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Original Article

INHIBITIVE ACTIVITY OF 17 MARINE ALGAE FROM THE COAST OF EL JADIDA-MOROCCO AGAINST *ERWINIA CHRYSANTHEMI*

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ABSTRACT

Objective: The objective of our work was to search for a new biopesticides extracted from marine algae found on the coast of El Jadida, Sidi Bouzid-Morocco.

Methods: Extracts of 17 species of algae (Rhodophyceae, Rhodophyceae, Chlorophyceae) collected from the coast of El Jadida, Morocco, were tested for their antibacterial activity against the bacterial strain *Erwinia chrysanthemi* that causes soft rot in potato (*Solanum tuberosum* L.).

Results: Of the 17 species studied, those belonging to the Phaeophyceae and Rhodophyceae were the most active, while Chlorophyceae have a low inhibition. Maximum inhibition of the growth of *Erwinia chrysanthemi* was obtained by extracts prepared in dichloromethane and methanol, and by dichloromethane extract. No activity was observed in the aqueous extracts.

Conclusion: The results obtained in this study clearly indicated that macroalgae from the coast of Sidi Bouzid can be used in the treatment of plant diseases especially soft rot of potato.

Keywords: Antibacterial Activity, *Erwinia chrysanthemi*, Potato, Algae, El Jadida (Sidi Bouzid, Morocco).

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the fourth most cultivated food plant in the world, after the three major cereals: wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.). Worldwide, it is grown on approximately 20 000 000 ha, with a production of the order of 300 000 000 t/y [1]. In Morocco, the potato is the most important vegetable in terms of area and production; with about 1.4 000 000 t produced, mainly for the local market. Exports are currently lower than during the 80s and 90s when they frequently exceeded 100 000 t/y. Conversely, imports continue to increase (from 40 000 to 60 000 t each y). Moroccan producers are becoming increasingly aware of the need to improve their production techniques, to better meet the demands of the local market and export market [2].

The production of potatoes is accompanied by severe diseases caused by bacterial phytopathogens leading to enormous losses in yield and poor quality worldwide. One of the most important diseases infecting potato is soft rot caused by pathogenic species of bacteria of the genus *Erwinia*: pectinolytic phytopathogenic enterobacteria that cause soft rot disease on a wide range of plant species. The use of pesticides to fight these diseases has increased significantly in recent years, regardless of the economic level of the country. This has led to advances in agriculture and increases in production, but continuous use of these chemical products poisons and pollutes the environment, negatively impacting agricultural production and reducing its sustainability [3, 4]. Numerous short- and long-term human health effects have been recorded [5]. Fauna and flora are adversely affected the decimation of beneficial agricultural predators of pests has led to the proliferation of several pests and diseases [4]. Despite the adverse impacts and costs, farmers continue to use pesticides in most countries at an increasing rate, whereas the use of biological methods of pest control has decreased.

The need to develop a sustainable agricultural system requires alternative methods to reduce the use of synthetic chemical pesticides for plant protection. Among the alternatives, the use of biocontrol agents or biopesticides has aroused interest because of their ecological advantages. The stimulation of natural plants defences is considered as one of the most promising strategies for crop protection [6-9]. This original biological approach does not exert direct effects on the pathogen [9-11] but stimulates natural defences in plants, leading to a systemic acquired resistance [6, 12].

