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# Antifungal effectiveness of fungicide and peroxyacetic acid mixture on the growth of *Botrytis cinerea*

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ABSTRACT

In the attempt to reduce the negative impacts of chemical pesticides on environment and consumer's health, a new plant treatment practice minimizing the amount of pesticides needed during pests and diseases treatments has been developed. Our approach is based on combining the biocide effects of fungicide with the peroxyacetic acid (PAA) one. In this paper, we focused on the *in vitro* study of the antifungal activity of this combination against *Botrytis cinerea*, the most redoubtable threat of tomatoes plants in Morocco. First, different concentrations of a peroxyacetic acid product (PERACLEAN®5) and two commercially available fungicides SWITCH and SIGNUM were tested separately for their inhibitory effects on the mycelial growth and spores germination of *B. cinerea*. 100% inhibition of fungal growth was achieved using 16.77 and 14.47 μg/ml of SIGNUM and SWITCH respectively and 1.5% of PERACLEAN®5. When combined with 0.5% of the peroxyacetic acid mixture (PERACLEAN®5), the pesticides 100% effective concentrations decreased to 0.5 μg/ml for both pesticides. Hence, this approach allowed us to suppress the pathogen while minimizing the amounts of applied fungicides by more than 95%.



## 1. Introduction

In Morocco, tomatoes represent an important branch of the Moroccan economy, with an annual production of approximately 1.3 million tons [1] and being the major exported vegetable with 64% of tonnage exported [2]. This commodity is faced to many threats; with one of the most redoubtable is *Botrytis cinerea* causing significant economic losses [3]. *Botrytis cinerea* Persoon: Fries (teleomorph Botryotinia fuckeliana, also known as “grey mould fungus”) causes serious pre- and post-harvest diseases in at least 200 plant species including agronomically important crops and harvested commodities, such as grapevine, tomato, strawberry, cucumber, bulb flowers, cut flowers and ornamental plants [4]. This fungus is difficult to control because it has a variety of modes of attack, diverse hosts as inoculum sources, and it can survive as mycelia and/or conidia or for extended periods as sclerotia in crop debris [5]. The control of this pathogen remains a challenge and is still based upon multiple applications of fungicides. This chemical control is effective and efficient but, at the same time, can leads to the development of pathogen resistance, chemical residues in fruit, phytotoxicity to other organisms or environmental and public health problems [6].

Different alternatives methods to control *Botrytis cinerea* in various crops have been reported in the literature during the last years, including i) biocontrol agents [7–11]; ii) biologically active natural products [12–14], (iii) GRAS-classified sanitizers [15,16] and even combination [17–20]. However, the effects of a sanitizer and fungicide mixture on grey mould caused by *B. cinerea* have never been reported before. Hence, in order to minimize the negative impacts of chemicals pesticides, we describe in this paper a new pest and diseases treatment approach that may allow farmers to reduce the applied amount of pesticide. Our approach is based on decreasing the pesticide 100% effective concentration by a synergistic action of a peroxyacetic acid preparation and commercially available fungicides.

