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Biofungicide for the Control of *Botrytis Cinerea* and *Fusarium Oxysporum*: a Laboratory Study

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The use of fungicides based on plant extracts for the inhibition of phytopathogens has become a sustainable alternative for the environment. In this sense, research has developed biofungicides based on extracts of *Allium cepa* (*A. cepa*), *Allium sativum* (*A. sativum*), *Zingiber officinale* (*Z. officinale*) and Domestic Residual Oil (DRO) for the inhibition of *Botrytis cinerea* (*B. cinerea*) and *Fusarium oxysporum* (*F. oxysporum*). The inhibition of the two phytopathogenic fungi was evaluated in vitro, measuring the growth of mycelium of the fungi inoculated in the potato dextrose agar (PDA) culture medium, subjected to four treatments with three different doses (10, 15 and 20 %). The treatment that totally inhibited the mycelial growth of *B. cinerea* and *F. oxysporum* was the biofungicide composed by *Z. officinale* and domestic residual oil with a dose of 15 %. Finally, it is concluded that the use of the biofungicide is favorable for the control of fungi and could be used as an environmentally friendly alternative.

1. Introduction

Solanum lycopersicum (*S. lycopersicum*) (tomato) is one of the most cultivated and used vegetables for food processing due to the main nutritional contributions it has (high content of vitamins and minerals) (Iglesias et al., 2017). During the cultivation period of *S. lycopersicum*, favorable climatic and agronomic conditions arise for the propagation of phytopathologies, among which are "gray rot" and "vascular withering" caused by the fungi *B. cinerea* and *F. oxysporum* (Boiteux et al., 2019; Terrones, 2015). To date, many of these diseases are attacked using chemical fungicides whose residues negatively alter the environment and human health (Borucka and Celiński, 2019; Ferreira et al., 2018; Oliveira, 2018). In order to implement sustainable agriculture, some researchers have evaluated the use of extracts from various plants in the control of phytopathogenic pests. In *S. lycopersicum* L. crops, the extract of *Junglannes* spp. and *Carya* sp. inhibits the fungus *F. oxysporum* allowing the adequate development of the plant (Jasso de Rodríguez et al., 2019). On the other hand, the extracts of *Trichilia heudelotii*, *Nesogordonia papaverifera*, *Celtis mildbraedii*, *Cola gigantea* and *Triplochiton scleroxylon* are effective in inhibiting *F. oxysporum* (Kamelé, 2019).
There are unconventional plants such as *Sesamun indicum*, whose extract is used to inhibit *B. cinerea* and *F. oxysporum* by presenting fungal and/or fungistatic characteristics (Fernández and Laurentin, 2016), or the ethanolic extract of *Azadirachta indica* (neem) which is used to inhibit *B. cinerea* (Gholamnezhad, 2019). Among the fungi that afflict *S. lycopersicum* L. (tomato) crops are *B. cinerea* and *F. oxysporum*. *B. cinerea* is an aggressive, versatile phytopathogenic fungus and is primarily responsible for the disease known as gray mold or strawberry rot. *B. cinerea* reproduction occurs in senescent and/or dead tissues of ornamental plants, fruits and vegetables and in high humidity environments (Abbey et al., 2019; Šernaitė et al., 2020). *F. oxysporum* is a fungus with high antioxidant activity that lives in the soil and is the phytopathogen that causes vascular withering and the phytopathogenic disease called Fusariosis (Ramírez et al., 2020).
Plant extracts have several secondary metabolites that make possible the mycelial inhibition of any fungus. These metabolites vary by species, for example, *A. cepa* extracts have secondary metabolites such as flavonols which are sulfur-rich compounds that prevent the proliferation of phytopathogenic microorganisms (Miralha Franco et al., 2018). *A. sativum* extracts have various biologically active components. These components usually contain sulfur compounds such as allicin, ajoene, allylmethyltrisulfide, diallyltrisulfide,

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diallyldisulfide, saponins, flavonoids, among other compounds that inhibit the growth of fungi and bacteria (De Falco et al., 2018; Fufa, 2019; Liaqat et al., 2019). On the other hand, the extracts of *Z. officinale* are composed mainly of oxygenated monoterpenes such as citral, ethyl ether verbenil, geranic acid, artemiseole, among other compounds that allow the inhibition of various fungi and phytopathogenic bacteria (Hussein and Joo, 2018).