Macroalgae and their extracts have a long tradition of being used in coastal agriculture as a soil conditioner to enhance crop productivity [13], and to influence respiration, photosynthesis, nucleic acid synthesis and ion uptake [14-18]. Consequently, the products can enhance nutrient availability, water-holding capacity, increase antioxidants, and enhance metabolism and increase chlorophyll production [17, 19-20] and managing abiotic and biotic stresses in crop plants. The Moroccan coast is particularly rich in algal biodiversity and constitutes a reserve of species of considerable economic, social and ecological potential. More than 150 000 macroalgae species are found in oceans of the globe, which include green, brown and red algae, but only a few of them are identified [21]. Bibliographic inventory of benthic algae of the Moroccan coast revealed the presence of 213 species including 93 Chlorophyceae and 110 Phaeophyceae [22]. The aim of our work was to search for a new biopesticides extracted from marine algae found on the Atlantic coast of the city of El Jadida, Sidi Bouzid, Morocco. In this study, tests were conducted to determine the antibacterial activity of 17 species of marine algae from three families: Rhodophyceae, Chlorophyceae and Phaeophyceae, extracted using methanol, dichloromethane, dichloromethane and methanol or water against *Erwinia* species.

MATERIALS AND METHODS

Algal materials

Seaweeds was collected by hand-picking during March to April 2013 from the coast of El Jadida (33 ° 33 °16'09''N, 8 °30' 8 °45'W) (fig. 1). The algae were cleaned, washed in distilled water, then dried at room temperature and crushed to a fine powder.

The algae investigated were identified from fresh species as; Chlorophyceae (green algae): *Codium elongatum, Ulva* sp., *Enteromorpha intestinalis*, phaeophyceae(brown algae): *Bifurcaria bifurcata, Fucus spiralis, Laminaria digitata, Sargassum vulgaris, Cystoseira ericoides, Cystoseira myriophylloides, Halopitys incurvus,* and Rhodophyceae (red algae): *Corallina elongata, Laurencia*

pinnatifida, Gracilaria cervicornis, Gymnogongrus norvegicus, Gelidium sesquipedale, Gelidium sp., *Plocamium coccineum.*

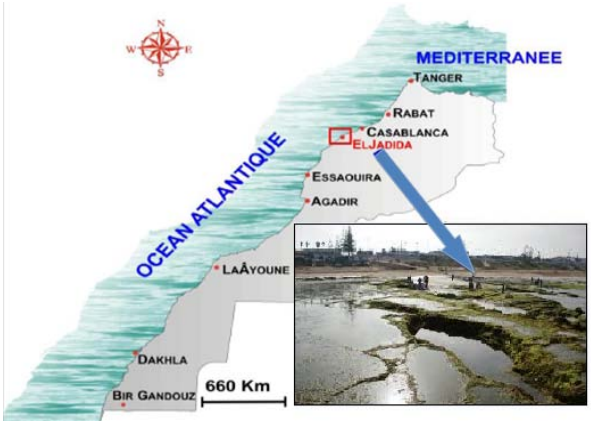

Fig. 1: Localization of the collection site of Sidi Bouzid

Preparation of extracts

The separate powder from each species of dried algae was extracted in different solvents, namely methanol, dichloromethane, dichloromethane and methanol (50:50), and water, as described by Caccamese and Azolina (1979) [23]. The resulting extracts were concentrated by drying in a rotary evaporator under reduced pressure (at 45 °C) until a crude extract was obtained, and was stored at 4 °C until utilization.

Isolation and culture of the pathogen

The isolate from fragments of potato plants showing symptoms of decay caused by *Erwinia* spp. were harvested and the fragments were placed in sterile bags and wet plastic to promote their development. The removal of fruiting bodies and the aerial mycelium from the fragments collected was performed under sterile conditions. Part of the infected tissue was removed, rinsed with water, or superficially disinfected using cotton soaked in 70 % ethanol. The tissues was placed in sterile saline (NaCl 0.85 %) 20 min to 2 h and then diluted to a ratio of 1:10 and 1:100 in sterile distilled water. Isolation and development of the bacteria are carried on the culture medium cct inoculated to exhaustion, then inoculated medium was incubated at 26 °C for 24 to 72 h. For purification, the colonies were picked using a sterile loop and streaked on petri dishes containing the cct culture media, Levan, (kb) King B or Crystal Violet Pectate (cvp). Purification was repeated several times in order to be assured of the purity of the bacteria [24]. After incubation the culture media were stored at 4 °C.

