Essential Tea Tree Oil Activity against *Bremia lactucae* in Lettuce

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**Abstract:** Downy mildew caused by the oomycete *Bremia lactucae* Regel is a serious disease of lettuce in field and greenhouse production. Here we report on the antifungal activity of essential Australian tea tree oil (TTO) derived from *Melaleuca alternifolia* against downy mildew in lettuce. Preventive treatments with Timorex Gold (STK Ltd., Petach Tikva, Israel), fungicide containing TTO, suppressed the development of *B. lactucae* on cotyledons and young lettuce plants. Epifluorescence microscopy showed that TTO had a moderate inhibitory effect on spore germination of the pathogen but a strong inhibitory effect on sporophore emergence and hence on sporulation. Timorex Gold (22.3 EC W/V) was as effective as copper hydroxide in controlling the disease in organic field plots. It was as effective as the fungicides Bellis (Boscalid + Pyraclostrobin; BASF, Germany) and Commet (pyraclostrobin; BASF, Germany) in conventional production. The results demonstrate that Timorex Gold effectively suppresses the development of downy mildew in lettuce in growth chambers as well as in the field, and thus suitable biopesticide for its control.

**Keywords:** biopesticides; downy mildew; disease control; *Lactuca sativa*; *Melaleuca alternifolia*

1. Introduction

Downy mildew caused by the oomycete *Bremia lactucae* Regel is the most serious disease in field and greenhouse lettuce production [1]. A low level of infection can downgrade the crop, causing significant losses at harvest, and can promote decay during postharvest transit and storage; whereas a high level of disease can render a crop unmarketable [2]. The pathogen produces moldy yellow lesions on the leaves, which become necrotic and make the heads unmarketable. *B. lactucae* spores infect through the epidermal cells and then develop coenocytic mycelia which grow intercellularly through the mesophyll, obtaining nutrients via haustoria that invaginate the plant plasmalemma [3]. Successful infection results in profuse sporulation on the leaf surface, which is responsible for the characteristic downy appearance of the disease. Current approaches to combating *B. lactucae* rely on genetic resistance and fungicides. Controlling the disease in organic production is dependent on a small number of approved products, including copper hydroxide. However, use of this heavy metal may have long-term consequences due to its accumulation in soil [4]. Copper fungicides also cause phytotoxicity in moisture conditions often in production. In conventional production, the disease can be controlled by synthetic chemicals, including protective and systemic fungicides such as phenylamide (e.g., metalaxyl/mefenoxam-based compounds, and phosphites). However, isolates that are insensitive to these compounds are common [5,6].

The global search for plant-protection solutions that are both effective and environmentally safe is driven by the need to supply food to the ever-growing world population, and the call for chemical load reduction. An alternative procedure for protecting lettuce plants against downy mildew was
shown by the non-protein amino acid DL-3-amino-n-butanoic acid (BABA) [7]. BABA is capable of inducing systemic resistance against numerous pathogens [8] and effectively controlled downy mildew development in lettuce in growth chambers and in the field [7].

The natural Australian tea tree oil derived from the Melaleuca alternifolia plant contains many components, mostly terpenes (p-cymene, terpinen-4-ol, terpinolene, 1,8-cineole, α-pinene, α-terpinene, γ-terpinene), sesquiterpenes, and their respective alcohol (monoterpene alcohol-terpineol) [9]. It has a maximum content of 15% of 1,8-cineole and a minimum content of 30% of terpinen-4-ol, which is the main active constituent of tea tree oil (TTO) [9]. TTO has been shown to be an effective antiseptic and bactericide [9–12], and more recently also an effective fungicide [13–16]. The fungicidal and antimicrobial activities of TTO against fungal pathogens are derived from its ability to inhibit respiration and disrupt the permeability barrier presented by the membrane structures of living organisms [9,11]. The natural fungicide Timorex Gold (22.3 EC W/V) was prepared with TTO as the active ingredient. This formulation enables using an emulsified TTO on plant tissue [17]. It was found effective against a broad range of plant-pathogenic fungi in numerous crops [16–19].

Preliminary studies showed that foliar sprays of Timorex Gold applied to lettuce plants effectively controlled downy mildew (Reuveni, unpublished data). In the present study we used epi-fluorescent microscopy in order to test the activity of pure tea tree oil and Timorex Gold against B. lactucae in lettuce. In addition, the efficacy of Timorex Gold against downy mildew was evaluated in growth chamber and field-grown lettuce plants. The study provided evidence that TTO inhibited B. lactucae in lettuce at various stages and suppressed downy mildew development in the field.