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Peroxyacetic acid (PAA) is a biodegradable product and a highly effective biocide, used in a wide range of applications and demonstrates excellent bactericidal and fungicidal activity against a wide range of microorganisms [21–24]. It had been used for purposes ranging from disinfestations of bulbs and nematodes to prevention of other horticultural diseases through disinfecting potting soil and cleaning irrigation equipment [25]. There are several registered products on the market, containing peroxyacetic acid, hydrogen peroxide and acetic acid in different proportions as stabilized mixtures. These mixtures are environmentally friendly products as their manufacture does not involve polluting processes; they have the same function as other chemicals, and after use and management, there is no toxic residue left in the environment [26]. They can be used in the application of green chemistry in agriculture and none of the products formed during the degradation process are harmful. Acetic acid, hydrogen peroxide, water, and oxygen, are biodegradable, not harmful or even secondarily useful [26]. Several studies have reported the antifungal activity of these mixtures against *Penicillium digitatum* and *Botrytis cinerea* [15], *Monilinia laxa* and *Rhizopus stolonifer* [27] and many other pathogens. However, to have a fungicide effect; 1.5–2% of the mixture based on 5% of PAA is needed, even if this concentration may suppress the pathogen, it may cause a severe damages when applied to the plant. In addition, the rapid decay of those products, limit their application as a biofungicide [28]. To cover these products limitations, we describe in this study, as stated above, a new approach based on the combination of a commercially available peroxyacetic acid mixture (PERACLEAN®5) with two commonly used fungicides against grey mould. This new practice presents several advantages over the conventional methods to control *B. cinerea*: i) It will minimize the 100% effective concentration of the two products, enhance fungicidal action and consequently reduce the well known negative impacts of pesticide; ii) Lengthen the period of effectiveness iii) Peroxyacetic acid being rapidly active will speed up the biocide action against the pathogen; iv) Being highly acid, Peroxyacetic acid will induce the acidification of the fungicide, necessary before application on field, so no additional acidifying product needed and iv) it's a cost effective way to limit the grey mould incidence. To the best of our knowledge, this paper is the first one describing this kind of approach for treatment against *B. cinerea.* The *in vivo* studies on the effect of this combination against tested fungus on tomatoes plant are in progress and will be published soon.

## 2. Materials and methods

### 2.1. Chemical products

A commercially available 5% peracetic acid (PAA) solution: PERACLEAN®5 by EVONIK INDUSTRIES AG used as disinfectant and consisting of 5% of peroxyacetic acid, 26.4% of hydrogen peroxide and 6.8% of acetic acid, and two commonly used fungicides by the Moroccan tomatoes farmers to control the grey mould disease caused by *Botrytis cinerea*: SIGNUM (26.7% Boscalide and 6.7% Pyraclostrobine) by BASF and SWITCH 62.5 (37.5% Cyprodinil and 25% Fludioxonil) by SYNGENTA were selected to study their antifungal efficacy, separately and in combination with PERACLEAN®5 against *B. cinerea.*

### 2.2. Botrytis cinerea inoculums

*Botrytis cinerea*, the causal agent of grey mould disease of tomatoes, was isolated from naturally infected tomatoes leaves obtained from the region of Agadir and identified in Laboratory of Applied Chemistry and Environment in National School of Applied Science in Agadir (ENSA/Agadir). The fungus was isolated as follows: circles were taken from infected areas on tomato leaves and rinsed with tap water three times then placed in Petri plates containing potato-dextrose agar medium (PDA). The plates were incubated then at 25 °C. A week later, the fungi were transferred to new plates and the transplanting was repeated several times to get a sufficient amount of fungi [29]. *B. cinerea* culture had been then maintained on potato dextrose agar (PDA) until use.

### 2.3. Antifungal assays

#### 2.3.1. Mycelial growth inhibition

The antifungal activities of the above chemicals were tested *in vitro* against mycelial growth of *B. cinerea*. The study was conducted in three steps: The first trial was established to assess the antifungal activity of PERACLEAN®5 against *B. cinerea*, the second, to determine the 100% effective concentration of SWITCH and SIGNUM and the third, to study the effectiveness of the fungicide and sanitizer combinations on mycelial growth of *Botrytis cinerea*.

Four concentrations of PAA in PERACLEAN®5: 0.5, 1, 1.5 and 2% (v/v) and four concentrations of SWITCH and SIGNUM 0.5, 1, 5 and 10 μg/ml were tested separately for their inhibitory effects on the mycelial growth of *B. cinerea in vitro* and then a combination of different concentrations of PERACLEAN®5 and the two fungicides, separately, was conducted as shown in Table 1.

For inhibition assays, *Botrytis cinerea* was inoculated on a PDA medium amended with the above mentioned chemicals concentrations according to the method described by Elbouchtaoui et al. [15],: The tested compounds were dissolved in ethanol and poured into an Erlenmeyer flasks containing sterilized potato-dextrose agar (PDA) medium to obtain final concentrations as mentioned above. After gentle stirring, the mixture was dispensed in sterilized Petri dishes (9 cm diameter). Dishes containing PDA free of chemicals served as controls and the final ethanol concentration was identical in both control and treated cultures. A 5 mm diameter disc of a 10 day old culture of *B. cinerea* was placed at the center of these Petri plates. Inoculated dishes were then sealed with parafilm and incubated at 25 ± 2° C. The average linear growth of tested fungus was calculated after 7 days when the control reaches full growth and growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control.

