Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE E TECNOLOGIE AGRARIE, AMBIENTALI E ALIMENTARI

Indirizzo Ecologia Microbica e Patologia Vegetale

Ciclo XXXI

Settore Concorsuale: 07/D1

Settore Scientifico Disciplinare: AGR/12

Effect and mechanism of action of extracts from algae and cyanobacteria in the control of fungal plant pathogens

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Esame finale anno 2019

Contents

Abstract

Fungal pathogens can attack plants in all cultivation systems and cause fruit decay in post-harvest. Their control was based on the use of synthetic products. Upon the EC regulation No. 1107/2009, concerning the placing of plant protection products on the market and repealing the Council Directives 91/414/EEC, many synthetic pesticides are currently banned for their non-target effects and environmental and health hazards. The use of many others has been restricted.

In agriculture, algae and cyanobacteria extracts are used for their stimulant effects on plant vigour and productivity but little is known on their effect against fungal pathogens.

The objectives of this thesis were to study: (i) the antifungal activity of water extracts from the cyanobacterium *Anabaena* sp., and from the algae *Ecklonia* sp., *Halopithys* sp. *Jania* sp. and *Chlorella* sp. against the fungal plant pathogens *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *lycopersici* and *Podosphaera xanthii*, *in vitro* and *in vivo* assays; (ii) quantification of compounds in the extracts; (iii) the antifungal activity against *B. cinerea* of polysaccharides obtained from the extracts (iiii) the plant defence responses by bioassay, by the increase of enzymatic activities related to the plant defence responses and by the accumulation of PR proteins; (iiiii) phytotoxic and biostimulant effects of extracts.

The treatment with extracts from *Anabaena* sp., *Ecklonia* sp., *Jania* sp. showed activity against all fungal pathogens by working directly to the pathogen and indirectly through the involvement of plant defence responses. Moreover, polysaccharides derived from the extracts played a role in *B. cinerea* control by working directly against the pathogen and indirectly by increasing plant defence responses. Considering these results, algae and cyanobacteria extracts may provide a useful tool for the disease management in sustainable agriculture, once their antifungal effects will be verified on plants in a larger scale experiment.

1. Introduction

The strategy for preventing, destroying, or controlling a harmful organism ('pest') or disease, or protects plants or plant products during production, storage and transport is mainly based on the use of ‘pesticides’. This term includes herbicides, fungicides, insecticides, acaricides, nematicides, molluscicides, rodenticides, growth regulators, repellents, rodenticides and biocides (EU Pesticides Database).

Upon the recent EC regulation No. 1107/2009, concerning the placing of plant protection products on the market and repealing the Council Directives 91/414/EEC, many synthetic pesticides are currently banned for their non-target effects and environmental and health hazards, and the use of many others has been restricted. In particular, these restrictions have made the management of fungal plant pathogens difficult and greatly stimulated the research of alternative solutions to chemicals. Moreover, under the same EC regulation, no chemically synthesized soil fumigants are allowed, making the control of soil-borne pathogens extremely difficult. With respect to this, the research on control of plant diseases is focusing on alternative means to chemical ones, considering also consumer demands of healthy foods obtained with cultivation system with low ambient impact.

The control of fungal plant diseases could be addressed through direct or indirect means. Direct means include mainly the use of synthetic products directly against the pathogen to reduce or break down its ability to damage the plant. They include also genetic, mechanic, physic and biological means, which can provide a complementary tool in IPM strategies. Direct means are applied on plant, seed or other vegetative propagation material, in soil tillage and during shelf-life of agricultural products. Biological strategies for plant protection are becoming an important tools to be applied in environmentally-friendly disease management, reducing the potentially adverse environmental effects of hazardous pesticides. These includes microbial fungicides based on the mycoparasites and beneficial bacteria. Contrary to pest/insect biocontrol, disease biocontrol is a quiet new concept, especially in relation to recent studies. The first microorganism applied against a plant pathogen is the bacterium *Agrobacterium radiobacter* race K84 against *Agrobacterium tumefaciens*, the causal agent of crown gall disease, registered in the United States in 1979, but not yet in Italy, in collaboration with the United States Environmental Protection Agency (Fravel, 2005). Currently in Italy, several products based on microbial fungicides are registered, such as the mycoparasites *Ampelomyces quisqualis* e *Coniothyrium minitans, Trichoderma harzianum, T. gamsii, T. asperellum*,

and such as the bacterial antagonists *Streptomyces* sp. *Bacillus subtilis*, *B. amyloliquefaciens* e *Pseudomonas chlororaphys*.

Indirect means are based on chemical and natural substances or microorganisms that can stimulate and/or increase the natural plant defences against diseases. Among chemical substances able to activate plant defence system against plant pathogens, acibenzolar-S-methyl is authorised in several European countries, such as Italy, Spain, France, Netherland, United Kingdom, Belgium, and Portugal (Reg. EC 1107/2009). In Italy, it is used against bacterial diseases on nut, peach and tomato, against downy mould on tobacco and against the fire blight on pear (Bion, Syngenta Crop Protection). Another chemical substance well known to stimulate plant defences is fosethyl aluminium that is commercialized in different formulations, e.g. Aliette (Bayer Crop Science) especially used against grapevine downy mildew (Scannavini et al., 2008; di Marco et al., 2011). Among natural inducers derived from plants, oligogalacturonides and cellodextrins showed to trigger plant defence responses in several crops, such as in grapevine against *Botrytis cinerea* (Davidsson et al., 2017; Aziz et al., 2003, 2006). Another example of natural inducer is laminarin, a polysaccharide derived from the brown seaweed *Laminaria digitate*, registered in France on wheat to control powdery mildew (Iodus 40®, Goëmar). Number of studies has shown that seaweed polysaccharides and derived oligosaccharides activate defence responses and protection against a broad range of pathogens in plants such as *B. cinerea* and *Plasmopara viticola* on grapevine (Aziz et al., 2003), *Erwinia carotovora* on tobacco (Klarzynski et al., 2000), *Colletotrichum lindemuthianum* on bean seedlings (Paulert et al., 2009) and against *Pseudomonas syringae* on tomato plants (Jaulneau et al., 2010).

Several researches were focused on the activity against plant pathogens of extracts obtained from algae and cyanobacteria by using organic solvents (Rizvi and Shameel, 2004; Kumar et al., 2008; Arunkumar et al., 2010; Sivakumar, 2014; Jiménez et al., 2011), while few studies have examined the antifungal activity of water extracts (Roberti et al., 2015; Righini et al., 2018).

Algae and Cyanobacteria in Agriculture

Algae and cyanobacteria have long been used in human consumption, as a source of natural compounds of pharmaceutical and cosmetic interest. Moreover, they are used for the production of biofuels and substrates for laboratory *in vitro* culture. In agriculture, extracts from algae and cyanobacteria are used for their stimulant effects on plant vigour and productivity but little is known on their effect against fungal pathogens. Fungal pathogens can attack plants in all cultivation systems and can cause fruit decay in post-harvest. Among soil-borne fungi that can infect the plant in soil cultivation, *Sclerotinia sclerotiorum, Rhizoctonia* spp., *Phytophthora* spp., *Pythium* spp., *Fusarium* spp., *Verticillium* spp. are the most important (Husaini and Neri, 2016; Pastrana et al., 2016). They affect the root system, hindering the nutrient and water absorption from soil. These pathogens cause severe symptoms such as yellowing, wilting, damping-off, root rot and collar rot and, in conventional cultivation systems, they are controlled by synthetic compound applications. Among foliar pathogens, fungus belonging to *Erysiphales* order are the causal agent of powdery mildew, a severe disease that causes important economic losses (Jarvis et al., 2002) requiring several fungicidal treatments (Romero et al., 2007). Fruits, such as strawberry, tomato and cucumber are perishable fresh food. The traditional strategy for controlling post-harvest decay relies on the application of fungicides during the crop growing cycle around flowering until harvest time. The fungi responsible of post-harvest decay are *Botrytis cinerea*, *Colletotrichum* spp., agent of grey mould and anthracnose, respectively, *Penicillium* spp., *Rhizopus* spp. and *Mucor* spp. (Husaini and Neri, 2016).