Identification of the pathogen

Soft rot *Erwinias* are facultatively anaerobic, flagellated, rod shaped and Gram negative. Although it is desirable to confirm these characters, it is possible to rely on their ability to grow and form cavities on Crystal Violet Pectate (cvp) to identify the more common soft rot causing *Erwinia carotovora* and *Erwinia chrysanthemi*. Only a few tests are necessary to confirm their identity. These are listed below:

Test*

- Catalase
- Oxidase
- Oxidation/fermentation
- Rotting of potato tuber slice

* Details given in table 1.

Identification at species and subspecies level is based on bacterial reaction pattern to a relatively small number of tests. As only three species or subspecies are commonly associated with potatoes, identification may be further simplified by applying a selected number of tests.

It is essential to use freshly growing pure cultures of the bacteria on nutrient agar (na) or Luria broth agar (lba) when performing the tests. The inclusion of bacteria known to give a positive or a negative reaction for a particular test is also useful [24, 25].

Antimicrobial bioassays

Antibacterial assays were carried out using the agar disc-diffusion assay [26]. Three colonies of each bacterium were removed with a wire loop from the original culture plate and were introduced into a test tube containing 5 ml of Nutrient broth. An overnight culture yielded a suspension of 10^6 bacteria per ml (evaluated by the absorbance value of 0.5 at 620 nm). This solution was diluted 100-fold and the bacterial density was then adjusted to 0.2×10^4 cells per ml with sterile water to inoculate Petri dishes containing Mueller-Hinton agar culture media. Plates were dried for about 30 min before inoculation and were used within 4 d of preparation. The organic extracts were tested using paper disks (6 mm diameter) impregnated with the solution. For the aqueous extract, the method of the wells is used. A volume of extracts obtained is deposited in wells on agar containing the appropriate test strain. After the temperature was equalized at 4 °C, the microorganisms were incubated overnight at 37 °C. Diameters of inhibitory zones were then measured. Discs impregnated with standard antibiotics such as, streptomycin were used at 50 or 100 µg/ml as reference in the test of antibacterial activity. In addition, control disks were prepared with each solvent and all tests were performed in triplicate. Representative halos were those measuring a diameter superior to 10 mm [27].

Antibacterial efficiency of extracts was evaluated according to the following scale:

Ø ≤ 8 mm: No-significant antimicrobial activity

8<Ø ≤ 12 mm: Moderate antimicrobial activity

12<Ø ≤ 14 mm: Significant antimicrobial activity

Ø>14 mm: Very significant antimicrobial activity

Statistical analysis

The data were statistically analyzed by applying a one-way ANOVA for comparison of mean values. All tests were considered to be statistically significant at *P<0.05.

RESULTS AND DISCUSSION

Identification tests

After incubation, bacterial growth was assessed and the colony diameter read off should be between 4.0 and 7.0 mm. Typical colonies have the characteristics described below:

• In the culture media King B (kb): colonies appear after 48 h, are white circular tending to spread out and not fluorescent under UV at 356 nm.

• On the culture media of Levan: colonies appear after 48 h, are whitish, circular convex, smooth and like mucous.

• On culture media of cct (Differential medium): colonies appear after to 72 h, are pale-purple, circular, strongly convex or convex then become smooth and mucous membranes. Their growth is slower than on King B (kb) and Levan culture media. The culture media of cct medium inhibits *Pseudomonas* spp., but not *Pantoea agglomerans*.

• On culture media of Crystal Violet Pectate (cvp): The bacteria form characteristic deep cup-like cavities or pits which are different from those formed by the pectolytic pseudomonads, which are shallower and wider.

The identification of the pathogen is the realization of a series of tests to confirm the characteristics described above. Suspect colonies are identified by different tests; this identification is

confirmed by the pathogenicity test on young tobacco leaf and slices of potato tuber. Only a few tests are needed to confirm their identity as *Erwinia*. They are listed below (table 1).

