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Activity of seaweed and cyanobacteria water extracts against *Podosphaera xanthii* on zucchini

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Abstract

The purpose of this research was to study the effects of water extracts of algae belonging to the *phylum Rodophyta*, *Heterokontophya*, *Chlorophyta* and of *Cyanobacteria* against *Podosphaera xanthii* on zucchini (*Cucurbita pepo*) cotyledons. Each extract was sprayed on the upper surface of cotyledons at the concentration of 0.5 %, then cotyledons were inoculated with six 10-µl drops of a spore suspension of the pathogen (1×10^6 spores/ml), and incubated on water agar in Petri dishes. Depending on the species, the extracts showed inhibition, stimulation or no effect on the disease severity caused by *P. xanthii* and on the pathogen sporulation density. Extracts of *Corallina* sp., *Halopithys* sp., *Sargassum* sp. and *Anabaena* sp. showed significant inhibitory effect on both parameters. This study suggests that seaweed and cyanobacteria water extracts applied on zucchini cotyledons may be considered in further experiments on plants as a useful preventative tool for the disease management in sustainable agriculture.

Keywords: seaweed; cyanobacteria; extracts; Podosphaera xanthii; zucchini; disease control

Riassunto

Nell'agricoltura tradizionale, da molto tempo, i patogeni delle colture sono gestiti con agrofarmaci di sintesi, il cui uso è attualmente regolamentato in maniera più sostenibile, rispetto al passato, per la salvaguardia della salute dell'uomo e dell'ambiente. Le nuove normative prevedono l'utilizzo di mezzi alternativi a tali prodotti, tra cui quelli costituiti da microrganismi fungini e batterici, alcuni dei quali sono registrati come agrofarmaci per la difesa delle colture dai patogeni. Altri prodotti composti da sostanze naturali, come gli estratti di alghe, sono noti per esercitare un'azione biostimolante della pianta, consentendole di superare stress abiotici e biotici, e quindi rappresentano una interessante possibilità di impiego nella difesa integrata delle colture. Pochi studi sono stati condotti per saggiare l'effetto delle alghe nei confronti di patogeni fungini fogliari. Lo scopo di questa ricerca è stato quindi quello di verificare su cotiledoni di zucchino (*Cucurbita pepo*) l'attività diretta di estratti di alghe rosse (*Phylum Rodophyta*), alghe brune (*Phylum Heterokontophyta*), alghe verdi (*Phylum Chlorophyta*) e cianobatteri (*Phylum Cyanobacteria*) contro il patogeno fungino *Podosphaera xanthii*, agente del mal bianco delle cucurbitacee. Le foglie cotiledonari sono state recise da piante sane, trattate per nebulizzazione con ciascun estratto di alghe e cianobatteri, quindi inoculate con 6 gocce di 10 µl ciascuna di sospensione sporale del patogeno (1×10^6 spore/ml) e incubate su agar acqua in capsule Petri per 9 giorni a 22-24 °C. L'effetto dei trattamenti è stato valutato sia come gravità di malattia (percentuale di area fogliare con

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sintomi di malattia/area inoculata), sia come densità di sporulazione (numero di spore/area fogliare inoculata). In funzione della specie saggiata, gli estratti hanno mostrato effetti di inibizione, stimolazione o nessun effetto sui parametri considerati. Gli estratti delle alghe rosse *Corallina* sp. ed *Halopithys* sp., dell'alga bruna *Sargassum* sp. e dei cianobatteri *Anabaena* sp. e *Spirulina* sp. hanno ridotto significativamente sia la malattia, sia la sporulazione. Al contrario, gli estratti di alghe verdi hanno dimostrato effetti nulli o hanno stimolato la malattia. Questo studio dimostra che trattamenti preventivi di cotiledoni di zucchino con estratti acquosi di alghe marine e cianobatteri possono essere considerati per ulteriori studi in strategie di gestione delle malattie in agricoltura sostenibile.

Parole chiave: alghe; cianobatteri; estratti; Podosphaera xanthii; zucchino; difesa da malattie