However, the recent EU restriction on synthetic pesticides made the management of fungal plant pathogens difficult and greatly stimulated the research of alternative solutions to chemicals. Applications of extracts from algae and cyanobacteria can help to limit disease spread in several crops and to develop effective alternatives to chemical treatment in crop protection and nutrition management. Indeed, crop nutrition and protection has become difficult due to recent restrictions in agrochemical use and the lack of effective alternatives, in particular for horticultural plants in open field and in soil or soilless cultivation under greenhouse conditions. The existing reports on this topic show the strong potential of algae and cyanobacteria extracts application on plants both as biostimulants and bioprotectants against fungal pathogens. Further investigation is needed to fully uncover the interesting and exploitable antifungal properties of extracts from algae and cyanobacteria to clarify the mechanism of action of extracts and singular components against pathogens.

Algae

Algae refer to a very diverse and large group of aquatic photosynthetic organisms. They include unicellular organisms such as the green alga *Chlorella*, and marine multicellular algae commonly named seaweeds, such as the brown alga *Sargassum* that can reach several meters in length. Algae are classified in several phyla among which Chlorophyta (green algae), Rhodophyta (red algae), and Ochrophyta are the major ones, with the brown algae (Phaeophyceae) being a large class of the Ochrophyta (Guiry, 2012). The geographic location and season of harvest influence the composition of the algae (Black, 1950; Painter, 1983; Westermeier et al., 2012) and their content of polysaccharides (Rioux et al., 2007; Schiener et al., 2015). Algae also contain essential nutrients, trace of metal mixtures such as Cu, Co, Zn, Mn, Mo, etc. (Cabrita et al., 2016), and plant growth regulators like auxins and cytokinins (Rayorath et al., 2008; Craigie, 2011).

Due to their composition, extracts from algae have long been used in agriculture to enhance soil fertility and crop productivity (Khan et al., 2009; Craigie, 2011; Arioli et al., 2015). Their commercial products usually consist of liquid formulations where algae are suspended in water, acids, or alkalis (Calvo et al., 2014) upon mechanical and physical processing by low temperature, milling, or high pressure (Hervé and Rouillier, 1977; Stirk and van Staden, 2006; Craigie, 2011; Pramanick et al., 2017). Liquid products can be applied on roots by immersion of nursery seedlings before transplantation, or to agricultural crops through soil irrigation, or spray treatments onto the leaves. These treatments showed beneficial effects on several crops such as tomato, apple, wheat, strawberry, and winter rapeseed (Crouch and van Staden, 1992; Basak, 2008; Kumar and Sahoo, 2011; Alam et al., 2013; Jannin et al., 2013). The extracts from the brown algae *Ascophyllum nodosum* and *Ecklonia maxima* were shown to be highly effective in increasing various plant parameters, e.g., vegetative and reproductive growth and yield, chlorophyll, and mineral elements content of leaves (Tab. 1). Besides their wide use as organic fertilizers and plant growth promoters, extract from algae can enhance the tolerance of crops to a wide range of abiotic and biotic stresses and extend the post-harvest shelf life of fruit (Craigie, 2011; Calvo et al., 2014; Ibrahim et al., 2014; Latique et al., 2014). Moreover, extract from algae have great potential as disease crop protectants, both through a direct effect against a number of fungal plant pathogens and indirectly through induction of resistance in plants by enhancing the activities of various defense-related enzymes that can help the plants to withdraw fungal invasion as focused in recent studies (Jayaraman et al., 2011; Hernández-Herrera et al., 2014a, b; Roberti et al., 2016; Esserti et al., 2017).

Algae contain several beneficial compounds for plant vigor, such as macro and microelements, amino acids, vitamins, polysaccharides, and hormones, such as cytokinins, auxins and auxin-like compounds, and abscisic acid (ABA)-like substances (Blunden, 1991; Crouch and van

Staden, 1993; Stirk et al., 2003; Rayorath et al., 2008; Craigie, 2011). These components are contained in the extract from algae upon extraction and their applications stimulate both plant growth and yield. Several types of extract from algae have been shown to display beneficial effects on plants alone or in mixed formulations. For example, extracts from *A. nodosum*, due to its high concentration in cytokinins, auxins, and other growth stimulants (Khan et al., 2009; Craigie, 2011), is contained in the majority of agricultural biostimulant products (Sharma et al., 2014). Marine algae themselves are rich in auxin and auxin-like compounds, as shown by Kingman and Moore (1982) who reported that 1 g of *A. nodosum* dry extract contains 50 mg of the indolacetic acid. Betaines, other components of extracts from *A. nodosum*, are involved in alleviating osmotic stresses induced by salinity and drought stresses through the decrease in chlorophyll degradation, as shown by Blunden et al. (1997) on tomato, dwarf French bean, barley and maize.

Biostimulant effect was also demonstrated by extract from *E. maxima* on several plants species, such as beans, pepper, and mung bean where seedling height, diameter of main stem, number of leaves, leaf chlorophyll content, and root development were increased (Arthur et al., 2003; Lötze and Hoffman, 2016; Kocira et al., 2018). The presence of plant growth regulators such as auxins, cytokinins, polyamines, gibberellins, abscisic acid, brassinosteroids and phlorotannins in the extract from *E. maxima* (Rengasamy et al., 2015) was found to be responsible for its stimulatory effect on the growth of agricultural crops (Stirk et al. 2004, 2014; Papenfus et al,. 2013). Rengasamy et al. (2015) obtained from 1 kg of *E. maxima* dry weight a total of 480 mg of eckol, a typical phlorotannin named after *Ecklonia* genus, suggesting that it may be a major component in *E. maxima*. These authors demonstrated that eckol applied on maize seeds highly increased shoot (55%) and root elongation (71%), and biochemical activities of α-amylase in seedling roots. The expression of α-amylase in cereal seeds is also regulated by plant hormones, e.g., gibberellin and abscisic acid at the transcriptional level (Ritchie and Gilroy, 1998), suggesting that eckol may act as a hormone. Alpha amylase enzyme drives the starch hydrolysis that generates simple sugars, fundamental for the biosynthesis of the primary cellular components of the developing embryos (Lovegrove and Hooley, 2000; Nanjo et al., 2004). Such activities are important in cereal crops, where growth and development after germination mainly depends on the mobilization of starch stored in the endosperm (Olsen et al., 1998).

Extract from algae contain a number of bioactive compounds with antimicrobial, antiviral, anticancer, antioxidant and antifungal properties that have been studied for several applications. The direct effect of extract from algae on fungal pathogens, or their possible resistance induction effect on plants have been thoroughly investigated. Among extract from algae, those from *A. nodosum* and *Ecklonia maxima* have been widely studied. Antifungal activity of extract from *A. nodosum* against pathogens

is documented on several plant species, e.g. on carrot against infections of *B. cinerea* and *Alternaria radicina* that were reduced by plant spray treatment with extract from algae (Jayaraj et al., 2008). The same authors also showed that the treatment increased the transcript levels of PR proteins related to the induced resistance of plant (Tab. 2). On cucumber plants, in greenhouse environment, spray treatment and/or root drench with *A. nodosum* showed to reduce disease incidence caused by *F. oxysporum*, *Alternaria cucumerinum, B. cinerea* (Jayaraman et al., 2011) and by *Phytophthora melonis* (Abkhoo and Sabbagh, 2016). Jayaraman et al. (2011) and Abkhoo and Sabbagh (2016) found that the extract from *A. nodosum* enhanced activities of various defence related enzymes such as chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, and lipoxygenase and enhanced levels of phenolic compounds (Tab. 2). When an extract from *A. nodosum* was applied in mixture with *Laminaria* sp., on strawberry a decrease of grey mould incidence was obtained. A mixture of an extract from *A. nodosum* with one of *D. potatorum* applied by soil treatment, suppressed the infection of broccoli caused by *Plasmodiophora brassicae*, probably resulting from the stimulation of resistance mechanisms in the plant in relation to the presence of polysaccharides in the extract (Wite et al., 2015). Indeed, extracts derived from *A. nodosum* contain several compounds, including polysaccharides and oligosaccharides, known to act as elicitor and signal transduction molecules in plants (Walters et al., 2005) and can induce expression of various defence-related proteins and proteinase inhibitors *in vivo* (Cluzet et al., 2004). In addition, the increase of lipoxygenase activity may be related to oxidation of membrane lipids that results in the production of several antifungal compounds, including phytoalexins, initiation of cell wall lignification, and signal transduction leading to resistance responses (Sutherland, 1991).