The relative growth inhibition of treatment compared to negative control was calculated by percentage, using the following formula:

**Table 1**
Tested combinations of fungicides (SWITCH or SIGNUM) with PERACLEAN®5.

| Treatments | PERACLEAN®5 (%) | Fungicide concentration (μg/ml) |
|---|---|---|
| $T_1$ | 0.5 | 0.5 |
| $T_2$ | 1 | 0.5 |
| $T_3$ | 1.5 | 0.5 |
| $T_4$ | 2 | 0.5 |
| $T_5$ | 0.5 | 1 |
| $T_6$ | 1 | 1 |
| $T_7$ | 1.5 | 1 |
| $T_8$ | 2 | 1 |
| $T_9$ | 0.5 | 5 |
| $T_{10}$ | 1 | 5 |
| $T_{11}$ | 1.5 | 5 |
| $T_{12}$ | 2 | 5 |
| $T_{13}$ | 0.5 | 10 |
| $T_{14}$ | 1 | 10 |
| $T_{15}$ | 1.5 | 10 |
| $T_{16}$ | 2 | 10 |

$$Inhibition\% = \frac{dc - dt}{dc} \times 100$$

Where dc and dt are the averages of 3 replicates of radial growth of fungus (mm) in the control and treated plates, respectively [30].

*2.3.2. Inhibition of spore germination*

Conidia of *B. cinerea* were obtained from 2 weeks old PDA cultures incubated at 25 °C in 12/12 h light/dark. Culture plates were vortexed in a tube containing 10 ml sterile distilled water and 0.05 ml Tween 80. A sterile magnetic stir bar was placed on the agar and set stirring for 5 min to lose the spores [31]. The suspension enriched in spores was filtered through two layers of sterile muslin cloth to eliminate mycelium. Finally the conidial concentration was determined using an hemocytometer and adjusted to $10^5$ spores per ml.

For inhibition tests, 1 ml of this spore suspension was added to a tube containing 9 ml of various concentrations of PERACLEAN®5 (0; 0.5; 1; 1.5; 2.0 and 2.5% (v: v)), fungicide (0.5, 1, 5 and 10 μg/ml) or the above-mentioned combinations, then each solution was incubated at 25° C for 15 min. 0.1 ml of each suspension was spread onto PDA medium and incubated for 6 days at 25 °C [15]. The germinated spores were counted and the inhibition of spore germination (P) in % was calculated according to the following formula:

$$P = A - B/A \times 100$$

Where A is the number of germinated conidia in the control and B is the number of germinated conidia after treatments.

## 2.4. Statistical analysis

Data were analyzed using MINITAB statistical software version 17. Treatment means were separated by Tukey's test at $P \leq 0.05$.

**Table 2**
Suppression efficacy of SIGNUM and SWITCH on *B. cinerea.*

| Fungicide | Applied concentration (μg ml$^{-1}$) | Disease suppression efficacy (%± SD*) |
|---|---|---|
| SWITCH | 0 | 0 ** |
| | 0.5 | 56.63 ± 3.86 c |
| | 1 | 61.40 ± 2.85 c |
| | 5 | 82.20 ± 3.23 b |
| | 10 | 98.05 ± 2 a |
| SIGNUM | 0 | 0 e |
| | 0.5 | 37.67 ± 4.06 d |
| | 1 | 50.88 ± 4.98 c |
| | 5 | 77.91 ± 1.93 b |
| | 10 | 91.29 ± 3.74 a |

* All data are the mean of three replicates ± standard deviation.
** Values followed by the same letter in each column are not significantly different from each other according to Tukey's test at $P \leq 0.05$.

