Phytoprotection

Assessing effects of a plant-derived pesticide on *Tetranychus urticae*, *Botrytis cinerea* and *Bombus impatiens*

Évaluation des effets d'un pesticide d'origine végétale sur *Tetranychus urticae*, *Botrytis cinerea* et *Bombus impatiens*

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Volume 103, Number 1, 2023

Received 2023-03-28; accepted 2023-05-16

URI: https://id.erudit.org/iderudit/1105561ar
DOI: https://doi.org/10.7202/1105561ar

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Publisher(s)

Société de protection des plantes du Québec (SPPQ)

ISSN

0031-9511 (print)
1710-1603 (digital)

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Cite this article

Gaudet, K., Faraone, N. & Hillier, N. K. (2023). Assessing effects of a plant-derived pesticide on *Tetranychus urticae*, *Botrytis cinerea* and *Bombus impatiens*. *Phytoprotection*, *103*(1), 42–51. https://doi.org/10.7202/1105561ar

Article abstract

Tetranychus urticae Koch (two-spotted spider mite) is an agricultural pest with a host range of over 1100 species of plants. *Tetranychus urticae* has rapidly developed resistance to a variety of synthetic chemical pesticides due to its high fecundity and short generation time. Plant essential oils have been recognized as a novel natural source of pest control that have a reduced impact to the environment and human health compared to synthetic pesticide application, and which may provide a viable alternative for managing *T. urticae*. The present study assessed the potential of a plant-derived product (product 102) as an acaricide, through topical and residual bioassays on a variety of plant species including common bean plant (*Phaseolus vulgaris* L.), lettuce (*Lactuca sativa* L.), tomato (*Solanum lycopersicum* L.), kale (*Brassica oleracea* L.), cucumber (*Cucumis sativus* L.), hops (*Humulus lupulus* L.) and hemp (*Cannabis sativa* L.). The results of our study indicate that *C. sativa* is not a suitable plant to host *T. urticae*. Product 102 was determined to be effective at preventing the growth of two known fungal species of economic concern (*Cladosporium herbarum* Persoon and *Botrytis cinerea* Persoon). By conducting acute contact toxicity tests, we also determined that product 102 is significantly less toxic to *Bombus impatiens* Cresson compared to the commonly used synthetic insecticide imidacloprid.

Assessing effects of a plant-derived pesticide on *Tetranychus urticae*, *Botrytis cinerea* and *Bombus impatiens*

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Received 2023-03-28; accepted 2023-05-16

PHYTOPROTECTION 103 : 42-51

Tetranychus urticae Koch (two-spotted spider mite) is an agricultural pest with a host range of over 1100 species of plants. *Tetranychus urticae* has rapidly developed resistance to a variety of synthetic chemical pesticides due to its high fecundity and short generation time. Plant essential oils have been recognized as a novel natural source of pest control that have a reduced impact to the environment and human health compared to synthetic pesticide application, and which may provide a viable alternative for managing *T. urticae*. The present study assessed the potential of a plant-derived product (product 102) as an acaricide, through topical and residual bioassays on a variety of plant species including common bean plant (*Phaseolus vulgaris* L.), lettuce (*Lactuca sativa* L.), tomato (*Solanum lycopersicum* L.), kale (*Brassica oleracea* L.), cucumber (*Cucumis sativus* L.), hops (*Humulus lupulus* L.) and hemp (*Cannabis sativa* L.). The results of our study indicate that *C. sativa* is not a suitable plant to host *T. urticae*. Product 102 was determined to be effective at preventing the growth of two known fungal species of economic concern (*Cladosporium herbarum* Persoon and *Botrytis cinerea* Persoon). By conducting acute contact toxicity tests, we also determined that product 102 is significantly less toxic to *Bombus impatiens* Cresson compared to the commonly used synthetic insecticide imidacloprid.

Keywords: bumblebees, essential oils, fungicide, topical toxicity, two-spotted spider mite.

[Évaluation des effets d'un pesticide d'origine végétale sur *Tetranychus urticae*, *Botrytis cinerea* et *Bombus impatiens*]

Tetranychus urticae Koch (tétranyque à deux points) est un ravageur agricole ayant plus de 1100 espèces de plantes hôtes. *Tetranychus urticae* a rapidement développé une résistance à une variété de pesticides chimiques synthétiques en raison de sa fécondité élevée et de son temps de génération court. Les huiles essentielles de plantes ont été reconnues comme une nouvelle source naturelle de lutte contre les ravageurs ayant un impact moindre sur l'environnement et la santé humaine par rapport à l'application de pesticides synthétiques, et qui peuvent constituer une alternative viable pour lutter contre *T. urticae*. La présente étude a évalué le potentiel d'un produit dérivé d'une plante (produit 102) en tant qu'acaricide, par le biais d'essais biologiques topiques et résiduels sur une variété d'espèces végétales, y compris le haricot commun (*Phaseolus vulgaris* L.), la laitue (*Lactuca sativa* L.), la tomate (*Solanum lycopersicum* L.), le chou (*Brassica oleracea* L.), le concombre (*Cucumis sativus* L.), le houblon (*Humulus lupulus* L.) et le chanvre (*Cannabis sativa* L.). Les résultats de notre étude indiquent que *C. sativa* n'est pas une plante appropriée pour *T. urticae*. Le produit 102 s'est avéré efficace pour empêcher la croissance de deux espèces fongiques connues et préoccupantes sur le plan économique (*Cladosporium herbarum* Persoon et *Botrytis cinerea* Persoon). En effectuant des tests de toxicité aiguë par contact, nous avons également déterminé que le produit 102 est significativement moins toxique pour *Bombus impatiens* Cresson que l'insecticide synthétique couramment utilisé, l'imidaclopride.

Mots-clés : bourdons, huiles essentielles, fongicide, toxicité topique, tétranyque à deux points.