The different identification tests of the bacteria tested were accomplished with the appointment of *Erwinia chrysanthemi* that is a gram-bacteria causing soft rot of potato (table 2).

Antibacterial activity

The results of the screening of antibacterial activities against bacteria *Erwinia chrysanthemi* are summarized in tables 3 to 5.

Table 1: Confirmation identity of kind *Erwinia*

Tests	*Erwinia* Reaction
Soft rot of potato	+
Test potash indicator Gram	-
Catalase	+
Oxidase	-
Oxidation/Fermentation	+/+
Rot slices of potato tuber	+

+: positive reaction,-: negative reaction.

Table 2: Identification of species and subspecies of *Erwinia*

Tests	*Erwinia* Reaction
Test growth na (nutrient agar) at 37 °C	+
Differentiation of *Erwinia* spp. By growth at different temperatures: 27 °C, 35.5 °C, 37 °C	+
Test at 35 °C growth	+
Sensitivity to streptomycin	+
Growth in 5% NaCl	-
Test of sucrose transformation	-
Utilization of organic acids: Citrate	+
Production of:	
Indole	+
Phosphatase	+
Acid production from:	
Lactose	-
Maltose	-
Trehalose	-
Sorbitol	-
Tobacco hypersensitivity test (hst)	**+**

+: positive reaction,-: negative reaction

Table 3: Antibacterial activity of different brown seaweeds extracts against *Erwinia chrysanthemi*

Solvent of extraction	*Diameter of inhibition (mm)						
	Grown algae						
	B10	B11	B12	B13	B14	B15	B16
CH_3OH	12±1.63	10±0.82	8±2.94	8±2.83	8±2.31	9±2.00	16±1.41
CH_2Cl_2	18±2.12	8±3.61	13±1.58	10±1.22	8±3.54	10±1.87	8±2.12
CH_2Cl_2/CH_3OH	15±3.67	12±1.87	15±3.08	12±1.41	8±2.55	7±2.12	12±1.87
Aqueous extract	-	-	-	-	-	-	-

(-): No activity; CH_3OH: Methanol; CH_2Cl_2: Dichlorométhane; B10: *Bifurcaria bifurcata*, B11: *Fucus spiralis*, B12: *Laminaria digitata*, B13: *Sargassum vulgaris,* B14: *Cystoseira ericoides*, B15: *Cystoseira myriophylloides*, B16: *Halopitys incurva*.

Of the seven brown algae tested, three species showed a positive activity against the bacteria test. An important activity (diameter of inhibition higher than 15 mm) was observed in the dichloromethane extract of *Bifurcaria bifurcata* and in the methanolic extract of *Halopitys incurvus*. The dichloro methane/methanol extract of *Laminaria digitata* and *Bifurcaria bifurcata* showed an inhibition of *Erwinia chrysanthemi* for a diameter lower or equal to 15 mm (table 3).

Table 4: Antibacterial activity of different green seaweeds extracts against *Erwinia chrysanthemi*

Solvent of extraction	*Diameter of inhibition (mm)		
	Green algae		
	G1	G2	G17
CH_3OH	8±2.94	10±0.82	9.3±0.58
CH_2Cl_2	10±0.71	10±1.58	9±1.73
CH_2Cl_2/CH_3OH	8±4.47	10±1.22	5.33±4.73
Aqueous extract	-	-	-

(-): No activity; CH_3OH: Methanol; CH_2Cl_2: Dichlorométhane; G1: *Codium elongatum*, G2: *Ulva sp*, G17: *Enteromorpha intestinalis.*

Of the three green algae tested, the presence of a positive activity on *Erwinia chrysanthemi* was observed in dichloromethane extract of *Codium elongatum*, with a diameter of inhibition lower or equal to 15 mm, the dichloromethane/methanol, dichloromethane and methanolic extract of *Ulva* sp. showed an inhibition for a diameter lower or equal to 15 mm (table 4).