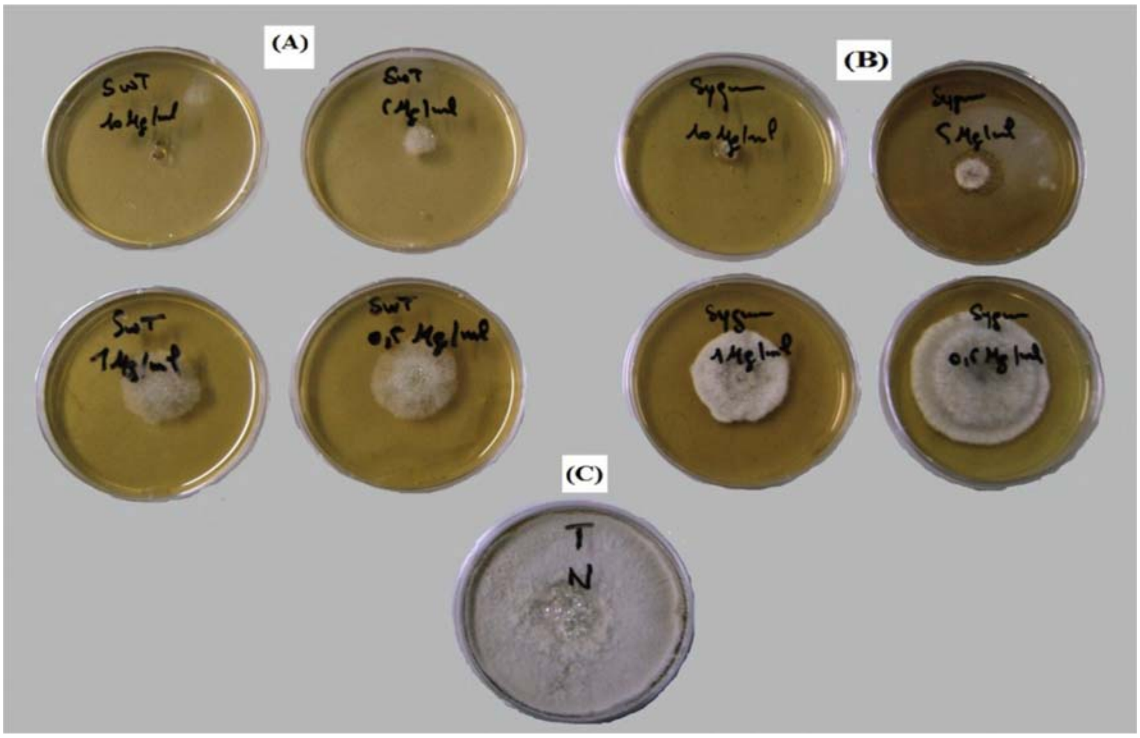


**Fig. 1.** Antifungal activity of different concentrations of SWITCH (A) and SIGNUM (B) on mycelial growth of *B. cinerea* compared to the control (C) after 7 days incubation.

**Table 3**
Inhibition of *B. cinerea* growth by different concentrations of PERACLEAN®5.

| Concentration (%) | 0 | 0.5 | 1 | 1.5 | 2 |
|---|---|---|---|---|---|
| Inhibition rates (% ± SD*) | 0d** | 73.59 ± 1.24 c | 94.36 ± 0.39b | 99.09 ± 0.93a | 99.83 ± 0.28a |

* All data are the mean of three replicates ± standard deviation.
** Values followed by the same letter in each column are not significantly different from each other according to Tukey's test at $P \leq 0.05$.