2. Materials and Methods

Plants. The susceptible lettuce (Lactuca sativa L.) cultivar Noga (Romaine type, Hazera Genetics, Mivhor, Israel) was used, unless stated otherwise. Plants were grown from seeds in 250 mL pots containing a peat-vermiculite mixture (1/1, v/v), ∼20 plants per pot. The plants were grown in a greenhouse (18–26 °C) and used 1 week after seeding, at the cotyledon stage. In some experiments, five true leaf plants grown in 250 mL pots, one plant/pot, were used. The plants were fertilized with 0.5% N: P: K (20: 20: 20) once a week.

Pathogen. Isolate IL60 of B. lactucae Regel obtained in 2010 from K. Sharaf (University of Haifa, Israel), was used in all growth chamber experiments [20] and maintained by repeated inoculation of detached cotyledons in the growth chamber. The pathogen is sensitive to phenylamides and CAA (Carboxylic Acid Amides) fungicides.

Chemicals. Tea tree oil was used as an emulsifiable concentrated formulation (Timorex Gold, 22.3 EC W/V; STK Bio-AG Technologies, Petah Tikva, Israel). TTO extracted from M. alternifolia plants (gratefully provided by STK Ltd., Petach Tikva, Israel) was used for comparison in the epi-fluorescent microscopy study. The following fungicides, registered for use against downy mildew in lettuce, were tested for comparison: Kocide 2000 (containing copper hydroxide, 53.8 dry flowable; DuPont) was used in growth chamber trials on young plants and as a standard in field trials conducted in Israel in organic-grown plots; Bellis (a premix containing boscalid + pyraclostrobin 200 + 800 WG; BASF, Germany) and Comet (containing pyraclostrobin 250 EC; BASF, Germany) were used as standards in a conventional field trial in Chile. The doses in laboratory and growth chamber experiments were in percentage (v/v) and in the field in L/ha as in product label.

2.1. Control of Downy Mildew in Growth Chambers

2.1.1. Application of Compounds

Timorex Gold was diluted in water to a series of concentrations and applied to five-leaf lettuce plants by spraying with handy glass sprayer onto the upper leaf surfaces until run-off (Tables 1 and 2). Plants were inoculated 1 day after the spray, incubated in a dew chamber overnight and then placed in
a growth chamber at 20 °C (12 h light/day, 100 µE·m⁻²·s⁻¹). Four replicate plants were used for each treatment and each experiment was conducted at least twice.

Table 1. Efficacy of formulated tea tree oil (TTO) applied prophylactically on downy mildew development induced by B. lactucae on young lettuce plants a.

| Treatment       | Affected Leaf Area % |
|-----------------|----------------------|
| (Concentration %, v/v) | Exp. 1 | Exp. 2 |
| Control Untreated | 56.9 a b | 30.3 a |
| Timorex Gold 0.5  | 5.3 b  | 1.0 b  |
| Timorex Gold 1.0  | 0.6 b  | 0.0 b  |
| Kocide 0.25       | 0.3 b  | 0.0 b  |

a Plants were sprayed with formulated TTO and 24 h later leaves were inoculated with a conidial suspension of B. lactucae as described in Section 2. b Mean values within columns followed by different letters are significantly (p < 0.05) different according to the Tukey–Kramer Test.

Table 2. Efficacy of formulated TTO applied prophylactically on downy mildew development caused by B. lactucae in young lettuce plants a.

| Treatment       | Affected Leaf Area % |
|-----------------|----------------------|
| (Concentration %, v/v) | Exp. 1 | Exp. 2 |
| Control Untreated | 43.8 a b | 70.0 a |
| Timorex Gold 0.125| 17.1 b | 26.3 b |
| Timorex Gold 0.25 | 11.0 bc | 23.0 b |
| Timorex Gold 0.5  | 10.6 bc | 5.5 c  |
| Timorex Gold 1.0  | 4.8 c  | 5.0 c  |
| Kocide 0.25       | 0.3 c  | 3.3 c  |

a Plants were sprayed with formulated TTO and 24 h later leaves were detached and inoculated with a conidial suspension of B. lactucae, b Mean values within columns followed by different letters are significantly (p < 0.05) different according to the Tukey–Kramer Test.