Extract from *E. maxima*, significantly controlled *Verticillium* wilt of pepper when applied in soil (Rekanović et al., 2010). On zucchini cotyledons, a spray treatment with *Ecklonia* sp. reduced the sporulation of *Podosphaera xanthii* the causal agent of powdery mildew of cucurbits (Roberti et al., 2016). The antifungal activity showed by extracts from *Ecklonia* species may be related to secondary bioactive compounds (e.g. phenols) with antioxidant activities as demonstrated against both human and plant pathogens. Among algae phenols, phlorotannins from *E. cava* exerted high antioxidant and anticancer activities against murin colon cancer cell line CT-26 (Athukorala et al., 2006), and those from *E. kurome* were effective against several strains of food-borne pathogenic bacteria, particularly against methicillin-resistant *Staphylococcus aureus* (Nagayama et al., 2002). These findings demonstrated that phlorotannins contained in extracts of *Ecklonia* species may be promising bioactive compounds also for medical application. Antifungal activity of extracts from others brown algae it is widely reported. An extract from *Sargassum* sp. inhibited mycelial growth of *Aspergillus* spp., *F. oxysporum* and *Penicillium* spp. (Mabrouk et al., 1985; Khallil et al., 2015). Applied on tomato plants,

an extract from *Sargassum filipendula* reduced disease severity caused by *Alternaria solani* and *Xanthomonas campestris* pv *vesicatoria* (Ramkissoon et al., 2017) and an extract from *Sargassum liebmannii* also reduced necrotic lesions induced by *A. solani* (Hernández-Herrera et al., 2014a). Both extracts increased the expression of levels of marker genes for defence signalling pathways (Tab. 2). The activity of extracts from *Sargassum* species is probably due to phenols and terpenes as well as fatty acids and volatile halogenated hydrocarbons. In fact, El Shafay et al. (2016) associated the antimicrobial activity against human multidrug resistant bacteria of extracts from *S. vulgare* and *Sargassum fusiforme* to these compounds. Moreover, Fernandes Peres et al. (2012) related the phenols and terpens found in extract from *Sargassum muticum*, *A. nodosum*, *Fucus spiralis*, *Stypopodium zonale*, and *Pelvetia canaliculata* to the inhibition of *C. lagenarium* growth showed by the extracts. In another study, extracts from *Cystoseira myriophylloides, Laminaria digitata* and *F. spiralis* applied on tomato plants reduced disease severity caused by *V. dahliae*, and increased the activity of defence enzymes such as polyphenol oxidase and peroxidase (Tab. 2; Esserti et al., 2017). A study carried out by De Corato et al. (2017) examined several extract from algae from both brown seaweeds, *L. digitata* and *Undaria pinnatifida*, and red seaweeds, *Porphyra umbilicalis*, *Eucheuma denticulatum* and *Gelidium pusillum*, by preventive and curative treatments on strawberries against *B. cinerea*. Preventive and curative treatment, extracts from *L. digitata*, *U. pinnatifida* and *P. umbilicalis*, strongly suppressed the disease severity and extract from *E. denticulatum* and *G. pusillum* poorly reduced disease development. In *in vitro* experiments showed antifungal activity against *B. cinerea* and, in particular, a total inhibition of pathogen growth and spore germination was showed by *L. digitata* and *U. pinnatifida*. Moreover, in the same study, a comparison was made among different extract fractions obtained by three solvents with a different affinity towards fatty acids (hexane), water-soluble polysaccharides (water) and phenolic compounds (ethanol). Only the hexane-soluble extracts showed inhibitory effect on mycelia growth and spore germination of *B. cinerea*, especially the extract from *L. digitata*, *U. pinnatifida* and *P. umbilicalis*. Overall, the extracts from brown algae showed higher antifungal activity with respect to those from red algae. This finding is consistent with the higher antioxidant activity shown by brown algae with respect to green and the red ones (Kelman et al., 2012). Despite the lowest antioxidant activity of red algae, the antifungal effect of extracts from *Corallina* sp. and *Halopithys* against *P. xanthii* was reported on zucchini cotyledons where an infection decrease was detected (Roberti et al., 2016). Moreover, an extract from *Gracilaria edulis* inhibited mycelial growth of *Macrophomina phaseolina*, *in vitro* (Ambika et al., 2014), and the one from *Kappaphycus alvarezii* sprayed on tomato seedlings infected by *M. phaseolina* increased transcription of pathogenesis related genes such as PR-1b1, PR-3 and PR-4 and the endogenous concentration ABA, IAA (auxin), salicylic acid (SA) and the cytokinin zeatin

(Agarwal et al., 2016). Accordingly, it is known that a high concentration of cytokinin leads to SA accumulation and activation of defence gene expression (Argueso et al., 2012).

Concerning the extracts from green algae, El-ghanam et al. (2015) showed that *Chlorella vulgaris* inhibited the disease severity rot caused by *B. cinerea*, on strawberry. Moreover, the extract from *C. vulgaris* highly inhibited the *B. cinerea* mycelial growth and sporulation in in vitro experiments. The inhibition of mycelial growth by an extract from *C. vulgaris* has also been demonstrated for *Aspergillus niger* (Ghasemi et al., 2007). Ahmed (2016) suggested a correlation between antifungal activities and antioxidant compounds, such as phenolics and flavonoids very abundant in *C. vulgaris* extract. The composition of other green microalga extracts, *Zygenma czundae*, *Zygenma stellinum* and *Zygenma tenue* revealed different content in saturated and unsaturated fatty acids, in sterols and terpenes, which were correlated to the antifungal activity of extract from algae on mycelia growth of *Curvularia lunata*, *Fusarium sporotrichoides*, *M. phaseolina*, *R. solani* and *Sclerotium rolfsii* (Ghazala and Shameel, 2005). Extracts from green macroalgae also showed direct antifungal activity, e.g. an extract from *Ulva lactuca* highly inhibited the mycelial growth of *A. niger*, *Penicillium digitatum* and *R. solani*. The *U. lactuca* fractions with highest antifungal activity was the one containing phthalic acid, aromatic compounds and fatty acids (Abbassy et al., 2014). In addition to the direct activity of extracts from *U. lactuca*, its capability to stimulate plant defence responses was shown. On tomato plants, Hernández-Herrera et al. (2014a) suggested that increase in expression of defence-related genes, such as phenylalanine ammonia lyase, following the application of an extract from *U. lactuca*, could justify the reduction of necrotic lesions caused by *A. solani* (Tab. 2).

Some studies focused on the antifungal activity of the two single compounds laminarin and bromophenol bis (2, 3-dibromo-4,5-dihydroxybenzyl) ether, extracted from *L. digitata*, *Rhodomela confervoides* and from *Leathesia* nana (Meszka and Bielenin, 2011; Liu et al., 2014; Feliziani et al., 2015). The application of laminarin on several strawberry cultivars controlled infection caused by *B. cinerea*, *P. aphanis* and *M. fragariae*, under field conditions (Meszka and Bielenin, 2011). Laminarin was also applied repeatedly as pre-harvest treatment on strawberry producing a reduction of decay incidence and severity caused by *B. cinerea* and *Rhizopus* sp. (Feliziani et al., 2015).

Bromophenol bis (2,3-dibromo-4,5-dihydroxybenzyl) ether (BDDE), extracted from the red alga *Rhodomela confervoides* and from the brown algae *Leathesia nana*, inhibited the mycelia growth, spore germination and the germ tube elongation of *B. cinerea* (Liu et al., 2014). The same was also capable to inhibit mycelial growth of *C. gloeosporioides*. Treatment by soaking of fresh strawberry fruits with BDDE reduced decay incidence by *B. cinerea*. Bromophenol BDDE was shown to interact with DNA of *B. cinerea* by binding in the minor groove as well as by intercalation (Liu et al., 2014).