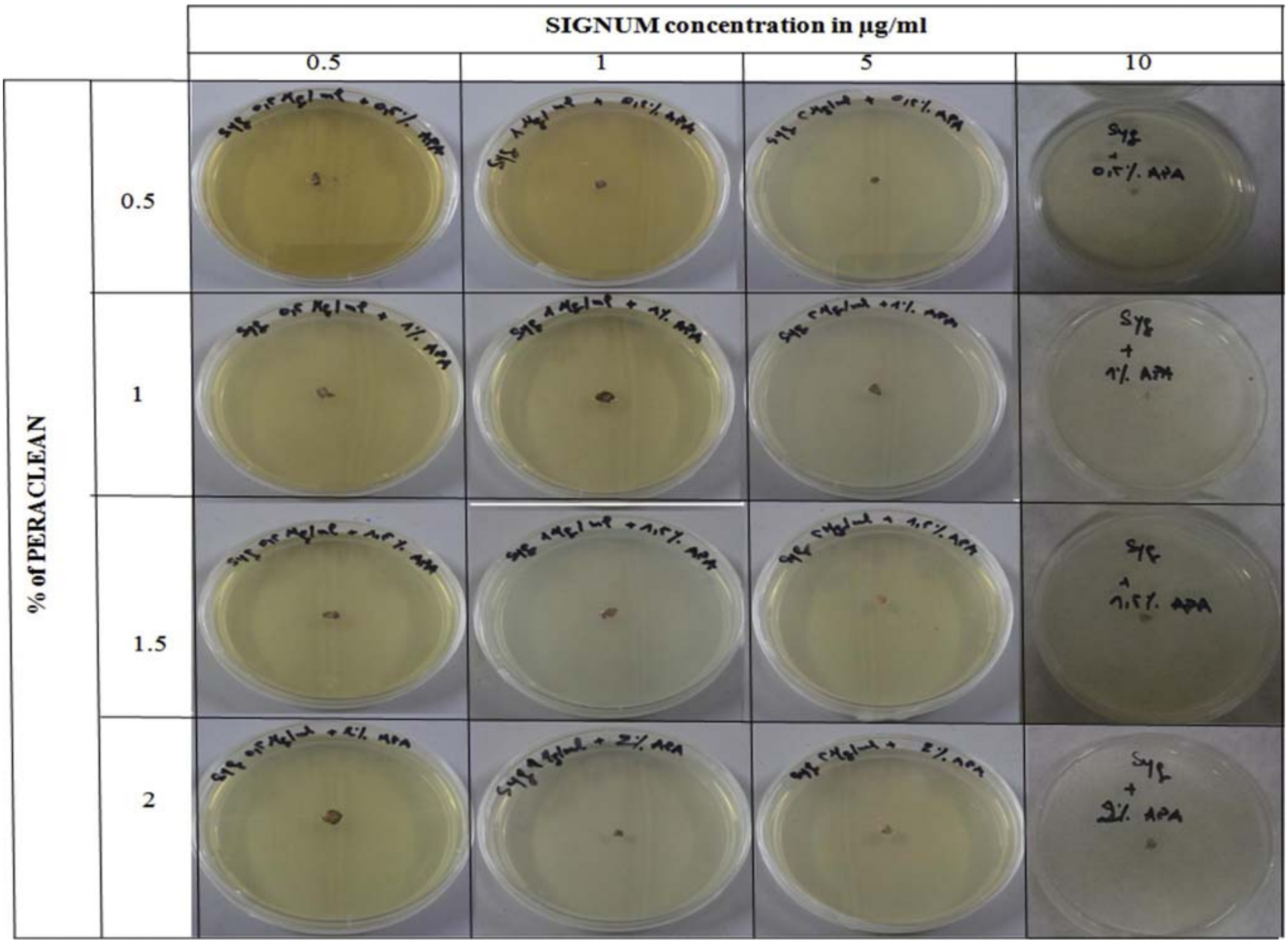


**Fig. 2.** Antifungal effect of SIGNUM and PERACLEAN®5 mixture on *B. cinerea* mycelial growth.

## 3. Results

### 3.1. Compatibility tests

The knowledge about compatibility of agrochemicals products before mixture and application on field is mandatory in order to avoid problems which may arise. Therefore, in this work and by consulting the labels of the two tested fungicides and PERACLEAN®5, no incompatibility warning was found between them. In addition, PERACLEAN®5 has demonstrated good compatibility with both fungicides as forming a uniform solution and no change in color, temperature or texture was observed.

### 3.2. Antifungal assays

The efficacy of the two commonly used pesticides; SIGNUM and SWITCH; as well as the peroxyacetic acid mixture PERACLEAN®5; at the above mentioned concentrations for inhibiting mycelial growth and spore germination of *B. cinerea*, under *in vitro* conditions, was evaluated. All the tests conducted in this study have been done in triplicate and the results are shown as mean values of three replicates of colony diameters or number of germinated conidia.