2.1.2. Inoculation

Spores of B. lactucae Regel were collected from freshly sporulating lettuce leaves into ice-cool double-distilled water. The spore concentration was adjusted to 2 × 10⁴ spores/mL and sprayed onto the upper leaf surfaces of the test plants until run-off, using a glass atomizer. The plants were subsequently placed in a dew chamber (100% relative humidity, 18 °C, darkness) for 15–20 h, and then transferred to a growth chamber at 18–20 °C (12 h light/day, 100 µE·m⁻²·s⁻¹). At 6–7 days post inoculation (dpi), the plants were placed in transparent plastic boxes (100% relative humidity) for 1 or 2 days to induce sporulation of the pathogen on the leaves.

In some experiments, leaves were detached from plants, 1 day after spray, placed in 9 cm Petri dishes containing wet filter paper adaxial side up, and spray inoculated in a similar manner. The inner surface of each Petri dish lid was gently sprayed with distilled water to maintain 100% humidity. The dishes were placed in darkness at 18 °C for 20 h and then transferred to a growth chamber under the above-mentioned conditions. Four plants, each containing four leaves, were used in each treatment. In addition, seedlings at the cotyledon stage were sprayed with Timorex Gold at various concentrations and were challenged with B. lactucae 4 h later. The plants were incubated at 20 °C, 12 h light/day in Perspex boxes and then transferred to a growth chamber under the above-mentioned conditions.

2.1.3. Microscopy of the Bremia–Lettuce Interaction

Detached cotyledons of lettuce were placed on moistened paper in Petri dishes and each was inoculated on the abaxial surface with a 10 µL droplet of water suspension containing ≈100 spores of B. lactucae isolate IL60 and a 10 µL droplet of each tested material. TTO extract was emulsified by mixing 4 mL of the pure material + 20 µL Tween 20 + 20 µL Tween 80 + 39 mL water (10% a.i.).
Cotyledons inoculated with a conidial suspension in water served as controls. Inoculated cotyledons were placed at 20 °C, 12 h light/day. At 2 and 5 dpi, four leaves were removed, clarified in boiling ethanol for 5 min, placed in 0.05% basic aniline blue pH 8.9 at 4 °C for 20 h, mounted on 20% glycerol, stained with 0.01% calcofluor, and examined with an Olympus A70 epi-fluorescent microscope (Tokyo, Japan) (Cohen et al. 2010). Materials were tested at final concentrations of 0, 312, 625, 1250, 2500, and 5000 ppm a.i. (note that some concentrations were not tested in some experiments).

2.2. Control of Downy Mildew in the Field—Trials in Organic Management

Four field experiments using lettuce plants were conducted in commercial organic-grown plots in 2009 in two regions of Israel. The first two trials were carried out in Mevo Modein using the cv ‘15002’ of the Romaine type produced by “Tiv Shtil”, Israel. Trials three and four were carried out in the Beit Shaan Valley using the cv “Limor” of the Crisphead lettuce and cv Noga 936 of the Romaine type, respectively. Timorex Gold at various rates, Kocide 2000 as a fungicide standard, and control untreated plants were evaluated in all trials. Fungicides were sprayed four to five times at specified days and intervals as specified for each trial, when symptoms of downy mildew were absent or evident on leaves. A ‘Still’ Backpack sprayer with a motorized engine and mist blower with a single nozzle type no. 3 was used to spray the fungicides with a spray volume of 300 L/ha. In all trials, the plants were evaluated for disease 6 days after the last application.

Disease had been evident in these plots in previous years. Methods of fertilization, drip irrigation, and other cultural practices for this crop grown in organic management were as recommended to commercial growers by the Extension Service of the Ministry of Agriculture, Israel. Treatments in all experiments were arranged in a randomized complete block design. Plots consisting of 4–6 m row length, each containing three sub-rows, were replicated four times.

2.2.1. Field Trials 1 and 2—Mevo Modein, Israel

Five treatments consisting of an untreated control, Timorex Gold at 0.75, 1.5, and 3.0 L/ha, and Kocide 2000 (Huston, TX, USA) at 0.75 kg/ha as a standard were evaluated in these trials. In trial 1, fungicides were applied five times to the cv ‘15002’ of the Romaine type produced by “Tiv Shtil” Ltd., Israel, on 1, 18, and 25 January and 1 and 8 February 2009, when symptoms of downy mildew were absent on leaves. In trial 2, fungicides were applied five times to a similar cultivar on 1, 8, 14, and 24 February and 2 March 2009.