Currently, the development of phytopathogenic pests is a constant problem in the agricultural industry. This productive activity uses 85 % of the pesticides produced worldwide, and their excessive use causes severe damage to the soil composition and the taxonomy of plants and fruits. These damages are caused by the different chemical compounds present in the pesticides, which disperse rapidly in biotic (fauna and flora) and abiotic (air, soil and water) environments, altering their balance and presenting themselves as a threat to human health (del Puerto Rodríguez et al., 2014). Likewise, in the report "Soil contamination: a hidden reality", it is expressed that the global consumption of fertilizers reached 200 million tons in 2018; being Chile and the United States the consumer countries of 50% of the total (Rodríguez Eugenio et al., 2019).

The elaboration of biofungicides is a promising alternative to conventional fungicides. Therefore, the objective of this research was to evaluate the fungicidal activity of four biofungicides obtained from *A. cepa*, *A. sativum*, *Z. officinale* and domestic residual oil on *B. cinerea* and *F. oxysporum*.

2. Methodology

2.1 Identification and isolation of fungi

The fungi (*B. cinerea* and *F. oxysporum*) were isolated from leaves, fruits and stems of *S. lycopersicum* crops in the Kantu nursery, located in Lurin, Lima, Peru. Petri dishes with Potato Dextrose Agar were used for identification and isolation.

2.2 Obtaining the biofungicide

Obtaining the biofungicide followed the manual "Antiamoebic and phytochemical screening of some Congolese medicinal plants" (Tona et al., 1998). For this, 3 Kg of each plant (*A. cepa*, *A. sativum* and *Z. officinale*) were disinfected with sodium hypochlorite and abundant distilled water, then dried and cut into small pieces and then liquefied separately. Each liquefied paste was deposited in glass containers adding 800 mL of 90 % ethanol, and then stored at 5 ºC. After 48 hours of refrigeration, the macerated products were filtered, stored in bottles and kept in an environment away from sunlight and at 20°C until they were used in the experimental tests (Figueroa Gualteros et al., 2019).

2.3 Characterization of the fungi

For the process of characterization of the fungi, *B. cinerea* and *F. oxysporum* cultures were submitted to a microscope, model Olimpus, with 400 X and 100 X magnification, and later images of the structures of each one of the phytopathogens were captured.

2.4 In vitro antifungal activity test

For the test of the antifungal activity the potato dextrose agar (PDA) culture medium was mixed with the biofungicides inside an Erlenmeyer of 250 mL. The different extracts were diluted in the culture medium in three different doses (Table 1).

Table 1: Dosage of treatments´

N°	Treatments	Dose (%)		
1	Biofungicide 1 (BIO1)	10	15	20
2	Biofungicide 2 (BIO2)	10	15	20
3	Biofungicide 3 (BIO3)	10	15	20
4	Biofungicide 4 (BIO4)	10	15	20

BIO1: *A. cepa* + DRO
BIO2: *A. sativum* + DRO
BIO3: *Z. officinale* + DRO
BIO4: *A. cepa* + *A. sativum* + *Z. officinale* + DRO

Subsequently, the diluted medium was distributed in Petri dishes according to the doses of the different treatments shown in Table 1, and incubated at a temperature of 25°C. After three days of incubation, the diameter of the mycelial growth (mm) of each fungus was measured using a digital vernier. Measurement was performed at three-day intervals during the twelve-day incubation, and all measurements obtained were

inserted into a data collection format for further processing to determine the percentage of mycelial growth inhibition (Iglesias et al., 2017). The percentage mycelial growth inhibition was calculated according to equation 1 (Luksiene et al., 2020).

$$ICM = \frac{CTT - CTE}{CTT} \times 100\% \quad (1)$$

From the equation, %ICM is the percentage of Mycelial Growth Inhibition, CTT the mycelial growth in the control treatment and CTE the mycelial growth in the evaluated treatment.

3. Result and discussion

3.1 Characterization of *B. cinerea* and *F. oxysporum*

Figure 1 show the microscopic structure of the fungi *B. cinerea* and *F. oxysporum*, respectively.

