

Antifungal activity of medicinal plants extracts against *Botrytis cinerea* the causal agent of gray mold on tomato

Btissam Senhaji^{1,3}, Bouchra Chebli¹, El Hassan Mayad^{1,2,3}, Zahra Ferji³

1. Biotechnology and Environmental Engineering Team, National School of Applied Sciences, PO Box: 1136/S, Agadir, Morocco.
 2. Laboratory of Plant Biotechnology, Faculty of Sciences, Ibn-Zohr University, PB. 8106 - Cité Dakhla Agadir, Morocco.
 3. Plant Protection Department/ Nematology, Horticultural Complex of Agadir, Hassan II Institute of Agriculture & Veterinary Medicine - Agadir, Morocco
- E-mail of the corresponding author: elhassan.mayad@gmail.ma

Abstract

Four aromatic and medicinal plants of the Souss-Massa region were tested for their efficiency in reducing postharvest gray mold of tomato fruits caused by *Botrytis cinerea* *in vitro* and *in vivo* experiment. This antifungal activity was tested using two types of extracts: organic plant extract and aqueous extracts. When they are used at 1000 ppm, the four organic plant extracts of *Asteriscus imbricatus* inhibit completely the growth of *B. cinerea*. However complete inhibition of the mycelia growth of the pathogen was observed at 2000ppm concentration by ether and chloroform extracts of *Pulicaria mauritanica*. Moreover, the organic extracts of *Lavandula dentata* showed a moderate antifungal effect; while the four organic extracts of *Globularia alpyum* have no effect on the studied fungus. The aqueous extract of *A. imbricatus* has inhibited completely the growth of *B. cinerea* at 20000 ppm. The aqueous extract of *P. mauritanica* showed a moderate antifungal effect, while the aqueous extracts *L. dentata* and the aqueous extracts of *G. alpyum* were ineffective against *B. cinerea*. The *in vivo* test shows that disease incidence decrease as the concentration of *A. imbricatus* and *P. mauritanica* extracts increase. This study has demonstrated that organics and aqueous extracts of these two plants are promising antifungal agents which could be used as bio-fungicide in tomato crops protection against *B. cinerea*.

Keywords: Antifungal activity, extracts, medicinal plants, *Botrytis cinerea*, postharvest.

1. Introduction

Tomato crop plays an important role in the Moroccan economy by the currencies that generate (billions dirhams per year), and by the employment opportunities it provides. But now, this culture is threatened by several parasites responsible for diseases that cause heavy economic losses. Among the most important tomato diseases that cause commercially significant losses in worldwide (Gullino, 1992) and in Morocco, are Gray mold. This disease is caused by *Botrytis cinerea* Pers. Fr. which is considered one of the most destructive tomato pathogens (Eden and *al.*, 1996; Dik and Elad, 1999); especially in greenhouses where the temperature and humidity are favorable to the development of the fungus (Elad, 1999). That pathogen is able to infect various plant parts such as fruits, flowers, leaves, stems and seeds (Elad, 1999). The attack by the fungus occurs through the formation of germ tubes from conidia which penetrate the cell walls at any level of plant (Pucheu and Mercier 1983). For a long time, chemical fungicides are the essential tools to control *B. cinerea* in pre-and post-harvest to ensure sufficient production (Leroux, 2004). But in recent years, fungicide treatments of postharvest fungus may have several side effects, including the development of resistant strains and environmental contamination. Consequently, several studies have been focused on the use of ecofriendly methods to control the fungal diseases. Some of these methods use antagonistic microorganisms while others are based on plant extracts or essential oil compounds and their derivatives (Wilson *et al.*, 1987; Shimoni, 1993; Arras *et al.*, 1995; Carta *et al.*, 1996; Cutler *et al.*, 1996; Anthonov *et al.*, 1997; Bhaskara Reddy *et al.*, 1998; Alilou *et al.*, 2008; Soylyu *et al.*, 2010). In the prosecution of Moroccan aromatic plants valorization especially local plants that are used as remedies in folk medicine, this study aimed at the assessment of the antifungal activity of different organic and aqueous extracts obtained from four local plant: *Asteriscus imbricatus* (*Asteraceae*), *Pulicaria mauritanica* (*Asteraceae*), *Lavandula dentata* (*Lamiaceae*) and *Globularia alpyum* (*Globulariaceae*). The choice of these plants is stimulated by their potential use in traditional medicine as antiseptic (Saadi *et al.*, 2013) and the lack of data according to their chemical composition and their biological activity against *B. cinerea* the causal agent of postharvest gray mold of tomato.

