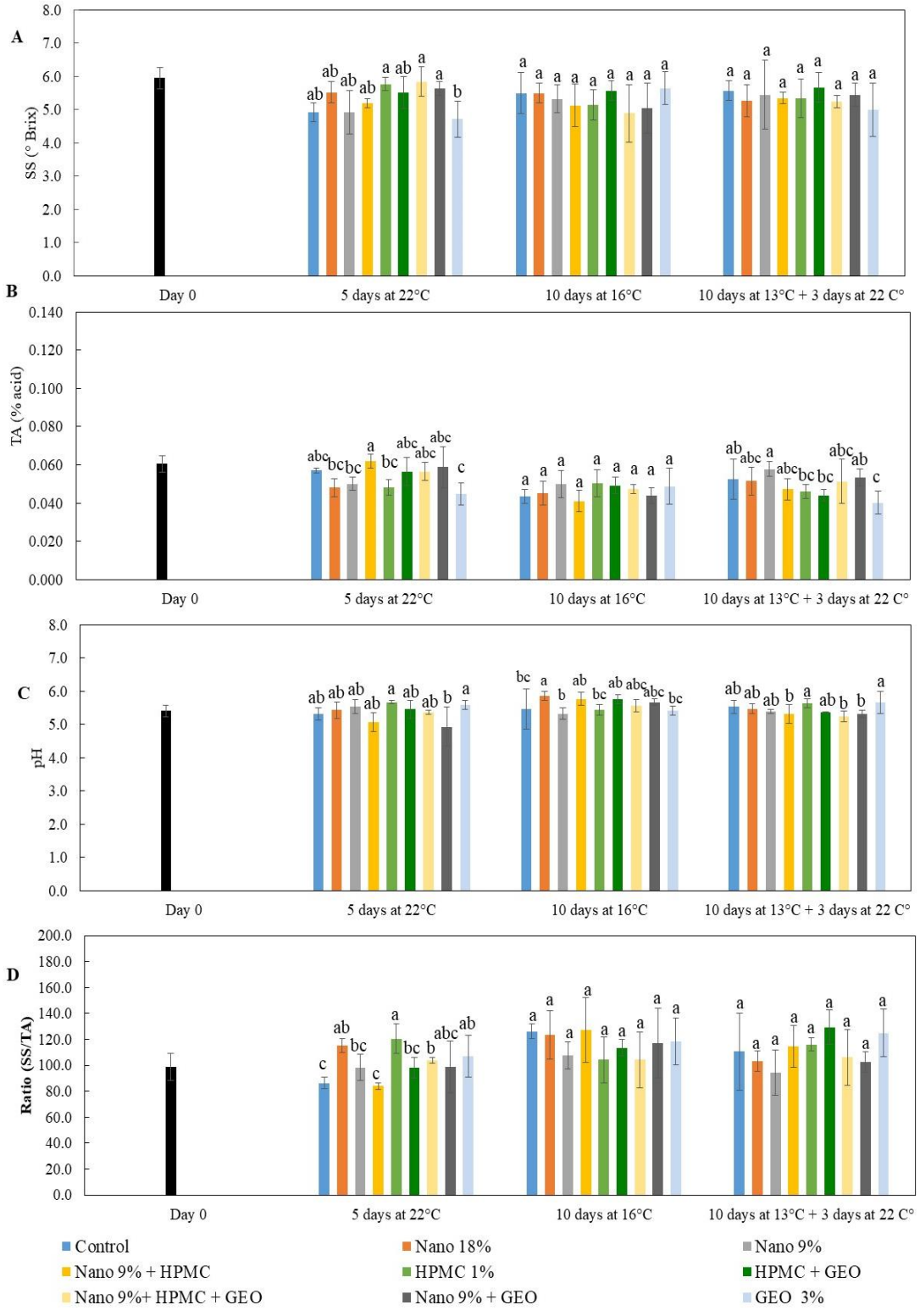

Figure 6. A) SS – solid soluble; **B**) TA – titratable acid; C) pH; **D**) ratio – SS/TA values for uncoated papaya and coated with different coatings at experiment 2. Columns with different letters are significantly different by Tukey or Games Howell (according to homogeneity of variances) applied after ANOVA one way ($p<0.05$) within storage period.

4.2.3 Flesh firmness

The flesh firmness of the fruits was influence by treatments (at $p>0.05$), as graphically represented in Figure 7. The Lot 1 from the Experiment 1 was the most affected, showing after 6 days stored at 22 °C, firmness reduction, from baseline (71 N), to an average value inferior to 30 N.

Several enzymes, such as polygalacturonase, β-galactosidase, and pectinesterase are related to ripening and the cell wall degradation occurs due to the pectin and hemicellulose hydrolysis. Such process is assumed as the main responsible for the pulp softening, with consequence decreasing papaya firmness during maturation (Fabi et al., 2007; Lazan et al, 1995).

In formulations having HPMC, a rapid loss is measured, with exception of the combination with Nanoemulsion at 9%. Samples coated with Nano 18% also retarded firmness loss, however the large standard deviations (error bars) in both measurements point to a high variability of coating effectiveness.

The fruit firmness has a close relationship to temperature. When stored at 13°C (Lot 2), papayas present a higher stability of firmness along the conservation period. Fluctuations in values between samples reflected by large standard deviations in the average values, were also recorded. The samples coated with Micro 9% + HPMC resulted in the highest averages with the smallest deviations, indicating better action upon firmness preservation.

After cold storage (13 days) and market simulation for 5 days at 22°C, the samples are significantly softer, confirming the effect of temperature in increasing ripening rate e consequently a significant loss of firmness are

registered (Figure 7A). Nano 9% + HPMC showed the highest firmness value, significantly different from HPMC and control.

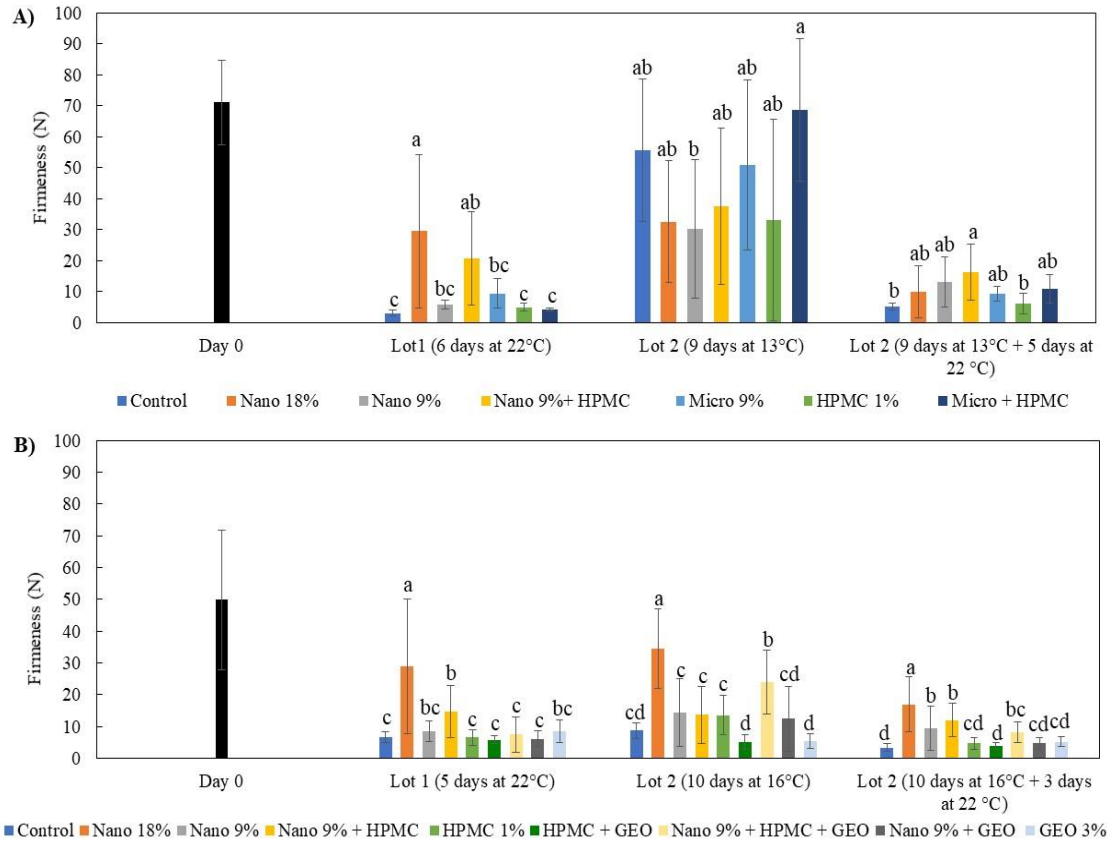

Figure 7. Flesh firmness (N) of 'Redland' papaya fruit coated with different coatings at experiment 1 (A) and experiment 2 (B). For each storage period, columns with different letters are significantly different by Duncan test ($p<0.05$), applied after ANOVA. Nano: carnauba wax nanoemulsion coating, Micro: carnauba wax microemulsion coating; HPMC: hydroxypropyl methyl cellulose coating and GEO: ginger essential oil nanoemulsion.

In Figure 7B are the results from Experiment 2. All samples in Lot 1 underwent reductions in the values of firmness. In these, the results are similar to those obtained for Experiment 1. Nano 18% coatings also resulted in a better reduction in softening of the papaya tissue, however large standard deviations were also registered.

Under refrigeration, after 10 days at 16 °C (Lot 2), the Nano 18% formulation was confirmed as the treatment with a better retention in firmness

(preserving 66% of the original value), followed by Nano 9% + HPMC (with 45 % of the initial value). All the other treatments presented a significant decrease, with firmness losses superior to 75%.