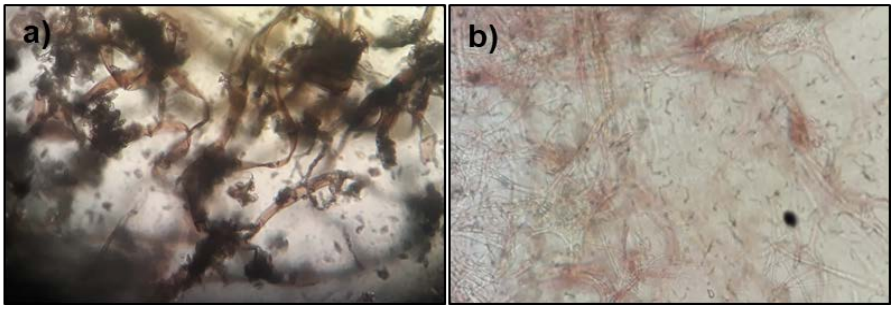

Figure 1: Imagen microscópica: a) B. cinerea y b) F. oxysporum

Macroscopically, *B. cinerea* develops cottony colonies of variable pigmentation, from greyish white to greyish brown. In Figure 1-a, it was observed microscopically that the mycelium is constituted by partitioned, cylindrical hyphae (Latorre, 2004). On the other hand, *F. oxysporum* develops cottony colonies with white and violet pigmentation. In Figure 1-b, it was observed microscopically that the mycelium is constituted by Microconidia that have hyalines in ellipsoidal to cylindrical form that can be straight or curved (Latorre, 2004).

3.2 Mycelial inhibition of *B. cinerea*

The percentages of *B. cinerea* inhibition are shown in Table 2. The biofungicide based on *Z. officinale* extract and DRO, achieved the total inhibition of the *B. cinerea* fungus at concentrations of 15 and 20 %. On the contrary, the less effective biofungicide was the one elaborated with *A. sativum* extract and DRO. The total inhibition carried out by the biofungicide based on *Z. officinale* and DRO was favorable due to the bioactive compounds of the extract such as gingerol, oxygenated monoterpenes, citral, etc (De Falco et al., 2018).

Table 2: Inhibition of mycelial growth of B. cinerea from the beginning of treatment (day 3) to the end of treatment (day 12)

Treatments	Mycelial inhibition (%)											
	10% dose				15% dose				20% dose			
	D3	D6	D9	D12	D3	D6	D9	D12	D3	D6	D9	D12
BIO1	98.57	72.68	47.66	46.28	99.59	93.39	86.86	82.82	100	100	100	100
BIO2	92.78	59.47	45.45	38.57	98.83	80.86	73.08	57.03	100	96.84	90.74	87.90
BIO3	100	81.87	64.89	57.72	100	100	100	100	100	100	100	100
BIO4	93.84	77.94	60.07	48.34	100	100	80.25	71.09	100	100	100	100

D3, D6, D9 y D12: day 3, day 6, day 9 y day 12, respectively

From Table 2, with the 10% dose, the treatment that achieved the highest percentage of inhibition (57.72 %) of *B. cinerea* in the last day of incubation was the BIO3 (*Z. officinale* extract and DRO) since it presents secondary compounds that prevent the spread of *B. cinerea* fungus (Ferreira et al., 2018). On the other hand, with the 15% dose, the biofungicide that totally (100 %) inhibited the fungus was also the BIO3. This inhibition was carried out from the third day of incubation until the last day of experimentation as a result of citral, verbenyl ethyl ether, geranic acid, among other compounds belonging to the species of *Z. officinale* that totally inactivate the growth of phytopathogens (Hussein and Joo, 2018). In other works, the crude extract of *Z. officinale* managed to inhibit *B. cinerea* by 17.5% at a dose of 0.5% (Hussein and Joo, 2018). The difference in the percentages of inhibition could be associated to the incubation temperature of the plates submitted during the experimental development, since the extract of *Z. officinale* presents synergistic properties (Sacchi Sneha, 2016) that allow them to inhibit the mycelial growth of *B. cinerea* at low temperatures of 21 to 25°C (Ramírez et al., 2020).

In treatments with 20% dose, the biofungicide that did not achieve total inhibition of *B. cinerea* was the BIO2 (*A. sativum* extract and DRO) because the bioactive compounds of the species do not effectively intervene in the mycelial inhibition of *B. cinerea* (Chen et al., 2018). On the other hand, the three remaining biofungicides achieved a total inhibition of the fungus in mention from the first day of incubation. Other researchers used *Thymus* extract at a dose of 30%, and obtained *B. cinerea* mycelial inhibition results of less than 63% (Šernaitė et al., 2020). (Hapon et al., 2017; Rodríguez-Rodríguez et al., 2017) succeeded in inhibiting *B. cinerea* fungus by 65% and 84.3%, respectively, with extracts of *Citrus limonia* and *Larrea divaricata*.

