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Postharvest control of gray mold of tomato using seaweed extracts

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Abstract

The study was carried out to evaluate the effect of brown alga crude extracts of *Cystoseira tamariscifolia* and *Bifurcaria bifurcata* against the mycelium (growth of) *Botrytis cinerea* inducing grey mold during tomato postharvest. The results showed that the hexanic extract (91,000ppm) and the aqueous one (49,600ppm) have a very important inhibitory potency against the tested fungi with a very small amount of 100 µL. The important activity was obtained with the hexanic extract of *C. tamariscifolia* against *B. cinerea* with an inhibition zone up to 2 ± 0.18cm. The treatment of tomato fruits inoculated with the fungal species, with the aqueous extract of the brown algae ensures their protection against the phytopathogenicity of the fungus. An important protection was obtained also with the aqueous extract of *B. bifurcata*. Metal contents analysis show that these two seaweeds are not harmful.

The practical use of seaweeds during postharvest opens a new way in the biological control. Their extracts could be an ecofriendly alternative for the protection of crops against pathogens.

1. Introduction

Post-harvest losses of fresh fruits and vegetables are due to fungal and bacterial infection [1, 2]. They are estimated for tomato at 4% during harvest and 62% along the distribution chain [3] that leads to severe economical losses.

Grey rot of tomato, caused by *Botrytis cinerea* Pers., is a severe disease worldwide on tomato where advanced storage technologies are available [4]. The primary means for controlling the above disease still relies mainly on the use of chemical fungicides. However, alternative control methods are needed regarding risks of fungicides on human health and the environment. Antimicrobial and biostimulant properties of seaweeds are explored for use in agriculture [5, 6, 7].

Although Morocco spans from the Mediterranean Sea to the Atlantic Ocean on the north and the west with a broad range of macroalga biodiversity, there is only few reports derived from antimicrobial activities of seaweeds [7, 8, 9], with relatively little attention to their potential antifungal properties during postharvest.

Thus, the current study represents an attempt to bridge the gap and was designed to evaluate the antifungal activities of two brown algae *Cystoseira tamariscifolia* and *Bifurcaria bifurcata* against the mycelium (growth of) *Botrytis cinerea* inducing grey mold during tomato postharvest.

2. Materials and methods

2.1. *Collections*

Cystoseira tamariscifolia and *Bifurcaria bifurcata* was collected on rocks at low tide in March 2016 on the coast of Sidi Bouzid, near El Jadida city (33° 13′ 52″ N, 8° 32′ 51″ W). The samples were washed in seawater at the sampling station, placed in plastic bags and transported to the laboratory in an icebox. At the laboratory, seaweeds were rinsed with seawater to remove sand and epiphyta and washed with distilled water to remove salt excess.

2.2. *Extraction*

The algae were dried at room temperature, cut into small pieces and ground in a thermomix robot. Sixty grams of each algal powdered sample were submitted to $CHCl_3$/EtOH (5/5) extraction by soaking for overnight at room temperature. The combined extracts were concentrated by evaporation under reduced pressure, to yield the final crude extract as dark green oil. The crude extract was then extracted with hexane.

The aqueous extract was obtained according to the protocol described in the literature [10] with some changes in seaweed powder quantity used in order to get a more concentrated extract.

The extracts were filtered through bacterial filters (Millipore, 0.45 µm) and stored at 4°C before being used.

2.3. *Isolation and culture of fungal strains*

The strain *Botrytis cinerea* was isolated from infected tomato and transferred aseptically to Potato Dextrose Agar (PDA) slants for maintenance of the culture. The fungus was further purified by single spore isolation and maintained on PDA. The pathogen was identified based on morphological characters.

2.4. *Antifungal test*

2.4.1. In vitro inhibition assays

The *in vitro* assays were carried out according to the marbles technique. In a first step the fungus suspensions of *Botrytis cinerea* ($3.8x10^5$ spores mL^{-1}) sowing in depth was made at 1 mL per petri plates, then we poured the culture medium super cooling (45°C to 50°C). Afterwards, the wells created by marbles were poisoned with seaweed extracts (50µL and 100µL). The solvent and water were considered as negative controls and the Fluconazole as the positive control. In this experience we tested two volumes (Table 1) and all tests were performed in triplicate. Growth of fungal isolates was scored after 4 days of incubation at 25°C. The zone of inhibition was calculated by measuring the radius of the inhibition zone around the well (in mm). The readings were taken in three different fixed directions.

Table 1: Tested volumes of hexane and aqueous extracts and their mass equivalence.