At the end of marketing condition (3 days at 22°C after 10 days stored at 16°C), the room temperature was decisive in accelerating the loss of firmness, independent of the formulation. In such condition, the Nano 18% still appears as the better coating. No positive effect was found in associating GEO to formulations concerning firmness maintenance.

4.2.4 Skin color

Change in visual color is a direct result from the fruit's ripening. In papaya, the alterations are characterized by an increase of lightness (L*), pointing to a gradual ripening of the skin. The enhance of L* value is a natural result from the synthesis of carotenoids and chlorophyll degradation (decrease in green color), characteristic of most fruit ripening (Schweiggert et al., 2011).

As summarized in Table 3, for Experiment 1 the reduction in lightness on papaya skin is evident for lot 2, in most treatments, in storage time and temperature conditions essayed. After cold storage, the neat HPMC coatings showed no effect on original lightness preservation, with values similar to the control. When incorporated nanostructured carnauba to the HPMC, some lightness is also maintained, as compared to control values. Statistical analysis showed a significant difference at $p<0.05$, particularly for those coated with the formulation having the higher concentration of carnauba

nanoparticles (18%) (Lot 1) and Nano 9%, (Lot 2 – 9 days at 13º C), Table 3. Different responses to light incidence, for different concentrations were expected considering the proportional number of nanoparticles dispersed in the coating and the complex chemical composition of carnauba wax (Trezza & Krochta, 2000).

In experiment 2, the samples of Lot 1 after 5 days at 22 °C, presents the highest values of lightness. For these also the coatings composed of Nano 18% and Nano 9% + GEO resulted in the low values, which can indicate a slowing in the ripening process. The other treatments resulted in intermediated lightness. For Lot 2, after 5 days of cold stored (16 °C), the fruit's skins had no differences (at $p<0.05$) among treatments. For a longer storage time (10 days at 16 °C), small significant differences are measured with the lowest lightness L* values on samples coated with Nano 9% and Nano 9% + HPMC + GEO formulations. After marketing simulation (10 days at 16 °C followed by 3 days at 22 °C), most treatments resulted in low values to L* compared to control, however, no significant changes were found between treatments, except for HPMC coating which resulted in the lowest L*, significantly different from Nano 9% and its association with GEO (Nano 9% + GEO).

Table 3. Skin lightness (L*) of Redland papaya fruit coated with different formulations in experiment 1 and 2.

Treatments	**Lightness**			
Experiment 1	**Lot 1 (6 days at 22 °C) Average ± SD**	**Lot 2 (6 days at 13 °C) Average ± SD**	**Lot 2 (9 days at 13 °C) Average ± SD**	**Lot 2 (14 days*) Average ± SD**
Control	59.8 ± 3.7 a	46.3± 6.8 a	46.7 ± 5.7 ab	54.8 ± 1.3 a
Nano 18%	46.8 ± 3.8 d	44.8 ±1.8 ab	45.8 ± 3.3 ab	51.2 ± 3.8 ab
Nano 9%	56.7 ± 4.8 ab	41.0 ± 2.0 b	40.5 ± 1.9 c	49.7± 3.4 ab
Nano 9% + HPMC	51.0 ± 3.5 cd	46.2 ± 3.7 a	47.5 ± 3.9 a	49.8 ± 6.2 ab
Micro 9%	53.5 ± 4.8 bc	43.5 ± 2.9 ab	44.0 ± 1.7 abc	54.5 ± 5.4 a
HPMC	59.8 ± 4.5 a	44.3 ± 3.1 ab	45.3 ± 2.7 ab	52.8 ± 6.3 a
Micro 9% + HPMC	54.2 ± 3.1 bc	42.8 ± 2.9 ab	42.7 ± 2.9 bc	47.0 ± 2.0 b
Experiment 2	**Lot 1 (5 days at 22 °C)**	**Lot 2 (5 days at 16 °C)**	**Lot 2^{z} (10 days at 16 °C)**	**Lot 2^{z} (13 days**)**
Control	58.9 ± 3.7 a	50.8 ± 6.4 a	54.2 ± 4.6 ab	58.0 ± 2.6 ab
Nano 18%	51.2 ± 4.9 b	47.1 ± 5.1 a	48.8 ± 2.8 ab	52.2 ± 6.8 bc
Nano 9%	54.2 ± 4.9 ab	51.8 ±3.2 a	51.9 ± 5.1b	60.6 ± 1.6 a
Nano 9% + HPMC	53.9 ± 1.6 ab	48.7 ± 4.7 a	53.2 ± 3.1ab	51.3 ± 4.1 bc
HPMC	53.6 ± 2.8 ab	46.5 ± 7.2 a	52.8 ± 5.1ab	48.6 ± 4.2 c
HPMC + GEO	55.6 ± 4.8 ab	51.0 ± 7.0 a	57.2 ± 2.7ab	54.3 ± 3.6 abc
Nano 9% + HPMC + GEO	49.4 ± 6.6 ab	46.9 ± 4.4 a	49.2 ± 5.2 b	52.3 ± 4.8 bc
Nano 9% + GEO	55.0 ± 4.6 b	48.1 ± 5.3 a	53.8 ± 4.9 ab	59.2 ± 2.3 ab
GEO	55.4 ± 7.8 ab	46.6 ± 7.7 a	57.9 ± 5.3 a	53.0 ± 5.5 abc

*9 days at 13 °C followed by 5 days at 22 °C (simulated marketing storage)
** 10 days at 16 °C followed by 3 days at 22 °C
Columns with different letters are significantly different by Duncan and Tukey ^{z} test ($p<0.05$) applied after ANOVA

During fruit ripening the level of different types of carotenoids determines the skin color and its intensity. In papaya ripening a relationship between the color of the peel and total carotenoids could be established through the Chroma quantification (Singh & Rao, 2011), as displayed in Table 4. During fruit storage, both Experiment 1 and 2 showed the tendency of chroma values to increase. The control samples and those coated with HPMC have the higher chroma as an indicative of a more advanced stage of maturation. The better conservative effect was attained by Nano 9% and Nano 18% coatings by presenting the lowest chroma values. This pattern is observed in all stored conditions. Nano 9% + HPMC, Micro 9% and Micro +

HPMC coatings also have some effect in delaying fruit maturation, however not as efficient as the nanoemulsions formulations.

In Lot 2 (Experiment 1), after 6 days at 13°C, the Nano 9% was the only coating that presented statistical differences among the treatments, slowing down the papaya ripening in this condition. After 9 days at 13°C, the differences between samples are reduced with slight benefits for samples coated with 9% carnauba emulsions (Table 4). After marketing simulation, the Micro 9% + HPMC coating performed better in maintaining the chroma at low values.

Concerning the Experiment 2, Lot 1 at 22°C, the control (uncoated fruits) and those coated with GEO, resulted in accelerated development of color yellow/orange (highest chroma). In these, the chroma value was lower for Nano carnauba-based coatings (Nano 18% and Nano 9% + HPMC + GEO). The other treatments were in intermediate values of chroma. After 5 days at 16°C no statistical differences were computed between samples. At the end of marketing period, the HPMC coating resulted in the lowest chroma, followed by Nano 18% and Nano 9% + HPMC. Based in color saturation evaluation (chroma), one can say that coatings of nanoparticulated carnauba, in association or not with HPMC, showed to be more efficient in preserving original papaya skin color along fruit maturing in almost all stored conditions here evaluated.

Table 4. Chroma (C*) variation of Redland coated papaya fruits under different stored conditions.

Treatments	Chroma			
Experiment 1	**Lot 1 (6 days at 22 °C) Average ± SD**	**Lot 2 (6 days at 13 °C) Average ± SD**	**Lot 2[z] (9 days at 13 °C) Average ± SD**	**Lot 2 [z] (14 days*) Average ± SD**
Control	53.4 ± 5.7 a	34.2 ± 5.1 a	33.6 ± 6.1 ab	44.7± 2.0 ab
Nano 18%	34.1 ± 4.6 d	32.1 ± 2.5 a	33.8 ± 4.0 ab	39.2 ± 4.4 abc
Nano 9%	47.7 ± 7.6 ab	24.0 ± 2.2 b	28.2 ± 3.0 c	36.6 ± 5.5 bc
Nano 9% + HPMC	39.7 ± 4.4 cd	35.1 ± 4.3 a	38.3 ± 4.4 a	38.1 ± 8.8 abc
Micro 9%	44.4 ± 7.9 bc	30.1 ± 3.4 a	28.9 ± 2.9 bc	43.6 ± 8.4 ab
HPMC	54.9 ± 8.1 a	34.4 ± 5.0 a	35.7 ± 3.7 a	45.3 ± 8.3 a
Micro 9% + HPMC	43.0 ± 4.6 bc	30.4 ± 3.1a	28.7 ± 4.0 bc	32.2 ± 3.2 c
Experiment 2	**Lot 1 (5 days at 22 °C)**	**Lot 2 (5 days at 16 °C)**	**Lot 2 [z] (10 days at 16 °C)**	**Lot 2 [z] (13 days**)**
Control	52.6 ± 5.6 a	40.2 ± 7.6 a	44.3 ± 5.7 ab	49.6 ± 4.1 a
Nano 18%	39.2 ± 5.5 bc	35.2 ± 6.3 a	34.9 ± 3.8 b	39.9 ± 9.0 bc
Nano 9%	44.3 ± 7.6 abc	41.1 ± 4.0 a	41.0 ± 6.3 ab	53.0 ± 3.7 a
Nano 9% + HPMC	42.5 ± 2.0 bc	36.7 ± 6.3 a	41.6 ± 3.9 ab	39.5 ± 6.1 bc
HPMC	45.9 ± 3.7 abc	35.3 ± 8.4 a	43.5 ± 6.7 ab	35.8 ± 4.7 c
HPMC + GEO	44.4 ± 5.8 abc	40.7 ± 8.2 a	49.1 ± 2.5 a	45.7 ± 5.0 ab
Nano 9% + HPMC + GEO	37.1 ± 9.3 c	34.9 ± 6.0 a	36.6 ± 6.4 b	40.8 ± 6.4 bc
Nano 9% + GEO	45.0 ± 7.0 abc	38.2 ± 5.6 a	44.1 ± 6.9 ab	52.5 ± 3.2 a
GEO	46.5.2 ± 9.5 ab	34.9 ± 10.1 a	49.9 ± 7.0 a	41.6 ± 9.4 bc

*9 days at 13 °C followed by 5 days at 22 °C (simulated marketing storage)
** 10 days at 16 °C followed by 3 days at 22 °C
Columns with different letters are significantly different by Duncan and Tukey [z] test ($p<0.05$) applied after ANOVA

By means of hue angle, it is possible to evidence the skin color transformation from green to yellow. That happens along time, in which the hue angle measured on papaya skin decreases during storage, exhibiting higher values at initial stages (related to green peel) and a tendency to low values at the end of storage period (development of yellow and orange color), (Schweiggert et al. 2011).