2.2.2. Field Trials 3 and 4—Beit Shaan Valley, Israel

Four treatments consisting of an untreated control, Timorex Gold at 1.5 and 3.0 L/ha, and Kocide 2000 at 0.75 kg/ha as a standard, were evaluated in these trials. Fungicides were applied four times to cv “Limor” of the Crisp head type in trial 3 and cv Noga 936 lettuce plants in trial 4, on 5, 9, 13, and 19 March 2009.

2.3. Control of Downy Mildew in the Field—Trial in Conventional Management

The cv “Journey” was used in this trial conducted at the Agriculture Experiment Station Sidal Ltd., in Pan Sugar, La Serena, Coquimbo region, Chile. Five treatments consisting of an untreated control, Timorex Gold at 1 and 1.5 L/ha, and Bellis at 0.4 kg/ha and comet at 0.5 kg/ha as fungicide standards, were evaluated in this trial. Fungicides were sprayed six times at 7-d intervals, when symptoms of downy mildew were evident on leaves. A ‘Still’ Backpack sprayer with a motorized engine and mist blower with a single nozzle type no. 3 was used to spray the fungicides with a spray volume of 300 L/ha. The plants were evaluated for downy mildew 14 days after the last application. Plots of 6 m row length were replicated four times.
2.4. Assessment of Downy Mildew Development on Leaves

2.4.1. In Growth Chamber Trials

For plants at the cotyledon stage, the number of symptomatic cotyledons was determined. For detached leaves or plants having true leaves, the proportion of infected leaf area on each plant or leaf was visually estimated at various days after inoculation.

2.4.2. In the Field

Lettuce plants which were naturally affected by downy mildew were visually assessed for the percentage of leaf area infected by *B. lactucae*. In trials conducted in Israel, three units of 1 m² each were randomly selected from the central part of each replicate plot and evaluated for the percentage of leaf area infected.

In the trial conducted in Chile, each of 10 randomly selected plants from each 4 m long unit in the center of each row was evaluated. The percentage of infected plants (incidence) and infected plant area (severity) was determined for each treatment. Treatments in all experiments were arranged in a randomized complete block design.

Ethical approval: This study does not contain any studies with human participants or animals performed by any of the authors.

3. Statistical Analysis

Experiments in growth chambers were conducted at least twice. One representative set of data is presented. The number of replicates per treatment varied according to the experiment: for intact 7-d old plants, there were four replicated pots with \( \approx 20 \) plants/pot per treatment; for adult plants, there were 4–10 replicated plants per treatment; for detached cotyledons, there were 7–12 leaves per treatment. The data were subjected to one-way analysis of variance (ANOVA) using the SAS JMP® 7.0 software (SAS Institute, Cary, North Carolina, USA). The means were separated using the Tukey–Kramer Test at \( \alpha = 0.05 \) and different letters were used to indicate significant differences between means.

4. Results

4.1. Control of Downy Mildew in Growth Chambers

Timorex Gold at 0.125% a.i. provided 87% control and 0.25% a.i. totally inhibited disease development on cotyledons (Figure 1).

![Figure 1](https://example.com/f1.png)

**Figure 1.** Efficacy of preventively applied Timorex Gold on the development of *Bremia lactucae* in lettuce cotyledons. Different letters on curves indicate a significant difference between means at \( p < 0.05 \).
4.2. Control of Downy Mildew in Young Plants

Timorex Gold was highly effective in protecting whole plants against downy mildew (Table 1). Plants at the five-leaf stage were sprayed with Timorex Gold at two concentrations, inoculated with B. lactucae 1 day later, and examined for infected leaf area at 8 dpi. In both experiments, 0.5% and 1% Timorex Gold provided 91–100% protection relative to control plants, with no difference from Kocide 2000 (Table 1). No signs of toxicity (chlorosis and necrosis) were observed on the treated plants.

Timorex Gold applied as a foliar spray to plants inhibited lesion development on detached leaves in a dose-dependent manner in two trials (Table 2). There was a reduction in the percentage of infected leaf area and sporulating leaf area at all tested concentrations (0.125–1%). A concentration of ≥0.5% was as effective as copper hydroxide, providing up to 93% protection compared to control untreated plants (Table 2).