#### 3.2.1. Mycelial growth inhibition

*3.2.1.1. Pesticides antifungal activity.* The inhibition rates of *B. cinerea* mycelial growth by the tested pesticides are presented in Table 2 and Fig. 1. As expected, *B. cinerea* colony growth decrease as pesticides concentration increased and according to the statistical data (data not shown), a complete inhibition of *B. cinerea* growth can be achieved using 16.77 and 14.47 µg/ml of SIGNUM and SWITCH respectively.

*3.2.1.2. PERACLEAN®5 antifungal activity.* The inhibition rates of *B. cinerea* growth by the previously cited concentrations of PERACLEAN®5 are presented in Table 3. A complete inhibition was achieved using 1.5% of PERACLEAN®5.

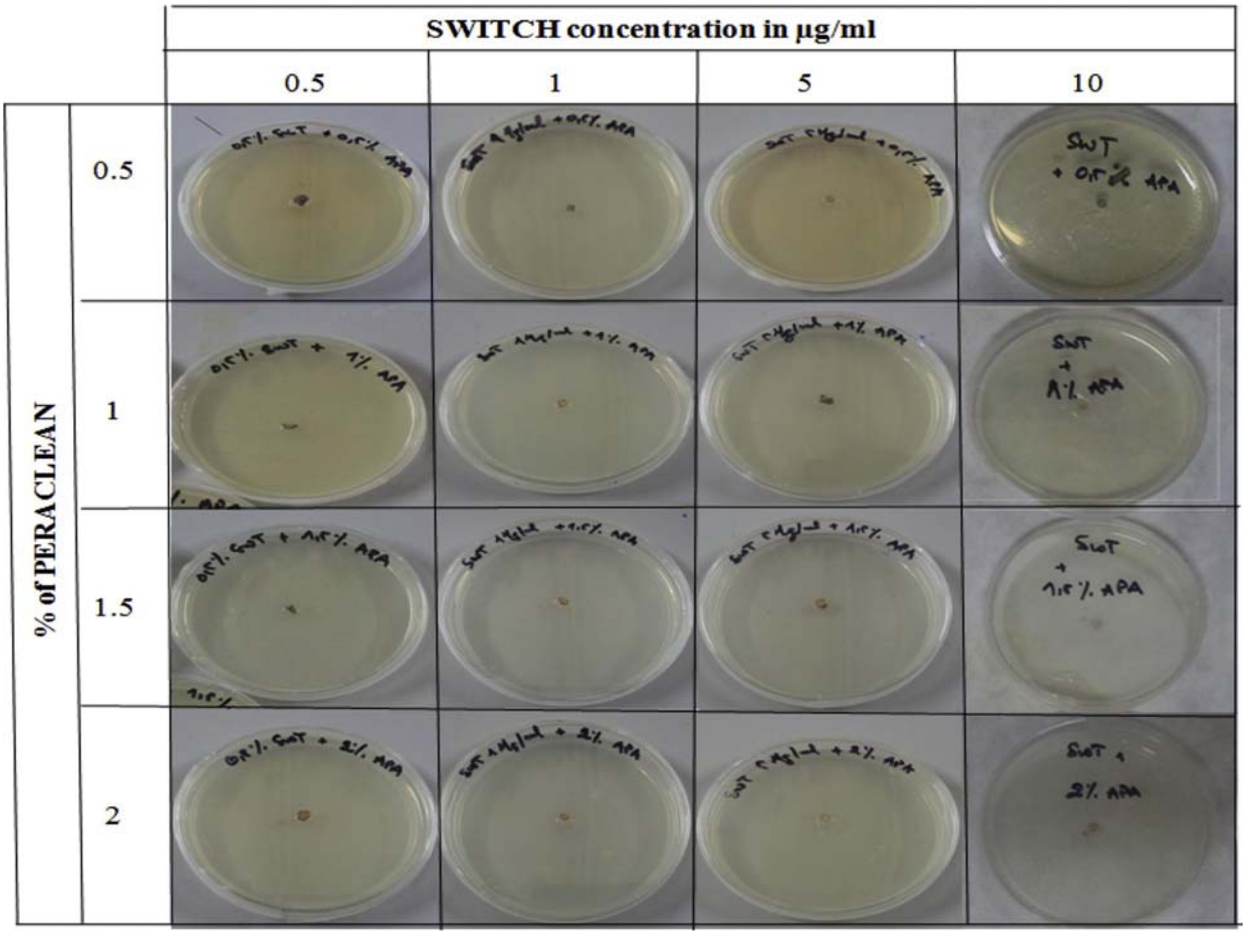


**Fig. 3.** Antifungal effect of SWITCH and PERACLEAN®5 combination on *B. cinerea* mycelial growth.

*3.2.1.3. Antifungal effect of PERACLEAN®5 and pesticide mixture.* When combining PERACLEAN®5 with the two tested fungicides, separately, as mentioned above, all the combinations of SWITCH or SIGNUM with PERACLEAN®5 were able to totally inhibit the linear growth of *B. cinerea* as shown in Figs. 2 and 3 and provide a greater response than if pesticide was applied alone. Even using 0.5% of PERACLEAN®5 with 0.5 µg/ml of tested fungicides, a complete inhibition of *B. cinerea* was observed. Hence, the 100% effective concentration of the two fungicides decreased by more than 95% from 16.77 and 14.47 µg/ml for SWITCH and SIGNUM respectively to 0.5 µg/ml for each when combined with the PAA based product.

*3.2.2. Inhibition of spore germination*

All concentrations of the tested products reduced the germination of spores of *B. cinerea*. A total inhibition was observed at a dose of 2.5% for 15 min of PAA. For the tested pesticides, when applied alone, 10 µg/ml of SIGNUM and SWITCH only permit 1.8 and 26.8% inhibition respectively after a contact time of 15 min as shown in Table 4. However, the synergistic action of PERACLEAN®5 with SIGNUM (or SWITCH) allowed a complete inhibition since the lowest concentrations (0.5% of PAA with 0.5% of pesticide) which demonstrate a good sporicidal action of this combination.

**Table 4**
Inhibition of *Botrytis cinerea* spore germination after 15 min contact with different concentrations of PERACLEAN, SWITCH and SIGNUM.