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INTRODUCTION

Natural based products are an excellent alternative to synthetic pesticides (A.K. Tripathi *et al*. 2009). Plant essential oils have been recognized as a viable natural source of pesticide that reduce the impact to the environment and human health compared to synthetic products (A.K. Tripathi *et al*. 2009). Unlike many synthetic pesticides, essential oils are relatively nontoxic to mammals, and non-persistent in the environment. Given the potential essential oils have in controlling pest species and being eco-friendly alternatives to synthetic pesticides, they have been increasingly used in commercial pesticides (Bakkali *et al*. 2008; Fierascu *et al*. 2020; Isman 2000, 2006; Isman and Machial 2006; Raveau *et al*. 2020; Regnault-Roger *et al*. 2012).

Tetranychus urticae Koch (Trombidiformes: Tetranychidae) (two-spotted spider mite) is an agricultural pest that has developed resistance to many pesticides and has a host range of over 1100 species of plants (Xu *et al*. 2018). Mites can rapidly evolve resistance to many synthetic pest control products due to their high fecundity and short generation time (Grbic *et al*. 2007). Plant-derived products are a viable alternative to synthetic pesticides and should be considered for the management of *T. urticae*.

Botrytis cinerea Persoon is a ubiquitous fungus responsible for grey mould on many economically important crops including vegetables (i.e., tomato, cucumber, and lettuce), ornamentals (i.e., roses and gerbera), bulbs (i.e., onions) and fruits (i.e., grapevine, strawberry, and kiwifruit) (Leroux 2007). For the past century, control *of B. cinerea* has relied heavily on synthetic fungicides. However, this is not regarded as a sustainable solution, as *B. cinerea* has shown rapid development of resistance to a variety of synthetic products (Elmer and Reglinski 2006). P. Tripathi *et al*. (2008) tested 26 essential oils against *B. cinera*, and among them *Chenopodium ambrosioides*, *Eucalyptus citriodora*, *Eupatorium cannabinum*, *Lawsonia inermis*, *Ocimum canum*, *O. gratissimum*, *O. sanctum*, *Prunus persica*, *Zingiber cassumunar* and *Z. officinale* exhibited the highest level of fungitoxic activity with 100% growth inhibition under laboratory conditions. Additionally, the study found that the antifungal potency of these oils was greater than that of some synthetic fungicides showing promising application of essential oils as fungicides.

Cladosporium herbarum Persoon is one of the most common environmental fungi to be isolated globally. The species occurs on herbaceous and woody plants and has frequently been isolated from air (Samson *et al*. 2000). *Cladosporium herbarum* is commonly associated with seeds of crops, including the common bean (*Phaseolus vulgaris* L.) (Dhingra *et al*. 2002). *Cladosporium herbarum* is not a pathogenic fungus, but it is of concern for human health being a known cause of asthma (Dhingra *et al*. 2002). Treatment of the seeds with fungicides can help eliminate pathogens and protect seedlings against various fungal diseases (Carvalho *et al*. 2011). Given that synthetic fungicides have a negative impact on the environment and human health, there is a need to develop more plant-derived fungicides (e.g., fungus killing) and fungistatic (e.g., fungus preventing) products (A.K. Tripathi *et al*. 2009).

In addition to testing the effectiveness of plant-derived products on pathogenic fungal species, it is important to examine the secondary effects on beneficial insects such as *Bombus impatiens* Cresson (Hymenoptera: Apidae) (bumblebees). Pollinators have been a key component of agriculture for centuries, with approximately 35% of human crops depending directly on pollinators (Codling *et al*. 2016; Klein *et al*. 2006). In recent years there have been increasing reports of overwintering losses of bee colonies and challenges in maintaining healthy colonies globally. This impact to bee population can be attributed to several factors, such as changes in climate, genetics, changes in available nutritional sources, parasites, and viruses. Recent studies suggest that one other factor may be the use of pesticides (Codling *et al*. 2016; Fairbrother *et al*. 2014).

Neonicotinoids are one of the most widely used classes of pesticides. The extensive use of neonicotinoids is mainly because they exhibit greater toxicity to invertebrates compared with vertebrates. Neonicotinoids are water soluble but show low toxicity toward fish. This class of pesticide is also seen as convenient because it is persistent in the environment, requiring less additional spraying, and it is versatile in the mode of application (Bonmatin *et al*. 2015; Codling *et al*. 2016). However, neonicotinoids accumulate in plant tissues, including pollen. Yamada *et al*. (2012) examined toxicity effects of common neonicotinoids and determined that concentrations as little as 1000 ng mL^{-1} of clothianidin and 400 ng mL^{-1} of dinitrofuran administered to multiple hives caused colony collapse disorder (CCD) or pre-CCD behaviour. Given the harmful impact neonicotinoids have on pollinators, there is a growing interest and urgency in adopting natural-based pesticides.

In this work, we have tested the properties of two novel essential oil-based products develop by a Canadian company (called 'product 101' and 'product 102') on different model pest species, looking at their potential as pesticides, fungicides, and the potential negative effects on non-target insect pollinators. Our study conducted a variety of acaricide experiments to test products 101 and 102 against *T. urticae*. Additionally, we performed fungistatic and fungicide experiments against *C. herbarum* and *B. cinerea*. Finally, we performed acute contact toxicity tests involving the essential oil-based product 102 on *B. impatiens*.

MATERIALS AND METHODS

Animals and plant care

Spider mites were provided by Vineland Research and Innovation Centre (Vineland Station, ON, Canada) to establish a colony at Acadia University, Wolfville (NS, Canada). The mite colony was maintained on common bean plants (*Phaseolus vulgaris* L.) in wire mesh cages (100 x 100 x 100 cm) located in a growth chamber (25 ± 2 °C, 16:8 L:D, 70 ± 5% RH). Bean plants were changed every 2 days, such that several of the oldest plants were replaced with 3-week-old plants. Replacement of bean plants provided a continuous food supply for the mite colony.