2. Materials and Methods

2.1. Collection and preparation of plant

The aerial parts of four plants species at the flowering stage were collected randomly during April in three different areas of Agadir (Cape Ghir, Imozzer Idaoutanane, Tmanar). A fresh sample of the plants was botanically authenticated and preserved for reference in the Mechanics and Process Laboratory, Energy and Environment, National School of Applied Sciences - Agadir, Morocco. This collected plant species are: *Asteriscus imbricatus*, *Pulicaria mauritanica* (Asteraceae), *Lavandula dentata* (Lamiaceae), *Globularia alypum* (Globulariaceae). Plant samples were cleaned, dried and reduced to a fine powder using an electric laboratory grinding mill and stored in the dark at 4°C until use (Talibi et al., 2012).

2.2. Pathogenic fungi isolation

The fungus was isolated from infected leaves of tomato and identified at the Laboratory of Mycology at Horticultural Complex of Agadir, Agronomy and Veterinary Institute Hassan II, Morocco. The fungi were isolated by picking circles from infected areas in tomato leaves and rinsed with tap water three times then placed in Petri plates containing PDA medium. The plates were incubated at 25 °C. After seven days, the fungi were transferred to new plates and the transplanting is repeated several times to get a sufficient amount of fungi (Chebli et al., 2003).

2.3. Preparation of plant extracts

2.3.1. Extraction using organic solvents

Hot extraction was performed using Soxhlet apparatus and a cellulose cartridge. The used solvents are in order of increasing polarity: petroleum ether, chloroform, ethyl acetate and methanol (Dohou et al., 2004). The final extracts were obtained after concentration and removal of the solvent by rotary evaporation of collected solution.

2.3.2. Aqueous extraction

The aqueous extraction was prepared by maceration for three hours under magnetic stirring. The mixture was filtered through Whatman paper N° 4. Obtained extracts were stored at 4°C and protected from light until use (Senhaji et al., 2005).

2.4. Evaluation of antifungal activity of plant extracts

2.4.1. Antifungal tests in vitro

The antifungal tests were conducted in solid medium testing a series of concentration 2000, 1000, 500, 250, 125, 100 and 50 ppm. For the aqueous extraction, a series of dilutions ranging from 25000 ppm to 1000 ppm was prepared. The obtained solutions were dispensed into Petri plates. Pathogen grown on PDA without any extract was used as control. Three plates for each concentration were prepared and inoculated aseptically with 7 mm diameter disks of the test fungus taken from actively growing edge of one week old culture and incubated at 25 °C for seven days. The percent mycelial growth inhibition (PI) was calculated using the following formula: "PI = (A - B/ A) * 100" where A = diameter of fungal colony (mean) in control and B = diameter of fungal colony (mean) with plant extract.

2.4.2. Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

To determine the fungistatic or fungicidal effect of the plant extracts on *Botrytis cinerea*, the fungal discs from treatment with no growth were transferred into fresh medium to test the revival of their growth under observation during nine days. Treatment in which mycelial growth did not occur after additional 9 days of incubation was considered fungicidal (Hervieux et al. 2002; Mecteau et al., 2002). The experiments were performed twice.