| Treatment | Concentrations | Germination inhibition (% ± SD*) |
|---|---|---|
| Peraclean % | 0 | 0 e** |
| | 0.5 | 41 ± 1.38d |
| | 1 | 60.5 ± 1.11c |
| | 1.5 | 82.43 ± 0.47b |
| | 2 | 97.96 ± 0.92a |
| | 2.5 | 99.53 ± 0.43a |
| Signum (µg/ml) | 0 | 0 d |
| | 0.5 | 0.1 ± 0.1d |
| | 1 | 0.5 ± 0.1c |
| | 5 | 0.91 ± 0.03b |
| | 10 | 1.8 ± 0.1a |
| Switch (µg/ml) | 0 | 0 d |
| | 0.5 | 0.26 ± 0.2d |
| | 1 | 3.6 ± 0.26c |
| | 5 | 13.23 ± 0.15b |
| | 10 | 26.8 ± 0.1a |

* All data are the mean of three replicates ± standard deviation.
** Values followed by the same letter in each column are not significantly different from each other according to Tukey's test at $P \leq 0.05$.

## 4. Discussion

Nowadays, there is a growing concern about the ecological problems caused by synthetic pesticides and their relevance to human health. Therefore, the development of new biopesticides or new eco-friendly practices has become a necessity. In the search for alternatives to chemical control, the uses of sanitizers classified as GRAS have been described in literature [15,16]. Among these products, Peracetic acid (PAA) seems to be very promoting. PAA is a biodegradable materiel that has been reported mainly for post-harvest disease control of fresh fruit and vegetables. It presents a greater stability, faster biocidal properties and could be used as postharvest disinfectant [27,32]. It has been used for purposes ranging from disinfestations of bulbs and nematodes to prevention of other horticultural diseases through disinfecting potting soil and cleaning irrigation equipment [25]. However, to have a fungicide effect; a high concentration of PAA is needed, even if this concentration may suppress the pathogen, it may cause a severe damages when applied to the plant. In addition, the rapid decay of this product, limit its application as a biofungicide [28]. In this work, a new environmentally friendly practice to suppress the incidence of *B. cinerea* using PAA has been described. The idea was to test the antifungal efficacy of a mixture of a biodegradable sanitizer containing Peracetic acid with commercially available fungicide in order to minimize to quantity of harmful applied chemicals.