Introduction

The term Algae includes several group of eukaryotic photosynthetic organisms not necessarily closely related. In this group, are included unicellular organisms as green algae e.g. *Chlorella* species, or multicellular algae, as brown algae e.g. *Sargassum s*pecies that can reach several meters in length. Algae are widely distributed and are able to live both in temperate and cold water and at different depth. The geographic location and the season of harvest (Black 1950; Painter 1983) influence algae composition such as the content in polysaccharides (Rioux et al. 2007), the color and the presence of pigments involved in the photosynthesis that discriminates between the *Phylum Chlorophyt*a (green algae), *Heterokontophyta* (brown algae) and *Rhodophyta* (red algae). Seaweed extracts can help the plants to better cope with biotic and abiotic stresses and offer potentiality for field application (Morot-Gaudry et al. 2009). Several benefic effects were observed such as improving of seed germination (Rayorath et al. 2008), plant growth, root development, nutrient elements transferring, resistance to the pathogens and yields (Stephenson 1966; Hankins and Hockey 1990; Craigie 2010). These effects were partly attributed to the content of essential nutrients and to the trace of metal mixtures (Cu, Co, Zn, Mn, Mo, etc.) which are necessary for the plants but not often present in the fertilizers (Craigie 2010; Rayorath et al. 2008). Seaweed extracts also contain a number of plant growth-regulators like cytokinins and auxins that resulted to increase yields and to improve heat tolerance in the plants (Zhang et al. 2010).
Cyanobacteria (blue-green algae) are prokaryotic microorganisms, widespread in all habitats, both in fresh and salt water (Lawton et al. 1991) and terrestrial soil. They are mostly photoautotrophic (Kulik 1995) able to fix nitrogen and produce a large variety of active secondary metabolites such as antibiotics, antiviral and antifungal compounds (Skulberg 2000). In a recent study we have demonstrated that application of a water extract of the strain *Anabaena* sp. BEA300B on zucchini cotyledons helps the earlier accumulation in the plant of enzymes correlated to induced systemic resistance as chitinase, ß-1,3-glucanase and peroxidase (Roberti et al. 2015).
Podosphaera xanthii (Castagne) U. Braun and Shishkoff *(Ascomycota*, *Erysiphales*) is one of the casual agents of powdery mildew, the major disease of plants belong to *Cucurbitaceae*, in particular zucchini plants, both in open field and greenhouse in Italy (Brunelli and Gengotti 2007) and worldwide (Peréz-García et al. 2009). This disease can markedly limit cucurbit production, so the control is needed. Traditionally, powdery mildew control by systemic synthetic fungicides is achieved (Mcgrath 2001; Brunelli and Gengotti 2007).
Currently, the research in plant disease control moves on methods with a low ambient impact. Microbial fungicides based on the mycoparasite *Ampelomyces quisqualis* is commercialized in several countries and other biological means have been proposed to control cucurbit powdery mildew (Romero et al. 2004), but little is known about the use of algae and cyanobacteria extracts against powdery mildew on zucchini.

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Roberti et al. (2015) also demonstrated that the extract of the cyanobacterium Anabaena BEA300B showed a direct antifungal activity on pathogen sporulation.
Considering that the control of fungal plant diseases is based on synthetic products, whose continuous use in the past brought several damages on human and animal health and to the agro-ecosystem, this research aimed to study the possibility to reduce powdery mildew disease on zucchini cotyledons through the preventative application of several seaweed and cyanobacteria extracts, as environmental-safe products complementary to crop nutrition and crop protection.

Materials and methods

Seaweed and cyanobacteria water extracts, plant material and pathogen

Algae and cyanobacteria were isolated by Banco Español de Algas (BEA), Marine Biotechnology Center, University of Las Palmas de Gran Canaria. The algae were collected from the sea (Fig.1a), partially dried in the greenhouse under environmental lighting for 12 h, dried at 60 °C and then grinded and pulverized with a mill (Fig.1b).