Volumes tested µL	Mass equivalence of hexanic extract per well (mg)	Mass equivalence of aqueous extract per well (mg)
50	32.7	49.5
100	65.4	99

2.4.2. In vivo assay

For Storage conditions of tomatoes we chose 7 kg of the variety *Calvi* with good state. After the washing of tomatoes, they were disinfected with alcohol at 90° and rinsed with sterile distilled water to remove any trace of disinfectant. 5 lots of about 1 kg each were created. For tested aqueous extracts every tomato has been dipped in a beaker containing 100 mL of the aqueous extract ($4.96g\ L^{-1}$), whereas 1 g of seaweed powder was applied to every lot of tomatoes. For controls we used sterile distilled water. After 24 h each tomato was inoculated with a spore suspension ($3.8x10^5\ mL^{-1}$) of a 7 day-old colony using a sterile vaporizer. Then all lots of tomatoes were distributed separately in basins disinfected with alcohol at 90° and rinsed with sterile distilled water. Then the basins were covered with pierced cling film to promote the growth of fungi and incubated at room temperature (15-18°C) for 15 days. The development of rots was evaluated by measuring the diameter of the expansion of infection for the 7th, 9th and 15th days, which allowed us to calculate the percentage of inhibition, using the following formula:

$$\text{Inhibition (\%)} = (D - Di) / D \times 100$$

Where D *is the diameter of rots of the untreated fruit and* Di *the diameter of rots of fruit treated with the extract.*

2.5. *Heavy Metal content*

The mineralization of the tow brown algae has been done according to the protocol described by Mazlani, 1995 [11]. 1g of samples was digested with 3 ml of nitric acid. After incubation at room temperature for 24 hours, 3 mL of distilled water was added. The tubes were then closed and incubated at 60°C for 24 hours. The amounts of metals (Cd, Cu, and Zn) in the filtrate were determined by Atomic Absorption Spectrophotometer.

2.6. *Statistical analysis*

All the experiments were carried out in triplicate and the mean values with standard deviation are presented. Standard deviations were found to be $\pm 1.5\%$. When the error bars are smaller than the symbols, they are not

shown. The data were tested for normality and homogeneity of variance, and tests for significance between treatments using a Statistica 6 program by measuring the index of Ficher ($p < 0.05$).

3. Results and discussion

3.1. *In vitro conditions of antifungal activity of algal extracts*

Results reported in figure 1 showed an inhibitory action on the mycelium development of *B. cinerea*. This action is related to the concentration of the hexanic extract; the higher activity on the mycelium of *B. cinerea* was obtained with the higher amount, 65.4mg per well. Also, we find a significant difference between *C. tamariscifolia* and *B. bifurcata* activities on this fungus ($F = 6.5666$, $P < 0.05$). Therefore, hexanic extracts exceed the activity of Fluconazole.

The antifungal effect of aqueous extracts of *C. tamariscifolia* and *B. bifurcata* on *B. cinerea* has shown a significant difference between the quantities tested ($F= 6.0925$; $P<0.05$), where the mass equivalent of the aqueous extract (99 mg per well) of *B. bifurcata* has given a significant antifungal activity (2.18 ± 0.5 cm) (Figure. 2).

The results of the antifungal activity showed that hexanic and aqueous extracts of *C. tamariscifolia* and *B. bifurcata* have produced a marked inhibition zone on *B. cinerea* mycelium growth. This finding was reported by some authors who showed the antimicrobial activity of these two macroalgae [7, 12, 13, 14, 15, 16, 17, 18]. This may be due to active components which are present in seaweed extracts. phenolics compounds were isolated from the brown algae *C. tamariscifolia* and *B. bifurcata* [19, 20]. Other terpenic substances have been isolated from species of *Cystoseira* genus [7, 12, 21, 22]. A meroditerpenoid metabolite has been isolated from the brown alga *C. tamariscifolia* and has shown an antifungal activity against three tomato pathogenic fungi, *Botrytis cinerea*, *Fusarium oxysporum* sp. *Mycopersici* and *Verticillium alboatrum* [7].

The efficiency of the hexanic extract compared with the same quantity of Fluconazole is a very promising finding for future applications. The literature also shows that red alga: *Solieria robusta* acts better against *Fusarium solani* than the commercial fungicide -Topsin-M [23]. Other authors have found that the methanolic extract of *Acanthaphora spicifera* has an effect almost similar to Amphotericin against three fungal species [24].