All plants involved in this study, including common bean plant (*Phaseolus vulgaris* L. var. 'Red Kidney'), lettuce (*Lactuca sativa* L. var. 'Little Gem Pearl'), tomato (*Solanum lycopersicum* L. var. 'Scotia'), kale (*Brassica oleracea* L. var. 'Darkibor'), cucumber (*Cucumis sativus* L. var 'Summer dance'), hops (*Humulus lupulus* L. var. 'Fuggle') and hemp (*Cannabis sativa* L. var. 'CFX-2'), were grown in phytotrons in the Harriet Irving Botanical Gardens (K.C. Irving Environmental Science Centre, Acadia University, NS, Canada), kept between 18 and 25 °C on a 12:12 L:D photoperiod. Plants were used in experiments when between 3 and 5-weeks old.

All *Bombus impatiens* Cresson (Hymenoptera: Apidae) obtained for this study were purchased from Koppert Biological Systems (Koppert Canada Limited, ON, Canada). All bees were selected from a single colony to provide similar origin and health. Bees provided from Koppert Biological Systems contained a queen, workers, and brood. The largest bees were selected for the study, and the bees were used within 6-weeks of arriving at Acadia University. The colony was maintained in a growth chamber (25 ± 2 °C, 16:8 L:D, 70 ± 5% RH). For nutrients, the colony was given 1 tsp of pollen balls daily (Hawkins Honey, ON, Canada), and cotton balls (Walmart Canada, Mississauga, ON, Canada) saturated with sugar solution (1:4 sugar water ratio) (Redpath Sugar Ltd, Toronto, ON, Canada) were replaced daily.

Chemicals

Products 101 and 102 were provided by Nutrilife Plant Products Limited (Abbotsford, BC, Canada). Products 101 and 102 were prepared at a 1:50 dilution according to the manufacturer's specifications. Product 102 is an experimental formulation adapted from Nutrilife SM-90 multi-purpose wetting agent, in combination with a coriander essential oil active ingredient. Product 102 has sulphonated castor oil, while product 101 has sulphonated canola oil, and both products have coriander seed oil. The specific amount of each component of the product 101 and 102 formulations cannot be disclosed since the formulations are company proprietary information. The product is under registration and it will be disclosed at the right time under the company's conditions. Vegol (Vegol® Crop Oil, Neudorff Commercial Canada, BC, Canada) was used as positive control in pesticide assays and was prepared at 1:50 concentration according to label recommendations. Green Earth Concentrate Lime Sulphur Insecticide-Fungicide Solution (Brantford, ON, Canada) was used as positive control in antifungal assays and was prepared at a 1:10 concentration based on label recommendations. Imidacloprid (positive control in pollinator experiments) was purchased from Sigma-Aldrich (Saint-Louis, MO, USA).

Acaricidal testing of products 101 and 102

Acaricidal testing of products 101 and 102 was conducted using both topical and residual toxicity tests, using a newly developed protocol. In both topical and residual testing, four different products were used: 1) control/water, 2) Vegol (1:50 dilution), 3) product 101 (1:50 dilution), 4) product 102 (1:50 dilution). Topical toxicity was tested on leaves from lettuce, tomato, kale, cucumber, hops and hemp plants. Residual toxicity was tested on leaves from hops, hemp, and bean plants.

For topical experiments, the mites were sprayed directly after being transferred onto the foliage, and mortality was assessed at 1, 2, 4, and 24 h time intervals. For residual experiments, foliage was sprayed prior to transferring the mites. Mites were transferred at 2, 24, 48, and 72 h post-treatment, and mortality was assessed at 24 h after transferring. A dissecting microscope was used (AmScope SM-1BSX-64S, Irvine, CA, USA) to assess for movement of mites when probed with a paintbrush to determine mortality. For the topical experiments, there were 5 mites per treatment type and the experiment was repeated 5 times (n = 25). For the residual experiments, there were 10 mites per treatment type and the experiment was repeated 5 times (n = 50). All mites involved in acaricidal studies were mature adults.

Preparing tomato juice agar plates

Agar plates were prepared (Gonzalez *et al.* 2020; He *et al.* 2011) within one week of conducting experiments. Agar solution consisted of one part tomato juice (Heinz, ON, Canada), four parts distilled water, and 2% (w/v) agar (Cape Crystal Brands, New Jersey, USA). Agar solution was brought to a boil on a hot plate and boiled for 5 min. The solution was cooled for one minute prior to pouring into plastic Petri dishes (100 mm x 15 mm) (FisherbrandTM, Fisher Scientific, ON, Canada). Approximately 20 mL were poured into each plate and left to cool for 30 min. Then the plates were sealed with Parafilm (FisherbrandTM, Fisher Scientific, ON, Canada) and stored in a fridge at 4 °C until the start of experiments.

Testing efficacy as a fungicide

To test the efficacy of product 102 as a fungicide, two different fungi were investigated: *Botrytis cinerea* Persoon and *Cladosporium herbarum* Persoon. For the fungicidal studies, we focused on product 102 as preliminary studies using 102 demonstrated it was a more effective acaricide. For both species of fungi, spores were plated and grown for 4 days in an incubator (25 ± 2 °C; 90% humidity) (Gonzalez *et al.* 2020; He *et al.* 2011). *Cladosporium herbarum* spores were obtained from purchased strawberries, and the collected spores were sequenced to confirm identification. *Botrytis cinerea* spores were provided by the Walker mycology lab at Acadia University (Wolfville, NS).

The fungal experiment testing efficacy of product 102 was conducted using two different approaches described by He *et al*. (2011) and Gonzalez *et al*. (2020): (1) inoculating the plates after treatment to test for the prevention of fungus growth; (2) inoculating the plates prior to treatment to test for the killing of fungus. To inoculate agar plates with fungus, a sterile cotton swab (Puritan Medical Products, Guilford, Maine, USA) was swiped for approximately 5 s on fungus culture and then T-streaked onto clean agar plates. Inoculation of plates was done in a fume hood, and we included a negative control group in our experimental design to help assess for any potential contamination.

For both experiments, there were 24 replicates per treatment group (i.e., product 102, reference fungicide group, and control groups). Each experiment included a positive and a negative control. The positive control plates were treated with distilled water and inoculated with fungus to assess normal fungus growth rate. The negative control plates were treated with distilled water, but not inoculated with fungus, to test for any potential contamination introduced during the experimental protocol. The treatment group of interest was product 102 (1:50 dilution), which was compared to a commercially sold reference fungicide (Green Earth Concentrate Lime Sulphur Insecticide-Fungicide Solution; Brantford, ON, Canada) (1:10 dilution).