For experiment 1, at end 6 days at room temperature (Lot 1), it is evident the color alteration for uncoated fruits (lower hue angle), compared to the other treatment (Table 5). For all samples, the coating resulted in some delay in color development, most evident for Nano 18% followed by Nano 9%

+ HPMC coatings. For Lot 2, after 6 and 9 days under refrigeration (13°C), color changes are almost indistinguishable between samples. However, after market simulation, most of the original color is preserved on nano carnauba-based coatings, with exception of Micro 9%+HPMC, indicating a better color maintenance. Control resulting in the lowest values in hue and worst color conservation.

Table 5. Hue angle (h*) measured on Redland papaya fruits, coated and uncoated, at different storage conditions (Experiment 1 and 2).

Treatments	**Hue**			
Experiment 1	**Lot 1 (6 days at 22°C) Average ± SD**	**Lot 2 (6 days at 13°C) Average ± SD**	**Lot 2 (9 days at 13°C) Average ± SD**	**Lot 2 (14 days*) Average ± SD**
Control	83.3 ± 5.5 d	108.6 ± 11.3 b	111.8 ± 10.3 a	88.6 ± 4.4 c
Nano 18%	111.2±6.6 a	114.8 ± 3.6 ab	112.3 ± 6.5 a	104.3 ± 4.3 ab
Nano 9%	91.2 ± 6.8 c	120.2 ± 1.6 a	118.1 ± 2.7 a	104.2 ± 6.0 ab
Nano 9% + HPMC	103.3 ± 5.1 ab	113.5 ± 5.7 ab	112.5 ± 4.5 a	104.6 ± 10.6 ab
Micro 9%	99.8 ± 9.5 bc	115.5 ± 3.5 ab	116.0 ± 3.8 a	100.9 ± 7.0 b
HPMC	94.0 ± 6.6 c	114.2 ± 5.4 ab	112.9 ± 4.9 a	101.0 ± 7.5 b
Micro 9% + HPMC	93.5 ± 6.4 c	116.1 ± 3.7 ab	116.9 ± 4.3 a	110.6 ± 2.4 a
Experiment 2	**Lot 1 (5 days at 22 °C)**	**Lot 2 (5 days at 16 °C)**	**Lot 2 (10 days at 16 °C)**	**Lot 2 (13 days**)**
Control	90.0 ± 5.2 b	106.0 ± 7.6 ab	96.9 ± 6.2 bcd	85.9 ± 6.7 d
Nano 18%	108.5 ± 10.6 a	114.9 ± 8.4 a	110.9 ± 3.1 a	100.4± 13.6 a
Nano 9%	98.2 ± 8.3 ab	102.8 ± 8.6 ab	97.6 ± 8.7 bc	83.2 ± 3.2 d
Nano 9% + HPMC	104.0 ± 3.9 a	109.1 ± 9.2 ab	99.0 ± 4.0 bc	101.6 ± 7.6 a
HPMC	101.0 ± 7.2 ab	112.2 ± 7.3 ab	100.2 ± 6.9 b	104.0 ±5.5 a
HPMC + GEO	97.1 ± 12.2 ab	101.4 ± 14.8 b	91.0 ± 6.1 cd	90.4 ± 7.2 bcd
Nano 9% + HPMC + GEO	110.2 ±13.3 a	113.8 ±7.4 a	108.7 ± 8.3 a	97.8 ± 9.0 ab
Nano 9% + GEO	97.4 ± 11.9 ab	109.7 ± 8.0 ab	99.2 ± 6.7 bc	86.5 ± 3.7 cd
GEO	102.6 ± 10.9 ab	112.6 ± 9.3 ab	89.1 ± 7.0 d	95.5 ± 5.4 cd

*9 days at 13 °C followed by 5 days at 22 °C (simulated marketing storage)
** 10 days at 16 °C followed by 3 days at 22 °C
Columns with different letters are significantly different by Duncan test ($p<0.05$) applied after ANOVA

In samples of Experiment 2, in Lot 1 and 2, the overall results were quite similar. The control samples turn yellow faster (low hue values) and Nano 18% can be considered the most effective coating in delaying orange color development, followed by Nano 9%+HPMC and Nano + HPMC + GEO coatings. Neat Nano 9% after marketing condition surprisingly resulted in low

hue, similar to that measured on control. HPMC coating also showed good response in reducing color changes during ripening. The overall appearance is shown at appendice 1 for experiment 1 (Figures 1 to 3) and experiment 2 (Figures 4 to 6).

4.2.5 Internal CO_2, O_2 and ethylene contents.

4.2.5.1 Internal CO_2 and O_2

On Experiment 1, the variation of internal gases (%), shows for Lot 1 (after 6 days at 22°C), an increasing of CO_2 with concomitant reduction in the O_2 level during storage (Figure 8A and B). The higher percentage of CO_2 with low O_2 was found in samples treated with Nano 9% + HPMC formulation. The lowest concentration of internal O_2 was measured in samples coated with Nano 18%, indicating the effect of the carnauba nanoparticles concentration in the formation of less permeable coatings (restricting the O_2 uptake). These results are in agreement to colorimetric analysis in which these samples showed the highest hue angle related to a delay in color development (reduced ripening).

For Lot 2, after 9 days at 13 °C, an increasing in internal O_2 is observed for all treatments with proportional reduction in the CO_2 content. The decrease in CO_2 concentration is expected in fruits under refrigerated storage, due to a reduction of respiration rates when in cold environments. In this storage condition the uncoated samples show high level of gas exchange, with high concentration of internal O_2 and low CO_2. At low temperature, the Nano 9% and Micro 9% were not effectives in providing a

protective O_2 barrier, however, Nano 18% and Nano 9% + HPMC showed to be more efficient for that. The control with low CO_2 internal content along with high O_2 is characteristic of a normal respiratory process.

After refrigeration for 13 days and 5 days at 22 °C, it is clear the influence of the temperature on papaya respiration. Low levels of internal O_2 are measured with a parallel growth on CO_2, mainly inside coated samples. Coatings with low permeability, which retain a large concentration of CO_2 could favor an anaerobic/fermentative environment and increase in the of ethanol production (Baldwin et al., 1994, Hagenmaier, 2000; Hagenmaier & Baker, 1994). Nevertheless, edible coatings normally present different degrees of permeability, even for a similar formulation. That occurs due to inevitable formation of irregular structures and thickness during film consolidation, as observed and commented by McHugh and Krochta, 1994.

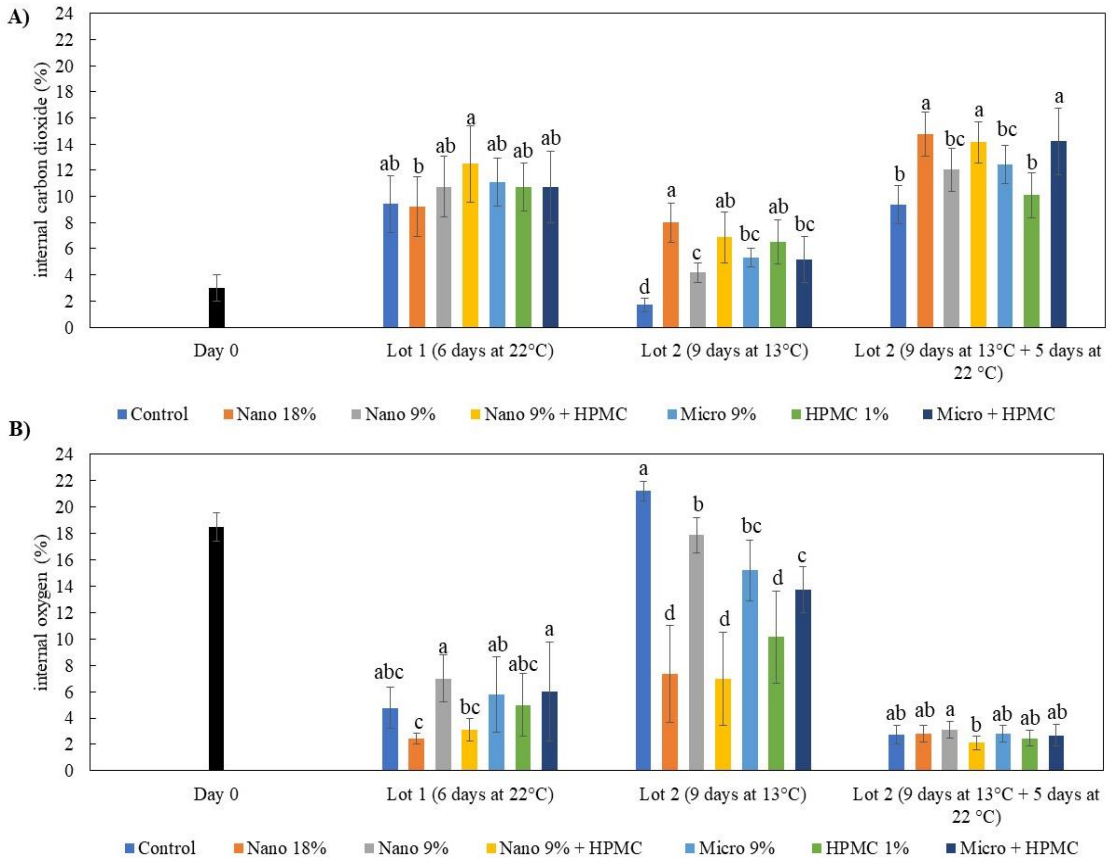

Figure 8. Internal CO_2 (A) and O_2 (B) of 'Redland' papaya fruit coated with different coatings at experiment 1. For each storage period, columns with different letters are significantly different by Duncan and Tukey ^{z} test ($p<0.05$), applied after ANOVA. Nano: carnauba wax nanoemulsion coating, Micro: carnauba wax microemulsion coating; HPMC: hydroxypropyl methyl cellulose coating.