2.5. Evaluation of antifungal activity in vivo on tomato fruits

According to the results of the *in vitro* test, only plant that showed a percent mycelial growth inhibition greater than 75% were selected for the *in vivo* test, which are *A. imbricatus* and *P. mauritanica* in this study. Healthy and uniform tomato fruits without symptoms of disease and no treated with pesticides were selected, then soaked in a solution of 10% bleach for 2 min and rinsed three times with tap water and dried in the open air. Preliminary tests were conducted to determine working conditions and concentrations used. The fruits were wounded with a sterile needle at three equidistant sites on the fruit to a 2 mm depth and width. The stock solutions were prepared to get final concentrations of 3000, 4000 and 5000 ppm for organic extracts and final concentrations: 3000, 4000 and 5000 ppm for the two plants studied. 20 µl of each concentration of extract were injected in each location of the wound using sterile distilled water for fruits control. After two hours, 20µl of spore suspension of *B. cinerea* (10⁵ spores ml⁻¹) were added to each wound. The spore concentration was determined using a Thoma cell. The inoculated fruits in 12 replicates per treatment were incubated at 25 °C for 7 days. The number of inoculated

active lesion sites was counted daily over a period of 7 days by counting from the third day of incubation. All treatments were arranged in a complete randomized block design and repeated twice. Disease incidence were calculated as follows:

Disease incidence (%) = [(number of rotten wounds / number of total wounds)] x 100.

2. 6. Statistical analysis:

The data were statistically analyzed using SPSS 16.0 software for analysis of variance (ANOVA) and mean comparison with Student-Newman-Keuls tests were used to segregate treatments which were significantly different at $P < 0.05$.

3. Results and discussion

3.1. In vitro antifungal activity of plant extracts against *Botrytis cinerea*

The results presented in Tables 1 and 2 respectively show the antifungal activity of organic and aqueous extracts of the four tested plants against *B. cinerea*. Most plant extracts tested reduced mycelial growth. However the antifungal effect of the extracts depends on plant species, type of extract and concentration. Indeed, for the organic extracts: the two plants belonging to the *Asteraceae* family were the most effective: *Asteriscus imbricatus* and *Pulicaria mauritanica*. The four organic plant extracts of *A. imbricatus* reduced completely mycelial growth of *Botrytis cinerea* at 1000 ppm. In a previous study, Allilou et al. (2008) found that at 2000 ppm, the essential oil of this plant *A. imbricatus* inhibited mycelial growth of *Penicillium digitatum* and *P. expansum* by 100% and by 97,01% for *B. cinerea*. Extracts of *P. mauritanica* showed also an interesting antifungal activity: 100% for ether and chloroform at a concentration of 2000 ppm. For ethyl acetate and methanol extracts, the antifungal effect was less effective. These results are in agreement with those of Znini et al. (2013) which showed that the essential oil of *P. mauritanica* (collected in the Errachidia area at the south eastern Morocco), inhibit mycelial growth of *Alternaria* spp. and *P. expansum* by at 100% and by 87,36 % for *Rhizopus stolonifer* at 2000 μ L/L. Other *Asteraceae* plants have demonstrated antifungal activity against *B. cinerea*: Essential oil from the Indian marigold *Tagetes patula*, in the daisy family of *Asteraceae*, completely inhibited *B. cinerea* growth at 10 μ l/ml (Romagnoli et al. 2005). Subsequent evaluation indicated that the essential oil of *Artemisia argyi* Lévl. et Vant inflorescence (wormwood) was also shown to inhibit *B. cinerea* growth (Wenqiang et al 2006).

The third plant *Lavandula dentata* showed a moderate antifungal effect at 2000ppm with an inhibition rate of 35% for the ether extract and 9% for the chloroform extract; while the inhibition rate was almost zero for ethyl acetate and methanol extracts. In a similar study Chebli et al. (2003) have found that essential oil of the same plant *L. dentata* inhibit mycelium radial growth of *B. cinerea* by 1,9% at 250ppm. Other *Lamiaceae* plants have demonstrated antifungal activity against *B. cinerea*: *Essential oils from Origanum compactum and Thymus glandulosus completely inhibited B. cinerea at 100 ppm* (Chebli et al., 2003). Moreover, in a recent study Soyulu et al. (2010) have found that the volatile and the contact phase of essential oil of *Lavandula stoechas L.* have completely inhibited *B. cinerea* mycelia growth at concentrations 1.6 μ g/ml and 25.6 μ g/ml respectively. While the fourth plant extracts of *Globularia alpym* have no effect. We also noted that for these extracts the antifungal activity increased with increasing concentration and decreasing polarity. For the aqueous extracts, the antifungal effect appears only at high concentrations. Among them, the highest activity was observed with the aqueous extract of *A. imbricatus* for which the percentage of inhibition is greater than 90% from the concentration of 15000 ppm. The second aqueous extract is that of *P. mauritanica*, for which the percentage of inhibition is 22% at the higher concentration. This antifungal effect was comparable to that reported by other findings: Askarne, et al. (2012) reported that the aqueous extracts of *Asteriscus odoratus* and *Halimiumum bellatum* (*Asteraceae*) inhibit mycelial growth of *Penicillium italicum* by 88,97% and 85,64% respectively using 10 mg of plants powders for 100 ml of PDA medium. In the same study the aqueous extract of *P. mauritanica* inhibit the same fungi by 10,77%. Concerning, the aqueous extracts of *L. dentata* and *G. alpym* no antifungal activity was recorded.