Table 5: Antibacterial activity of different red seaweeds extracts against *Erwinia chrysanthemi*

Solvent of extraction	*Diameter of inhibition (mm)						
	Red algae						
	R3	R4	R5	R6	R7	R8	R9
CH_3OH	18±1.41	8±2.94	8±2.94	10±0.82	9±3.56	11±1.15	14±1.15
CH_2Cl_2	12±1.73	8±4.74	11±1.00	12±1.87	9±3.08	10±1.22	11±2.24
CH_2Cl_2/CH_3OH	16±1.22	8±4.06	17±1.58	8±4.47	12±1.87	10±1.22	11±2.24
Aqueous extract	-	-	-	-	-	-	-

(-): No activity; CH_3OH: Methanol; CH_2Cl_2: Dichlorométhane; R3: *Corallina elongata*, R4: *Osmundea pinnatifida*, R5: *Gracilaria cervicornis,* R6: *Gymnogongrus crenulatus*, R7: *Gelidium corneum,* R8: *Gelidium sp*, R9: *Plocamium cartilagineum*.

For red algae, an important activity against *Erwinia chrysanthemi* was observed in the methanolic extract of *Corallina elongata* with a diameter superior to 15 mm. The dichloromethane extract of *Corallina elongata*, and *Gymnogongrus norvegicus* showed an inhibition of bacteria with a diameter of 12 mm. Concerning the dichloromethane/methanol extract of this red algae, extract of *Corallina elongata* and *Gracilaria cervicornis* showed an important activity (diameter of inhibition higher than 15 mm) (table 5). For aqueous extracts, no antibacterial activity against *Erwinia chrysanthemi* was detected (table 3 to 5).

Plants are able to defend themselves against pathogens with a variety of preformed structures and inducible reactions. Inducible reactions essentially require the perception of signal molecules, which may results from pathogen attack or an external chemical treatment. This kind of recognition leads to triggering of a plethora of reactions, which result in augmentation of resistance to the invading pathogens. This enhanced state of resistance is effective against a broad range of pathogens and parasites, including fungi, bacteria, viruses, nematodes, parasitic plants, and even insect herbivores [10, 28]. The reactions include stimulation of the phenylpropanoid pathway, production of defense-specific signal molecules such as salicylic acid, jasmonates, and accumulation of antimicrobial compounds/proteins such as phytoalexins and pathogenesis-related proteins [29]. The chemical stimuli or elicitors, which bring about these induced reactions, are diverse and include oligosaccharides, polysaccharides, lipids, glycoproteins, peptides, and proteins [30, 31]. Many elicitors generally enhance non-host plant resistance. It has become a fascinating crop protection strategy by mimicking pathogen attack using non-specific elicitors [8, 31], and many such elicitors from synthetic and natural sources are being examined for efficacy in crop disease control. Treatment of plants with various agents, including plant extracts, can induce resistance to subsequent pathogen attack both locally and systemically [9]. Seaweeds are known for their high original polysaccharides some of which have various degrees of sulfating. Brown algae, in particular, are already used in fertilization for a long time successfully. Some have been work specific to the eliciting properties of their constituents that are now entering products commercial. In the literature most macroalgal polysaccharides and derived polysaccharides activate defense responses of plants and protection against a range of pathogens by activating salicylic acid, jasmonic acid and/or ethylene signaling pathways at a systemic level [32].

CONFLICT OF INTERESTS

Declared None

CONCLUSION

This screening reports an important antibacterial activity in the algae collected from the coast of Sidi Bouzid. Results obtained in the present study revealed that *Corallina elongata* and *Bifurcaria bifurcata* are the potential producers of antibacterial activity against *Erwinia chrysanthemi* that can limit or minimize the effect of soft rot of potato. As a results, it can be concluded that macroalgae from the coast of Sidi Bouzid can be used in the treatment of plant diseases especially soft rot of potato.

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