In Experiment 2, it is possible to observe that the use of neat GEO as coating did not form any barrier to gases, as values statistically equal to the uncoated controls (Figure 9). When kept along 5 days at room temperature, the Nano 18% coating significantly reduced the internal content of O_2 as compared to control samples. When stored for 10 days at 16 °C, the internal gas concentrations are found to be similar to those measured in Experiment 1 (9 days at 13 °C). More accentuated fluctuations are recorded to samples which underwent 3 days market simulation after 10 days stored at 16°C. CO_2 and O_2 content vary among samples. Control and GEO coating resulted in

the lower level of CO_2 and the highest measured for the Nano 18% coating, with the others taking intermediates positions. Lipid-based coatings, such as carnauba wax, are more hydrophobic than HPMC, and more effective against moisture/water loss, as confirmed by the firmness measurements and expected to acts better as barrier to gas exchange (Assis et al., 2008; Lin & Zhao, 2007). Based on these analyses one can draw the conclusion that nanoemulsion and microemulsion of carnauba, as protective coating on papaya, reduce respiration rate delaying ripening in several condition of storage. On the other hand, if it causes CO_2 accumulation, may allow fermentation that can result in off-flavors.

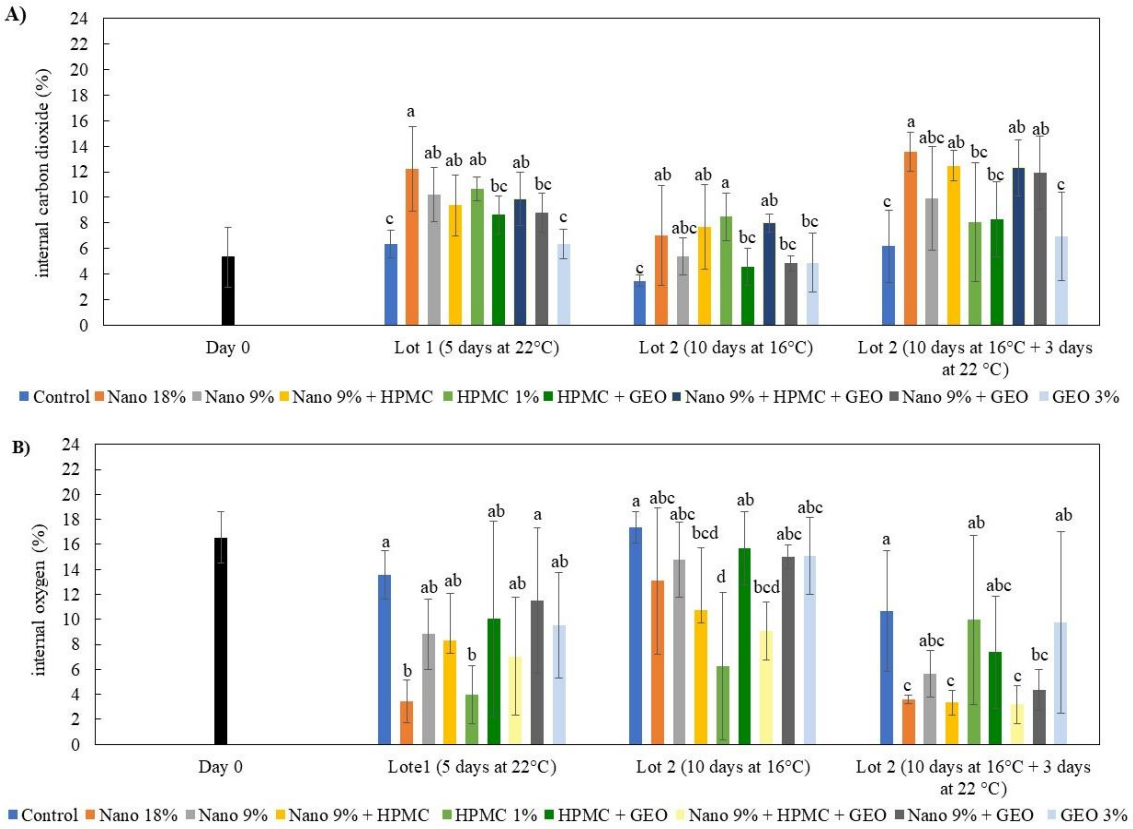

Figure 9. Internal CO_2 (A) and O_2 (B) of 'Redland' papaya fruit coated with different coatings at experiment 2. For each storage period, columns with different letters are significantly different by Duncan test ($p<0.05$), applied after ANOVA. Nano: carnauba wax nanoemulsion coating, Micro: carnauba wax microemulsion coating; HPMC: hydroxypropyl methyl cellulose coating and GEO: ginger essential oil nanoemulsion.

4.2.5.2 Ethylene

Papaya is a climacteric fruit with endogenous ethylene gas (C_2H_4) playing a fundamental role in the regulation of the ripening process. Generally, the ethylene production increases simultaneously with the maximum respiration rate up to the climacteric peak, from which C_2H_4 levels started to decrease (Singh & Rao, 2011).

As measured for Experiment 1 (Figure 10 A), the ethylene production is significantly increased during storage after at 22 °C. After 6 days the level rises from a baseline of 0.46 ppm to an average concentration of 5 ppm (Lot

1). Despite of the high error bars in the individual results, the numerical levels of ethylene was not statically different in coatings. However, Nano 18% showed a trend to lower values than other treatments, along with the lowest O_2, as measured before. It is well-known that ethylene production is oxygen dependent, and low levels of internal O_2 may cause a reduction in ethylene production (Kader, 1983).

At low-temperature storage (Lot 2), as expected, the physiological activities are reduced and consequently also the ethylene generation. All samples in this condition (after 9 days at 13 °C) presented similar levels of ethylene (mean value was 1.2 ppm), comparable to the baseline, with slight statistical differences at $p<0.05$. Highlighting just HPMC coating that showed a tendency to a little higher production. When submitted to market simulation (additional 5 days at 22 °C) after 9 days at 13 °C, the ethylene levels naturally increase, reaching an average concentration of 4.4 ppm. For these, the carnauba Nano 9% + HPMC seems to perform better with inferior levels of ethylene.

In Experiment 2, ethylene increases after storage at 22 °C for 5 days was also expected. The initial level of 1.69 reached 4.48 ppm after the period. In fruits coated with HPMC + GEO highest level of ethylene were measured, which may be related to advanced maturity staged. The other coatings positioned in intermediate levels. Cold temperature was essential to slow-down ethylene production to an average content of 2.8 ppm after 10 days at 16 °C and after simulated marketing conditions (additional 3 days at 22 °C), the levels increased again, reaching values of 5.86 ppm (Figure 10B).

Experiment 2 had cold storage temperature changed by 13 °C to 16°C It was observed that after 9 days at 13 °C the level of ethylene reached 0.6 ppm amongst treatments, and after 10 days at 16 °C the average was 2.8 ppm, what is considerable for an increment of just 3 °C.