3.2. MIC and MFC

The table 3 shows the MIC and MFC values of selected plant extract which inhibited the mecyliar growth. This test showed that 2000ppm is the fungicidal concentration for the three organics extracts: Petroleum ether extract and Chloroform extract of *Asteriscus imbricatus* and for the Petroleum ether of *P. mauritanica*. The fungicidal concentration for the aqueous extract of *A. imbricatus* is 25000 ppm.

3.3. Antifungal activity of plant extracts on infected tomato fruits against *B. cinerea*

The results of *in vivo* tests after seven days of incubation showed that both of the organic extracts and the aqueous extract of *A. imbricatus* and *P. mauritanica* reduced significantly the incidence of gray mold. The results of *in vivo* antifungal activity of *A. imbricatus* and *P. mauritanica* organics extracts against *B. cinerea* spores on

infected tomato fruits are shown in figure 1. According to the results of the statistical analysis, the organic extracts were divided into three groups: The first group combines the petroleum ether and chloroform extracts that have reduced the incidence of disease by more than 85% at 5000ppm. The second group is represented by the ethyl acetate extract, which has reduced the incidence of disease by 70% at the same concentration. The third group is represented by the methanol extract, which has reduced the incidence of disease by almost 15% at 5000ppm. Thus the figure 2 shows the effect *in vivo* of aqueous extracts of *A. imbricatus* and of *P. mauritanica* on gray mold disease respectively. Indeed the aqueous extract of *A. imbricatus* has reduced the incidence of disease by 100% at 50000 ppm, this reduction did not exceed 70% for *P. mauritanica* aqueous extract in the same concentration. For all the extracts, the incidence of gray mold increases when the concentration of the extracts decreases. The results of *in vivo* test confirm that the antifungal activity depends on the type and on the concentration of extract. Under *in vitro* conditions, *A. imbricatus* and *P. mauritanica* extracts had great potential to reduce the mycelial growth of *B. cinerea*. However, the antifungal effects *in vivo* conditions were not as strong as those *in vitro*. These last have shown the need for high concentrations of plant extracts. But higher concentrations of plant extracts may have adverse effects on fruit such as drying and browning. Although there are no signs of phytotoxicity on tomato fruit for highest concentration *in vivo* experiments, it is interesting to perform the experience using whole tomato plants.

4. Conclusion

In conclusion, in this work we evaluated the antifungal activity of organic and aqueous extracts of four medicinal plants in order to find the natural product(s) with fungicidal activity against *B. cinerea* in postharvest. The organics and aqueous extracts of *A. imbricatus* and of *P. mauritanica* reduce significantly growth of *B. cinerea* mycelia on solid media and control infection of tomato fruits by this pathogen. Due to their miscibility with water, these plant products can be easily used with the irrigation water to reduce the damages caused by *B. cinerea* on the tomato crops. However, further studies need to be conducted to identify the active compounds responsible for the antifungal effect of each plant and to evaluate the cost and efficiency of these extracts on wide range of diseases in commercial greenhouses.