Besides the above mentioned biostimulation role, the hormone component of extacts from algae can directly influencing the plant defence responses to pathogens by disrupting the delicate hormone-based cross kingdom communication occurring at early stages of plant-pathogen interaction (Chanclud and Morel, 2016). The phytohormones contained in algae, IAA, gibberellin and cytokinins, are all known to be produced by both fungal pathogens and plants (Chanclud and Morel, 2016) and have dramatic effects on their growth and development, indirectly influencing plant pathogen responses. On the other hand, auxins are known to directly influencing the outcome of fungal pathogen infections, being susceptibility or resistant factor depending on the type of pathogen parasitism (bio- or necrotrophism) and plant tissues (Nafisi et al., 2015). For example, IAA induces susceptibility to *B. cinerea* in *Arabidopsis* leaves (Savatin et al., 2011), but in tomato leaves and eggplant fruit, pre-treatment with IAA or naphthaleneacetic acid (i.e. NAA a synthetic auxin) reduces *B. cinerea* infections (Sharon et al., 2007). Thus, the activity of algal extracts against fungal pathogens of strawberry, can also be exerted through the endogenous content of algae hormones, especially auxin. More knowledge is needed in order to understand the contribution of these components to the role of algae in plant protection.

Journal of Applied Phycology
https://doi.org/10.1007/s10811-018-1478-2

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Use of algae in strawberry management

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Received: 21 December 2017 / Revised and accepted: 9 April 2018

Tab. 1 Examples of application and beneficial effects on plants of extracts from algae.

Extract from algae	Plant	Treatment[1]	Beneficial effect	Reference
Ochrophyta				
Aschophyllum nodosum	pepper	F	length, fruit diameter, yield	Eris et al., 1995
	apple	F	shoot growth, blooming, flowering	Basak, 2008
	okra	F	plant height, fresh and dry weight, yield	Zodape et al., 2008
	eggplant	F	yield	Bozorgi, 2012
	onion	S	shoot growth, number of shoots per plant, yield	Dogra and Mandradia, 2012
	saffron	F	flower tube length	Bozorgi et al., 2012
	mango	S	leaf chlorophyll content accelerating scion shoot height gain	Morales-Payan, 2013
	rapeseed	S	root and shoot growth	Jannin et al., 2013
	strawberry	S	root and shoot growth, yield	Alam et al., 2013
	peach	F	leaf area, leaf chlorophyll, leaf content of mineral elements	Al-Rawi et al., 2016
Ecklonia maxima	lettuce	S	leaf content of mineral elements, yield	Crouch et al., 1990
	tomato	S	leaf surface area, fresh weight, number of flowers	Crouch and van Staden, 1992
	pepper	S+F	number and size of fruits	Arthur et al., 2003
	apple	F	vegetative growth, prolonged blooming, yield	Basak, 2008
	loquat	S+F	seedling height, diameter of main stem, number of leaves per seedling, chlorophyll content, leaf dry weight	Al-Hawezy, 2015
Sargassum ilicifolium	fenugreek	Se+S+F	shoot growth, fresh biomass, content of pigments, carbohydrate, proteins, amino acids, polyphenols and nitrogen	Pise and Sabale, 2010
Sargassum wightii	wheat	D	seed germination, growth, yield	Kumar and Sahoo, 2011
Durvillaea potatorum + A. nodosum	broccoli	D	leaf number and area, stem diameter	Mattner et al., 2013
Rhodophyta				
Gracilaria corticata	fenugreek	Se+S+F	shoot growth, fresh biomass, content of pigments, carbohydrates, proteins, amino acids, polyphenols and nitrogen	Pise and Sabale, 2010
Hypnea musciformis	peanut	S	seed germination, fresh and dry weight, root and shoot length, number of branches, leaf area and chlorophyll, content of fruit proteins	Selvam and Sivakumar, 2014
Chlorophyta				
Ulva fasciata	fenugreek	Se+S+F	shoot growth, fresh biomass, content of pigments, carbohydrates, proteins, amino acids, polyphenols and nitrogen	Pise and Sabale, 2010

[1]Foliar spray (F), soil irrigation (S), seedling dipping (D) and seed treatment (Se) (Righini et al., 2018).

Tab. 2. Effective applications of algae extracts against fungal pathogens correlated to the increase of plant defence responses.

Alga	Plant	Increase of plant defence responses	Pathogen	Reference
Ascophyllum noodosum	Cucumber	Enzymes: chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, and lipoxygenase. Genes: chitinase, lipoxygenase, glucanase, peroxidase, and phenylalanine ammonia lyase	*Alternaria cucumerinum, Botrytis cinerea Didymella applanata, Fusarium oxysporum,*	Jayaraman et al., 2011
A. nodosum	Cucmber	Enzymes: β-1,3-glucanase, peroxidase, polyphenol oxidase. Genes: lipoxygenase, phenylalanine ammonia lyase, galactinol synthase,	*Phytophthora melonis*	Abkhoo and Sabbagh, 2016
A. nodosum	Carrot	Enzymes: peroxidase, polyphenoloxidase, phenylalanine ammonia lyase, chitinase, b-1,3-glucanase. Genes: PR-1, chitinase, lipid transfer protein, phenylalanine ammonia lyase, chalcone synthase, non-expressing pathogenesis-related protein, PR-5	*Alternaria radicina, B. cinerea*	Jayaraj et al., 2008
Cystoseira myriophylloides, Fucus spiralis, Laminaria digitata	Tomato	Enzymes: polyphenol oxidase, peroxidase	*Verticillium dahliae*	Esserti et al., 2017
Gelidium serrulatum, Sargassum filipendula, Ulva lactuca,	Tomato	Genes: PR-1, PIN II (marker gene of the jasmonate mediated defence pathway)	*Alternaria solani, Xanthomonas campestris pv vesicatoria*	Ramkissoon et al., 2017
Caulerpa sertularioides, Padina gymnospora,, Sargassum liebmannii, Ulva lactuca	Tomato	Genes: wound-induced proteinase inhibitor I, wound-induced proteinase inhibitor II, wound-inducible carboxypeptidase, phenylalanine ammonia lyase (PAL), cysteine proteinase changes were observed for PI - II	*A. solani*	Hernández-Herrera et al., 2014a
Kappaphycus alvarezii	Tomato	Hormones: ABA, IAA, SA and zeatin hormones. Genes: PR-1b1, PR-3 and PR-5	*Macrophomina phaseolina*	Agarwal et al., 2016

Cyanobacteria

Cyanobacteria, also known as cyanophyta or blue-green algae, are one of the most diverse and widely distributed Prokaryotes (Walter et al., 2017) included in Bacteria superkingdom (Oren and Garrity, 2014). Their origin dates back to 3 billion years ago, durind the transition from anoxygenic to oxygenic conditions through photosynthesis (Schirrmeister et al., 2011).Their evolution in these conditions led their development in unicellular and multicellular, photosynthetic and non-photosynthetic (i.e., Melainabacteria) (Schirrmeister et al., 2011; Di Rienzi et al., 2013; Soo et al., 2014), free-living, symbiotic, toxic and predatory organisms (Soo et al., 2015). Moreover they occupy many niches such as terrestrial, planktonic, and benthic habitats (Walter et al., 2017) and they are able to utilise N2 through the process of biological nitrogen fixation (Prasanna et al. 2012).

They are divided in eight orders: *Gloeobacterales, Synechococcales, Spirulinales, Chroococcales, Pleurocapsales, Oscillatoriales, Chroococcidiopsidales* and *Nostocales* (Komárek et al., 2014).

