*Article*

# Testing of Eight Medicinal Plant Extracts in Combination with Kresoxim-Methyl for Integrated Control of *Botrytis cinerea* in Apples

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Academic Editor: Nieves Goicoechea

**Abstract:** *Botrytis cinerea* is a fungus that causes gray mold on many fruit crops. Despite the availability of a large number of botryticides, the chemical control of gray mold has been hindered by the emergence of resistant strains. In this paper, tests were done to determine the botryticidal efficacy of selected plant extracts alone or combined with kresoxim-methyl. In total, eight South African medicinal plants *viz Artemisia afra*, *Elyptropappus rhinocerotis*, *Galenia africana*, *Hypoxis hemerocallidea*, *Siphonochilus aetheopicus*, *Sutherlandia frutescens*, *Tulbaghia violacea* and *Tulbaghia alliacea* were screened. *Allium sativum*, a plant species known to have antifungal activity, was included in the *in vivo* studies. For the *in vitro* studies, synergistic interactions between the plant extracts and the kresoxim-methyl fungicide were tested with radial growth assays. Data indicated synergistic inhibitory effects between the fungicide and the plant extracts. Next, different doses of plant extracts combined with kresoxim-methyl were used for decay inhibition studies on Granny Smith apples. Synergistic and additive effects were observed

for many of the combinations. Even though this study was done using only one strain of *B. cinerea*, results showed that the tested indigenous South African plant species possess natural compounds that potentiate the activity of kresoxim-methyl.

**Keywords:** *Botrytis cinerea*; gray mold; kresoxim-methyl; medicinal plants; plant extracts; strobilurin; fungicides

## 1. Introduction

Growth of fungal pathogens leads to considerable economic losses during postharvest handling, transportation and storage of crops [1,2]. As a solution, synthetic fungicides have been used globally since the 1950s to protect major crops from damage by phytopathogenic fungi [3,4]. Many modern fungicides are effective and exhibit relatively low mammalian toxicity [5]. Disconcertingly, however, as a result of fungicide overuse, resistant strains of these pathogens are now appearing with alarming frequency [6,7]. This progression of resistance has now become a major problem globally, particularly where high resistance factors have been reported and the frequencies of mutant phenotypes in the population are high. This phenomenon greatly lowers the efficacy of active ingredients in the fungicides, resulting in an increase in the cost of chemical control and potentially resulting in environmental damage if repeated treatment is required [4].

*Botrytis cinerea* (Pers. ex. Fr) is a ubiquitous fungus causing gray mold on various crops, even when the most advanced postharvest technologies have been applied [8]. It is considered an important pathogen of vegetables, ornamental plants and fruits [4,6,9]. Together with blue mold, caused by *Penicillium* spp., gray mold is one of the main postharvest diseases of apples in storage. Gray mold can spread from fruit to fruit and whole bins of fruit can potentially become infected, often leading to severe economic losses [10]. In spite of the availability of numerous botryticides, the chemical control of gray mold has been encumbered by the emergence of resistant strains [4,9,11–14]. Of greatest concern, multiple drug resistant (MDR) strains have recently been isolated from French and German vineyards. MDR populations increase the risk of gray mold rot and, more worryingly, impede the effectiveness of current strategies for the management of fungicide resistance [6,15].

In recent years, researchers have focused on the discovery of plant-derived fungicides which are considered safer than synthetic fungicides. Plant extracts with fungistatic or fungicidal activities have shown potential as effective synthetic fungicide alternatives for postharvest control of various plant diseases of fruit [16–20]. Also, biofungicides applied in mixtures with synthetic fungicides, result in a reduction of the amounts of active ingredients applied and could possibly prevent the development of fungicide resistance [21,22]. In this study, tests were done to determine the botryticidal efficacy of selected plant extracts alone or in combination with kresoxim-methyl fungicide (Stroby®, BASF, Ludwigshaven, Germany). In total, eight South African medicinal plant species including *Artemisia afra*, *Elyptropappus rhinocerotis*, *Galenia africana*, *Hypoxis hemerocallidea*, *Siphonochilus aetheopicus*, *Sutherlandia frutescens*, *Tulbaghia violacea* and *Tulbaghia alliacea* were screened. *Allium sativum* (commercial garlic), a plant with known antifungal characteristics [23–25], was included in the *in vivo* studies. For the *in vitro* studies, synergistic interactions between the plant

extracts and the kresoxim-methyl fungicide were tested with radial growth assays. Next, different doses of plant extracts combined with kresoxim-methyl were used for decay inhibition studies on Granny Smith apples. Taken together, results showed that the tested indigenous South African plants possess natural compounds that potentiate the activity of kresoxim-methyl. This report offers an attractive prospect for the development of alternative strategies for controlling *B. cinerea*.