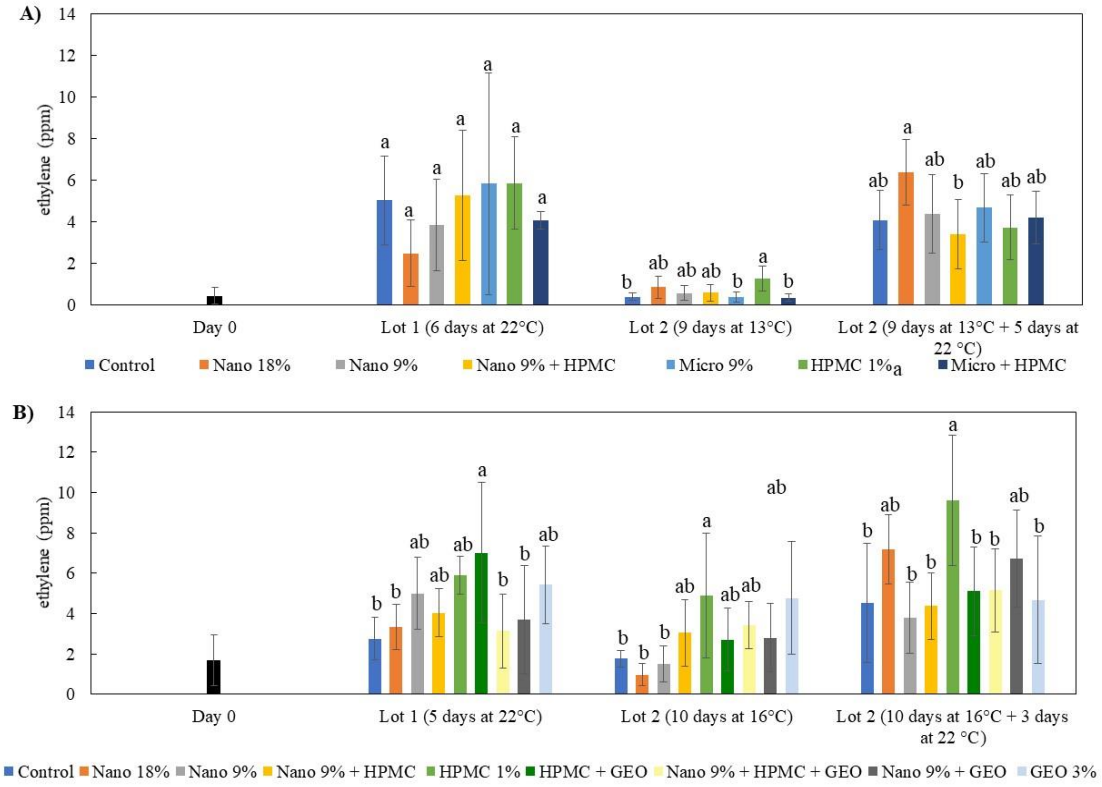

Figure 10. Internal ethylene (ppm) of 'Redland' of papaya fruits with different coatings, as measured for Experiment 1 (A) and Experiment 2 (B). For each storage period, columns with different letters are significantly different by Duncan and Tukey [z] test ($p<0.05$), applied after ANOVA.

4.2.6 Sensory analyzes

The evaluation of fruits by panelists was conducted in samples from Experiment 2, Lot 1 after 5 days at room temperature and Lot 2 after 10 days at 16 °C + 3 days at 22 °C (13 days). For each treatment the data was obtained from all measured attributes (sweetness, sourness, papaya flavor,

fermented flavor, and another off-flavor), and separately analyzed. The intensity scores are presented in Figures 11 and 12, as the averaged for each attribute within type of coating. Mean values of all attributes did not differ significantly, according to the Mann-Whitney U Wilcoxon test applied for two independent groups (samples from Day 5 and from Day 13). Such indicates that no differences were perceptible by panelist among all treatments.

The initial aim of the present sensory evaluation was a tentative to verify the perception in recognizing any off-flavors developed in coated papayas, what was not evidenced. By reducing the group of treatments and increasing the number of panelists, maybe small differences could be pointed out. Undesirable flavor due to internal synthesis of ethanol, acetaldehyde or butanoic acid was expected, although not noticed.

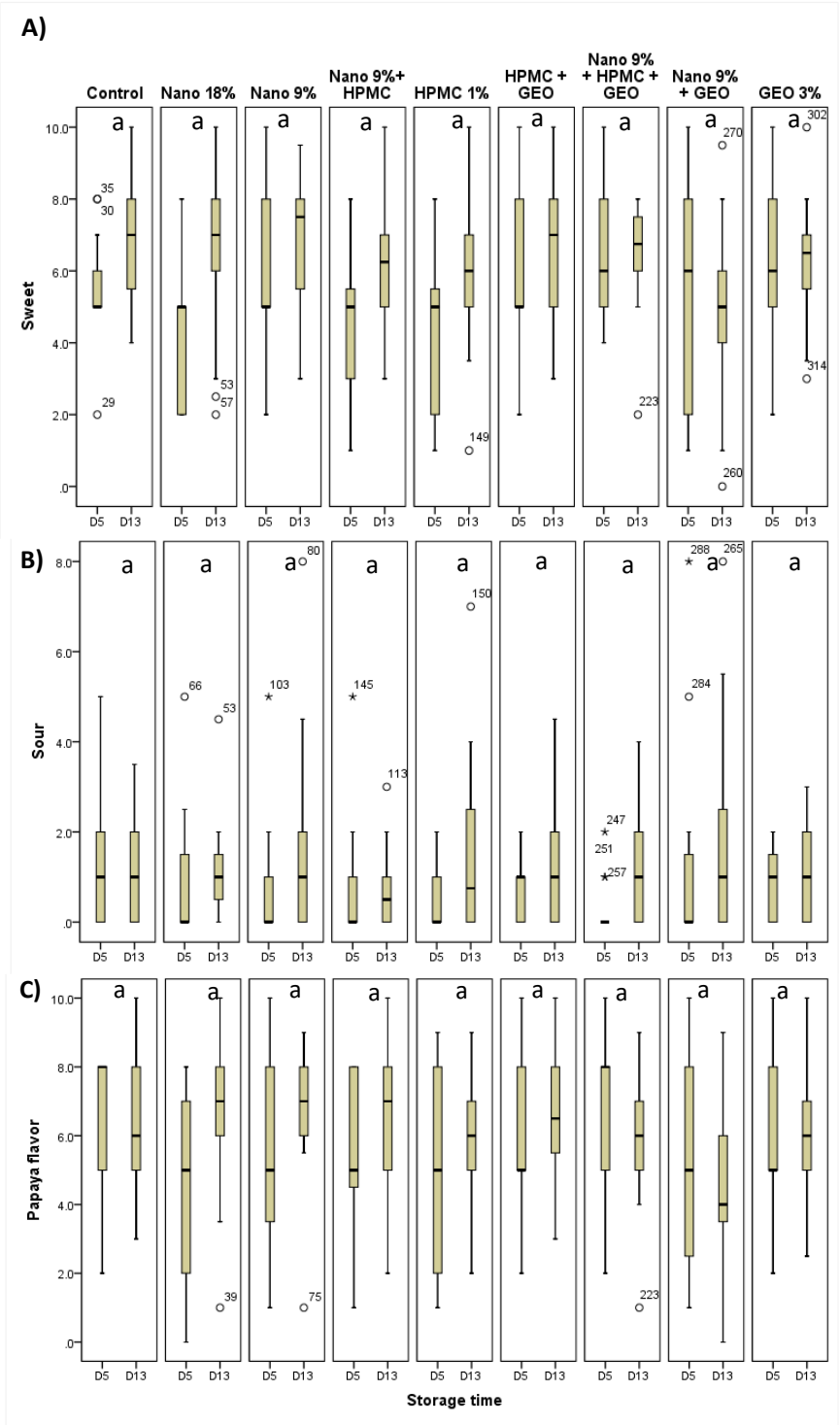

Figure 11. Sensory analyze score of 'Redland' papaya fruit coated with different coatings at experiment 2 for **(A)** sweetness, (**B)** sourness and **(C)** papaya flavor at the end of Lot 1 storage (D5: 5 days at 22 °C) and Lot 2 (D13: 10 days at 16 °C followed by 3 days at 22 °C). For each treatment, columns with same letters are not significantly different by Mann-Whitney U Wilcoxon ($p<0.05$) noun parametric ANOVA test applied for 2 independent samples (Day 5 and Day 13). Nano: carnauba wax nanoemulsion coating; HPMC: hydroxypropyl methyl cellulose coating and GEO: ginger essential oil nanoemulsion.

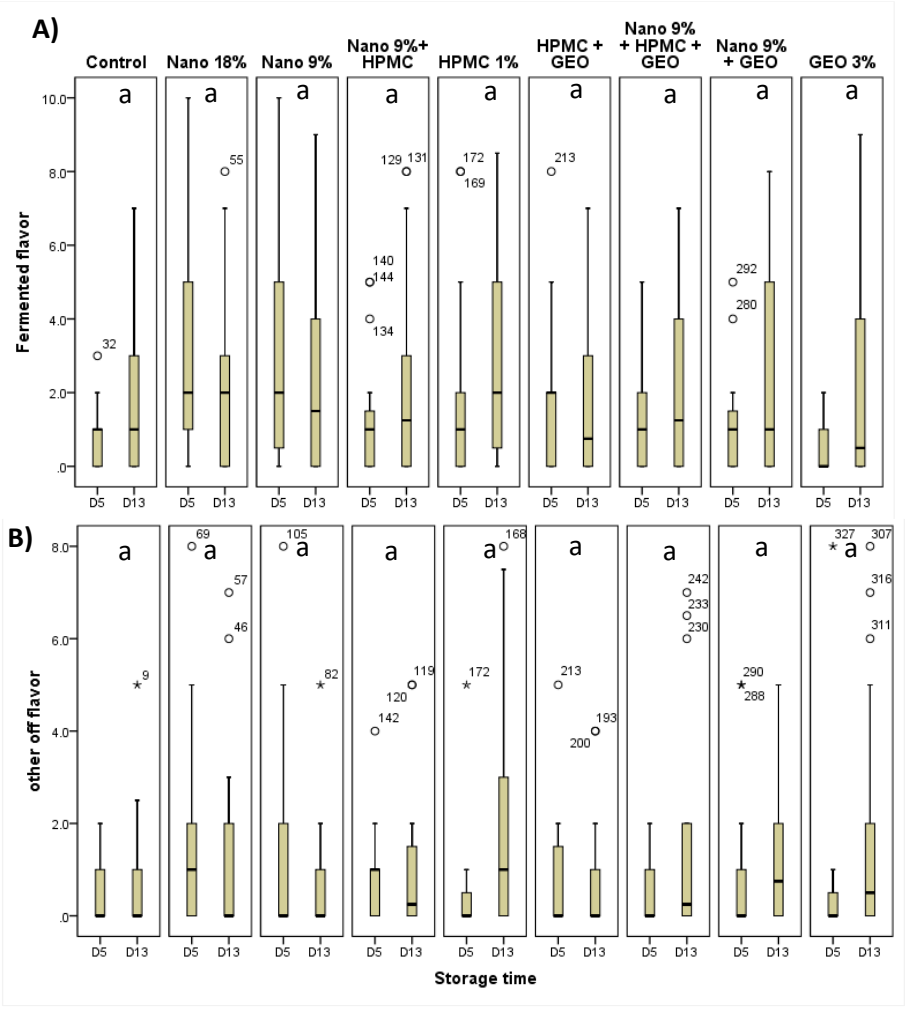

Figure 12. Sensory analyze score of 'Redland' papaya fruit coated with different coatings at Experiment 2 for **(A)** fermented flavor and **(B)** other off-flavor at the end of Lot 1 storage (D5: 5 days at 22 °C) and Lot 2 (D13: 10 days at 16 °C followed by 3 days at 22 °C). For each treatment, columns with the same letters are not significantly different by Mann-Whitney U Wilcoxon ($p<0.05$) noun parametric ANOVA test applied for 2 independent samples (Day 6 and Day 13). Nano: carnauba wax nanoemulsion coating; HPMC: hydroxypropyl methyl cellulose coating and GEO: ginger essential oil nanoemulsion.