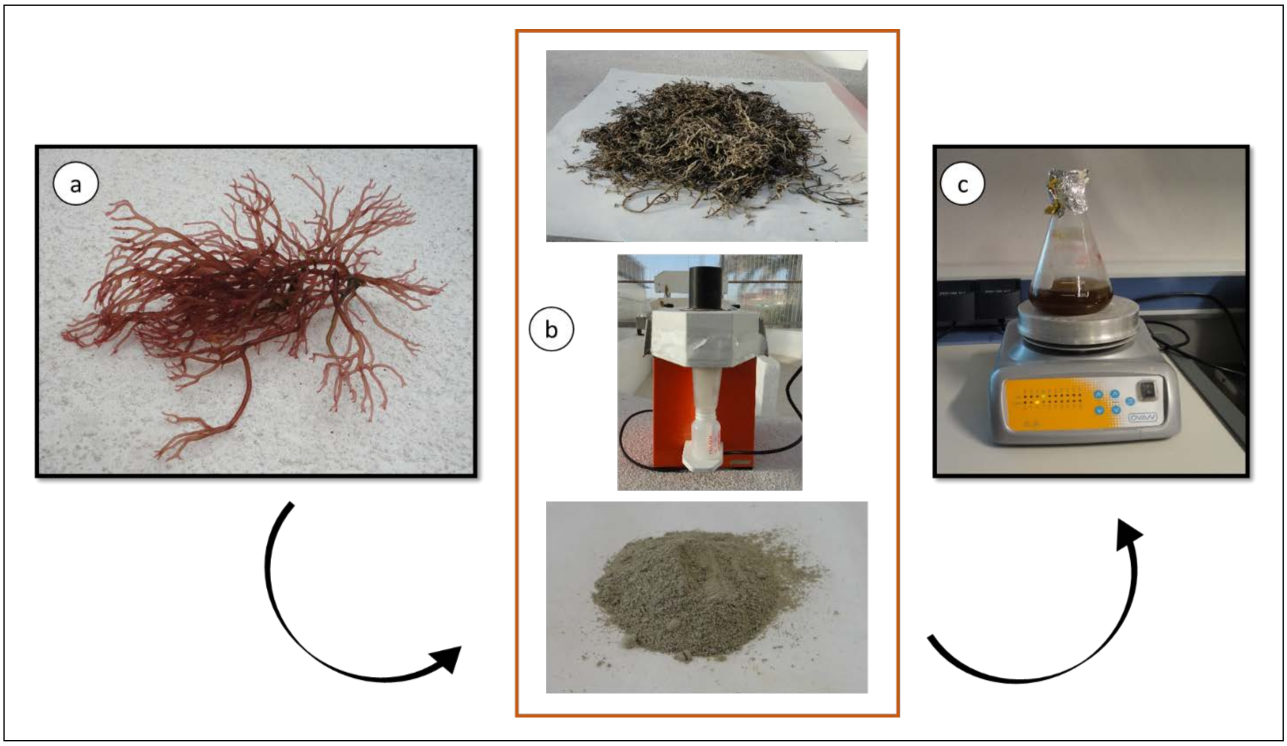

Fig. 1. Preparation of seaweed water extracts: a) fresh seaweed; b) in order from the top, seaweed dried at 60 °C, mill and seaweed pulverized by the mill; c) seaweed water extraction.
Fig. 1. Preparazione degli estratti acquosi di alghe: a) alga fresca; b) in ordine dall'alto, alga essiccata a 60 °C, polverizzatore e alga polverizzata; c) estrazione in acqua.

Cyanobacteria were cultivated in a low cost bioreactor (Almeida et al. 2009) for 20 days, centrifuged (12,000 rpm), lyophilized and then maintained at 4 °C. The water extracts of algae and cyanobacteria were obtained by suspending each powder in sterile distilled water (0.5 %) under continuous stirring at 50 °C for 12 h (Fig.1c), and then filtered through sterile filter paper before use. This extraction procedure was that provided the best plant responses without causing phytotoxic effects in preliminary tests.

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Tab. 1. Types, *Phylum*[1] and species of the algae and cyanobacteria used in the experiments.
Tab. 1. Tipi, *Phylum*[1] e specie di alghe e cianobatteri utilizzati negli esperimenti.

Green Algae *(Chlorophyta)*	**Red Algae *(Rhodophyta)***	**Blue-green Algae *(Cyanobacteria)***
Cymopolia barbata	*Corallina* sp.	*Microcoleus* sp.
Chlorella sp.	*Gracilaria cornea*	*Anabaena* sp.
Ulva rigida	*Grateloupia* sp.	*Phormidium* sp.
Brown Algae (*Heterokontophyta*)	*Halopithys* sp.	*Spirulina* sp.
	Hypnea sp.	
Cystoseira sp.	*Solieria* sp.	
Ecklonia sp.		
Lobophora variegata		
Sargassum sp.		

[1] Classification from Ruggiero et al. 2015.
[1] Classificazione da Ruggiero et al. 2015.

Zucchini plants var. Giambo F1 (Semencoop s.r.l., Cesena, FC, Italy) were grown in a substrate consisting of a sterile mixture of peat moss, sand and vermiculite (2:1:1 v:v:v) in pots (10 cm diameter). Plants were grown under a greenhouse at 22–24 °C (day), 16–18 °C (night), 14 h photoperiod and 60–80 % relative humidity.
The pathogen *P. xanthii* was obtained from zucchini leaves showing symptoms of powdery mildew collected at the experimental farm of the University of Bologna, located in the Po Valley, province of Bologna, Northern Italy (44°38056.2800N; 11°29024.0000E). Infected leaves were vigorously shaken on 15-day-old healthy plants of zucchini, maintained at the above conditions. The pathogen was identified based on morphological features of spore germination (Zaracovitis 1965) and on the presence of fibrosin bodies (Lebeda 1983). The pathogen was used to prepare the spore suspension (1×10^6 spores/ml) for leaf inoculation.

Treatments with extracts and pathogen inoculation

Zucchini cotyledons were cut off from 12-day-old healthy plants, sterilized with sodium chloride (1 % active Cl) for 3 minutes, washed three times with sterile distilled water and dried with adsorbent sterile paper. Four cotyledons were placed in each Petri dish (13 cm diam.), in 3 replicates, with the bottom surface in contact with the surface of an agarised medium containing Difco agar 1.5 %, glucose 10 g/l, benomyl 1 g/l, prochloraz 10 mg/l and streptomycin sulphate 0.5 g/l. Cotyledons were sprayed with each seaweed and cyanobacteria water extract separately until the surfaces were wet (5 ml of extract 0.5 % per cotyledon). Control cotyledons were sprayed with sterile distilled water. When cotyledons were dry, they were inoculated on the adaxial surface (Fig. 2) with six 10-µl drops of a spore suspension of *P. xanthii* (1×10^6 spores/ml) in a sterile laminar flow cabinet, according to Moret et al. (2009) with modifications. Plates were kept in a growth chamber at 22-24 °C under 12h/12h light/dark cycle, 95-100 % relative humidity, the essential conditions to achieve spreading lesions. The experiment was performed three times with similar results.