## 2. Results

### 2.1. Effect of Plant Extracts Alone and in Combination with Kresoxim-Methyl on the Growth of B. Cinerea in vitro

Firstly, the *in vitro* inhibitory effects of eight medicinal plant extracts alone and in combination with kresoxim-methyl on *B. cinerea* growth were studied (Table 1). In the absence of kresoxim-methyl, most of the plant extracts tested produced weak inhibitory effects (>50% inhibition). Only *G. africana* and *E. rhinocerotis* extracts showed radial growth inhibition >50%, at all doses tested and 500 mg·mL$^{-1}$, respectively. In general, the mycelium growth of *B. cinerea* decreased with increasing plant extract doses. *In vitro* studies with methanol extracts of *A. afra*, *A. sativum*, *E. rhinocerotis*, *H. hemerocallidea*, *S. aethiopicus*, *S. frutescens*, *T. alliacea*, and *T. violacea* showed weak antifungal properties against *B. cinerea*. The strongest antifungal activity in the radial growth bioassay was observed for *G. africana* extracts.

Although kresoxim-methyl (2.5 mg·mL$^{-1}$) in combination with *G. africana* extract at doses of 125.0, 250.0 and 500.0 mg·mL$^{-1}$ showed statistically significantly high inhibition levels compared to the kresoxim-methyl control, it only produced additive interactions (SR 0.5–1.5). The *E. rhinocerotis* extracts showed optimal botryticidal activity at 250.0 and 500.0 mg·mL$^{-1}$, while *A. sativum*, *S. aethiopicus*, *T. alliacea*, *H. hemerocallidea* and *T. violacea* showed optimal botryticidal activity at 125.0 and 250.0, but not at 500.0 mg·mL$^{-1}$. For the other combination treatments synergistic interactions (SR > 1.5; *p* < 0.5) were observed for *S. aethiopicus*, *S. frutescens* and *T. alliaceae* extracts at 62.5 mg·mL$^{-1}$, for *A. afra*, *A. sativum*, *H. hemerocallidea*, *S aethiopicus*, *T. alliacea* and *T. violacea* at 125.0 mg·mL$^{-1}$, and for *A. afra*, *A. sativum*, *H. hemerocallidea*, *T. alliacea* and *T. violacea* at 250.0 mg·mL$^{-1}$. Antagonistic effects (SR < 0.5) were primarily observed for the 500.0 mg·mL$^{-1}$ plant extract and 2.5 mg·mL$^{-1}$ Stroby combinations.

Total radial growth inhibition (100%) was only observed for the combinations of 5.0 mg·mL$^{-1}$ kresoxim-methyl and 250 mg·mL$^{-1}$ *T. alliacea* and *T. violacea* (Table 2). Most of the plant extracts and 5.0 mg·mL$^{-1}$ kresoxim-methyl combinations showed additive interactions. Synergistic interactions for plant extracts and the 5.0 mg·mL$^{-1}$ kresoxim-methyl combinations were observed for *T. alliacea* and *T. violacea* at 62.5 mg·mL$^{-1}$, for *A. afra*, *S. frutescens*, *T. alliacea* and *T. violacea* at 125.0 mg·mL$^{-1}$, and for *T. alliacea* and *T. violacea* at 250.0 mg·mL$^{-1}$. No synergistic effects were observed for the 500.0 mg·mL$^{-1}$ plant extract and 5.0 mg·mL$^{-1}$ kresoxim-methyl combinations. However, when the plant extracts were combined with kresoxim-methyl, significant reduction in mycelium growth of the fungus was observed for almost all of the combinations. The inhibitory effects in combinations with kresoxim-methyl were especially significant for extracts of *A. afra*, *E. rhinocerotis*, *G. africana*, *H. hemerocallidea*, *S. aethiopicus*, *S. frutescens*, *T. alliacea*, and

*T. violacea*. Using the Abbott method, we were able to calculate mathematical synergistic ratios for all the plant extracts at their respective doses and in combination with kresoxim-methyl. With the exception of *G. africana* and *E. rhinocerotis*, all the plant extracts showed synergism (>1.5) for the *in vitro* study. *Siphonochilus aethiopicus*, *S. frutescens*, *T. alliacea* and *T. violacea* showed the most potent synergistic interaction at the lowest extract dose (*i.e.*, 62.5 mg·mL$^{-1}$) used for the *in vitro* study.

*2.2. In Storage: Effect of Plant Extracts Alone and in Combination with Kresoxim-Methyl on the Growth of B. cinerea on Apples*

In this study, single extracts of the medicinal plant species *A. afra*, *E. rhinocerotis*, *G. africana*, *H. hemerocallidea*, *S. aethiopicus*, *S. frutescens*, *T. alliacea*, and *T. violacea* exhibited weak or no antifungal properties against *B. cinerea in vivo*. However, when low doses of the plant extracts were combined with a subinhibitory concentration of kresoxim-methyl, synergistic and additive interactions were observed. Synergistic effects were especially obvious for the lowest extract doses of *E. rhinocerotis*, *H. hemerocallidea*, *S. frutescens*, *T. alliacea* and *T. violacea* (Table 2). Significant differences in the inhibition of decay progression for the plant extract and kresoxim-methyl (0.005 mg·mL$^{-1}$) combinations compared to the control were observed for *A. afra* (15.63 mg·mL$^{-1}$), *G. africana* (62.5 mg·mL$^{-1}$), *E. rhinocerotis* (1.95, 3.91 mg·mL$^{-1}$), *H. hemerocallidea* (1.95, 3.91, 15.63, 31.25 mg·mL$^{-1}$), *S. frutescens* (1.95, 3.91, 7.81 mg·mL$^{-1}$), *T. alliacea* (1.95, 3.91 mg·mL$^{-1}$) and *T. violacea* (1.95, 3.91, 7.81, 31.25, 62.0 mg·mL$^{-1}$) (*p* < 0.05). Synergistic interactions were observed for the plant extract doses of *E. rhinocerotis* (7.81 mg·mL$^{-1}$), *H. hemerocallidea* (7.81, 15.63, 31.25 mg·mL$^{-1}$), *S. frutescens* (3.91 mg·mL$^{-1}$), *T. alliacea* (7.81 mg·mL$^{-1}$) and *T. violacea* (15.63 mg·mL$^{-1}$). Kresoxim-methyl, in combination with the higher plant extract dose of 62.5 mg·mL$^{-1}$, produced primarily additive interactions.

