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EVALUATION OF THE *IN VITRO* ANTIFUNGAL POTENTIAL OF NATURAL EXTRACTS AND SYNTHETIC PESTICIDES ON THE GROWTH AND DEVELOPMENT OF THE PATHOGEN *BOTRYTIS CINEREA*

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ABSTRACT

This study was carried out to evaluate the in vitro antifungal potential of natural extracts from marc, spontaneous flora plants, and synthetic pesticides. The biological control of pathogens is a modern alternative because does not represent a danger to humans and the environment. Thus, natural alternatives with a fungistatic or fungicidal effect on the pathogen Botrytis cinerea were identified. The results of the in vitro tests clearly indicated a significant inhibitory percentage on the growth rate of the Botrytis cinerea rot mycelium (62.38%), when was used the extract obtained from the Cabernet Sauvignon marc at a concentration of 15%, compared to the other two natural extracts used (Salvia officinalis and Chelidonium majus), where the diameter of the colonies recorded values close to the untreated control variant. Regarding the effect of the synthetic pesticides studied, the best results were recorded with fosetyl-aluminum where a 100% inhibition percentage on the Botrytis cinerea pathogen was recorded.

INTRODUCERE

Since ancient times, agriculture has directly influenced the world economy, thanks to the production of fruits, vegetables and grains that represent an essential source of income both nationally and internationally. However, plants are constantly threatened by microorganisms – fungi, bacteria and viruses – and harmful insects. Pathogens cause significant damage to agricultural crops.

According to the FAO, about one-third (over 1.3 billion tons) of the total annual amount of food grown and produced for human consumption worldwide is wasted. Total global losses for fruits and vegetables range from 43 to 65%. In addition to wasteful practices, which can lead to unnecessary losses of edible fruits and vegetables, a major cause of wastage can be attributed to microbial degradation (Dean et al. 2012, Shomron et al. 2022).

Botrytis cinerea Pers.: Fr [teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel] is one of the most destructive pathogens of vegetables and fruits both in the field and in storage after harvest (Jarvis 1980, Elad & Evensen 1995, Benito et al. 2000). In the specialized literature are many studies focused on the development of biological control strategies against the phytopathogenic agent due to its resistance to commonly used synthetic fungicides (Abbey et al. 2018). The pathogen *B. cinerea* causes the appearance of the disease called gray rot that affects a wide variety of horticultural crops such as: grapevine, apple, strawberries, tomatoes, beans, etc., being one of the main phytopathogens found in greenhouses (Elad et al. 2004). Currently, chemical compounds are used for pre- and post-harvest prevention, among the best-known are thiabendazole, phenylpyrrole, benomyl and fludioxonil. Because the previously listed compounds are local inhibitors, special attention must be paid to their use but also to the identification of new alternatives to these (Fernandez-Ortuno et al. 2013). For example, to reduce the amount of inoculum, responsible for the production of new infections, diseased fruits are removed from the affected plants (Gholamnezhad 2019).

Interest in organic farming has grown in the last period, which has led to numerous studies on phytoparasites control and combating with the help of plant extracts and oils. Phytoprotection products development of natural origin for horticultural crops as an alternative to synthetic fungicides is in the center of attention, because they have a low impact on the environment compared to conventional pesticides (Tomazoni et al. 2018). The algae extracts have proven to be a possible substitute for synthetic pesticides, as evidenced by the study of Shomron and collaborators (2022) who evaluated the antifungal potential of *Ulva rigida* extracts and observed that they induced resistance of table grapes at *B. cinerea* infection.

Therefore, the purpose of this study consisted in the valorization of native plant species, marc obtained from the winemaking process as well as synthetic pesticides to inhibit the mycelial hyphae of the *B. cinerea* phytopathogen.