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Fig. 2. Drops of spore suspension of *Podosphaera xanthii* (1×10^6 spores/ml) deposited on the adaxial surface of cotyledons after the treatment with seaweed and cyanobacteria extracts.
Fig. 2. Gocce di sospensione sporale di *Podosphaera xanthii* (1×10^6 spore/ml) depositate sulla pagina superiore di cotiledoni dopo il trattamento con estratti acquosi di alghe marine e cianobatteri.

Disease evaluation

The effect of the extracts on the disease was evaluated 9 days after pathogen inoculation both visually and at the optical microscope as disease severity, which represent the percentage of area showing symptoms of *P. xanthii* disease (white, powdery spots) and as sporulation density expressed as number of spores/mm^2 of inoculated area. For sporulation density detection, each cotyledon was washed with 5 ml of distilled water to remove the spores. Four drops of 10 µl of the spore suspension removed from treated and no treated cotyledons, were observed at the optical microscope and spores were counted.

Statistical methods

Data of each set of experiments were pooled since analysis of variance demonstrated that the experiment factor did not significantly interact with the treatment factor in any experimental set. Data in the figures are means of percentage of the repeated experiments. Percentage data were arcsine transformed before statistical analysis and original data are showed in the figures. Data collected from the experiments were analyzed using One-way ANOVA and means were compared with LSD test, $P < 0.05$, Statgraphic Plus Version 2.1 (Statistical Graphics Corp., USA 1996).

Results

Seaweed and cyanobacteria water extracts showed reduction, stimulation of diseased severity and of sporulation density, or no activity. Among the green algae, no species were able to reduce significantly the infected area and the pathogen sporulation. On the contrary, *Chlorella* sp. stimulated the infected area and *Ulva rigida* increased the pathogen sporulation (Fig.3).

Brown algae extracts showed some significant inhibition effects. *Sargassum* sp. reduced significantly both the infected area (55.1 %) and the sporulation (84.0 %) with respect to the inoculated control, while the treatment with *Cystoceira* sp. extract reduced the infected area only. On the contrary, *Ecklonia* sp. stimulated the infected area compared to the inoculated control. The other extracts did not show any significant effects (Fig.4).

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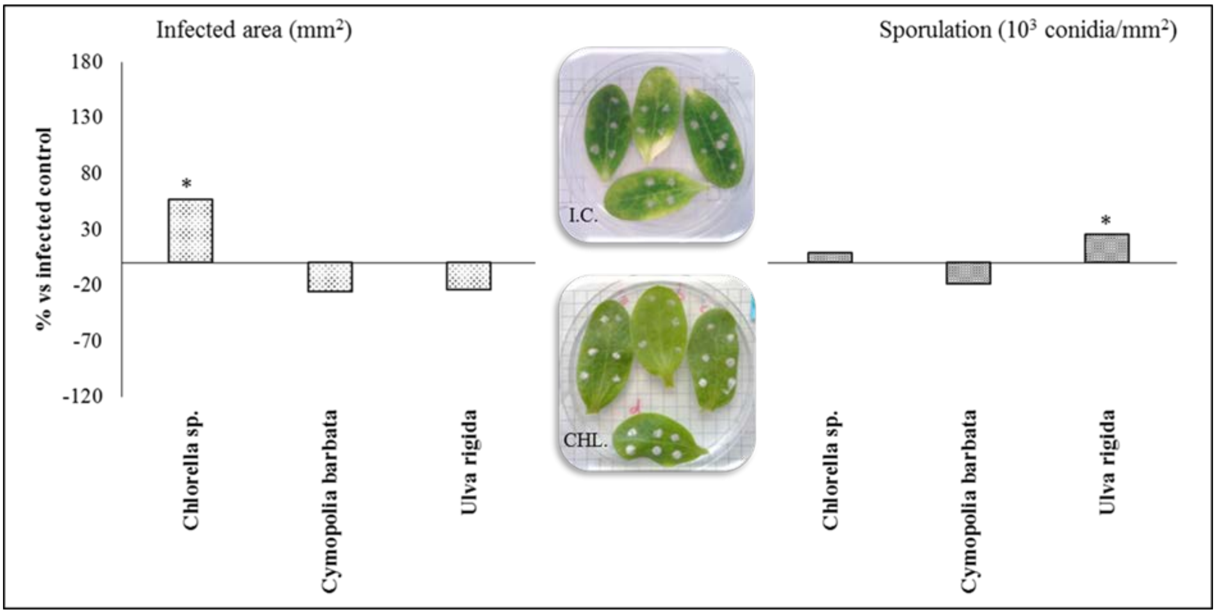