**3. Experimental Secion**

*3.1. Pathogen Preparations*

Non-resistant *Botrytis cinerea* was isolated from the surface of "Granny Smith" apples infected with gray mold disease and maintained on potato-dextrose agar (PDA) at 25 °C for 14 days (Disease Management Division, Agricultural Research Council (ARC) Infruitec—Nietvoorbij, South Africa). Fresh inoculum was prepared by transferring spores from stock cultures to PDA and incubating these at 25 °C in the dark under a white fluorescent light with a 12:12 light:dark photoperiod.

**Table 1.** Inhibition of radial growth and synergy ratio (SR) between the kresoxim methyl fungicide and different plant extract doses against *Botrytis cinerea in vitro*.

| | Radial Growth Inhibition (%) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Plant Extract Dose (mg·mL⁻¹) | | | | Plant Extract Dose + 2.5 mg·mL⁻¹ Stroby® | | | | | Plant Extract Dose + 5.0 mg·mL⁻¹ Stroby® | | | | |
| | 62.5 | 125 | 250 | 500 | 0 | 62.5 | 125 | 250 | 500 | 0 | 62.5 | 125 | 250 | 500 |
| Stroby® | | | | | 37 | | | | | 72.5 | | | | |
| *A. afra* | 0.9 | −0.5 | 2.6 | 15.3 | - | 22.6* | 38.3 * | 34.6 * | 17.2 ** | - | 76.7 * | 98.5 */** | 80.9 * | 39.3 */** |
| | | | | | | **1.2** | **2.3** | **1.7** | **0.5** | | **1.4** | **1.9** | **1.4** | **0.6** |
| *A. sativum* | −0.4 | 25.7 | 8.9 | 12.1 | | 52.0 * | 74.5 */** | 74.6 */** | 26.4 * | | 75.7 * | 96.8 */** | 99.8 */** | 77.5 * |
| | | | | | | **3.1** | **1.8** | **2.9** | **0.9** | | **0.8** | **1.2** | **2.3** | **1.2** |
| *E. rhinocerotis* | 17.8 | 27.6 | 47.4 | 62 | | 0.7 ** | 44.7 | 88.1 */** | 74.4 ** | | 29.6 ** | 79.8* | 85.8 * | 73.7 |
| | | | | | | **0** | **0.1** | **1.4** | **0.9** | | **0.4** | **1** | **0.9** | **0.6** |
| *G. africana* | 67.1 | 71.4 | 76.9 | 74 | | 61.7 ** | 81.8 ** | 87.0 ** | 84.3 ** | | 58.1 | 73 | 83.8 | 90.8 |
| | | | | | | **0.7** | **0.9** | **0.9** | **0.9** | | **0.5** | **0.6** | **0.6** | **0.7** |
| *H. hemerocallidea* | 7.2 | 11.4 | 28.6 | 24 | | 13.7 | 52.6 * | 79.3 */** | 49.3 * | | 87.3 * | 89.8* | 94.7 */** | 61.6 * |
| | | | | | | **0.6** | **1.9** | **1.8** | **1.2** | | **1.4** | **1.4** | **1.2** | **0.8** |
| *S. aethiopicus* | −4.6 | 22.6 | −6.8 | 26.8 | | 43.5 ** | 90.0 */** | 78.8 */** | 27.5 | | −2.4 ** | 19.6 ** | 28.4 */** | 0.7 */** |
| | | | | | | **3.5** | **2.3** | **7.9** | **0.6** | | **0.1** | **0.3** | **0.6** | **0** |
| *S. frutescens* | −3.4 | 3.4 | −0.2 | 8.8 | | 49.2 * | 12.4 ** | 26.1 * | 16.8 ** | | 59.7* | 93.9 */** | 80.0 * | 22.8 */** |
| | | | | | | **3.6** | **0.6** | **1.5** | **0.6** | | **1.2** | **1.7** | **1.5** | **0.4** |
| *T. alliacea* | −7.5 | 3.2 | 5 | 18.6 | | 38.0 * | 79.3 */** | 62.3 */** | 23.6 | | 71.3 * | 90.0* | 100.0 */** | 71.1 * |
| | | | | | | **4** | **4** | **2.8** | **0.7** | | **1.6** | **1.6** | **1.7** | **1** |
| *T. violacea (EC)* | −0.9 | −3.6 | −10.1 | 32.2 | | −3.6 */** | 19.5 * | 37.6 * | 52.4 * | | 47.7 * | 84.4 */** | 88.1* | 48.1 ** |
| | | | | | | **0.2** | **1.4** | **1.4** | **1.1** | | **0.9** | **1.7** | **1.4** | **0.6** |
| *T. violacea* | 0.7 | 11.8 | 9.6 | 19.6 | | 59.6 */** | 68.6 */** | 76.1 */** | 35.4 | | 97.5 */** | 97.5 */** | 100.0 */** | 92.5 */** |
| | | | | | | **3.4** | **2.4** | **2.9** | **1** | | **1.8** | **1.5** | **1.6** | **1.3** |