The research on cyanobacteria highlights their potentiality as source of biofertilizers and of bioactive compounds to be applied both for the biocontrol of fungal plant pathogen and as inducer of plant systemic resistance. In the last few years, considering the harmful effect of chemical fertilizers and pesticides on the environment, the research is focusing on alternatives to chemicals, such as cyanobacteria. The capacity of cyanobacteria to assimilate the N2 through the process of nitrogen fixation have long being used for rice fertilization (Yanni, 1991; Jha et al., 1999; Kannaiyan, 2000; Sinha et al., 2002; Jha and Prasad, 2006). There have been a number of studies of the beneficial effect of cyanobacteria as reported in Tab. 3. Along with their capacity of fixing atmospheric N, they improved plant growth and crop yield since they add organic matter to soil (Zaccaro et al., 1999; Maqubela et al., 2009), thus improving soil structure (De Caire et al., 2000; De Cano et al. 2002; Pandey et al., 2005; Maqubela et al., 2009). The positive effect on crop yield was due to their release of various substances such as vitamins, amino acids, polypeptides, antibacterial and antifungal substances and polymers, especially exopolysaccharides (Zaccaro et al., 1999; Singh et al., 2005; Maqubela et al., 2009). They are also able to synthesise phytohormones, such as IAA and gibberellin-like substances as *Anabaena* sp. and *Scytonema hofmanni* (Prasanna et al., 2008, 2009; Rodríguez et al., 2006). Beneficial effects of cyanobacteria application are reported on *Lupius termis* treated with the extract of *Cylindrospermum muscicola* and *A. oryzae* (Haroun and Hussein, 2003). They recorded an enhancement of shoot length, total leaf area and biomass. The extracts also increased the photosynthetic activity, content of nitrogenous compounds and carbohydrates in the shoot of the plant. Similar observation on morphological and biochemical parameters was done by Osman et al. (2010) on pea plant. They obtained an increase of root and shoot length, dry weights, leaf area and several constituents (vitamins, carbohydrates, elements, pigments, ecc.) of seedlings grown in soil

treated with *Nostoc entophytum* and *Oscillatoria angustissima*. *Anabaena* sp. and *Nostoc* sp. applied to the soil increased plant height, root length, dry and fresh weight and leaf number of tomato, cucumber and squash (Shariatmadari et al., 2013). On broad bean, soil treatment with *Nostoc minutum* and *Anabaena spiroides* increased shoot length up to 19.1 % (Al-Sherif et al., 2015). Values of dry weight, seed weight per plant and seed number per plant were similar to those obtained with chemical fertilizer. Soil treatment with species of *Nostoc, Anabaena, Calothrix, Haplosiphon, Oscillatoria, Lyngbya, Phormidium* enhanced soil microbial biomass, available nitrogen and increase rice and wheat seed germination, root and shoot growth, weight and yield (Obana et al., 2007; Prasanna et al., 2013; Pimratch et al., 2015). On rice seedlings, applying *N. carneum* together with *N. commune* was observed an increasing of 28.8 % of root length. Moreover, the application of the two cyanobacteria resulted in better rice production in terms of quantity and quality (Chittapun et al., 2018). An increase of seed germination of *Sorghum durra* was showed also by seed application of extracts from *A. oryzae* and *Synechococcus* sp. while an opposite effect was observed on seed germination of *Helianthus annuus* (Essa et al., 2015). Again on *H. annus* spray and soil treatment with *Oscillatoria annae* increased both plant and yield parameters such as number of leaves, leaf length and breadth, shoot, flower head weight and diameter and number of seeds per flower and seeds weight (Bhuvaneshwari et al., 2011). On willow, spray treatment with *M. aeruginosa* and *Anabaena* sp. increased plant height, total shoot length, fresh and dry weight and enhanced the plant physiological performance in comparison to a commercial product based on extract from brown algae and to treatment with gibberellic acid and indole-3-butyric acid (Grzesik et al., 2017).

Cyanobacteria has identified as one of the most promising source of natural bioactive compounds with antimicrobial (Burja et al., 2001), anti-protozoal (Simmons et al., 2008), anticancer (Russo and Cesario, 2012), antiviral, antibacterial, and antiproliferative activities (Dixit and Suseela, 2013) (Fig. 1). Several authors reported their antifungal activity against fungal plant pathogens (Moon et al., 1992; Prasanna et al., 2008; Radhakrishnan et al. 2009; Manjunath et al. 2010; Roberti et al., 2016). Most of the studies in vitro on the antifungal activity of extracts from cyanobacteria against plant pathogens have regarded *Anabaena* and *Nostoc* species (Tab. 4). Frankmolle et al. (1992a, b) reported that crude ethanolic extracts from *Anabaena laxa* inhibited the growth of different fungus, plant pathogens: included *Aspergillus oryzae, Candida albicans, Penicillium notatum, Saccharomyces cerevisiae* and *Trichophyton mentagrophytes*. From a screening conducted by Prasanna et al. (2008) resulted that twentythree strains of *Anabaena* inhibited the growth of *Fusarium moniliforme* while seventeen strains inhibited that of *A. solani*. Moreover, *Nostoc muscorum* is known to be effective against *R. solani* (de Caire et al., 1990) and several *Anabaena* and *Calothrix* strains exhibit fungicidal activity against species of *Pythium, Fusarium* and *Rhizoctonia* (Moon et al., 1992;

Prasanna et al., 2008; Radhakrishnan et al., 2009; Manjunath et al., 2010). Among all the compounds produced by these microorganisms hydrolytic enzymes and the homologues for chitosanase, endoglucanase and benzoic acid were identified and correlated to their activity against fungus (Gupta et al., 2010, 2011; Natarajan et al. 2012; Prasanna et al., 2010). Chitosanases are known to selectively degrade chitosan/chitin by hydrolysis of the β-1, 4-glycosidic bonds that link N-acetyl glucosamine residues of chitin and form the basis for antifungal activity. In addition to enzymes, they are also able to produce phenolic compounds that was demonstrated to inhibit *Candida albicans* growth (de Cano et al., 1990). Moreover, the terpenoid noscomin, extracted *N. commune* showed antibacterial activity against *Bacillus cereus, Staphylococcus epidermidis*, and *Escherichia coli (*Jaki et al., 2000). Several studies have regarded also the activity of *Microcystis aeruginosa* extracts against fungi. Khalid et al. (2010) reported that extracts from *M. aeruginosa* showed high antifungal activity against 7 human pathogens and 5 plant pathogens. The same cyanobacteria also demonstrated to inhibit the growth of *Aspergillus flavus A. niger, Fusarium verticillioides* and, *F. proliferatum* (Marrez and Sultan, 2016). Analyses on the composition and bioactivities of single compounds revealed that the 2,6-di t-butyl-4-methyl phenol (BHT), hexadecanoic acid and methyl ester were the main responsible of the antifungal activity.

Some studies have regarded also the activity of cyanobacteria against pathogens on plants. For example Alwathnani and Perveen (2012) reported that application of *Nostoc linckia* on tomato plants reduced disease incidence due to *F. oxysporum* f. sp. *lycopersici*. In vivo studies showed that F. oxysporum was very sensitive to cyanobacteria species (Rizk, 2006). Again on tomato, application of *N. com*mune on seed infected with *F. oxysporum* showed to decrease the average number of infected seedlings (Kim and Kim 2008). Application on zucchini cotyledons of a strain of *Anabena* sp. reduced symptoms of powdery mildew caused by *P. xanthii* and caused a systemic accumulation of enzyme activities correlated to induced systemic resistance, such as Endochitinase, *β*-N-acetylhexosaminidase, chitin 1,4- *β* -chitobiosidase, *β* -1,3-glucanase and peroxidases (Roberti et al., 2015, 2016). Like all micro- and macroalgae, cyanobacteria also produce copious amounts of polysaccharides. Several studies carried out in the last decade reported that polysaccharides and derived oligosaccharides from marine macroalgae are able to elicit defence responses and to enhance protection in cultivated plants against different pathogens, inducing oxidative burst and activating signalling pathways at systemic level, which lead to the accumulation of pathogenesis-related (PR) proteins and other antimicrobial compounds (Vera et al., 2011). Prasanna et al (2015) demonstrated that inoculating a biofilm composed by *Anabaena* sp. the activity of defense enzymes such as peroxidase, phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) increased in maize

roots and shoots within the levels of accumulation of Zn in flag leaf. Moreover, cyanobacterial treatments enhanced glomalin-related soil proteins and polysaccharides in soil.

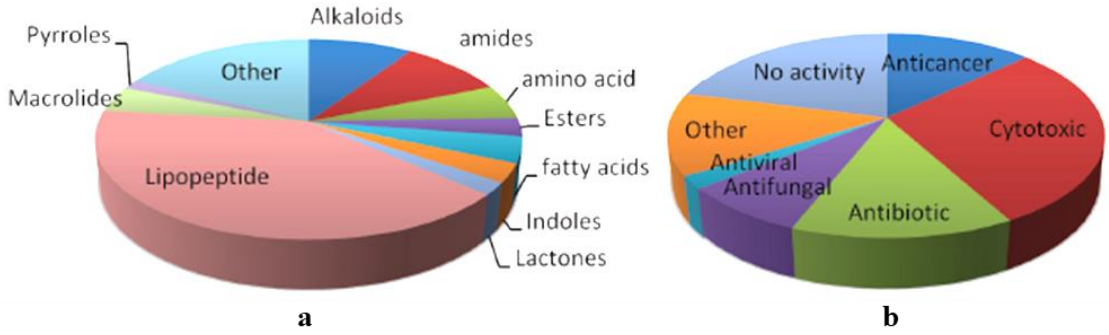

Fig. 1 Compounds isolated from cyanobacteria (a) and their main biological activity (b) (Encarnação et al., 2015, adapted from Burja et al., 2001).