4.3 Effect of coatings on disease development.

4.3.1 Natural diseases severity for Experiments 1 and 2

According to visual assessment, a high severity of natural decay was recorded on samples from Experiment 1 during storage of Lot 1 and Lot 2, Figure 13. By storing at room temperature (Lot 1) (Figure 13 A), all samples

undergo deterioration over time, however the uncoated fruits resulted in a higher severity compared to other treatments. Although the reasons are not clear, maybe due to the structural heterogeneities formed during coating, the results indicate that the associating of HPMC to carnauba micro and nanoemulsions impact negatively in the reduction of natural decay. Pure carnauba coatings (Nano 18%, 9%, and Micro) performed quite better in preventing papayas peel from diseases development, even at room temperature (Figure 13A).

For Lot 2, after 9 days at cold temperature storage (13 °C), no visual difference in the level of severity was possible to be identified among treatments. When submitted to simulated marketing conditions (Day 14), the uncoated papayas have the highest diseases severity.

In these conditions the Nano 9% followed by Nano 9 % + HPMC coatings resulted in the lowest severity (Figure 13B) evidencing a positive effect in protecting papaya peel from natural diseases.

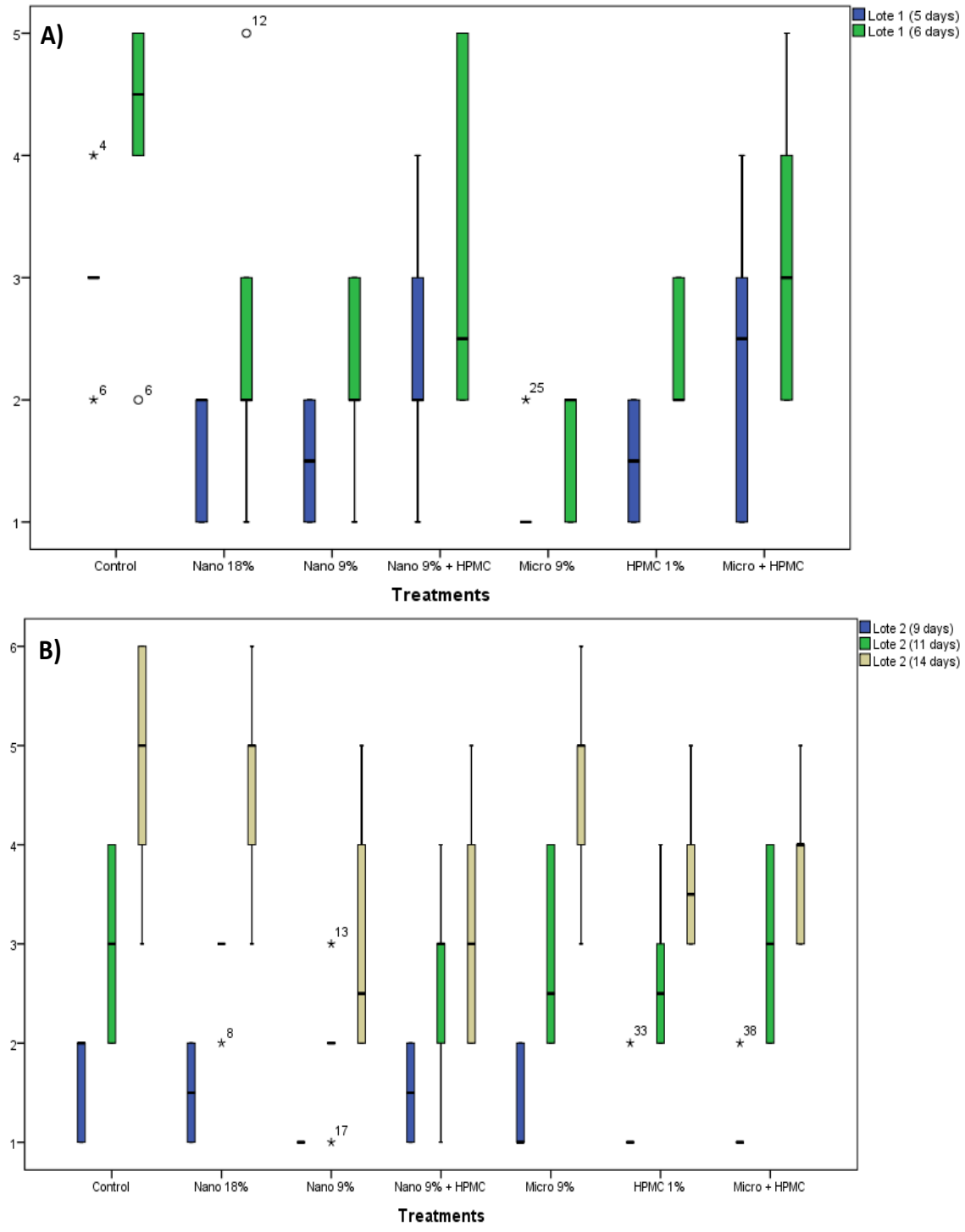

Figure 13. Natural diseases severity of 'Redland' papaya fruits with different coatings from Experiment 1, as ranked by visual assessment, after **(A)** room temperature storage (22 °C) and **(B)** cold storage followed by simulated marketing condition (9 days at 13 °C + 5 days at 22 °C). Nano: carnauba wax nanoemulsion coating; Micro: carnauba wax microemulsion coating; HPMC: hydroxypropyl methyl cellulose coating.

The same trend was observed to samples from Experiment 2, either in Lot 1 or Lot 2. In Figure 14A are shown the results of Lot 1 after 5 days at room temperature. The fruits coated with neat GEO and HPMC, or in their combinations, presented higher intensity of diseases than those observed to uncoated fruits. Coatings containing carnauba wax nanoemulsion with and without GEO, was evaluated with inferior diseases severity.

For Lot 2, after 5 days at 16 °C, the fruits did not showed differences in severity intensities (Figure 14B). After 10 days, the samples coated with Nano18%, Nano 9% + HPMC or Nano 9% + HPMC + GEO resulted in the lowest diseases severity. Tendency for a more intense fungal severities was observed in the control samples (uncoated fruits), as well as in HPMC coatings with or without GEO, and oil alone treatment. It is interesting to note that the combination of HPMC with GEO, has not resulted in any reduction on diseases severity. The lowest diseases severity, after simulated marketing conditions, was observed to papayas coated with Nano 18% and Nano 9% + HPMC + GEO.