The synergism ratio for percentage inhibition (values in bold) was based on the Abbott formula as described by [26]. Radial growth inhibition values of the plant extract and Stroby® combinations with one asterisk (*) are significantly different ($p > 0.05$) from the respective values of the plant extract doses without Stroby® in the same row. Radial growth inhibition values of the plant extract and Stroby® combinations with two asterisks (**) are significantly different ($p > 0.05$) from the value of the Stroby® dose (2.5 or 5.0 mg·mL⁻¹) in the column.

**Table 2.** Percentage inhibition of grey mold decay on Granny Smith apples inoculated with conidia of *B. cinerea*, and the relative level of synergism (SR) of mixtures containing 0.0 and 0.005 mg·mL$^{-1}$ of kresoxim-methyl and different doses of medicinal plant extracts.

| | | Decay Inhibition (%) | | | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Plant Extract Dose (mg·mL$^{-1}$) | | | | | | Plant Extract Dose + 0.005 mg·mL$^{-1}$ Stroby® | | | | | |
| | 1.95 | 3.91 | 7.81 | 15.63 | 31.25 | 62.5 | 0 | 1.95 | 3.91 | 7.81 | 15.63 | 31.25 | 62.5 |
| Stroby® | | | | | | | 57.4 | | | | | | |
| *A. afra* | −16.9 | 11.2 | 12.4 | 30 | −13.8 | 19.2 | | 53.9* / 1.3 | 88.0* / 1.3 | 72.6* / 1.1 | 93.6 */** / 1.1 | 36.6 * / 0.8 | 49.6 * / 0.7 |
| *E. rhinocerotis* | 49.1 | 10.4 | −9.6 | 86 | -6.2 | −13.9 | | 100.0 */** / 0.9 | 96.0 */** / 1.4 | 77.5 * / 1.6 | 52.3 * / 0.4 | 26.9 * / 0.5 | 37.4 * / 0.9 |
| *G. africana* | 10.6 | 19.7 | 1.6 | 37.6 | 59.2 | 74.1 | | 86.7* / 1.3 | 70.9 * / 0.9 | 70.3 * / 1.2 | 72.2 * / 0.8 | 82.4 / 0.7 | 96.3 ** / 0.7 |
| *H. hemerocallidea* | 8.4 | 28.5 | −6.1 | -0.1 | -3.4 | 24.6 | | 92.5 */** / 1.4 | 95.9 */** / 1.1 | 85.6 * / 1.7 | 94.0 */** / 1.7 | 94.0 */** / 1.8 | 70.0 * / 0.9 |
| *S. aethiopicus* | −3.9 | 4.1 | 27.9 | 23.8 | 32.4 | 16.8 | | 47.8* / 0.9 | 83.3 * / 1.4 | 72.8 * / 0.9 | 75.4 * / 0.9 | 81.6 * / 0.9 | 30.3 / 0.3 |
| *S. frutescens* | 8.9 | 5.1 | 12.2 | 8.6 | 3.6 | 16.1 | | 90.9 */** / 1.4 | 100.0 */** / 1.6 | 93.1 */** / 1.4 | 69.1 * / 1.1 | 86.2 * / 1.4 | 35.3 / 0.5 |
| *T. alliacea* | 16.6 | 24 | −11.1 | 13.6 | 18.2 | −8.7 | | 100.0 */** / 1.4 | 96.2 */** / 1.2 | 76.6 * / 1.7 | 73.4 * / 1 | 60.2 * / 0.8 | 68.8 * / 1.4 |
| *T. violacea* | 15.8 | 46.4 | 40.8 | −8.24 | 11.2 | 20.5 | | 100.0 */** / 1.4 | 93.2 */** / 0.9 | 100.0 */** / 1 | 81.9 * / 1.7 | 100.0 */** / 1.5 | 24.5 ** / 0.3 |

The synergism ratio for percentage inhibition (values in bold) was based on the Abbott formula as described by [26]. Decay inhibition mean values of the plant extract and Stroby® combinations with one asterisk (*) are significantly different ($p > 0.05$) from the respective values of the plant extract doses without Stroby® in the same row. Decay inhibition mean values of the plant extract and Stroby® combinations with two asterisks (**) are significantly different ($p > 0.05$) from the value of the Stroby® dose (0.005 mg·mL$^{-1}$) in the column.