MATERIALS AND METHODS

In the process of obtaining the extracts, the marc obtained from the Cabernet Sauvignon variety winemaking process was used, and the aerial parts of the *Salvia officinalis* and *Chelidonium majus* plant species. The solvent used was a binary mixture of pharmaceutical ethyl alcohol: distilled water in a ratio of 50:50. Before being subjected to extraction, the plant material was primary processed by: drying and grinding for 5 minutes at 6000 RPM in pulses. The relative humidity of the material determined with the help of the thermobalance had the value of 4.27% for the Cabernet Sauvignon mulberry, 6.94% for *Salvia officinalis* and 6.25% for *Chelidonium majus*.

Microwave Assisted Extraction (MAE)

To obtain the extracts, a microwave system, model NEOS-GR (Microwave Extraction System from Milestone Inc), was used according to the following experimental design: 20 g of plant material were immersed in 200 ml of solvent (distilled water: pharmaceutical ethyl alcohol), at a power of 220W for 10 minutes. Extracts were centrifuged at 6000 RPM for 5 minutes, then filtered and kept in brown glass vials at +4˚C until use.

The efficacy of natural extracts and synthetic substances was tested on the phytopathogen *B. cinerea* grown on potato-dextrose agar (PDA) medium. In each Petri dish, 15 ml of medium were poured into which the natural extracts were added in different concentrations as well as synthetic pesticides, three repetitions were performed for each experimental variant. The control variant consisted of potato-dextrose agar (PDA) medium without addition of fungicides or natural extracts. Thus, a mycelial disc of approximately 5-6 mm was taken from the plant material and placed in the center of each Petri dish, later being incubated at room temperature.

Considering that the diameter of the colonies was not in the form of a circle, they were measured in two directions and the average value was recorded. Percent inhibition of mycelial growth was calculated using the formula by Vincent (Vincent 1947).

$$I = \frac{C - T}{C} x100$$

Where,
I = Per cent inhibition
C = Radial growth in control
T = Radial growth in treatment

To establish a notable inhibitory percentage, four different concentrations of natural extracts were used, namely: 0.5%; 2%; 9% and 15% respectively, the observations being recorded at 3, 5, 7, 10 and 14 days respectively. Thus, for the validation of the results as well as reporting to the conventional protection methods, *in vitro* tests were carried out using fungicides, namely fosetyl-aluminum 0.3% and metiram 0.3%.

Data analysis

Data were processed using one-way ANOVA, followed by Šídák's multiple comparisons test. Processed data were expressed as mean ± standard error (SE). This analysis was performed using GraphPad Prism 9.0.0.0 software.

RESULTS AND DISCUSSION

Dynamics of mycelial hyphae growth on culture medium

The phytopathogen used in the present study was isolated on PDA (potato-dextrose agar) medium, the mycelial dynamics was fast, having a radial development from the point of inoculation. The diameter of the phytopathogen *B. cinerea* colonies averaged 2.9/3 cm at 3 days and 7/7 cm at 5 days (Figure 1).

Figure 1. *B. cinerea* on PDA plate

Antifungal activity

Compared to synthetic products, in the case of natural extracts, the fungicidal or fungistatic effect on the phytopathogenic agent is directly proportional to the concentration used. Thus, in order to establish a notable inhibitory percentage on the dynamics of the pathogen studied, four concentrations of the extracts obtained from marc, *Salvia officinalis* and *Chelidonium majus* were used: 0.5%, 2%, 9% and 15%, respectively.

Figure 2 shows the antifungal action of the three natural extracts on the mycelial hyphae of the fungus *B. cinerea*.