When testing fungal growth inhibition, plates were treated with water, product 102, or the Green Earth product, and left to absorb the treatment for 1 h. The plates were treated in a fume hood, and we left the plates half covered with lids until inoculation. To treat plates, a spray bottle (Silgan plastics, CO, USA) contained each of the different treatments and a single spray application (approximately 1 mL) was given to each plate. We chose to treat the plates as described because we wanted to recreate normal application conditions. Then the plates were inoculated with fungus (except for negative control), sealed with Parafilm, and placed in the incubator (25 ± 2 °C; 90% humidity). Plates were checked daily for 7 days following inoculation. Approximate

percent fungus cover was recorded daily for each plate. To measure percent cover, we took a photo of each plate, and then a 1 cm-grid was used to help visually assess percent fungus cover on a computer.

When testing fungicidal activity, all plates except the negative control group were inoculated with fungus and placed in incubator for 4 days to allow for fungal growth. After 4 days (all plates had at least 50% cover), plates were treated with product 102, Green Earth product, or water. Plates were then resealed in Parafilm and placed in incubator. Plates were checked daily for 7 days following treatment, and percent fungus cover was recorded.

Impact of product 102 on *Bombus impatiens* mortality

To determine the effects of product 102 on *B. impatiens*, contact toxicity tests were performed following a protocol adapted from Medrzycki *et al*. (2013). For each experiment replication, there were 5 bees for each treatment group, and a total of 5 replicates were run (n = 25). Product 102 was tested at 5 different concentrations (0.1:50, 0.5:50, 1:50, 5:50, 10:50). Imidacloprid was used as the positive control group at 2 ng μL^{-1}. We applied 10 µL of the imidacloprid solution for a total amount of 20 ng, which is the determined topical LD_{50} for a bumblebee species (*Bombus terrestris*) (Marletto *et al.* 2003). Distilled water was used as negative control.

Bees were individually picked from the colony by using forceps and grabbing them by a back leg, and then placing a single bee in a 50 mL Falcon tube (FisherbrandTM, Fisher Scientific, ON, Canada). Prior to treatment, each bee was anaesthetized using carbon dioxide, where just enough carbon dioxide was blown into the Falcon tube to arrest the movement of the individual bee. The anaesthetized bee was immediately placed on a glass Petri dish (Corning Inc., Arizona, USA), and the Petri dish was placed in a polystyrene foam box containing crushed ice to minimize movement during treatment. Then 10 µL of the assigned treatment solution was applied to the thorax of the bee using a micropipette.

Treated bees were kept in well-ventilated wooden cages (20.5 x 15 x 13.5 cm) with a syringe of 5 mL of sugar water (1:1 sugar water ratio). All wooden cages were kept in a growth chamber (25 ± 2 °C, 16:8 L:D, 70 ± 5% RH). Bees with different treatments were kept in separate boxes and there was a total of 5 bees per box. Mortality was recorded at 4, 24 and 48 h after treatment.

Statistics

All statistical analyses were done using R version 4.0.3 (R Core Team 2020).

We ran a generalized linear model (glm) to assess if plant type, treatment, and/or time had a significant impact on mite mortality for both topical and residual toxicity tests ($\alpha = 0.05$).

We performed a Shapiro-Wilk normality test on the fungus growth rate for each treatment group, and determined the data were not normally distributed. Therefore, to analyze whether fungus growth rate was significantly different among treatment groups, a Kruskal-Wallis rank sum test was performed for each of the different fungus experiments, followed by a Dunn multiple comparison with the Bonferroni method ($\alpha = 0.05$). These statistical tests were chosen given that the data were not normally distributed.

To determine if the concentration of product 102 significantly impacts mortality of bees at 4 h, the data for bee mortality at 4 h was fitted to a binomial model with Probit link. Additionally, LD_{50} of product 102 at 4 h was calculated with Probit analysis. The same statistical analyses were performed for bee mortality at 24 h and 48 h.

To determine if time and concentration significantly impact bee mortality, the data were fitted to a linear mixed model by maximum likelihood.

RESULTS

Topical toxicity

No significant difference in mite mortality for different treatment types was observed (Fig. 1). Interestingly, hemp (*C. sativa*) plants significantly impacted mite mortality as determined from the generalized linear model ($t = 12.38$; $Pr(>|t|) < 0.001$). Additionally, a significant difference in mite mortality for the control group on hemp compared to the control group on other plant species ($t = -5.11$; $Pr(>|t|) < 0.001$) was detected. The average mite mortality was higher in the control group on hemp plants compared to other plant species (Fig. 1). Time at 4 h post-treatment significantly impacted mite mortality ($t = 3.04$; $Pr(>|t|) < 0.01$) and at 24 h ($t = 4.34$; $Pr(>|t|) < 0.001$) (Fig. 2).

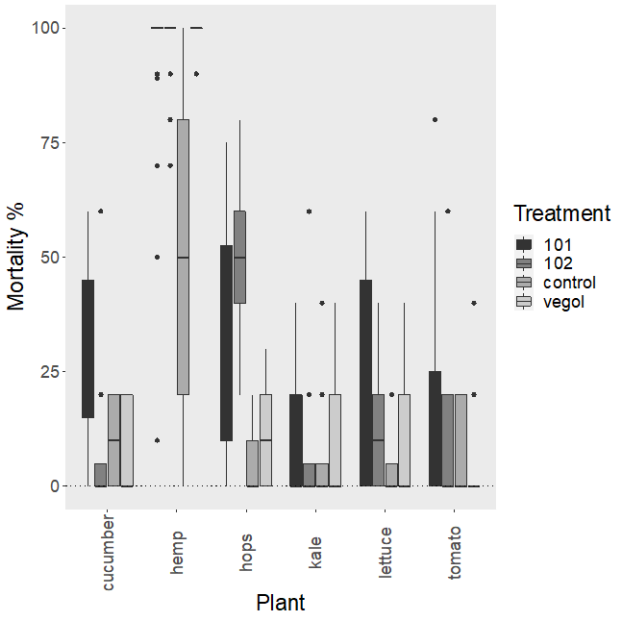

Figure 1. Mortality of *Tetranychus urticae* following topical treatment of Nutrilife product 101 and 102, control (water), and Vegol® Crop Oil on a variety of plant species (cucumber, hemp, hops, kale, lettuce, tomato), where mortality was recorded at 1hr, 2 h, 4 h, and 24 h post-treatment. For each plant species tested, n = 5, except for hemp (n = 25). The bar represents the median, the box represents the interquartile range, and the whiskers represent the maximum and minimum values that are not outliers.