Tab. 3- Beneficial effects of extracts from cyanobacteria on crops

Cyanobacteria	Crop	Beneficial effects	Reference
Anabaena sp.	maize	Concentration of Zn in flag leaf, variation of glomalin-related soil proteins and polysaccharides in soil, activity of defense enzymes in roots and shoots (peroxidase, PAL and PPO)	Prasanna et al., 2015
Anabaena sp.	willow	height, total shoot length, shoot number and length, shoot fresh and dry weight, chlorophyll content in leaves	Grzesik et al., 2017
Anabaena sp.	rice	higher phosphorus and potassium in seeds, seed weight, yield	Pimratch et al., 2015
Anabaena oryzae	sunflower	percentage of germination, shoot and root length, fresh and dry weight of seedling, enzyme activities of peroxidase, polyphenoloxidase and catalase	Essa et al., 2015
A. oryzae	sorghum	percentage of germination, shoot and root length, fresh and dry weight of seedling, enzyme activities of peroxidase, polyphenoloxidase and catalase	
Anabaena spiroides + *Nostoc minutum*+	bean	plant dry weight, shoot length, seed content of nitrogen, phosphorous, and potassium, shoot content of nitrogen and phosphorous	Al-Sherif et al., 2015

Anabaena vaginicola	cumber	Root length, plant height, root fresh and dry weight, fresh stem and leaf weight	Shariatmadari et al., 2013
A. vaginicola	tomato	Root length, plant height, leaf number, root dry weight, stem and leaf fresh weight, stem and leaf dry weight	
A. vaginicola	squash	Root length, plant height, leaf number, root fresh and dry weight, stem and leaf dry weight	
Cylindrospermum majus + Westiellopsis prolifica	rice	Yield	Jha and Prasad, 2006
M. aeruginosa	willow	height, total shoot length, shoot number and length, shoot fresh and dry weight, chlorophyll content in leaves	Grzesik et al., 2017
Nostoc calcicola	cucumber	Root length, plant height, root fresh and dry weight	Shariatmadari et al., 2013
N. calcicola	tomato	Root length, plant height, root fresh and dry weight, stem and leaf dry weight	
N. calcicola	squash	root length, plant height, leaf number, root Fresh weight	
N. carneum + N. commune	rice	root length, root and shoot dry weight, pike per plant	Chittapun et al., 2018
Nostoc carneum	rice	shoot dry weight, pike per plant, grains per spike	
N. commune	rice	root length, root and shoot dry weight, pike per plant	
Nostoc ellipsosporum	sunflower	percentage of germination, shoot and root length, fresh and dry weight of seedling, enzyme activities of peroxidase, polyphenoloxidase and catalase	Essa et al., 2015
Nostoc entophytum	pea	Germination percentage, root depth, shoot length, photosynthetic pigment fractions, carbohydrate and protein contents of seeds	Osman et al., 2010
N. ellipsosporum	sorghum	shoot and root length, fresh and dry weight of seedling, enzyme activities of peroxidase and catalase	Essa et al., 2015
Oscillatoria angustissima	pea	Germination percentage, root depth, shoot length, photosynthetic pigment fractions, carbohydrate and protein contents of seeds	Osman et al., 2010
Oscillatoria annae	sunflower	number of leaves, leaf length and breadth, shoot, root and stalk length, flower head weight and diameter, fresh and dry weight of	Bhuvaneshwari et al., 2011

		shoot and root, number of seeds per flower and seeds weight	
Synechococcus sp	sunflower	percentage of germination, shoot and root length, fresh and dry weight of seedling, enzyme activities of peroxidase, polyphenoloxidase and catalase	Essa et al., 2015
Synechococcus sp	sorghum	percentage of germination, shoot and root length, fresh and dry weight of seedling, enzyme activities of peroxidase, polyphenoloxidase and catalase	

Tab. 4 Antifungal activity of cyanobacteria extracts on colony growth of several plant pathogens.

Cyanobacteria	Extract solvent	Pathogen	Reference
Anabaena sp.	ethyl Acetate, dichloromethane	*M. phaseolina, F. monoliforme, A. solani, Pythium aphanidermatum, Fusarium solani*	Prasanna et al., 2008
A. laxa		*F. oxysporum f. sp. lycopersici, Fusarium moniliforme*	
Anabaena variabilis		*F. oxysporum f. sp. lycopersici, Fusarium moniliforme*	
A. variabilis	methanol, ethanol, water, acetic acid, acetone and butanol	*A. niger, A. solani.*	Tiwari and Kaur, 2014
Calothrix sp.	water, methanol or petroleum ether	*A. alternata, B. cinerea, C. gloeosporioides, F. oxysporium P. capsici, P. ultimum*	Kim, 2006
Fischerella sp,	methanol, aqueous supernatant	*A. niger, A. fumigatus*	Ghasemi et al., 2003
Microcystis aeruginosa		*F. oxysporum, M. phaseolina, P. aphanidermatum, P. oedochilum, R. solani*	Khalid et al., 2010
M. aeruginosa	aqueous, ethanol, ethanol, acetone, chloroform, ethyl acetate and hexane	*A. flavus, F. verticillioides , F. proliferatum*	Marrez and Sultan, 2016
Nodularia sp.	water, methanol or petroleum ether	*A. alternata, B. cinerea, C. gloeosporioides, F. oxysporium*	Kim, 2006
Nostoc sp.	water, methanol or petroleum ether	*A. alternata, B. cinerea, C. gloeosporioides, F. oxysporium, P. capsici, P. ultimum, R. stolonifer*	

Nostoc calcicola	ethanol	*F. solani, Penicillium chrysogenum*	Yadav, *et al.*, 2016
Nostoc commune	methanol	*F. oxysporum f. sp. lycopersici*	Kim and Kim, 2008
Nostoc entophytum	acetone, chloroform, methanol and water	*R. solani*	Osman et al., 2011
Nostoc linckia		*F. oxysporum f. sp. lycopersici*	Alwathnani and Perveen, 2012
N. muscorum	acetone, chloroform, methanol and water	*R. solani*	Osman et al., 2011
Oscillatoria sp.	water, methanol or petroleum ether	*A. alternata, B. cinerea, Colletotrichum gloeosporioides, F. oxysporium, Phytophthora capsici; Pythium ultimum*	Kim, 2006
Phormidium autumnale		*F. oxysporum f. sp. lycopersici*	Alwathnani and Perveen, 2012
Stigonema sp	methanol, aqueous supernatant	*A. niger, A. fumigatus*	Ghasemi et al., 2003
Spirulina platensis	methanol, ethanol, water, acetic acid, acetone and butanol	*A. niger, Alternaria solani.*	Tiwari and Kaur, 2014
Synechococcus elongates	methanol, ethanol, water, acetic acid, acetone and butanol	*A. niger, Alternaria solani.*	

Aims

Water extracts from algae and cyanobacteria may be considered environmentally safer than the extracts obtained with organic solvents. The objectives of this thesis are to study: (i) the antifungal activity of water extracts from cyanobacteria and brown, red and green algae against several fungal plant pathogens both *in vitro* and *in vivo* assays; (ii) the antifungal activity of polysaccharides obtained from the water extracts of cyanobacteria and algae; (iii) the plant defence responses by bioassay, by the increase of enzymatic activities related to the plant defence responses and by the accumulation of PR proteins; (iiii) phytotoxic and biostimulant effects of water extracts from cyanobacteria and brown, red and green algae.

The main characteristics of the algae and the cyanobacyeria used in the experiments are presented below.

The following studies are structured as a manuscript to submit in a scientific journal.