Fig. 3. Effect of treatment of zucchini cotyledons with water extracts of green algae on the percentage of infected area and on the sporulation density of *Podosphaera xanthii*. Percentages were calculated with respect to the inoculated control that was treated with water only. The asterisk on columns indicates significant difference between a treatment and the inoculated control according to LSD test (P < 0.05). On images, I.C. = Inoculated Control, CHL. = *Chlorella* sp.
Fig. 3. Effetto del trattamento dei cotiledoni di zucchino con estratti acquosi di alghe verdi sulla percentuale dell'area infetta e sulla densità di sporulazione di *Podosphaera xanthii*. Le percentuali sono state calcolate rispetto al testimone inoculato e trattato solo con acqua. Gli asterischi sulle colonne indicano differenza significativa tra un trattamento ed il controllo inoculato, in accordo con il test LDS (P < 0.05). Nell'immagine, I.C. = Controllo Inoculato, CHL. = *Chlorella* sp.

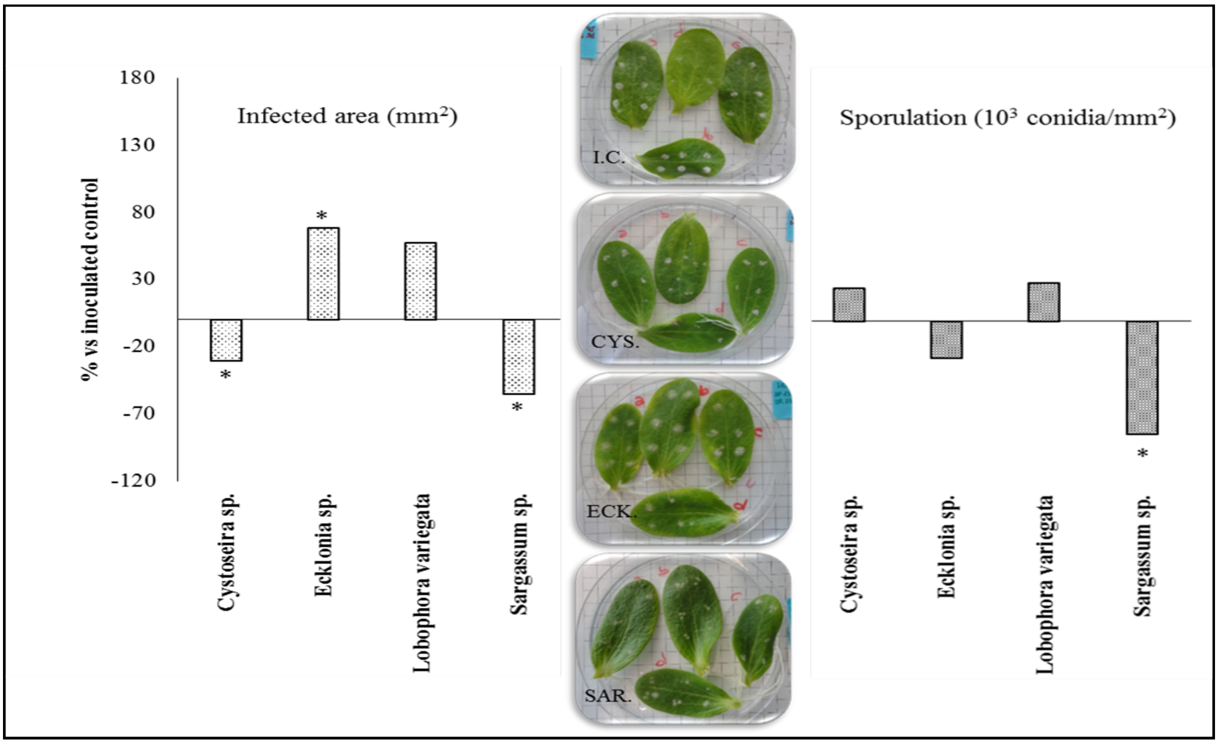

Fig. 4. Effect of treatment of zucchini cotyledons with water extracts of brown algae on the percentage of infected area and on the sporulation density of *Podosphaera xanthii*. Percentages were calculated with respect to the inoculated control that was treated with water only. The asterisk on columns indicates significant difference between a treatment and the inoculated control according to LSD test (P < 0.05). On images, I.C. = Inoculated Control, CYS. = *Cystoseira* sp., ECK. = *Ecklonia* sp., SAR. = *Sargassum* sp.