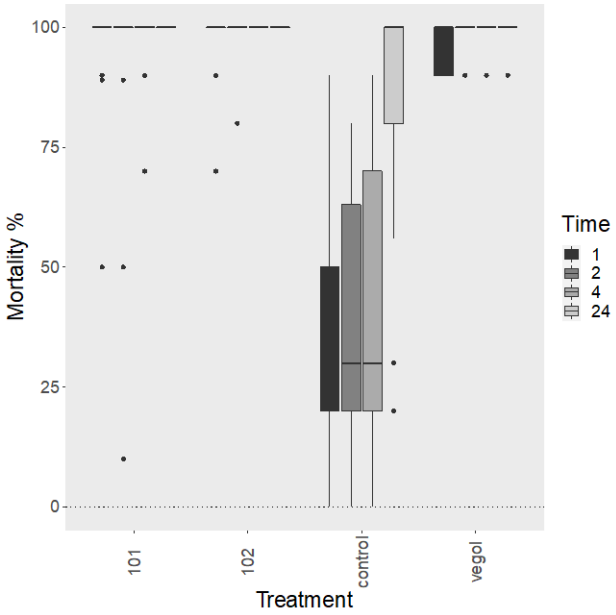

Figure 2. Mortality of *Tetranychus urticae* following topical treatment of Nutrilife product 101 and 102, control (water), and Vegol® Crop Oil on hemp (*Cannabis sativa*), where mortality was recorded at 1 h, 2 h, 4 h, and 24 h post-treatment (*n* = 25). The bar represents the median, the box represents the interquartile range, and the whiskers represent the maximum and minimum values that are not outliers.

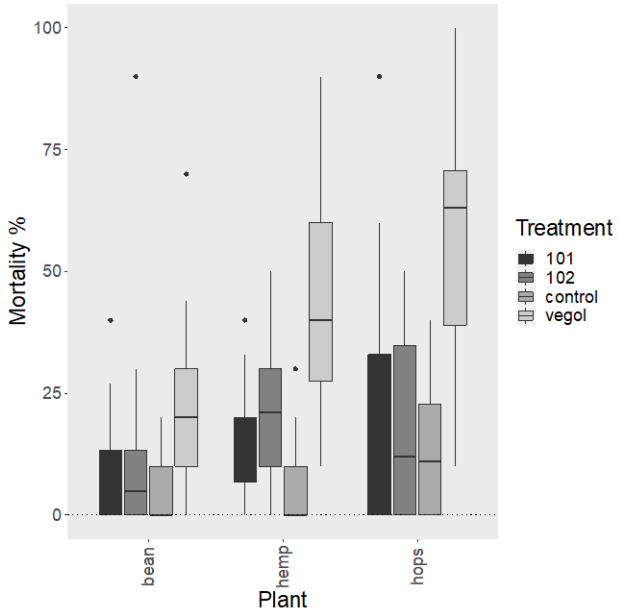

Figure 3. Mortality of *Tetranychus urticae* following residual treatment of Nutrilife product 101 and 102, control (water), and Vegol® Crop Oil on a variety of plant species (bean, hemp, and hops), where mites were transferred at 2 h, 24 h, 48 h, and 72 h post-treatment. Mortality was recorded 24 h after transferring mites. For each plant species tested, *n* = 50. The bar represents the median, the box represents the interquartile range, and the whiskers represent the maximum and minimum values that are not outliers.

Table 1. Dunn multiple comparison with the Bonferroni method for growth rate of *Cladosporium herbarum* and *Botrytis cinerea* for each treatment group as recorded for 7 days following a pre-inoculation treatment, where prevention of fungal growth was determined (*n* = 24).

Species	Comparison	Z	P.adj
C. herbarum	Control (-) *vs.* Control (+)	-17.3	< 0.0001
C. herbarum	Control (+) *vs.* Green Earth	17.3	< 0.0001
C. herbarum	Control (-) *vs.* Product 102	-11.0	< 0.0001
C. herbarum	Control (+) *vs.* Product 102	6.3	< 0.0001
C. herbarum	Green Earth *vs.* Product 102	-11.0	< 0.0001
B. cinerea	Control (-) *vs.* Control (+)	-17.7	< 0.0001
B. cinerea	Control (-) *vs.* Green Earth	-10.6	< 0.0001
B. cinerea	Control (+) *vs.* Green Earth	7.2	< 0.0001
B. cinerea	Control (-) *vs.* Product 102	-11.9	< 0.0001
B. cinerea	Control (+) *vs.* Product 102	6.0	< 0.0001

Residual toxicity

Overall, we detected no significant difference in mite mortality for treatment types or time (Fig. 3). Vegol applied on hops plants significantly impacted mite mortality ($t = 2.07$; $Pr(>|t|) < 0.05$). Mite mortality for Vegol was higher on average for residual tests performed on hops (Fig. 3).

Efficiency of product 102 as a fungicide

There was a significant difference in growth rate between treatment groups for *C. herbarum* growth inhibition (Kruskal-Wallis rank sum test; $X^2 = 440.84$; DF = 3; $P < 0.0001$). The growth rate of the positive control group was significantly higher than the growth rate of the negative control group, Product 102, and Green Earth (Table 1; Fig. 4A).