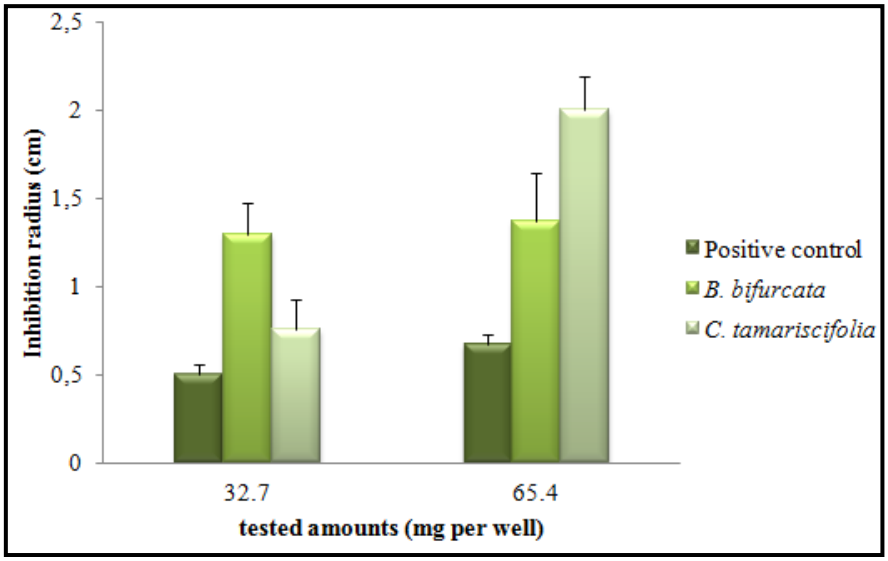

Figure 1: Evaluation of antifungal activity (inhibition radius) of *C. tamariscifolia* and *B. bifurcata* hexanic extracts against *B. cinerea*. (Average [n=3] ± standard deviation).

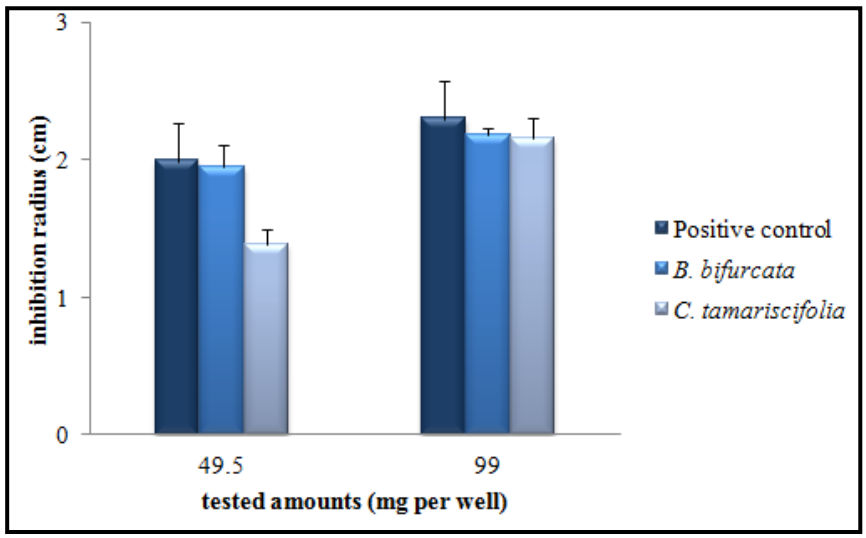

Figure 2: Evaluation of antifungal activity (inhibition radius) of *C. tamariscifolia* and *B. bifurcata* aqueous extracts against *B. cinerea*. (Average [n=3] ± standard deviation).

3.2. *Antifungal activity on infected tomatoes*

The *in vivo* test shows the changes of decay percentage values of treated and untreated tomato fruits during their storage (Figure. 3). Treatments significantly reduced the infection symptoms on tomatoes compared to those detected in the negative controls during the 9 first days. But after 11 days of the storage period, only the effect of *B. bifurcata* treatment persists with a high efficiency of the aqueous extract where the percentage inhibition during the 15th day of incubation is 50 ± 7%. The effect of *C. tamariscifolia* treatment is effective only during the 11 first where the reduction of the injury severity caused by *Botrytis cinerea* exceeds 60%. According to these results, we can conclude that the application of the aqueous extract of the two brown alga species tested on tomatoes has shown a protective activity against *B. cinerea* compared to the treatment with their powder. This may be due to the higher concentration of the active compound in aqueous extracts, which can contribute in the stimulation of defense mechanisms. It has been reported that the brown alga *Laminaria digitata* has a protective power against *Botrytis cinerea* and *Plasmopara viticola* by a stimulation of defense mechanisms [6]. Many authors have shown the antifungal activity of the algal extracts. For instance, the green alga *Ulva linza* extract has a protective effect against *Penicillium digitatum* [25], the activity of the brown alga *C. tamariscifolia* against phytopathogenic microbes was demonstrated [18]. Also, the antifungal action of some red algae against fungi, especially *B. cinerea* has shown promising results [26].