Algae and cyanobacteria used in the experiments

In the experiments carried out during the PhD research activity, algae and cyanobacteria were all provided by BEA (Banco Español de Algas), Gran Canaria, Spain. *Anabaena* sp. strain BEA 0300B used for the experiment was isolated from a coastal humid basaltic wall in Ajuy, Gran Canaria, Spain. The strain was cultivated in a low cost bioreactor for 20 days, with constant air bubbling, in a temperature-controlled room (25°C) then collected after centrifugation (12,000 rpm) and finally lyophilised and maintained at 4 °C. The same procedure was used for *Chlorella* sp. The macroalgae *Ecklonia* sp., *Halopithy* sp. and *Jania* sp. were provided as dry thallus, harvested in different season, March, May and October, respectively.

Rhodophyta

Rhodophyta are red algae, probably one of the oldest groups of eukaryotic algae (Lee, 2008). They have chlorophyll *a*, accessory photosynthetic pigments such as phycoerythrin, phycocyanin and allophycocyanins arranged in phycobilisomes, floridean starch as a storage product and thylakoids occurring singly in the chloroplast, and they lack flagellated cells. Most of the seaweeds belongs to Rhodophyta, including about 4000 algal species. They are present at all latitudes, mainly in temperate and tropical regions and only few species live in polar and sub Polar Regions (Woelkerling, 1990).
The size of red algae can vary according to the geographical region, for example the larger species occur in cool-temperate areas, whereas in tropical seas the Rhodophyceae (except for massive calcareous forms) are mostly small, filamentous plants. It is possible to find them at more than 200 m deep due to their accessory pigments, such as phycobiliproteins that allow the photosynthesis process in this condition. Few species are found in fresh water, where they do not reach as great a size as the red seaweeds (Eloranta et al., 1996).

Jania sp.

Taxonomic classification: Empire Eukaryota, Kingdom Plantae, Subkingdom Biliphyta, Phylum Rhodophyta, Subphylum Eurhodophytina, Class Florideophyceae, Subclass Corallinophycidae, Order Corallinales, Family Corallinaceae, Subfamily Corallinoideae, Tribe Janieae, Genus *Jania* (Schneider and Wynne, 2007).
The genus *Jania* includes red seaweeds belonging to the family *Corallinaceae* with hard, calcareous, branching skeletons (Fig. 2). It has been theorized that calcification of red algal thalli evolved as a protection against grazing by organ isms such as limpets, although it has also been pointed out that grazing is beneficial to the coralline algae in that the grazers remove epiphytes from the red algal thallus (Pueschel and Miller, 1996). The colours of these algae are most typically pink, or some other

shade of red, but some species can be purple, yellow and white. Members of the genus can be found in sheltered reef habitats, often in crevices or other shaded areas (Guiry, 2018; De Kluijver et al., 2011). For example, *Jania rubens* is commonly distributed in the Mediterranean, Atlantic and Eastern Pacific Ocean and in western Pacific (Bressan and Babbini, 2003; Mateo-Cid et al., 2013; Mendoza-Gonzales et al., 2014).

Fig. 2 *Jania rubens* var. *corniculata* (Calcherb-TSB-Herbarium Algarium, Universitatis Tergestinae Nova collectio - G. Bressan).

Halopithys sp.

Taxonomic classification: Empire Eukaryota, Kingdom Plantae, Subkingdom Biliphyta, Phylum Rhodophyta, Subphylum Eurhodophytina, Class Florideophyceae, Subclass Rhodymeniophycidae, Order Ceramiales, Family Rhodomelaceae,Tribe Amansieae, Genus *Halopithys* (Schneider and Wynne, 2007).

Halopithys sp. has cylindrical, cartilaginous, and dark red fronds that can reach 3 cm in length. The main branches can be alternate or subdichomous, simple or pectinate in lower parts, or even much branched above, often curved and hooked (Kim, 2015; algaebase; Fig. 3).

Species of this genus, such as H. *incurva*, are found on rocks, mid-littoral pools to subtidal, in the Mediterranean and Atlantic seas, from the south England to Canary Islands (Kim, 2015)

Fig. 3 *Halopithys incurva* (asturnatura.com).

Phaeophyceae

Phaephyceae are brown algae including 250 genera with about 1,500 species (van den Hoek, 1995). Most of them are marine organisms that prefer cold and well-oxygenated water where they play an important role both as food and as habitat. Brown algae present variable sizes from the filamentous epiphytes *Ectocarpus* up to the giant *Macrocystis,* that can reach 60 m in length and forms the so called 'kelp forest' (Cock et al., 2011).

The cell wall is composed by cellulose and alginates. Due to their viscosity, alginates are used in the food industry for toothpaste, soaps, ice cream and tinned meats (La Barre and Bates, 2018). They contain chlorophyll *a* and *c* and their colour can range from olive green to various shades of brown, in relation to the content of fucoxanthin, a carotenoid first isolated from the marine brown seaweeds *Fucus, Dictyota,* and *Laminaria* (Peng et al., 2011). Laminarin, low-molecular-weight polysaccharide, is the principal reserve of brown algae, and its content varies depending on species, harvesting season, habitat and method of extraction (Kadam et al., 2015). Brown algae are also used in agriculture as organic fertilizers since long time. Nowadays, a number of commercial products based on seaweed extracts are available to be used in agriculture and horticulture fertilization. *Ecklonia*, *Laminaria* and *Ascophyllum* species are the most use in these products due to their content of plant growth hormones such as auxins and gibberellins.

Ecklonia sp.

Taxonomic classification: Empire Eukaryota; Kingdom Chromista; Phylum Ochrophyta; Class Phaeophyceae; Subclass Fucophycidae; Order Laminariales; Family Lessoniaceae; Genus *Ecklonia* (Silberfeld et al., 2014).

To the genus *Ecklonia* belong algae species that grow in warm temperate regions, dominating extensive kelp beds in Australia, New Zealand, South Africa, and the north western Pacific (Steinberg and Kendrick, 1999; Choat and Schiel, 1982; Field et al., 1980). Some species, such as *E. radiata*, are also found in the northern hemisphere, but until today it has been described only from Oman on the Arabian Peninsula (Bolton and Anderson, 1994), while *E. cava* mainly inhabits coastal Japan and Korea (Wijesinghe and Jeon, 2012). The brown thallus of this genus can reach 1-15 m in length, with a conical holdfast branched, and each branch can produces a single plant (Fig. 4). Stipes is terete, solid or hollow, and can range from 2 cm to 12 m long, terminated by two tuft of sporophylls (spore-producing blades). The sporophylls are arranged in scrolls, the youngest ones being mere spikes, which become progressively larger and more distinct. The mature sporophylls are frequently ruffled and have distinct teeth along their margins (Druehl and Clarkston, 2016).

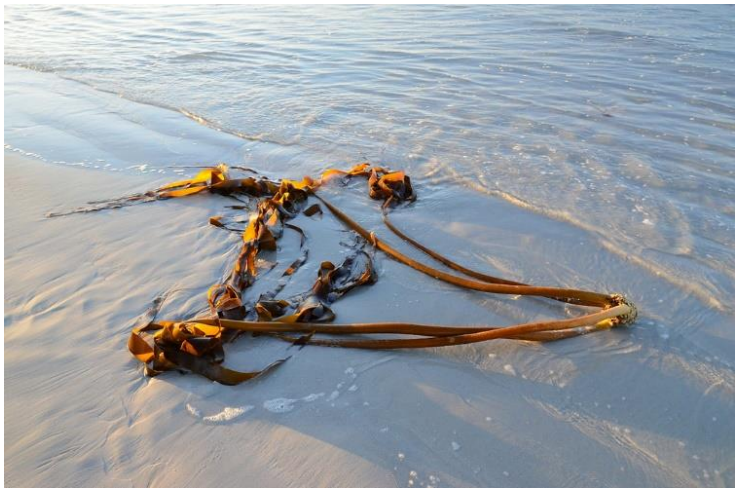

Fig. 4 *Ecklonia* sp. (Flickr website).

Chlorophyta

Chlorophyta Phylum, or green algae, includes both unicellular and multicellular species. Some of them establish symbiotic relationships with fungi to form lichens, protozoa and sponges but the majority of species are free-living (Díaz et al., 2016; Germond and Nakajima, 2016; Lubov et al., 2013). This phylum comprises about 9000 species, most of them live in but some species are adapted to a wide range of land environments (Graham et al., 2009; Leliaert et al., 2012). Like the land plants green algae contain chlorophyll *a* and chlorophyll *b* and starch in their plastids as storage compound (van den Hoek et al., 1995; Busi et al., 2013)

This phylum includes several classes: Bryopsidophyceae, Charophyceae, Chlorophyceae, Pedinophyceae, Pleurastrophyceae, Prasinophyceae, Trebouxiophyceae and Ulvophyceae.