## 3.2. Preparation of Plant Extracts

The plant parts of the following medicinal plant species used in traditional medicine practices in South Africa were obtained for the preparation of plant extracts: *Artemisia afra* Jacq. (fresh leaves), *Elytropappus rhinocerotis* (Lf) Less (fresh leaves), *Galenia africana* (L.) (dried leaves), *Hypoxis hemerocallidea* (Fisch. and C.A. Mey) (fresh corms), *Siphonochilus aethiopicus* (Schweif.) BL Burt (fresh rhizomes + fleshy roots), *Sutherlandia frutescens* (L.) R. Br. (dried leaves), *Tulbaghia alliacea* (fresh corms), *Tulbaghia violacea* Harv. (fresh leaves + rhizomes from Western Cape greenhouse plants), *Tulbaghia violacea* from the Eastern Cape (fresh leaves + rhizomes from "wild" plant). *Allium sativum* (bulbs) was included in the *in vivo* studies. Identity of the various plant species was authenticated and assigned a voucher number by Frans Weitz (Herbarium, Department of Botany, University of the Western Cape, Bellville, South Africa).

Where fresh plant organs were used, the organs were homogenized in a Waring blender and 50 g of the macerated plant was then extracted overnight in 100 ml methanol (MeOH; 99.8% Sigma Aldrich) in a closed conical flask at room temperature to obtain 50% (w/v) (500 mg·mL$^{-1}$) crude, viscous liquid extract. Where dried leaves were used, the leaves were powdered in a hammer mill and 50 g extracted overnight in a closed conical flask at room temperature in MeOH to obtain 50% viscous liquid extracts. Each crude methanol extract was filtered through Whatman No. 4 qualitative filter paper and stored at 4 °C until used. For the *in vitro* and apple fruit bioassays two-fold dilution series of the 50% crude plant extracts were prepared with deionized sterile water as described below.

## 3.3. In vitro Assay

A 1 ml suspension of the plant extracts and kresoxim-methyl (Stroby® WG fungicide, 500 g·kg$^{-1}$, BASF, Ludwigshaven, Germany), single or combinations of both products, were spread evenly onto a solidified PDA surface in 9 mm Petri dishes and allowed to air dry under sterile conditions in a laminar flow to evaporate the solvents. Controls consisted of sterile water and methanol. The 50% methanolic plant extracts were diluted in sterile water in 10 ml doses of 0.0, 62.5, 125.0, 250.0, 500.0 mg·mL$^{-1}$, with kresoxim-methyl (0.0, 2.5 and 5.0 mg·mL$^{-1}$), respectively. *In vitro* studies showed that assays with 2.5 and 5.0 mg·mL$^{-1}$ kresoxim-methyl provided the highest inhibitory effects with plant extracts in the radial growth experiments. Each plate was inoculated with 3-mm mycelial plugs removed from the margins of actively growing 14-day-old *B. cinerea* cultures, and placed upside down on the PDA surface. Radial growth was assessed 5 days after incubation at 22–23 °C.

## 3.4. Postharvest Assay on Apples

The apple cultivar, Granny Smith, were removed from cold storage, surface-sterilized with 70% EtOH for 2 min and air-dried. Each apple was wounded (5 mm in diameter and 3 mm in depth) three times halfway between the calyx and the stem end. A 20-μL drop of each plant extract and kresoxim-methyl was placed in the wounds and allowed to air-dry for two hours before application of a 20-μL conidial suspension (1 × 10$^4$ spores mL$^{-1}$); the 20-μL drops had final plant extract doses of 0.0, 1.95, 3.91, 7.81, 15.63, 31.25 and 62.5 mg·mL$^{-1}$, with or without kresoxim-methyl at 0.0 and 0.005 mg·mL$^{-1}$. Controls consisted of sterile water and methanol without the compounds to be tested.

Fruits were stored in commercial cardboard boxes at 20 °C in a high humidity (95% RH) walk-in incubator. Diameter of *B. cinerea* decay lesions was determined after a 7-day incubation period.

*3.5. Statistical Analysis*

The *in vitro* experimental design was completely randomized. Each mycelial plug in a Petri dish constituted a replicate. To assess differences in the mycelial growth of *B. cinerea* among the treatments the percentage inhibition were calculated from the radial growth as:

$$\% = 100 - [(\text{treatment/control}) \times 100] \tag{1}$$

All analyses were carried out using SAS Version 8.2 [27]. For the apple bioassay each treatment was performed on three apples, each with triplicate wounds. Percentage inhibition was determined for each replicate and averaged for the three apples. Data for percentage decayed fruit were subjected to a standard analysis of variance. The analysis of variance was performed using SAS Version 8.2 [27]. Student *t*-Least Significant Difference was calculated at the 5% significant level to compare treatment means of percentage inhibition. The synergism ratio for percentage inhibition was based on the Abbott formula [28] as described by Gisi [26]:

$$C_{exp} = (A + B) - (AB/100) \tag{2}$$

In this study, $C_{exp}$ was the expected efficacy of the mixture, with A and B the control levels given by kresoxim-methyl and the medicinal plant extract, respectively. The synergy ratio (SR) between the observed ($C_{obs}$) and expected ($C_{exp}$) efficacies of the mixture was calculated as:

$$SR = C_{ob}/C_{exp} \tag{3}$$

An SR >1.5 indicates a synergistic interaction between compounds; 0.5–1.5 indicates an additive interaction between compounds; <0.5 indicates an antagonistic interaction between compounds [29].