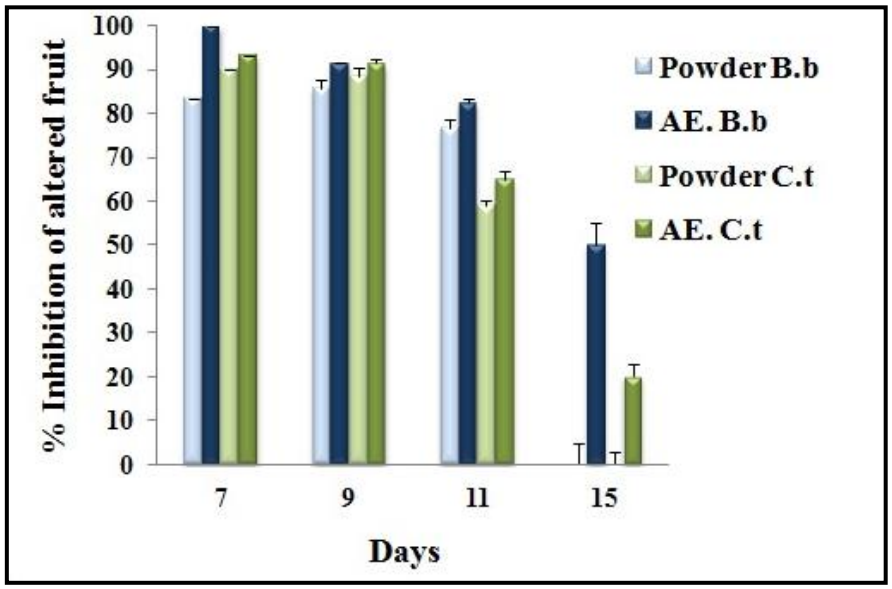

Figure 3: Effect of *C. tamariscifolia* (C.t.) and *B. bifurcata* (B.b.) aqueous extracts (A. E) (4.96 g L^{-1}) and powder (1 g per fruit) on the infection of tomato fruit caused by *B. cinerea* (Average [n=7] ± standard deviation).

3.3. *Heavy Metal content*

The results of the heavy metal contents shown that the two algae C. *tamariscifolia* and *B. bifurcata* accumulate heavy metals with different percentages (Table 2). The metallic contents Zn and Cu observed in the two brown algae do not appear to be high enough to lead to a potential risk of metal contamination at the agricultural level. Therefore Sidi Bouzid station can be considered an uncontaminated site by these two types of metals. The Cd is one of the most toxic metals in algae as in many other living beings. This slightly higher content may be related to ocean currents responsible for upwelling, especially rich in trace metals, including Cd [27].

The relative abundance of metals in *C. tamariscifolia* and *B. bifurcata* increases in the order Cd <Cu <Zn. These results are in agreement with those reported by other authors [28] who showed that the algae of El Jadida city accumulate more Fe> Zn> Mn> Cu> Cd. However, these metals, in general, have lower values than those recorded in other geographical areas.

With the intensification of urbanization, socio-economic activities and industrial activities, the rate of heavy metals will probably increase, and could lead to contamination risks of the final algae product.

Table 2: Metal content (µg per g dry weight) of *C. tamariscifolia* and *B. bifurcata* compared to the limit values of polluted sites in different algae (Average [n=3] ± standard deviation).

Samples	Zn (µg per g)	Cu (µg per g)	Cd (µg per g)
C. tamariscifolia	20.00 ± 0.17	2.10± 0.01	0.50 ± 0.00
B. bifurcata	29.50 ± 1.04	1.40 ± 0.03	0.20 ± 0.00
Metal concentrations in different algae For contaminated sites **[29, 30, 31, 32, 33]**	42 to 160	14 to 134	0.2 to 1

4. Conclusion

The three products (hexanic extract, aqueous extracts, and powder) from the two brown algae *C. tamariscifolia* and *B. bifurcata* showed their antifungal activity against *Botrytis cinerea*.

Among these products the ecological aqueous extract should be considered as a potential source of bioactive compounds and could serve as a biological control in Morocco coastal regions known for their production and tomato storage (e.g. Agadir and Dakhla). This may encourage the use of natural products as substitution of harmful chemical fungicides used along the agricultural food chain. Therefore, the investigation of heavy metal concentrations in the algae species may provide useful information on the transfer of potentially toxic elements from abiotic compartments (water and sediments) to higher consumers, including human beings.

Additional studies need to be performed to define and characterize, at the chemical and biochemical levels, the preferential effect of algal extracts on microorganisms that have adverse effects. A determination of the molecules really liable for this antifungal activity is also necessary to promote the product.

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