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Fig. 4. Effetto del trattamento dei cotiledoni di zucchino con estratti acquosi di alghe brune sulla percentuale dell'area infetta e sulla densità di sporulazione di *Podosphaera xanthii*. Le percentuali sono state calcolate rispetto al testimone inoculato e trattato solo con acqua. Gli asterischi sulle colonne indicano differenza significativa tra un trattamento ed il controllo inoculato in accordo con il test LDS (P < 0.05). Nell'immagine, I.C. = Controllo Inoculato, CYS. = *Cystoseira* sp., ECK. = *Ecklonia* sp., SAR. = *Sargassum* sp.

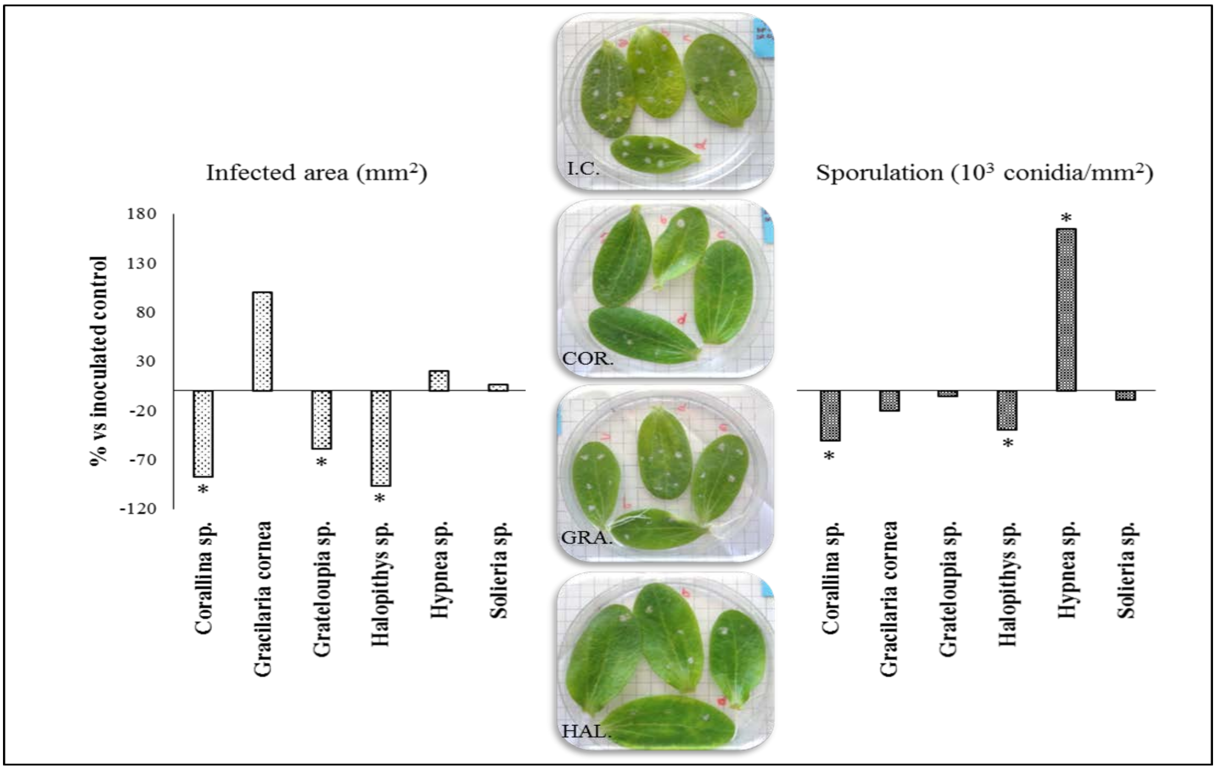

Fig. 5. Effect of treatment of zucchini cotyledons with water extracts of red algae on the percentage of infected area and on the sporulation density of *Podosphaera xanthii*. Percentages were calculated with respect to the inoculated control that was treated with water only. The asterisk on columns indicates significant difference between a treatment and the inoculated control according to LSD test (P < 0.05). On images, I.C. = Inoculated Control, COR. = *Corallina* sp., GRA. = *Grateloupia* sp., HAL. = *Halopithys* sp.

Fig. 5. Effetto del trattamento dei cotiledoni di zucchino con estratti acquosi di alghe rosse sulla percentuale dell'area infetta e sulla densità di sporulazione di *Podosphaera xanthii*. Le percentuali sono state calcolate rispetto al testimone inoculato e trattato solo con acqua. Gli asterischi sulle colonne indicano differenza significativa tra un trattamento ed il controllo inoculato in accordo con il test LDS (P < 0.05). Nell'immagine, I.C. = Controllo Inoculato, COR. = *Corallina* sp., GRA. = *Grateloupia* sp., HAL. = *Halopithys* sp.