4.3. Control of Downy Mildew in the Field

Four trials were conducted in commercial organic field-grown lettuce plants. Four or five foliar applications of Timorex Gold at various rates, or copper hydroxide as an organic standard product, significantly reduced the percentage of infected area on leaves (Table 3). Kocide 2000 provided slightly, but not significantly, better results in the second trial, compared to Timorex Gold at 1.5 and 3.0 L/ha. Applications of Timorex Gold at 0.75–3 L/ha were similarly effective and provided 46–69% reduction in infected leaf area, compared with control non-treated plants (Table 3). No phytotoxicity to the foliage was observed as a result of foliar applications of Timorex Gold.

Table 3. Efficacy of formulated TTO in controlling downy mildew in field-grown lettuce plants.

| Treatment and Rate/ha | Affected Leaf Area % |
|-----------------------|----------------------|
|                       | Trial 1 | Trial 2 | Trial 3 | Trial 4 |
| Control Untreated     | 20.3 a   | 27.2 a  | 20.6 a  | 28.1 a  |
| Timorex Gold 0.75 L   | 11.0 b   | 13.6 b  | n.t c   | n.t c   |
| Timorex Gold 1.5 L    | 8.2 b    | 9.6 bc  | 11.7 b  | 15.0 b  |
| Timorex Gold 3.0 L    | 6.3 b    | 8.8 bc  | 11.1 b  | 13.9 b  |
| Kocide 2000 0.75 kg   | 4.4 b    | 6.0 c   | 11.1 b  | 10.5 b  |

a Field-grown plants were sprayed with each material at a given rate and downy mildew that developed naturally was assessed as described in Section 2. Untreated plants served as controls. b Mean values within columns followed by different letters are significantly (p < 0.05) different according to the Tukey–Kramer Test. c n.t—not tested.

In the trial conducted in Chile in conventional field-grown lettuce plants under relatively low disease pressure, Timorex Gold at 1 and 1.5 L/ha provided high and satisfactory disease control and was as effective as both commercial fungicides used as standards (Table 4).

Table 4. Efficacy of formulated TTO in controlling downy mildew in field-grown lettuce plants in Chile.

| Treatment and Rate/ha | Disease Incidence % | Disease Severity % |
|-----------------------|---------------------|--------------------|
| Control Untreated     | 75.0 a              | 1.5 a              |
| Timorex Gold 1 L      | 5.0 b               | 0.05 b             |
| Timorex Gold 1.5 L    | 10.0 b              | 0.15 b             |
| Bellis 0.4 kg         | 5.0 b               | 0.05 b             |
| Comet 0.5 L           | 0.0 b               | 0.0 b              |

a Field-grown plants in conventional farm were sprayed with each product at a given rate and downy mildew that developed naturally was assessed as described in Section 2. Untreated plants served as controls. b Mean values within columns followed by different letters are significantly (p < 0.0001) different according to the Tukey–Kramer Test.

4.4. Microscopy of B. lactucae–Lettuce Interaction as Affected by Timorex Gold and Pure TTO

The effect of Timorex Gold on the development of the pathogen in detached cotyledons at 2 and 5 dpi is shown in Figures 2 and 3. In control inoculated cotyledons, most conidia germinated and
produced infection structures in the tissue. Timorex Gold at 625 and 1250 ppm a.i. had a minor effect, but at 2500 ppm it prevented spore germination, ingress of the pathogen into the tissue, and mycelium growth or formation of haustoria (Figure 2, Table 5). At 5 dpi, extensive spread of mycelia in the mesophyll, emergence of sporophores from stomata and abundant sporophores with sporulation were observed in the control cotyledons (Figure 3). Timorex Gold strongly suppressed sporophore formation and hence spore production, at a low dose of 625 ppm. No sporophores developed, and thus no sporulation was observed, at 1250 and 2500 ppm (Figure 3, Table 5). The data suggest that Timorex Gold has a greater inhibitory effect on sporophore formation than on spore germination (Table 5).

Figure 2. Fluorescence micrographs showing the effect of formulated TTO (625, 1250, and 2500 µg/mL a.i.) on germination and penetration of B. lactucae into lettuce leaves. Conidia and germ tubes fluoresce blue; infection structures inside the leaf fluoresce yellow. Bar = 50 µm. Photos were taken at 2 d after inoculation.