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Table 1. *In vitro* effects of *Asteriscus imbricatus*, *Pulicaria mauritanica*, *Lavendula dentata* and *Globularia alpym* organic extracts on mycelial growth of *Botrytis cinerea* : (PE): Petroleum ether, (CHL): Chloroform, (ACT): Ethyl acetate (MT): Methanol

Concentration (ppm)	<i>Asteriscus imbricatus</i>				<i>Pulicaria mauritanica</i>				<i>Lavendula dentata</i>				<i>Globularia alpym</i>			
	PE	CHL	ACT	MT	PE	CHL	ACT	MT	PE	CHL	ACT	MT	PE	CHL	ACT	MT
50	15,82f	2,05f	2,43e	1,9f	0g	0f	0d	0b	0d	0c	0a	0a	0a	0a	0a	0a
65	32,27e	17,7e	11,72d	4,89e	1,96f	1,18f	0d	0b	0d	0c	0a	0a	0a	0a	0a	0a
125	41d	43,92d	22,93c	17,38d	5,49e	5,1e	0d	0b	0d	0c	0a	0a	0a	0a	0a	0a
250	66,39c	67,47c	67,71b	52,82c	13,73d	9,8d	0d	0b	0d	0c	0a	0a	0a	0a	0a	0a
500	95,5b	93,82b	91,98a	92,44b	29,02c	21,96c	3,14c	0b	8,6c	0c	0a	0a	0a	0a	0a	0a
1000	100a	100a	100a	100a	56,08b	41,96b	10,2b	0b	21,56b	3,92b	0a	0a	0a	0a	0a	0a
2000	100a*	100a	100a	100a	100a	100a	39,22a	3,92a	35,29a	9,01a	0a	0a	0a	0a	0a	0a

*Means followed by different letters in each column are significantly different at $P < 0.05$ according to Newman and Keuls test.

Table 2: *In vitro* effects of *Asteriscus imbricatus*, *Pulicaria mauritanica*, *Lavendula dentata* and *Globularia alpym* aqueous extracts on mycelial growth of *Botrytis cinerea*.

Percentage inhibition of <i>Botrytis cinerea</i> mycelia growth %				
Concentration of aqueous extracts (ppm)	<i>Asteriscus imbricatus</i>	<i>Pulicaria mauritanica</i>	<i>Lavendula dentata</i>	<i>Globularia alpym</i>
1000	12,54d*	0e	0a	0a
2500	21,56cd	0e	0a	0a
5000	31,37c	0e	0a	0a
10000	54,11b	4,70d	0a	0a
15000	96,86a	9,80c	0,39a	0a
20000	100a	14,50b	0,39a	0a
25000	100a	21,96a	0,78a	0,39a

* Each value represents the mean of three replicates. Means followed by different letters in each column are significantly different at $P < 0.05$ according to Newman and Keuls test.

Table 3: Minimal inhibitory concentrations (MICs) and minimal fungicidal concentrations (MFCs) for the plant extracts against *Botrytis cinerea*

Extracts		MIC (ppm)	MFC (ppm)
<i>Asteriscus imbricatus</i>	Petroleum ether	1000	2000
	Chloroform	1000	2000
	Ethyl acetate	1000	>2000
	Methanol	1000	>2000
	Aqueous extract	20000	25000
<i>Pulicaria mauritanica</i>	Petroleum ether	2000	2000
	Chloroform	2000	>2000