Among red algae, *Corallina* sp. and *Halopithys* sp. significantly reduced both the infected area (87.8 % and 96.8 %, respectively) and sporulation (51.2 % and 30.2 %, respectively) in comparison to the inoculated control. *Grateloupia* sp. markedly decreased only the infected area (59.4 %). *Hypnea* sp. significantly stimulated the sporulation (Fig.5).

The extract of Cyanobacteria *Anabaena* sp. and *Spirulina* sp. significantly reduced the infected area by 26.6 % and 35.5 %, respectively. All the cyanobacteria extracts significantly reduced pathogen sporulation, with respect to the inoculated control (*Microcoleus* sp. 38.4 %, *Anabaena* sp. 53.7%, *Phormidium* sp. 32.4 %, *Spirulina* sp. 15.5%) (Fig.6).

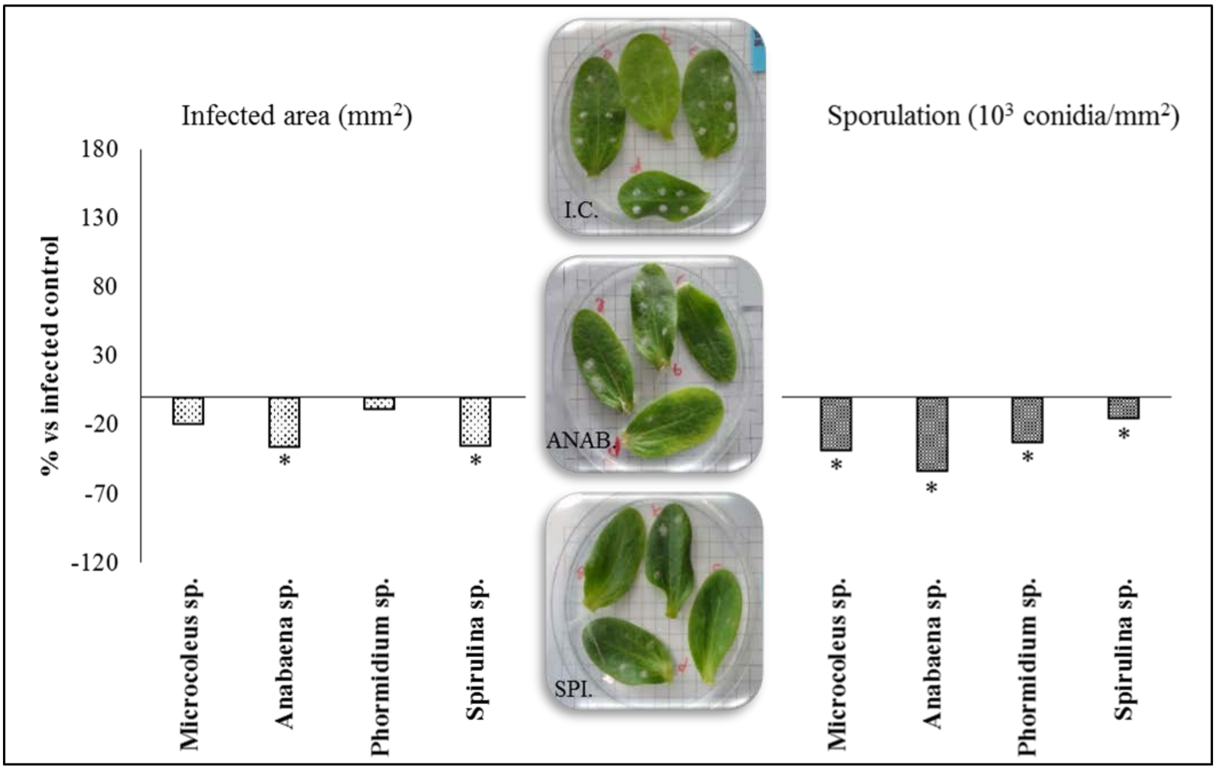

Fig. 6. Effect of treatment of zucchini cotyledons with water extracts of *Cyanobacteria* on the percentage of infected area and on the sporulation density of *Podosphaera xanthii*. Percentages were calculated with respect to the inoculated control that was treated with water only. The asterisk on columns indicates significant difference between a treatment and the inoculated control according to LSD test ($P < 0.05$). On images, I.C. = Inoculated Control, ANAB. = *Anabaena* sp., SPI. = *Spirulina* sp.

Fig. 6. Effetto del trattamento dei cotiledoni di zucchino con estratti acquosi di cianobatteri sulla percentuale dell'area infetta e sulla densità di sporulazione di *Podosphaera xanthii*. Le percentuali sono state calcolate rispetto al testimone inoculato e trattato solo con acqua. Gli asterischi sulle colonne indicano differenza significativa tra un trattamento ed il controllo inoculato in accordo con il test LDS ($P < 0.05$). Nell'immagine, I.C. = Controllo Inoculato, ANAB. = *Anabaena* sp., SPI. = *Spirulina* sp.