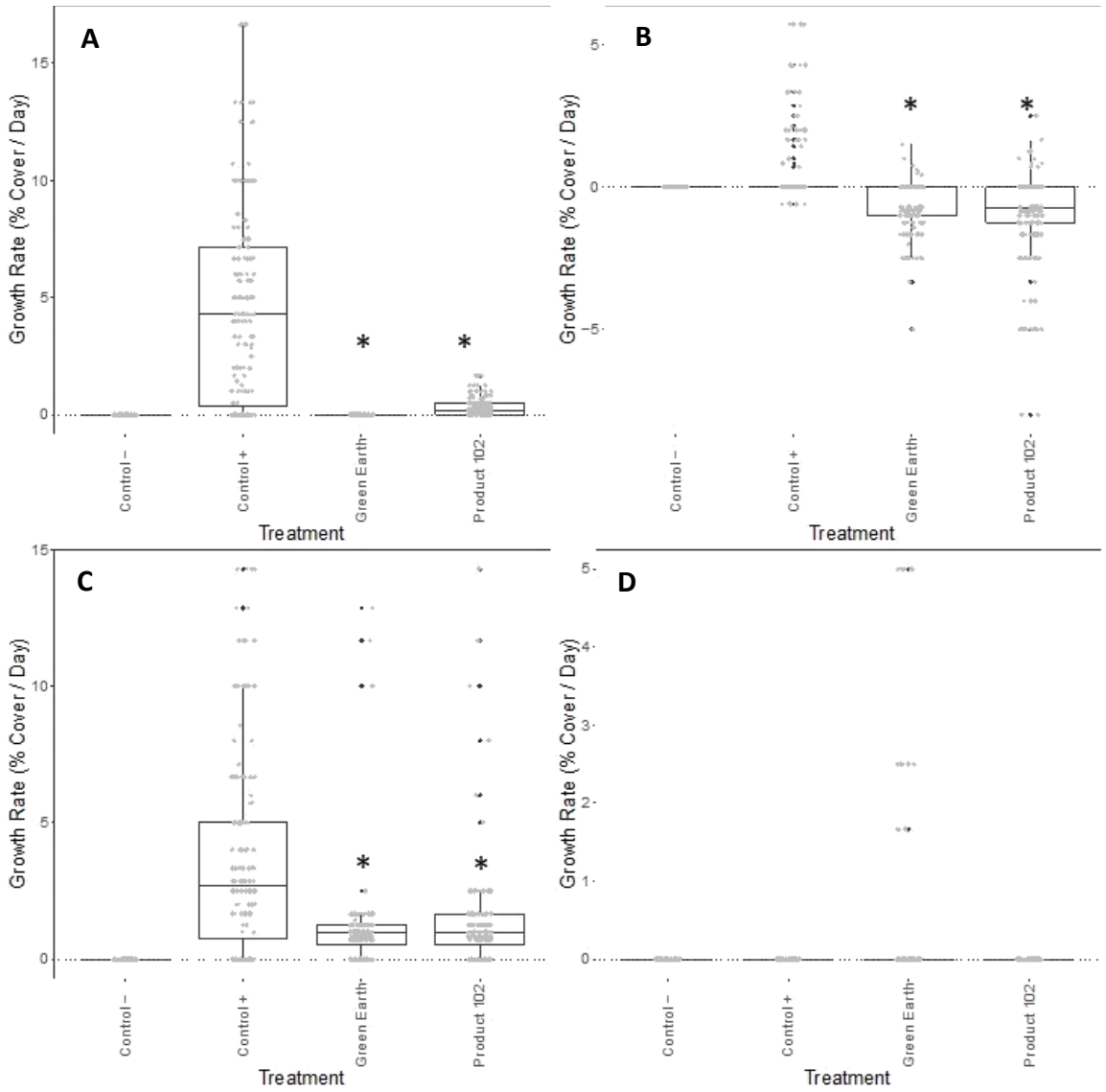

Figure 4. Growth rate of *Cladosporium herbarum* and *Botrytis cinerea* for each treatment group as recorded for 7 days following a pre- or post-inoculation treatment, where prevention of fungus growth or killing of fungus was determined (*n* = 24). (A): The prevention of *Cladosporium herbarum* growth. **(B):** The killing of *Cladosporium herbarum*. **(C):** The prevention of *Botrytis cinerea* growth. **(D):** The killing of *Botrytis cinerea*. The bar represents the median, the box represents the interquartile range, and the whiskers represent the maximum and minimum values that are not outliers.

Table 2. Dunn multiple comparison with the Bonferroni method for growth rate of *Cladosporium herbarum* and *Botrytis cinerea* for each treatment group as recorded for 7 days following a post-inoculation treatment, where fungus was grown for 4 days prior to treatment and antifungal activity was determined (*n* = 24).

Species	Comparison	Z	P.adj
C. herbarum	Control (-) *vs.* Green Earth	8.3	< 0.0001
C. herbarum	Control (+) *vs.* Green Earth	10.8	< 0.0001
C. herbarum	Control (-) *vs.* Product 102	10.0	< 0.0001
C. herbarum	Control (+) *vs.* Product 102	12.6	< 0.0001
B. cinerea	Control (-) *vs.* Green Earth	-5.4	< 0.0001
B. cinerea	Control (+) *vs.* Green Earth	-5.5	< 0.0001
B. cinerea	Green Earth *vs.* Product 102	5.5	< 0.0001

There was a significant difference in growth rate between treatment groups for *C. herbarum* killing (Kruskal-Wallis rank sum test; $X^2 = 226.25$; DF = 3; $P < 0.0001$). The growth rates of product 102 and Green Earth were significantly different from the growth rate of both the positive and negative control groups (Table 2). The growth rates of product 102 and Green Earth were negative, indicating that the products kill the fungus (Fig. 4B).

There was a significant difference in growth rate between treatment groups for *B. cinerea* growth inhibition (Kruskal-Wallis rank sum test; $X^2 = 326.37$; DF = 3; $P < 0.0001$). The growth rate of the positive control group was significantly higher from the growth rate of the negative control group, product 102, and Green Earth (Table 1; Fig. 4C).