## 4. Discussion and Conclusions

In recent years there has been a drive to find alternatives to chemical fungicides considered as safe and with lower risk to human health and the environment. Satisfactory results have been reported using biocontrol or antagonistic microorganisms [30] and natural compounds including various plant extracts [31,32]. Natural plant protectants with antimicrobial activity have been studied since they generally tend to have low mammalian toxicity, low environmental impact and wider public acceptance [5,20]. Secondary plant metabolites are often active against a small number of specific target microbial species and are biodegradable to nontoxic products, making it potentially valuable in integrated pest management programs [2]. Also, identifying synergistic combinations of fungicides and natural plant compounds could result in control strategies with high biological activity, low dose rate application and a low risk of pathogen-resistance development [5,20]. In this study, the reason for the reduced *B. cinerea* sensitivity to the combinations of kresoxim-methyl and plant extracts at higher doses has not yet been elucidated. We speculate that this could be indicative of competitive inhibition resulting from constituents in the plant extracts competing for the mode of action sites of kresoxim-methyl. This would effectively impede the complete inhibition of mitochondrial respiration, since the binding site of kresoxim-methyl is the ubihydroquinone cytochrome-c oxidoreductase [33]. In fact, more recent studies

with plant extracts showed that polyphenols could stimulate and/or enhance mitochondrial function [34–36]. Whether polyphenols act directly or indirectly to enhance mitochondrial functions and whether this requires binding of polyphenols to a specific receptor remain to be investigated. Studies with arbuscular mycorrhizal fungi showed that plant root-exuded factors cause changes in fungal mitochondrial morphology, orientation, and overall biomass and rapidly induces the expression of certain fungal genes and, in turn, respiratory activity before intense branching [37].

Interestingly, in this study the extract from *T. violacea* from the Western Cape showed higher inhibitory and synergistic activities in combination with Stroby® compared to the *T. violacea* extracts from the Eastern Cape and the *A. sativum* extracts. We do not know the reason for this observation as yet. However, due to the close relationship between *Allium* and *Tulbaghia* species within the *Alliaceae* family the biological and chemical characteristics of the two species seems to be comparable [38]. Interestingly, even though the *E. rhinocerotis* extract showed synergistic effects in the *in vivo* study, it did not produce a similar effect in the *in vitro* study. This could be a result of the type of biological formulation used, which could have affected the efficacy of the extract in the *in vitro* study [39,40].

As yet, we do not have an explanation for the lower ratios of synergistic reactions for the plant extract and kresoxim-methyl combinations *in vivo* compared to *in vitro*. However, this paper provided evidence that compounds in the tested plant extracts contributed additively and/or synergistically with the fungicide, kresoxim-methyl. In the *in vitro* radial growth assays, synergistic inhibitory effects were observed between kresoxim-methyl and plant extracts. Finally, in decay inhibition studies on Granny Smith apples synergistic and additive effects were observed for many of the combinations. Interestingly, the antifungal activities of the plant extracts in combination with the kresoxim-methyl did not appear to be dose-dependent. Although only one strain of *B. cinerea* was used in this study, we showed that sub-inhibitory concentrations of the kresoxim-methyl fungicide and very low dose rates of plant extracts can act in synergy for the potential control of gray mold in apples.

## Acknowledgments

## Author Contributions

J.A.K. conceptualized the experiments. C-L.K. and F.V. performed the experiments. J.A.K., F.V. and B.C.F. analyzed the results. B.C.F. and J.A.K. wrote the manuscript.

## Conflicts of Interest

The authors declare no conflict of interest.

## References

1. Zhang, H.; Li, R.; Liu, W. Effects of chitin and its derivative chitosan on postharvest decay of fruits: A review. *Int. J. Mol. Sci.* **2011**, *12*, 917–934.

2. Camele, I.; Altieri, L.; De Martino, L.; de Feo, V.; Mancini, E.; Rana, G.L. *In vitro* control of post-harvest fruit rot fungi by some plant essential oil components. *Int. J. Mol. Sci.* **2012**, *13*, 2290–2300.

3. Knight, S.C.; Anthony, V.M.; Brady, A.M.; Greenland, A.J.; Heaney, S.P.; Murray, D.C.; Powell, K.A.; Schulz, M.A.; Spinks, C.A.; Worthington, P.A.; *et al.* Rationale and perspectives on the development of fungicides. *Ann. Rev. Phytopathol.* **1997**, *35*, 349–372.

4. Leroux, P.; Gredt, M.; Leroch, M.; Walker, A.S. Exploring mechanisms of resistance to respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold. *Appl. Environ. Microbiol.* **2010**, *76*, 6615–6630.

5. Shao, X.; Cheng, S.; Wang, H.; Yu, D.; Mungai, C. The possible mechanism of antifungal action of tea tree oil on *Botrytis cinerea*. *J. Appl. Microbiol.* **2013**, *114*, 1642–1649.