Discussion and conclusion

Algae and cyanobacteria represent an interesting source of natural products with a broad spectrum of biological activities such as antimicrobial (Bouhlal et al. 2010), antiviral (Kim and Karadeniz 2011), antifungal (De Felício et al. 2010), antiallergic (Na et al. 2005), anticoagulant (Dayong et al. 2008), anticancer (Kim et al. 2011), antifouling and antioxidant activities (Devi et al. 2011). According to the literature, most of the researches focused the microbiological activities of extracts against human pathogens such as fungi, yeasts, bacteria and viruses (Richardson 1993; Burjia et al. 2001; Newman et al 2003; Raja et al. 2013). Few reports considered the effect of algae and cyanobacteria extracts against plant pathogens *in vitro* (Kulik 1995; Rizvi and Hameel 2003; Kumar et al. 2008; Arunkumar et al. 2010; Omar et al. 2012; Sivakumar, 2014). Very few researches regarded *in vivo* experiments that showed inhibitory effect of the extracts against phytopathogens such as *Botrytis cinerea* on tomato (Jemenez et al. 2011), *P. xanthii* on zucchini plants (Roberti et al. 2015), *Erysiphe polygoni* on turnips and *Botrytis cinerea* on strawberry (Stephenson 1966).

In this paper, we demonstrated that some water extracts of seaweed and cyanobacteria reduced the infected area and the sporulation of *P. xanthii* on zucchini cotyledons. Among the tested red algae, extracts derived from *Corallina* sp. and *Halopythis* sp. were the most active. The antifungal activity of other species of red

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algae was showed against *Botrytis cinerea* on detached tomato leaves (Jiménez et al. 2011). The antimicrobial activity was related to a great variety of sulphated galactans synthesized by red seaweeds, which are the major components of the extracellular matrix (Damonte et al. 2004; Aruna et al. 2010; Matsihiro et al. 2005; Pujol et al. 2006; Souza et al. 2012). *Corallinales* are also characterized by the presence of calcium carbonate in their cell walls (Silva and Johansen 1986) that is known to exert a repellent action. Little information is available about biological active compounds of *Halopithys* sp. Some studies reported that *H. incurva* extracts contain phenolic compounds showing antioxidant and chemo-preventative activities (Oumaskour et al. 2013; ZBAKH et al. 2014). Among the brown algae we tested, only the extract from *Sargassum* sp. was able to reduce disease symptoms and sporulation. Our data were consistent with the inhibition activity exerted by *Sargassum* extract against the plant pathogen *Aspergillus flavus* (Mandal et al. 2007). We hypothesize that polysaccharides such as laminarina, fucoidans and alginates, which are contained in many brown seaweeds (Chizhov et al. 1998; Duarte et al. 2001; Patankar et al. 1993), can be involved in the biological activities (Vera et al. 2011).

Cyanobacteria are considered one of the most promising group of microorganisms for their bioactive compounds. *Anabaena* sp. and *Spirulina* sp. extracts were the most effective against *P. xanthii* disease. It has been showed that both species produce numerous secondary metabolites, toxins and others compounds as lipopeptides, aminoacids, fatty acids. Among the lipopeptides, some of them have demonstrated cytotoxic, antitumoral, antiviral, antibiotic activity and some effects as herbicides and antifungal against pathogens (Burja et al. 2000; Patterson et al. 1994). Frankmölle et al. (1992a) reported that extracts of *A. laxa* inhibited the growth of fungi as *Aspergillus oryzae*, *Candida albicans*, *Penicillium notatum* and *Saccharomyces cerevisiae*. This activity was attributed to the laxaficine (Frankmölle et al. 1992b). Moreover, Kellam et al. (1988) showed that *A. variabilis* extract inhibited the growth of the cellulolytic fungus *Chaetomium globosum*.

Extracts from green algae did not showed any inhibitory effects on *P. xanthii* disease. Similarly, Kumar et al. (2008) recorded no activity *in vitro* by extract of two green seaweeds, *Chaetomorpha antennina* and *Enteromorpha flexuosa*, against *Pseudomonas syringae*. On the contrary, Omar et al. (2012) found *in vitro* activity of green algae such as *Ulva reticulata* and *Enteromorpha prolifera* against *Aspergillus flavus*, *A. fumigatus* and *A. niger*.

In this study, sporadic cases of increasing of disease symptoms or pathogen sporulation were found. This behaviour may be due to the composition of the substances in the seaweed extract, such as polysaccharides and mineral salts that fungi can utilize for their growth (Basu et al. 2015).

In conclusion, this study shows that some seaweed and cyanobacteria water extracts applied on zucchini cotyledons exert inhibition effect against the pathogen *P. xanthii*, reducing both disease symptoms and fungus sporulation. Once these effects will be verified on zucchini plants in a larger scale experiment, these extracts may provide a useful preventative tool to apply in environmentally- friendly disease management, reducing the potentially adverse environmental effects of hazardous pesticides.

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