There was a significant difference in growth rate among treatment groups for *B. cinerea* killing (Kruskal-Wallis rank sum test; $X^2 = 45.20$; DF = 3; $P < 0.0001$). The growth rate of Green Earth was significantly different from the growth rate of the negative control group, positive control group, and product 102 (Table 2). Green Earth seemed to allow for fungal growth (Fig. 4D), indicating that Green Earth was not effective at killing *B. cinerea*. Product 102 was also not effective as a fungicide, but it was effective as fungistatic product.

Contact toxicity tests of product 102 on Bombus impatiens

Concentration of product 102 significantly impacted mortality of bees at 4 h (glm; family = binomial; link = probit; $Pr(>|z|) < 0.001$) (Fig. 5). LD_{50} of product 102 at 4 h is 25.4% (V/v) ± 6.8%. There was not a significant impact of concentration of product 102 at 24 h (Fig. 5). LD_{50} of product 102 at 24 h is 13.7% (V/v) ± 5.6%. Concentration of product 102 significantly impacted mortality of bees at 48 h (glm; family = binomial; link = probit; $Pr(>|z|) < 0.05$) (Fig. 5). LD_{50} of product 102 at 48 h is 11.8% (V/v) ± 4.1%.

Time and concentration of product 102 significantly impacted bee mortality, where mortality increased over time and with increasing the concentration of product 102 (Fig. 5). All concentrations of product 102 have a significant impact on bee mortality (Table 3; Fig. 5). Imidacloprid significantly impacted bee mortality at all time intervals (Table 3; Fig. 5).

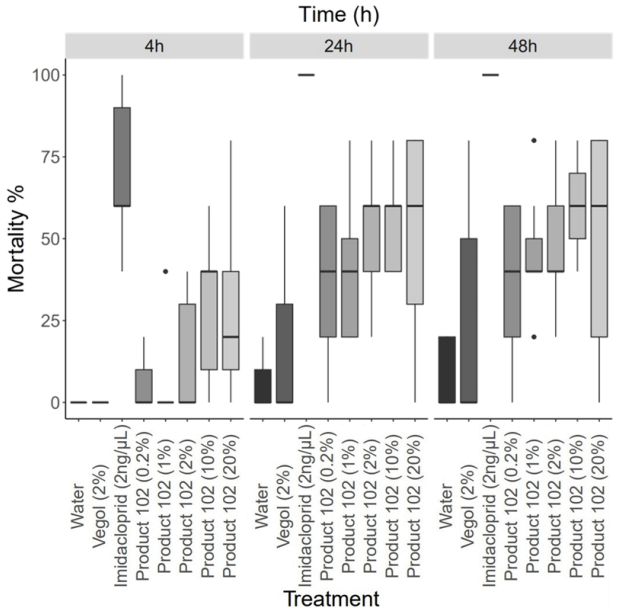

Figure 5. Mortality of *Bombus impatiens* Cresson from contact toxicity of several treatment groups, where 10 µL of solution was applied to the thorax. Mortality is shown for 4 h, 24 h, and 48 h after treatment ($n = 25$). The bar represents the median, the box represents the interquartile range, and the whiskers represent the maximum and minimum values that are not outliers.

Table 3. Fixed effects of linear mixed model fit by maximum likelihood, where t-tests use Satterthwaite's method ("lmerModLmerTest"), for bee mortality data at 4 h, 24 h and 48 h, where *n* = 25. Product 102 was tested at concentrations 0.2% v/v, 1% v/v, 2% v/v, 10% v/v and 20% v/v.

Level	Std. Error	df	t value	Pr(>|t|)
Conc. 0.2%	0.05475	833	3.479	0.000529 ***
Conc. 1%	0.05475	833	4.523	6.99e-06 ***
Conc. 2%	0.04741	833	4.720	2.76e-06 ***
Conc. 10%	0.05475	833	7.654	5.39e-14 ***
Conc. 20%	0.05475	833	7.132	2.14e-12 ***
Conc. Imida.	0.05475	833	15.656	< 2e-16 ***
Time 24 h	0.03353	833	7.563	1.04e-13 ***
Time 48 h	0.03353	833	7.990	4.50e-15 ***

Significant codes: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, . = $P < 0.1$.

DISCUSSION

We found that product 102 has fungicidal activities, as well as exhibited a low contact toxicity to *B. impatiens*. The results of the topical and residual toxicity tests do not demonstrate any significant acaricidal effect of product 101 or 102 (essential oil-based products) on mites. However, previous studies conducted in our lab demonstrate the repellent effect of product 102 on *T. urticae* (data not shown).

Interestingly, the results of our study indicate that *C. sativa* is not a suitable host plant to *T. urticae*. A study done by Górski *et al*. (2016) examined the residual toxic effects of hemp essential oil against *T. urticae* when treating bean plants with the oil. Górski *et al*. (2016) determined that the essential oil produced from hemp has a significant effect on *T. urticae* mortality, where the strongest effect was observed when testing at the highest concentration of 0.1% (v/v). Additionally, hemp essential oil has shown repellent properties against the cabbage butterfly (*Pieris brassicae* L.) and the Japanese beetle (*Popillia japonica* Newman) (MacPortland 1997; Rothschild and Fairbairn 1980). Secondary metabolites, predominantly cannabinoids and terpenes, play an important role in the plant's defence system, which can be triggered by biotic or abiotic stresses. When infestations of *T. urticae* feed on *C. sativa,* this can trigger cannabinoids and terpenes to be secreted by the trichomes of the plant (Kostanda and Khatib 2022). Future studies should investigate the acaricidal and repellent properties of Cannabis essential oil for the management of *T. urticae*. Although there is some support that *C. sativa* exhibits acaricidal properties to *T. urticae*, including our study, *T. urticae* does feed on *C. sativ*a and causes significant plant damage (Park and Lee 2002). A possible explanation of how *T. urticae* feeds on *C. sativa* while being susceptible to the plant and/or essential oil is that *T. urticae* might develop a host plant adaptation to Cannabis over time (Hu *et al*. 2022). Intraspecific genetic variation in host preference has been shown in multiple studies for *T. urticae*, as is true of many other polyphagous pests (Fry 1989; Futuyma and Peterson 1985; Gould 1979; Via 1990). When the species encounters a new host plant and/or a plant treated with an acaricide, it could rapidly select for a genotype carrying a set of genes whose expression best buffer against the chemicals of the new hostile environment (Dermauw *et al.* 2013). Dermauw *et al.* (2013) provided an explanation for *T. urticae*'s ability to evolve with *C. sativa*. Additionally, *T. urticae*'s polyphagy indicates an outstanding ability to adapt its digestive physiology and to overcome a wide range of defensive chemicals produced by host plants (Bensoussan *et al.* 2018).