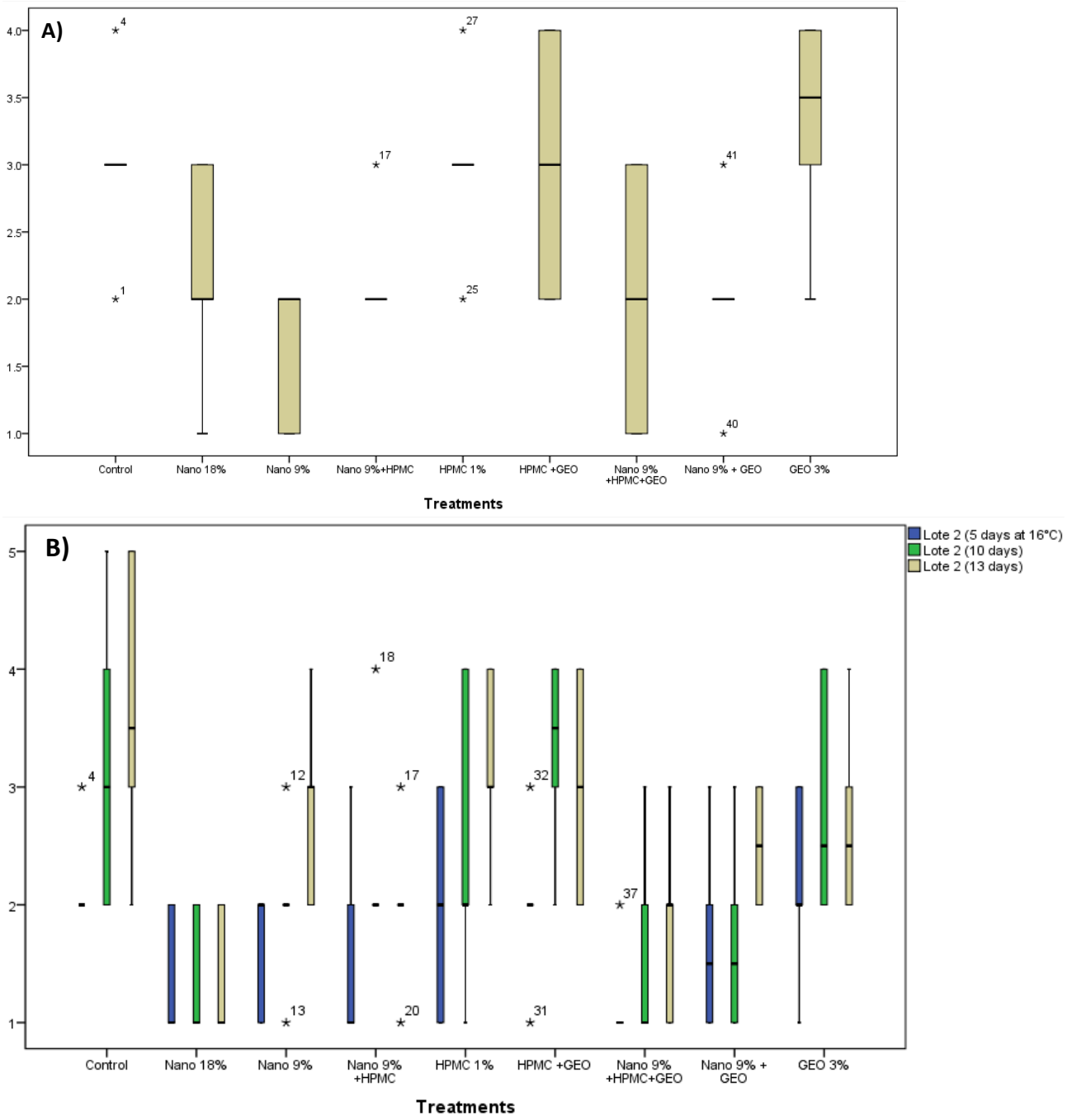

Figure 14. Natural diseases severity of 'Redland' papaya fruit coated with different coatings at Experiment 2 after 5 days at 22 °C **(A)** and **(B)** cold storage followed by simulated marketing condition (10 days at 16 °C + 3 days at 22 °C). For **A** there is no significantly differences by Kruskal-Wallis test for 9 independent samples. For each treatment in **B**, there is no significantly differences by Friedman test for 3 related samples (5 days, 10 days and 13 days. Nano: carnauba wax nanoemulsion coating; Micro: carnauba wax microemulsion coating; HPMC: hydroxypropyl methyl cellulose coating and GEO: ginger essential oil nanoemulsion.

4.3.2 Protection against inoculated *Colletotrichum gloeosporioides* fungus.

In Figure 15 is compared the intensities of natural decays in papayas with fruits inoculated with *C. gloeosporioides*, (Lot A: natural diseases and Lot B: diseases on inoculated samples). The results showed no statistical differences considering the Friedman test run for 3 groups of samples (after different times of incubation: Day 4, Day 6 and Day 11). However, independent of the formulation, all treatments showed a tendency in reducing the severity of natural post-harvest decay. The Nano 18% coatings appears to be more effect, followed by Nano 9% and Nano 9% + HPMC + GEO coating (Figure 15A).

The analyses carried out on inoculated fruits with *C. gloeosporioides*, (Figure 15B) confirmed the preventive effect of carnauba nanoemulsion coatings (at 9 and 18% particles dilutions) in inhibiting the development of *C. gloeosporioides* on papayas. Contrary to what was expected, by having active agents in its composition, the ginger essential oil (GEO) did not present any antifungal action against the inoculated microorganism at 1×10^{6} spores mL^{-1}.

The synergic action of GEO in association to carnauba wax nanoemulsion, as observed in the previous experiment (natural decay), was not repeated on inoculates fruits.

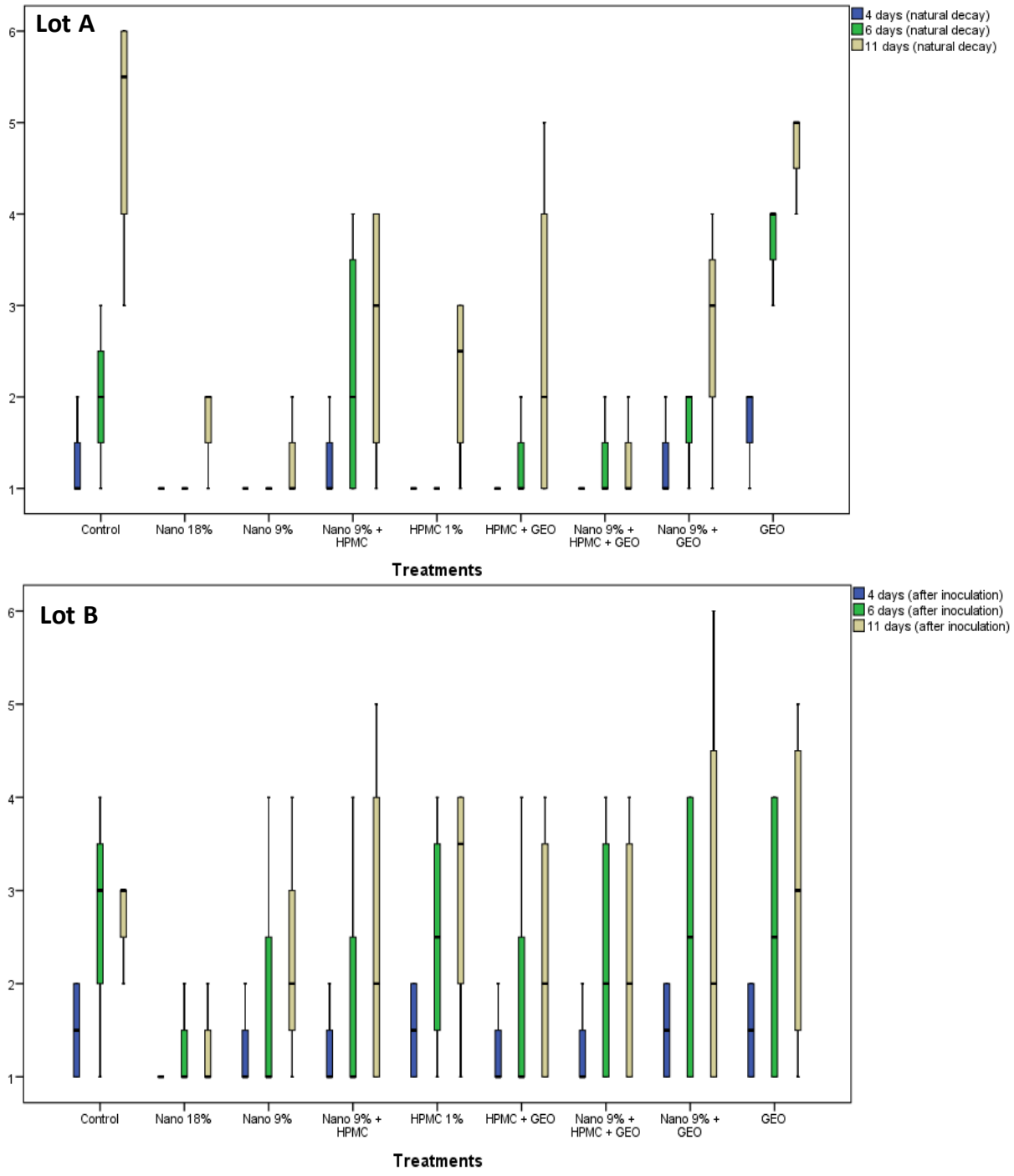

Figure 15. Disease severity of 'Redland' papaya fruit coated with different coatings storage for 11 days at 22 °C. **Lot A -** natural diseases severity for noun inoculated fruits and **Lot B** - Inoculated fruits with *Colletotrichum gloeosporioides* for severity decay. Non-parametric ANOVA were applied and there is no statistical difference by Friedman test for 3 related samples (Day4, Day6 and Day11) for lot A or B.

5. Conclusions

The coatings promote extension of postharvest shelf life and quality maintenance. The mostly effective formulation in retarding the ripening process was the carnauba 18% nanoemulsion suspension, which presented beneficial effect in terms of reducing loss of firmness, as well as in the formation of a gas barrier slowing fruit respiration, either in room temperature or under refrigeration. Coating with nanoemulsions (9% and 18%) also resulted in better skin color maintenance at the different storage conditions evaluated.

HPMC combined to nanoemulsions resulted effective protection with clear delay on the papaya ripening. Such effect can be attributed to the presence of particulate carnauba in the coatings. The carnauba emulsions also proved a better barrier to gas exchange, providing low internal O2 levels, which contributes to slowing the ripening process.

According to sensory evaluations changes on flavor were not perceived by the panelists. Treatment with ginger essential oil (GEO) did not acted was expected. No substantial protection or antimicrobial activity was achieved. When associated to carnauba nanoemulsions a positive effect was observed in reducing natural diseases occurrence over time. However no synergic activity was not record on inoculated C. gloeosporioides fungus on fruits.

In short, by considering the overall results, it is evident that the application of carnauba nanoemulsions-based coatings, results in a positive

effect in improving significantly the quality and shelf life, by reducing weight loss, color changes and slowing maturity in papayas.

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Considerações Finais

Os óleos de gengibre resultaram em maior atividade antifúngica que os extratos, sendo os mais indicados para serem empregados no controle de fungos associados a revestimentos. A combinação de nanoemulsão e GEO, em revestimentos protetores, foi mais eficaz em experimentos *in vitro* que *in vivo*.

A incorporação de GEO nos revestimentos promoveu a redução de doenças naturais, quando combinado com a nanoemulsão de cera de carnaúba. A nanoemulsão de carnaúba resultou em maior conservação da qualidade pós-colheita e do sabor das tangerinas nas condições experimentais utilizadas. As coberturas com nanoemulsões de cera de carnaúba foram mais adequadas na proteção e na manutenção da qualidade pós-colheita dos mamões em condições de armazenamento e de mercado.