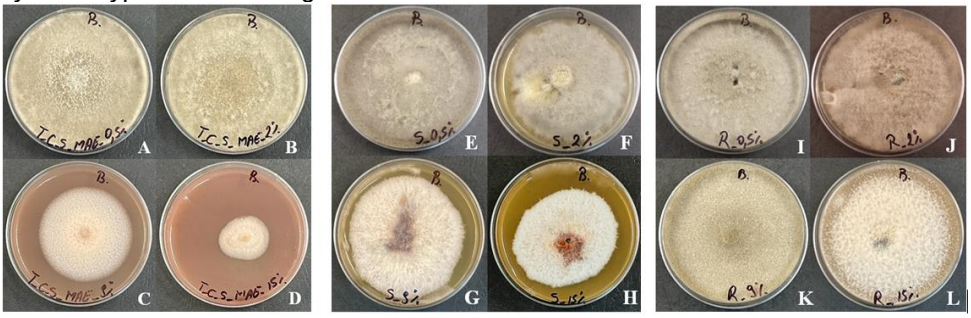

Figure 2. Growth of *B. cinerea* isolate on PDA amended with ethyl extract of Cabernet Sauvignon (A=0,5%; B=2%; C=9%; D=15%), *Salvia officinalis* (E=0,5%; F=2%; G=9%; H=15%) and *Chelidonium majus* (I=0,5%; J=2%; K=9%; L=15%).

The statistical analysis of the results after 14 days showed the highest percentage of inhibition of the mycelium growth of the phytopathogen *B. cinerea* in the case of using the 15% marc extract compared to all other variants, the differences being highly significant ($p < 0.0001$) (Figure 3).

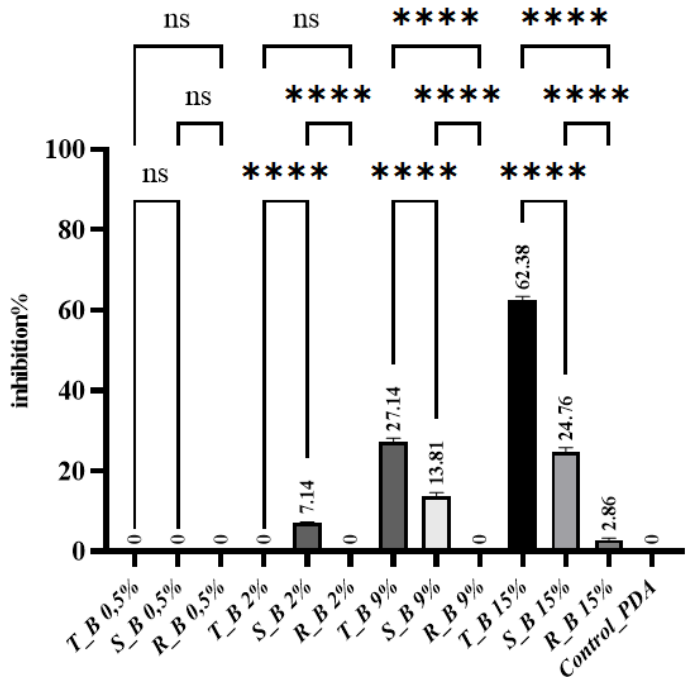

Figure 3. Effect of concentrations of the three types of extracts on the pathogenic fungus *B. cinerea*. The data are expressed as the mean ± SD values of three independent experiments performed in triplicate, and the values of p were calculated by the one-way ANOVA method followed by Šídák's multiple comparison test $^{****}p < 0.0001$; $^{ns}p > 0.9999$ (T_... - Cabernet Sauvignon marc extracts S_... - sage extract; R_... - celandine extract, B - *B. cinerea*).

The sensitivity of the pathogen to the natural extracts presented above was compared to that to the synthetic pesticides, fosetyl-aluminum and metiram (Figure 4). The concentrations used in the two synthetic pesticides were 0.3% according to the manufacturer's instructions.

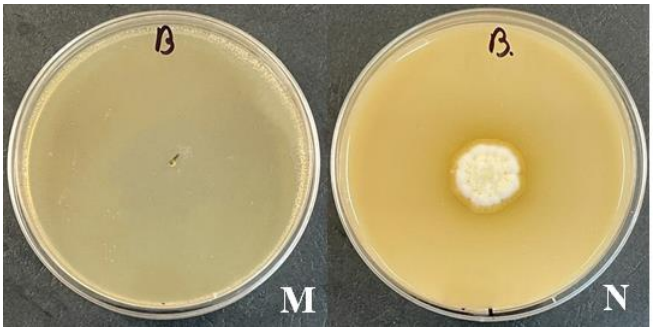

Figure 4. Antifungal effect of fungicides *in vitro* (M) fosetyl-aluminum 0,3%; (N) metiram 0,3%.