6. Kretschmer, M.; Leroch, M.; Mosbach, A.; Walker, A.S.; Fillinger, S.; Mernke, D.; Schoonbeek, H.J.; Pradier, J.M.; Leroux, P.; de Waard, M.A.; *et al.* Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus *Botrytis cinerea*. *PLoS Pathog.* **2009**, *5*, e1000696, doi:10.1371/journal.ppat.1000696.

7. Gabriolotto, C.; Monchiero, M.; Negre, M.; Spadaro, D.; Gullino, M.L. Effectiveness of control strategies against *Botrytis cinerea* in vineyard and evaluation of the residual fungicide concentrations. *J. Environ. Sci. Health Part B* **2009**, *44*, 389–396.

8. Spadaro, D.; Gullino, M.L. State of the art and future prospects of the biological control of postharvest fruit diseases. *Int. J. Food Microbiol.* **2004**, *91*, 185–194.

9. Kim, Y.K.; Xiao, C.L. Stability and fitness of pyraclostrobin- and boscalid-resistant phenotypes in field isolates of *Botrytis cinerea* from apple. *Phytopathology* **2011**, *101*, 1385–1391.

10. Notes on Apple Diseases Blue Mould and Grey Mould in Stored Apples. Available online: http://www.omafra.gov.on.ca/english/crops/pub360/notes/applemould.htm (accessed on 5 November 2013).

11. Walker, A.S.; Micoud, A.; Remuson, F.; Grosman, J.; Gredt, M.; Leroux, P. French vineyards provide information that opens ways for effective resistance management of *Botrytis cinerea* (grey mould). *Pest Manag. Sci.* **2013**, *69*, 667–678.

12. Billard, A.; Fillinger, S.; Leroux, P.; Lachaise, H.; Beffa, R.; Debieu, D. Strong resistance to the fungicide fenhexamid entails a fitness cost in *Botrytis cinerea*, as shown by comparisons of isogenic strains. *Pest Manag. Sci.* **2012**, *68*, 684–691.

13. Samuel, S.; Papayiannis, L.C.; Leroch, M.; Veloukas, T.; Hahn, M.; Karaoglanidis, G.S. Evaluation of the incidence of the G143A mutation and cytb intron presence in the cytochrome bc-1 gene conferring QoI resistance in *Botrytis cinerea* populations from several hosts. *Pest Manag. Sci.* **2011**, *67*, 1029–1036.

14. Bardas, G.A.; Veloukas, T.; Koutita, O.; Karaoglanidis, G.S. Multiple resistance of *Botrytis cinerea* from kiwifruit to SDHIs, QoIs and fungicides of other chemical groups. *Pest Manag. Sci.* **2010**, *66*, 967–973.

15. Leroch, M.; Plesken, C.; Weber, R.W.; Kauff, F.; Scalliet, G.; Hahn, M. Gray mold populations in german strawberry fields are resistant to multiple fungicides and dominated by a novel clade closely related to *Botrytis cinerea*. *Appl. Environ. Microbiol.* **2013**, *79*, 159–167.

16. Zabka, M.; Pavela, R.; Gabrielova-Slezakova, L. Promising antifungal effect of some Euro-Asiatic plants against dangerous pathogenic and toxinogenic fungi. *J. Sci. Food Agric.* **2011**, *91*, 492–497.

17. Liu, X.; Wang, L.P.; Li, Y.C.; Li, H.Y.; Yu, T.; Zheng, X.D. Antifungal activity of thyme oil against *Geotrichum citri-aurantii in vitro* and *in vivo*. *J. Appl. Microbiol.* **2009**, *107*, 1450–1456.

18. Askarne, L.; Talibi, I.; Boubaker, H.; Boudyach, E.H.; Msanda, F.; Saadi, B.; Ait Ben Aoumar, A. Use of Moroccan medicinal plant extracts as botanical fungicide against citrus blue mould. *Lett. Appl. Microbiol.* **2013**, *56*, 37–43.

19. Talibi, I.; Askarne, L.; Boubaker, H.; Boudyach, E.H.; Msanda, F.; Saadi, B.; Ben Aoumar, A.A. Antifungal activity of Moroccan medicinal plants against citrus sour rot agent *Geotrichum candidum*. *Lett. Appl. Microbiol.* **2012**, *55*, 155–161.

20. Soylu, E.M.; Kurt, S.; Soylu, S. *In vitro* and *in vivo* antifungal activities of the essential oils of various plants against tomato grey mould disease agent *Botrytis cinerea*. *Int. J. Food Microbiol.* **2010**, *143*, 183–189.

21. De Lapeyre de Bellaire, L.; Essoh Ngando, J.; Abadie, C.; Chabrier, C.; Blanco, R.; Lescot, T.; Carlier, J.; Côte, F. Is chemical control of *Mycosphaerella* foliar diseases of bananas sustainable. In Proceedings of the International Symposium on Recent Advances in Banana Crop Protection for Sustainable Production and Improved Livelihoods, White River, South Africa, 10–14 September 2007; Jones, D., van den Bergh, I., Eds.; ISHS Acta Horticulturae: White River, South Africa, 2009; pp. 161–170.