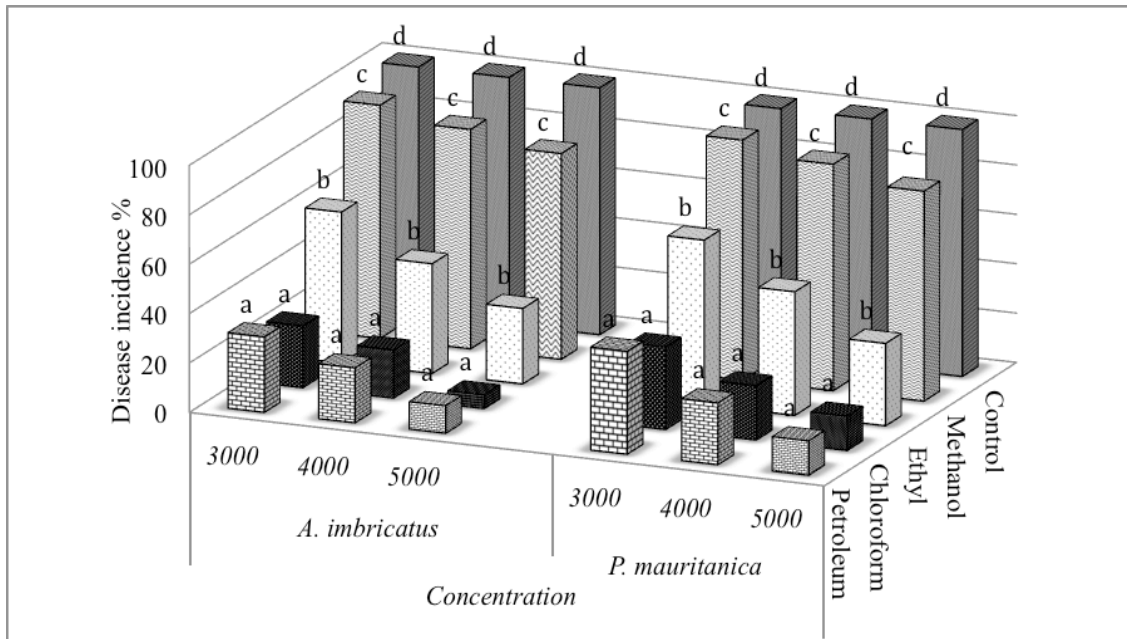


Figure 1. *In vivo* antifungal activity of *Asteriscus imbricatus* and *Pulicaria mauritanica* organic extracts against *Botrytis cinerea* spores on infected tomato fruits seven days after incubation.

* Significant differences ($P < 0.05$) between means were indicated by different letters above histograms bars.

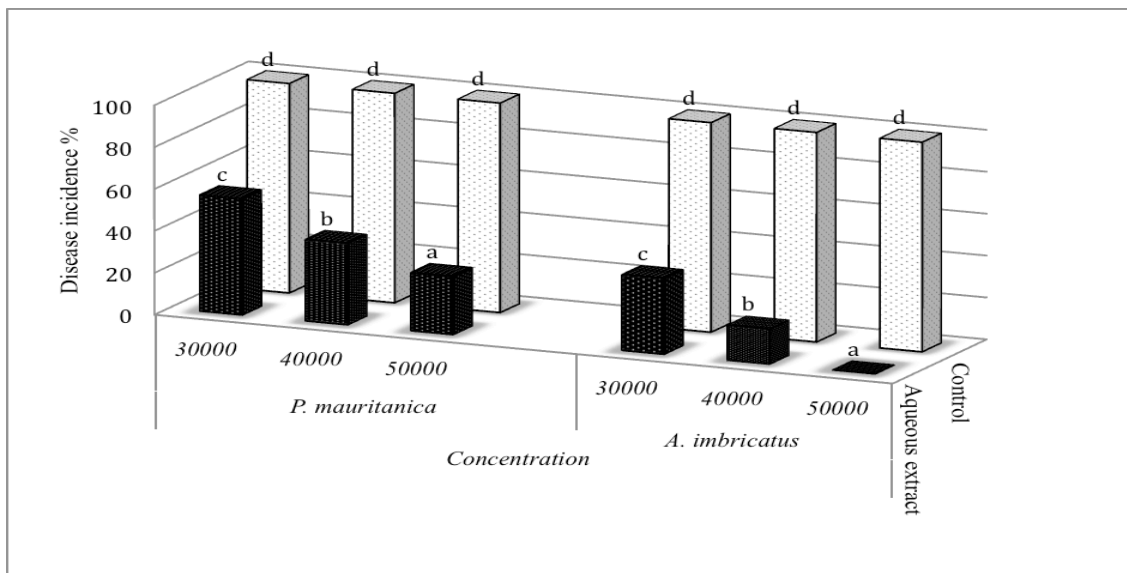


Figure 2. *In vivo* antifungal activity of *Asteriscus imbricatus* and *Pulicaria mauritanica* aqueous extract against *Botrytis cinerea* spores on infected tomato fruits seven days after incubation.

* Significant differences ($P < 0.05$) between means were indicated by different letters above histogram bars.