In the case of using synthetic pesticides, fosetyl-aluminum led to 100% inhibition of the mycelium of the pathogen *B. cinerea*. Good results were also observed in the case of the metiram fungicide where the percentage of inhibition was 73.33% (Figure 5).

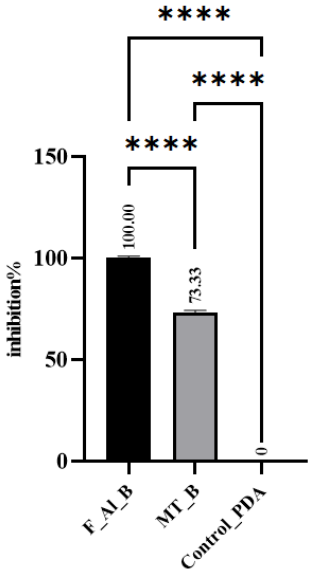

Figure 5. The effect of synthetic pesticides on the pathogenic fungus *B. cinerea* (B). The data are expressed as the mean ± SD values of three independent experiments performed in triplicate, and the values of p were calculated by the one-way ANOVA method followed by Šídák's multiple comparison test ****p < 0.0001 (F_Al – fosetyl-aluminum, MT – metiram, PDA – potato-dextrose agar)

Thus, it has been demonstrated that natural extracts can present real antifungal properties that can later be exploited on an industrial scale in agriculture, representing a sustainable alternative to synthetic pesticides, since the procurement of the raw material and the obtaining process do not present high production costs.

In this study, the natural extract obtained from marc showed the highest percentage of inhibition (62.38%), and the one from rostopasca the lowest percentage of inhibition (2.86%). Analyzing the specialized literature, the value of the percentage of inhibition varies significantly depending on the plant species from which the extracts are obtained but also on the extraction methods used. The in vitro studies by Mendoza et al. (2013a) demonstrated the antifungal activity of extracts obtained from a mixture of marc varieties (Cabernet Sauvignon, *Carmènere* and *Syrah*) against the phytopathogen *B. cinerea*, the inhibition percentage being 50%. Also, Mendoza et al. (2013b), observed a variation in the inhibitory effect produced by marc extracts obtained from Cabernet Sauvignon, Syrah and Carmenere varieties located in the Misiones de Rengo vineyard, as well as Cabernet Sauvignon and Syrah located in the Miguel Torres vineyard. Thus, the

best inhibitory effect on the pathogen *B. cinerea* was obtained with the extract from the Cabernet Sauvignon variety from the Misiones de Rengo vineyard.

Other research has highlighted the antifungal properties of *Salvia officinalis* essential oil against two phytopathogens that cause significant damage to horticultural crops, namely *B. cinerea* and *Fusarium sambucinum*. Following in vitro testing of *Salvia officinalis* essential oil, a strong fungicidal effect was demonstrated at a concentration of 10 µL (Rguez et al. 2019). In other studies, the extract of Chelidonium majus was used to determine the antifungal potential and it was found that the extract obtained from this type of plant material inhibited the mycelial growth of the *B. cinerea* species grown on Czapek agar medium, drastically reducing its development, in a percentage of 93.07% at a concentration of 120 µl/ml compared to the control variant (Pârvu et al. 2008).

CONCLUSION

Ecological agriculture is gaining more and more ground compared to conventional practices, therefore, plant extracts are a sustainable alternative that shows promising results in controlling and combating phytopathogenic agents. The results of the present study highlighted the high effectiveness of the extract obtained from the Cabernet Sauvignon marc using the concentration of 15% compared to the other natural extracts obtained from *Salvia officinalis* and *Chelidonium majus* against the growth and development of the pathogen *Botrytis cinerea*. Regarding synthetic pesticides, it can be said that fosetyl-aluminum showed 100% fungicidal activity on the phytopathogen studied.

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*** GraphPad Prism 9.0.0.0., https://www.graphpad.com/.