Product 102 was determined to be effective at inhibiting growth and killing *C. herbarum*, which is one of the most common fungi isolated from the environment. A review completed by Whiley *et al*. (2018) investigated the antifungal properties of a variety of essential oils against common indoor fungal species. The review found that clove oil, tea tree oil, oregano, thyme, and lemon essential oils are all effective antifungal agents against a large number of fungi isolated from indoor environments. Clove oil has been most extensively researched and has demonstrated as an effective fungicide against common species such as *Aspergillus versicolor*, *A. niger*, *A. fumigatus*, *Cladosporium sphaerospermum*, *C. cladosporioides*, *Penicillium chrysogenum*, *P. aurantiogriseum*, *P. digitatum*, *P. simplicissimum*, and *Ulocladium chartarum* (Levinskaitė and Paškevičius 2013). Our study found similar findings where product 102 (essential oil-based) was effective at inhibiting growth and killing *C. herbarum*.

Product 102 was found to be effective at preventing the growth of *B. cinerea*, but it was not effective at killing the fungus once present. Bouchra *et al*. (2003) tested the essential oils of *Origanum compactum* L. and *Thymus glandulosus* L. against *B. cinerea*. The study determined that the mycelium growth of *B. cinerea* was completely inhibited at the concentration of 100 ppm. The inhibitory effect was mainly associated with the two most abundant components, thymol and carvacrol. Several other authors have also studied the antimicrobial activity of thymol and carvacrol (Arras and Usai 2001; Curtis *et al*. 1996; Kim *et al*. 1995).

There is limited knowledge regarding the antifungal mechanism of action of essential oils (Singh and Chittenden 2010; Verma *et al*. 2011). The active components of essential oils are mainly phenols, terpenes, aldehydes, and ketones (Ceylan and Fung 2004) and it is generally concluded that these functional groups act against cell membranes of microorganisms. That many essential oils are hydrophobic contributes to their ability to accumulate in the cell membrane, which disrupts cell structure and increases cell permeability (Lv *et al*. 2011). The mode of action of most antifungal compounds, including those antifungal essential oils which have been studied, is based on targeting either the formation or the function of ergosterol, an important component of the fungal cell membrane (Hector 1993; Pinto *et al*. 2009). This membrane interaction weakens the cell structure and increases permeability, which causes cell leakage and eventually cell lysis. Shao *et al*. (2013) determined that this is the mechanism of action for tea tree oil preventing the growth of *B. cinerea*, where the cell wall structure of *B. cinerea* was reported to have lost its ultrastructure and showed rupturing. This could be a putative mode of action for product 102 against *C. herbarum* and *B. cinerea*.

Whiley *et al*. (2018) noted that there are limited studies that scale up laboratory results and assess the efficacy of essential oils indoors. One of the leading indoor air quality concerns is the presence of fungi, which have been associated with increased risk of adverse health effects such as respiratory conditions and allergies (Arthur, 2005; Bird *et al.* 2012; Nevalainen *et al*. 2015). Future studies could focus on larger scale testing (within home and commercial buildings) of essential oils against common indoor fungi species, including realistic application methods and the potential for long-term antifungal persistence (Whiley *et al*. 2018).

We determined that the LD_{50} for *B. impatiens* of product 102 for 4, 24 and 48 h is 25.4%, 13.7%, and 11.8% (v/v) respectively. Given that the recommended concentration for product 102 application is 1:50 (2% v/v) the results of our study suggest that product 102 is unlikely to cause mortality to *B. impatiens* when applied at the recommended concentration within greenhouses or residential areas. Additionally, product 102 is significantly less toxic to *B. impatiens* compared to synthetic pesticides such as imidacloprid (average LD_{50} of 2 ng μL^{-1}) (Marletto *et al.* 2003). Matos *et al*. (2021) compared the toxicity of synthetic insecticides deltamethrin and imidacloprid to the essential oil of *Lippia sidoides* Cham (rosemary pepper) against the stingless bee *Nannotrigona aff. Testaceicornis* Lepeletier (Hymenoptera: Apidae). The study determined that *L. sidoides* essential oil and its major component thymol have low lethal and sublethal toxicity to *N. aff. Testaceicornis*. Our study suggests the use of less hazardous alternatives to neurotoxic pesticides to protect our pollinators.

Overall, product 102 is effective at preventing the growth of two known fungal species of economic and health concern (*C. herbarum* and *B. cinerea*). Additionally, the product is significantly less toxic to *B. impatiens* when compared to a frequently used neonicotinoid insecticide. Our study provides support for the use of essential oil-based products for fungal management, as well as a safe alternative to synthetic insecticides for protecting pollinators.

ACKNOWLEDGEMENTS

We would like to acknowledge that this study was conducted in Mi'kma'ki, the ancestral and unceded territory of the Mi'kmaq. We would like to thank Nutrilife Plant Products Limited for supporting our research and providing materials. We would like to thank the K.C. Irving Environmental Science Centre for the use of growth chambers, phytotrons and research space. We would like to thank Sarah Adams and Dr. Allison Walker for their assistance and advice when culturing species of fungi. Additionally, we would like to thank Wendy Hillier, Luca Voscort, Sarah Hobbs, and Tyler Peskett for their assistance with data collection.

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