3.3 Mycelial inhibition of *F. oxysporum*

The percentages of *F. oxysporum* inhibition are shown in Table 3. The biofungicide based on *Z. officinale* extract and DRO, and the biofungicide composed of the three extracts and the DRO achieved total inhibition of the fungus with doses of 15 and 20%. On the contrary, the remaining biofungicides (*A. strain* + DRO and *A. sativum* + DRO) achieved the total inhibition of *F. oxysporum* only with the dose of 20%.

Table 3: Inhibition of mycelial growth of F. oxysporum from the beginning of treatment (day 3) to the end of treatment (day 12)

Treatments	Mycelial inhibition (%)											
	Dose 10%				Dose 15%				Dose 20%			
	D3	D6	D9	D12	D3	D6	D9	D12	D3	D6	D9	D12
BIO1	88.20	71.04	61.80	53.54	100	91.51	89.52	87.96	100	100	100	100
BIO2	96.49	85.66	75.30	71.83	100	93.07	91.10	88.06	100	100	100	100
BIO3	87.43	76.04	67.65	64.89	100	100	100	100	100	100	100	100
BIO4	100	93.41	90.28	85.61	100	100	100	100	100	100	100	100

D3, D6, D9 y D12: day 3, day 6, day 9 y day 12, respectively

In Table 3, the biofungicide that achieved the highest mycelial inhibition (85.61%) of *F. oxysporum*, at 10% doses, was BIO4 (*A. cepa* + *A. sativum* + *Z. officinale* + DRO) because it has organosulfurized components such as alkaloids, phenolic acids, flavonoids and gingerol that inhibit mycelial growth of the fungus (Miralha Franco et al., 2018). In other investigations, the authors were able to inhibit *F. oxysporum* by 58% from the seventh day of treatment with a 30% dose of *Syzygium aromaticum* oil (Faggiani et al., 2015).

Regarding the treatments BIO3 (*Z. officinale* + DRO) and BIO4 (*A. cepa* + *A. sativum* + *Z. officinale* + DRO), with doses of 15%, these biofungicides were effective in totally inhibiting the mycelial growth of *F. oxysporum* from the third day of incubation due to the action of its bioactive components such as citral, verbenyl ethyl ether, geranic acids and artemisiole (Gholamnezhad, 2019). In other works, *Juglans mollis* (4000 mg L^{-1}), *Juglans microcarpa* (5000 mg L^{-1}) and *Carya ovata* (5000 mg L^{-1}) ethanolic extracts showed the highest antifungal effect against *F. oxysporum* (Jasso de Rodríguez et al., 2019). Already, for the treatments with doses of 20%, the total inhibition of *F. oxysporum* was observed from the third day of treatment. In other studies, panthropical plants such as *Oxalis barrelieri* L., *Stachytarpheta cayennensis* L. and *Euphorbia hirta* L. inhibited mycelial growth of *F. oxysporum* by 80 and 100%, with application doses of 1.25 to 20 mg L^{-1} (Mekam et al., 2019).

The mycelial inhibition of *B. cinerea* and *F. oxysporum* was possible based on vegetable extracts and domestic residual oil, being an eco-friendly alternative to counteract phytopathogenic pests (Arici et al., 2019). The biologically active compounds of the plant extracts could provide new alternatives for the development of natural acaricides and fungicides that could be included as a set of strategies for an integrated pest management program in order to produce the least damage to the environment (Jasso de Rodríguez et al., 2019; Valdés-pérez et al., 2016).

4. Conclusions

The efficacy of biofungicides based on plant extracts (*A. cepa, A. sativum* and *Z. officinale*) and domestic residual oil for mycelial inhibition of *B. cinerea* and *F. oxysporum* was favorable, obtaining values of 85.08% and 91.88%, respectively. It was observed that the inhibition of *B. cinerea* and *F. oxysporum* fungi started from the third day of exposure, and the best results were found at the 15% dose of BIO3 and 20% of the other biofungicides, with inhibition values higher than 87.90%.

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