Figure 3. Fluorescence micrographs showing the effect of formulated TTO (625, 1250, and 2500 µg/mL a.i.) on sporulation of B. lactucae into lettuce leaves. Sporophores and spores fluoresce blue. Bar = 250 µm. Photos were taken at 5 d after inoculation.
When detached cotyledons were inoculated with spores mixed with pure TTO, a significant effect was observed on the penetration of the pathogen into the tissue (Figure 4). Spores did germinate on treated cotyledons even at the highest concentration used, but no infection vesicles were produced (Figure 4). Microscopical observations made at 5 dpi revealed that pure TTO is highly inhibitory (Minimum Inhibitory Concentration (MIC) = 625 ppm) to sporophore formation and hence to sporulation (Figure 5).

Table 5. The effect of Timorex Gold on conidial germination at 24 h and sporophore formation at 5 dpi of *B. lactucae* on detached cotyledons of lettuce *a*.

| Treatment Concentration, ppm a.i. | Percent Germinating Spores 24 h | Number Sporophores/Cotyledon 5 dpi |
|----------------------------------|----------------------------------|-----------------------------------|
| 0                                | 66 a                             | 55 a                              |
| 312                              | 50 ab                            | 17 b                              |
| 625                              | 40 b                             | 15 b                              |
| 1250                             | 23 c                             | 2 c                               |
| 2500                             | 6 d                              | 0 d                               |
| 5000                             | 0 e                              | 0 d                               |

*a* The effect of Timorex Gold at various concentrations on germination and sporophores production of *B. lactucae* on detached cotyledons was determined. Mean values within columns followed by different letters are significantly 

\( p < 0.0001 \) different according to the Tukey–Kramer Test. Parts per million (ppm); Dpi (Days post inoculation).

**Figure 4.** Fluorescence micrographs showing the effect of pure TTO (1250, 2500, and 5000 µg/mL a.i.) on germination and penetration of *B. lactucae* in lettuce leaves. Conidia and germ tubes fluoresce blue; infection structures inside the leaf fluoresce yellow. Bar = 20 µm. Photos were taken at 2 d after inoculation.

**Figure 5.** Fluorescence micrographs showing the effect of pure TTO (625, 1250, and 2500 µg/mL a.i.) on sporulation of *B. lactucae* in lettuce leaves. Sporophores and spores fluoresce blue. Bar = 50 µm. Photos were taken at 5 d after inoculation.
5. Discussion

The ultimate value of any chemical compound as a disease-control agent depends on the mode of action of its molecule on one or more stages of the pathogen’s life cycle. Important stages in the life cycle of *B. lactucae* involved in host infection and disease development include germination of conidia, penetration into the host, mycelial growth in the host, and sporulation [3]. A product that inhibits any of these stages would thus reduce the ability of *B. lactucae* to cause disease. In conventional production, the disease can be controlled by synthetic chemicals, including protective (e.g., copper hydroxide, chlorothalonil) and systemic fungicides such as phenylamidine (e.g., metalaxyl/mefenoxam), strobilurins (e.g., azoxystrobin), and CAA (e.g., dimethmorph)-based compounds. However, isolates that are insensitive to these compounds are frequent [5,6]. An alternative procedure for protecting lettuce plants against downy mildew was shown by the non-protein amino acid DL-3-amino-n-butanoic acid (BABA) [7]. In contrast, control of the disease in organic growth depends on a limited number of approved products with copper hydroxide being the most commonly used.

This paper presents the activity of TTO against *B. lactucae* in lettuce. The mode of action of TTO differs from those of synthetic fungicides such as phenylamides [6] or strobilurines such as pyraclostrobin. Strobilurin analogs inhibit mitochondrial respiration by blocking electron transfer at the cytochrome bc1 complex, also known as complex III. The fungicidal activity of TTO against fungal and oomycete pathogens arises from its ability to disrupt the cell membrane’s permeability [11,21,22]. In yeast cells and isolated mitochondria, TTO components destroy the cellular integrity, inhibit respiration and ion transport, and increase membrane permeability [12,21,22]. Our previous data, obtained with the aid of transmission electron microscopy, showed that TTO disrupted the fungal cell wall and cell membrane of *Mycosphaerella fijiensis* at stages 4 or 5 of fungal development in the intracellular space of the banana leaf mesophyll [19]. A similar effect was reported by Shao et al. [13] on the mycelial morphology and ultrastructure, cell wall, and membrane of *Botrytis cinerea* in in vitro experiments.