Our *in vitro* assays showed that rising concentration of all tested chemicals reflected negatively on both linear growth and spore germination of *Botrytis cinerea*. When used alone, 1.5% of peroxyacitic acid based sanitizer PERACLEAN®5 permits the complete suppression of *B. cinerea in vitro*. The obtained value was less than the one described by Elbouchtaoui et al. [15], using a similar product (Perydroxan) where 2.5% of peroxyacetic acid mixture has been reported to totally suppress the fungal pathogen, this small deviation may be due to the highest content of Hydrogen Peroxide in PERACLEAN®5 (26.4%) compared with Perydroxan (25%). To have a sporicidal action, a higher dose of peroxyacetic acid was required to inhibit *B. cinerea* spores germination which was in agreement with the value reported by Elbouchtaoui et al. [15], (2.5%) and also with the data in the literature where a higher dose of peroxyacetic acid was reported to be required to kill spores than vegetative bacteria [21]. Even if this compound presents a good antifungal activity against this pathogen, it can't be used alone as fungicide product for tomato grey mold disease control due to its quick biodegradability and severe damages that may cause to the plants using high concentration of peroxyacetic acid.

In the other hand, when applied alone, a total inhibition of mycelial growth of *B. cinerea*, *in vitro*, was achieved using 16.77 and 14.47 μg/ml of SIGNUM and SWITCH respectively, while a slight sporicidal effect was observed for these two compounds with only 1.8 and 26.8% inhibition of spore germination was obtained using 10 μg/ml of both pesticides, these values were in concordance with these reported by Rahman et al. [33], where 10 μg/ml and 5 μg/ml of SIGNUM and SWITCH allowed 1.6% and 18.3% inhibition of spore germination respectively. In general, at lower concentrations, SWITCH seems to have a greater sporicidal and fungicidal effect than SIGNUM.

When combining the PAA based product with the fungicide, the effective concentrations of both chemicals decreased significantly to 0.5 μg/ml for both pesticides and 0.5% for PERACLEAN®5, demonstrating a good antifungal and sporicidal effect against *B. cinearia*. Hence, in this *in vitro* study, this combination allowed us to minimize the applied pesticides concentrations necessary to fully inhibit *B. cinerea* by more than 95%. The *in vivo* studies on tomatoes plants are in progress and if similar results were found, this new approach will be a promising practice able to suppress pathogens on plants in a healthy and environmentally friendly way by decreasing the required amounts of chemical pesticides.

## 5. Conclusion

A new agricultural treatment practice minimizing the amount of pesticides necessary to fully inhibit *B. cinerea* by more than 95% was described in this paper. This approach is based on a beneficial combination of a sanitizer containing peroxyacetic acid (PERACLEAN®5) with fungicide. The *in vitro* study of antifungal and sporicidal activities of this combination against *Botrytis cinerea* isolated from infested tomatoes plants was described in this paper. When applied alone, a complete inhibition of the mycelial growth of *B. cinerea* by two commercially available fungicides SWITCH and SIGNUM and the peroxyacetic acid mixture PERACLEAN®5 was achieved using 16.77 and 14.47 μg/ml of SIGNUM and SWITCH respectively and 1.5% of PERACLEAN®5. The combination of these two pesticides separately with PERACLEAN®5 enhances their inhibitory effect against the fungus and provides a greater response than if the pesticide was applied alone and the 100% effective concentration of both chemicals decreased by more than 95% to 0.5 μg/ml when combined with PAA. The *in vivo* studies on the effect of this combination against *B. cinerea* on tomatoes are in progress and will be published soon. If similar results as the *in vitro* tests were obtained, this approach will be an innovative technique which will allow farmers to minimize the used pesticides quantity by more than 95% and hence, reducing the well known negative impact of chemical products on environment and consumer's health.

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