22. Schmitt, A.; Seddon, B. Biocontrol of plant pathogens with microbial BCAs and plant extracts—Advantages and disadvantages of single and combined use. In Proceedings of the 14th International Reinhardsbrunn Symposium 2004, Friedrichroda, Thuringia, Germany, 25–29 April 2004; BCPC: Atlon, UK, 2005; pp. 205–225.

23. Goncagul, G.; Ayaz, E. Antimicrobial effect of garlic (*Allium sativum*). *Recent Pat. Anti-Infect. Drug Discov.* **2010**, *5*, 91–93.

24. Low, C.F.; Chong, P.P.; Yong, P.V.; Lim, C.S.; Ahmad, Z.; Othman, F. Inhibition of hyphae formation and SIR2 expression in *Candida albicans* treated with fresh *Allium sativum* (garlic) extract. *J. Appl. Microbiol.* **2008**, *105*, 2169–2177.

25. Iciek, M.; Kwiecien, I.; Wlodek, L. Biological properties of garlic and garlic-derived organosulfur compounds. *Environ. Mol. Mutagen.* **2009**, *50*, 247–265.

26. Gisi, U. Synergistic Interactions of fungicides in mixtures. *Phytopathology* **1996**, *86*, 1273–1279.

27. SAS Institute Inc. *SAS/STAT User's Guide*, Version 8; SAS Campus Drive: Cary, NC, USA, 1999; Volume 2.

28. Abbott, W.S. A method of computing the effectiveness of an insecticide. *J. Am. Mosq. Control Assoc.* **1987**, *3*, 302–303.

29. Gisi, U.; Binder, H.; Rimbach, E. Synergistic interactions of fungicides with different modes of action. *Trans. Br. Mycol. Soc.* **1985**, *84*, 299–306.

30. Fiume, F.; Fiume, G. Biological control of *Botrytis* gray mould and Sclerotinia drop in lettuce. *Commun. Agric. Appl. Biol. Sci.* **2005**, *70*, 157–168.

31. Martinez-Romero, D.; Serrano, M.; Bailen, G.; Guillen, F.; Zapata, P.J.; Valverde, J.M.; Castillo, S.; Fuentes, M.; Valero, D. The use of a natural fungicide as an alternative to preharvest synthetic fungicide treatments to control lettuce deterioration during postharvest storage. *Postharvest Biol. Technol.* **2008**, *47*, 54–60.

32. Rial-Otero, R.; Gonzalez-Rodriguez, R.M.; Cancho-Grande, B.; Simal-Gandara, J. Parameters affecting extraction of selected fungicides from vineyard soils. *J. Agric. Food Chem.* **2004**, *52*, 7227–7234.

33. Sauter, H.; Ammermann, E.; Benoit, R.; Brand, S.; Gold, R.E.; Grammenos, W.; Köhle, H.; Lorenz, G.; Müller, B.; Schirmer, U.; Speakman, J.B.; Wenderoth, B.; Wingert, H. Mitochodrial respiration as a target for antifungals: Lessons from research on strobilurins. In *Antifungal Agents—Discovery and Mode of Action*; Dixon, G.K., Copping, L.G., Holloman, D.W., Eds.; BIOS Scientific Publishers: Oxford, UK, 1995; pp. 173–191.

34. Davinelli, S.; Sapere, N.; Visentin, M.; Zella, D.; Scapagnini, G. Enhancement of mitochondrial biogenesis with polyphenols: Combined effects of resveratrol and equol in human endothelial cells. *Immun. Ageing* **2013**, *10*, 28, doi:10.1186/1742-4933-10-28.

35. Rehman, H.; Krishnasamy, Y.; Haque, K.; Thurman, R.G.; Lemasters, J.J.; Schnellmann, R.G.; Zhong, Z. Green tea polyphenols stimulate mitochondrial biogenesis and improve renal function after chronic cyclosporin a treatment in rats. *PLoS ONE* **2013**, *8*, e65029.

36. Rasbach, K.A.; Schnellmann, R.G. Isoflavones promote mitochondrial biogenesis. *J. Pharmacol. Exp. Ther.* **2008**, *325*, 536–543.

37. Tamasloukht, M.; Sejalon-Delmas, N.; Kluever, A.; Jauneau, A.; Roux, C.; Becard, G.; Franken, P. Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. *Plant Physiol.* **2003**, *131*, 1468–1478.

38. Lyantagaye, S.L. Ethnopharmacological and phytochemical review of *Allium* species (sweet garlic) and *Tulbaghia* species (wild garlic) from southern Africa. *Tanzan. J. Sci.* **2011**, *37*, 58–71.

39. McLean, K.L.; Swaminathan, J.; Frampton, C.M.; Hunt, J.S.; Ridgway, H.J.; Stewart, A. Effect of formulation on the rhizosphere competence and biocontrol ability of Trichoderma atroviride C52. *Plant Pathol.* **2005**, *54*, 212–218.

40. Adandonon, A.; Aveling, T.A.S.; Labuschagne, N.; Tamo, M. Biocontrol agents in combination with *Moringa oleifera* extract for integrated control of Sclerotium-caused cowpea damping-off and stem rot. *Eur. J. Plant Pathol.* **2006**, *115*, 409–418.