Os resultados mostraram que a aplicação de nanoemulsão, isolada ou combinada com HPMC e GEO, tem ação na manutenção da qualidade de tangerinas e mamões.

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Apêndice - Aspecto geral da coloração dos mamões nos experimentos 1 e 2, capítulo 4.

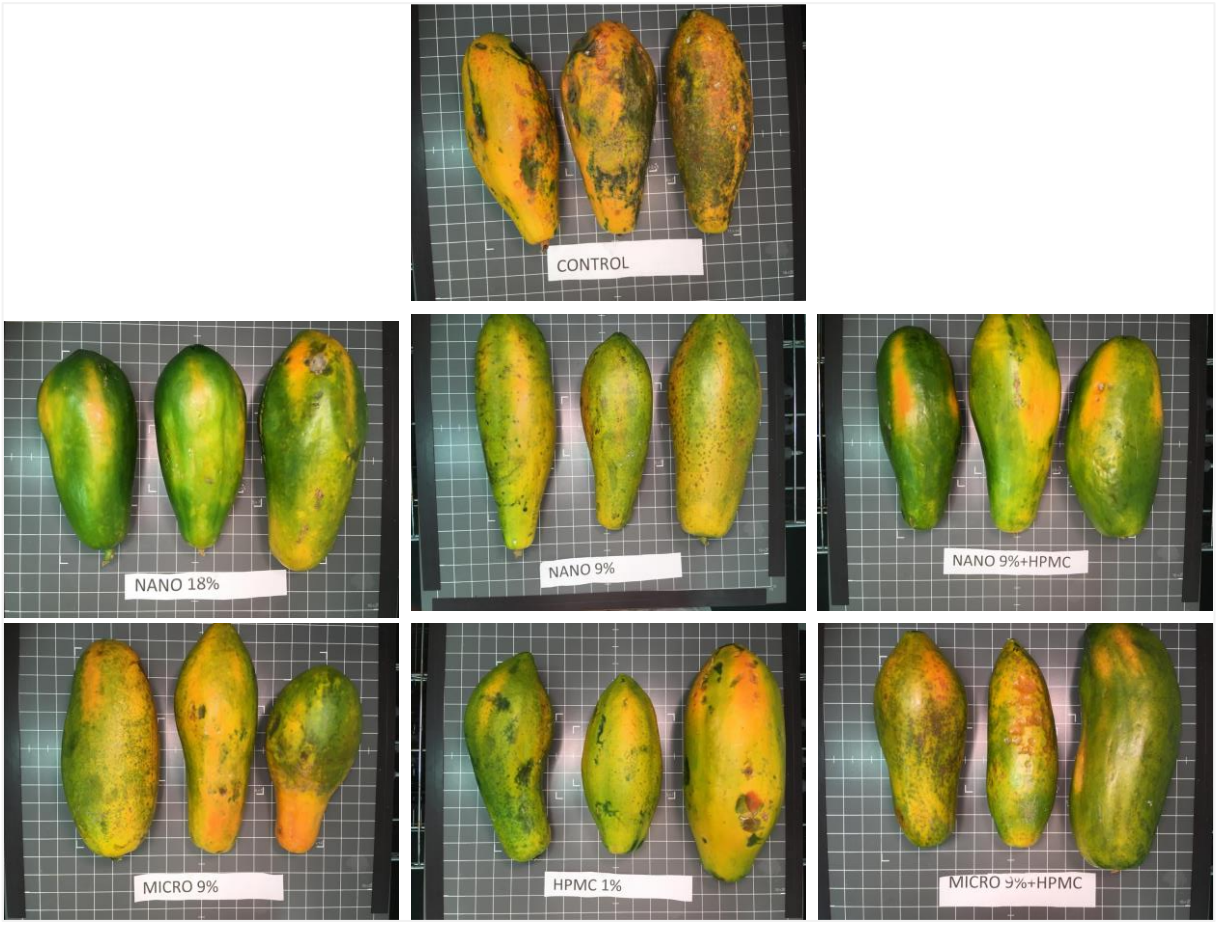

Figure 1. 'Redland' papaya fruits coated with different coatings at Experiment 1 after room temperature storage (6 days at 22 °C) Nano: carnauba wax nan- emulsion coating; Micro: carnauba wax microemulsion coating; HPMC: hydroxypropyl methyl cellulose

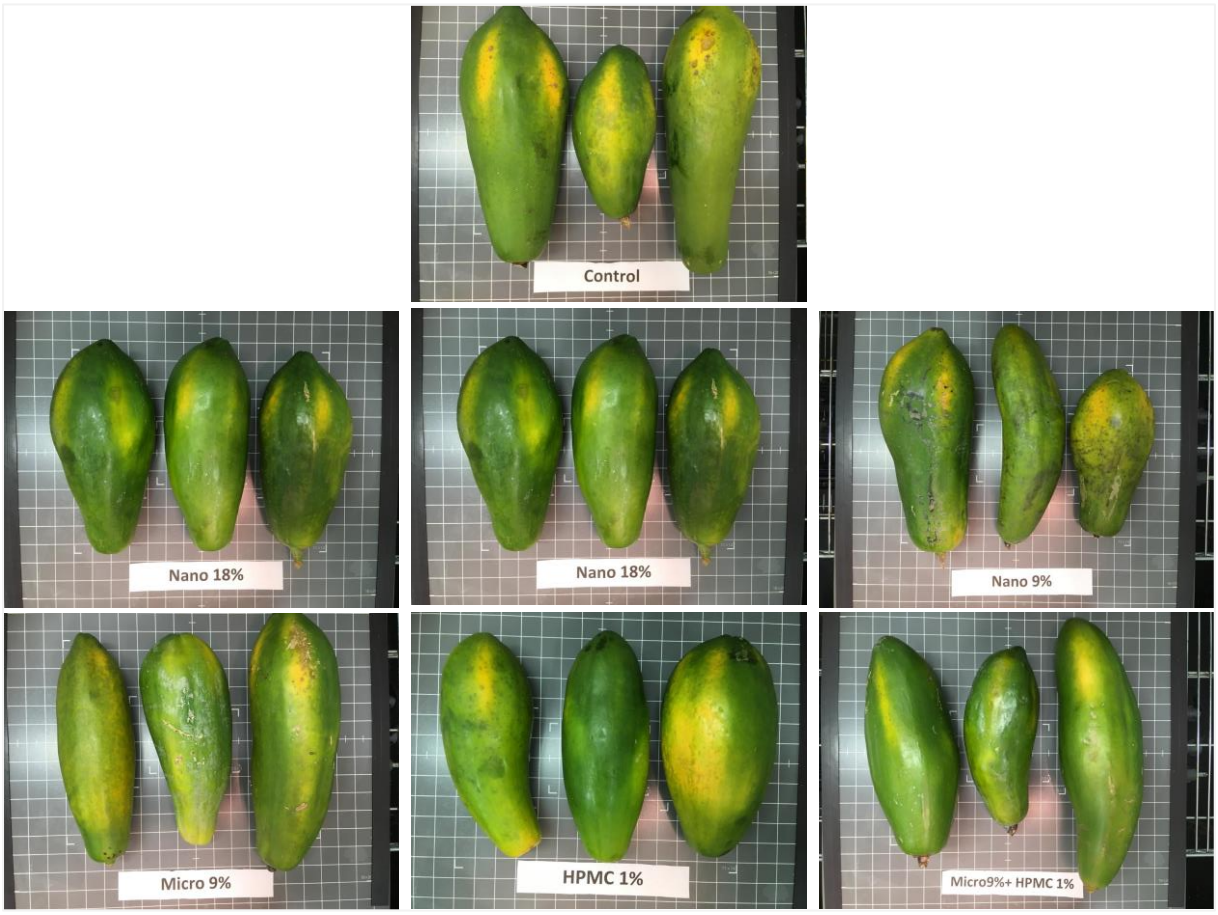

Figure 2. 'Redland' papaya fruits coated with different coatings at Experiment 1 after cold storage (9 days at 13 °C). Nano: carnauba wax nano-emulsion coating; Micro: carnauba wax microemulsion coating; HPMC: hydroxypropyl methyl cellulose coating.

Figure 3. 'Redland' papaya fruits coated with different coatings at Experiment 1 after simulated marketing condition (9 days at 13 °C + 5 days at 22 °C). Nano: carnauba wax nano-emulsion coating; Micro: carnauba wax microemulsion coating; HPMC: hydroxypropyl methyl cellulose coating and GEO: ginger essential oil nano-emulsion.

Figure 4. 'Redland' papaya fruits coated with different coatings at Experiment 2 after room temperature (5 days at 22 °C). Nano: carnauba wax nano-emulsion coating; Micro: carnauba wax microemulsion coating; HPMC: hydroxypropyl methyl cellulose coating and GEO: ginger essential oil nano-emulsion.

Figure 5. 'Redland' papaya fruits coated with different coatings at Experiment 2 after cold storage (10 days at 16 °C). Nano: carnauba wax nano-emulsion coating; Micro: carnauba wax microemulsion coating; HPMC: hydroxypropyl methyl cellulose coating and GEO: ginger essential oil nano-emulsion.

Figure 6. 'Redland' papaya fruits coated with different coatings at Experiment 2 after simulated marketing condition (10 days at 16 °C + 3 days at 22 °C). Nano: carnauba wax nano-emulsion coating; Micro: carnauba wax microemulsion coating; HPMC: hydroxypropyl methyl cellulose coating and GEO: ginger essential oil nano-emulsion.