Epifluorescence microscopy revealed that Timorex Gold at 625 and 1250 ppm a.i. had a minor effect, whereas at 2500 ppm it prevented spore germination and thus infection (Figure 2, Table 5). Timorex Gold at higher doses seems to adversely affect the penetration of *B. lactucae* into the epidermal cells of lettuce. The failure of *B. lactucae* to penetrate may result from reduced synthesis and secretion of hydrolytic enzymes by the pathogen, which are necessary for penetration, either as a direct effect on the fungus or through the host tissue by inhibition of the “signal” mechanism for the secretion of these enzymes. Formation of infection structures in the epidermal cells was totally inhibited at a high dose of 2500 and 5000 ppm of Timorex Gold and pure TTO, respectively (Figures 2 and 4). These doses are compatible with those used in the field (Tables 3 and 4). Both Timorex Gold and pure TTO inhibited sporophore formation, and hence spore production, in a dose-dependent manner (Figures 3 and 5, Table 5). In spite of sporophores formation, no conidiation occurred at ≥625 ppm of TTO. The data suggest that Timorex Gold is more inhibitory to mycelial development and sporophore formation than to spore germination. This might be related to the ability of TTO to penetrate into the host tissue and to intracellular space of the leaf mesophyll as demonstrated in previous study [19]. It is therefore concluded that TTO is effective against *B. lactucae* mainly after penetration, and especially against hyphal growth (colonization) in the mesophyll. As a result, sporulation was greatly reduced at 5 dpi. Previous studies [13,19,21,22] support this conclusion.

A similar study with BABA against *B. lactucae* in lettuce was conducted by Cohen et al. [20]. They showed that BABA did not affect spore germination, appressorium formation, or penetration of *B. lactucae* into the host. It allowed the formation of primary and secondary vesicles in the epidermal cells, but inhibited the emergence of the infective hyphae from these primary invading structures of the pathogen, thus localizing the pathogen solely to the penetrated epidermal cells [20].

Timorex Gold inhibited lesion development on treated leaves and limited the expansion of lesions (Figure 1, Tables 1 and 2), thus severely restricting the potential of *B. lactucae* to infect plant tissue and cause disease. Timorex Gold was effective when applied as a foliar spray to growth-chamber-grown young plants or to field-grown plants. It effectively controlled downy mildew in field trials in
organic-production lettuce plants conducted in Israel (Table 3) and was as effective as copper hydroxide (Table 3). Copper has been in use in agriculture to control oomycetes, fungi, and bacteria for over a century. It plays important roles in integrated pest management, but is essential in organic farming, where disease management depends almost exclusively on its use. However, use of this heavy metal may have long-term consequences due to its accumulation in the soil. This led the European Union to establish maximum limits on copper in organic farming. Strategies for reducing copper inputs include preventive phytosanitary measures, use of forecasting models, use of resistant varieties, and natural alternatives to copper-based products [4]. Our results on organic growth (Table 3) suggest that use of ‘Timorex Gold’ can be an alternative for this heavy metal, with the same plant protection effectiveness. Furthermore, Timorex Gold provided disease control similar to synthetic fungicides in a field trial conducted in conventional-grown plants (Table 4).

TTO-based products can be an important tool for inclusion in conventional fungicide programs, in which to manage resistance development during the season. It can be rotated in applications with products to which \textit{B. lactucae} populations have shown a loss of sensitivity. In this way, the population of individuals less sensitive to synthetic systemic fungicides such as phenyl amide and/or strobilurins can be reduced.

This paper provides substantial evidence that Timorex Gold is an effective biopesticide for controlling downy mildew in lettuce both organic and conventional systems.

6. Conclusions

This study provides further novel information on the mode of activity of the Australian tea tree oil (TTO) against downy mildew pathogen in lettuce. Epifluorescence microscopy revealed that Timorex Gold, a TTO-based biofungicide, and TTO had a greater inhibitory effect on sporophore formation and sporulation than on spore germination. In addition, Timorex Gold effectively controlled growth-chamber-grown young lettuce plants or field-grown plants, mainly with low-moderate disease pressure. These results together with the efficacy of TTO in controlling fungal pathogens make it an important component for disease control in crop protection.

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