Chlorella sp.

Taxonomic classification: Empire Eukaryota; Kingdom Plantae; Subkingdom Viridiplantae; Infrakingdom Chlorophyta; Phylum Chlorophyta; Subphylum Chlorophytina; Class Trebouxiophyceae; Order Chlorellales; Family Chlorellaceae; Genus *Chlorella* (Bock et al.,2011).

Chlorella species are unicellular microorganisms that grow both in fresh and salt water and in the soil. Some species have a great phylogenetic importance since contain sporopollenin, a substance typical of the pollen granules of the plants (Atkinson et al., 1972; Fig. 5). The cells can be spherical or ellipsoid, single or forming colonies. Reproduction occurs by autospores that are released through disruption of mother cell wall (Guiry, 2018).The genus Chlorella includes species in which the "rigid cell wall" is composed by polysaccharides formed mainly by glucose and mannose and others in which the cell wall is composed mainly by glucosamine. The sugar composition of the cell wall matrix of the first group is dominated by mannose and fucose whereas in the second group galactose, fucose, and sometimes xylose are the main sugars found (Loos and Meindl, 1982; Takeda, 1991). Species such as *C. vulgaris* are widely used for fish diets and human food supplements: they are rich in lipid-soluble and B-group vitamins, such as B1, B2 (riboflavin), B6 (pyridoxal), and B12 (Wells et al., 2017).

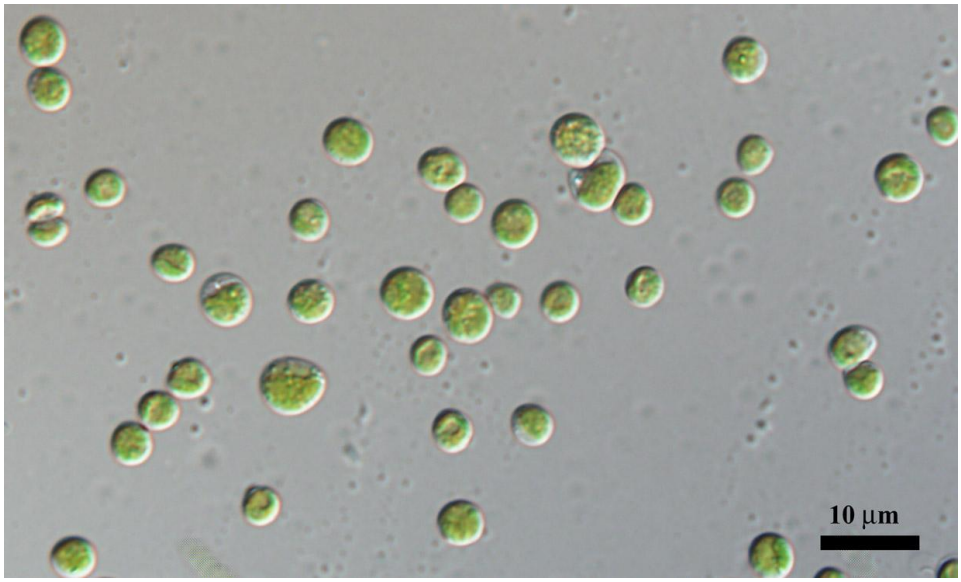

Fig. 5 *Chlorella vulgaris* cells. (CCALA website).

Cyanobacteria

Cyanobacteria, known also as blue-green algae, are a large group of photosynthetic prokaryotes (Walter et al., 2017) included in Bacteria superkingdom (Oren and Garrity, 2014 Schirrmeister et al., 2011; Di Rienzi et al., 2013). They can be found in different environments such as terrestrial and aquatic (oceans, fresh water) and in extreme conditions, desert or hypersaline and thermal water

(Walter et al., 2017). They are able to utilise N2 through the process of biological nitrogen fixation (Prasanna et al., 2012) through multiple specialized cell types, including nitrogen-fixing heterocysts, spore-like akinetes, and the cells of motile hormogonia filaments (Kumar et al., 2009; Golden and Yoo, 2003). The phylum includes about 2000 species in 150 genera, with a wide range of shapes and sizes. They often live in colonial aggregates that can take on a multitude of form, or can be free-living or have symbiotic relatioship (Soo et al., 2015).

Cyanobacteria are called "blue-green algae" due to the blue pigment phycocyanin, which, together with chlorophyll *a* and other pigments, captures light for photosynthesis (Saad and Atia, 2014). Aquatic cyanobacteria are also known for their 'blooms' in both fresh and marine waters (Bláha et al., 2009). These blooms cause a decrease of dissolved oxygen levels in water and create a significant water quality problem, especially because many cyanobacterial species are capable of synthesizing a wide range of odours, noxious compounds or potent toxins (Sivonen and Jones, 1999).

They are divided in eight orders: Gloeobacterales, Synechococcales, Spirulinales, Chroococcales, Pleurocapsales, Oscillatoriales, Chroococcidiopsidales and Nostocales (Komárek et al., 2014).

Anabaena sp.

Taxonomic classification: Empire Prokaryota; Kingdom Eubacteria; Subkingdom Negibacteria; Phylum Cyanobacteria; Class Cyanophyceae; Subclass Nostocophycidae; Order Nostocales; Family Nostocaceae; Genus *Anabaena* (Komárek et al., 2014).

Anabaena genus belongs to Nostocaceae family and is known for its high nitrogen fixing activity and symbiont relationship with different plants. For example, the genus *Anabaena azollae* lives in symbiosis with the aquatic ferns of the genus *Azolla*, in which the cyanobacterium found cavities in the leaves (Bocchi and Malgioglio, 2010). *Anabaena* genus includes filamentous cyanobacteria and some of them produce toxins (Sivonen and Jones, 1999; Fig. 6). The most frequently found toxins in cyanobacterial blooms worldwide are hepatotoxic cyclic peptides such as microcystins and nodularins (Sivonen and Jones, 1999). Massive presence of cyanobacteria that contain neurotoxins (anatoxin-a, anatoxin-a(S) and saxitoxins) has been found in Australia, Europe and North America (Sivonen and Jones, 1999). Some species of cyanobacteria, included *Anabaena, Nostoc and Spirulina*, are consumed as food due to their high protein and fibre content (Anusuya et al., 1981; Anupama, 2000).

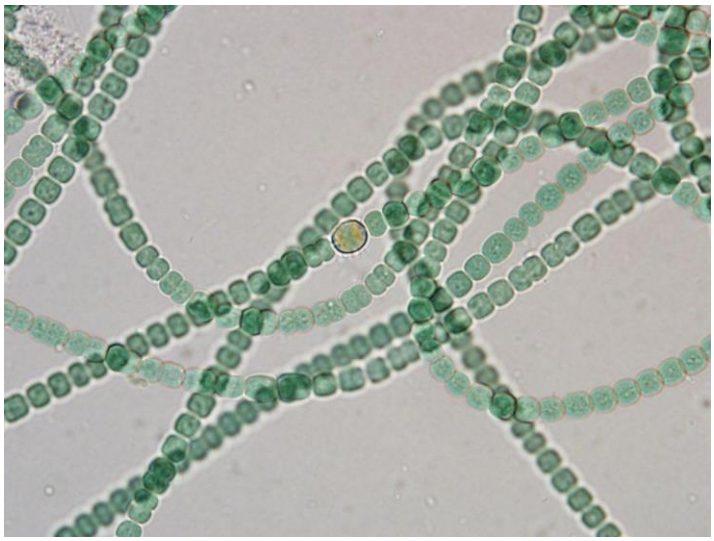

Fig. 6 *Anabaena variabilis* (algaeresearchsupply.com).

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Bion, Syngenta Crop Protection https://www.syngenta.it/sites/g/files/zhg301/f/bion_50_wg_-_etichetta_clp.pdf?token=1507118685

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2. Antifungal activity of water extracts from algae and cyanobacteria against *Rhizoctonia solani* on tomato

The effect of water extracts from the macroalgae, *Jania* sp., *Ecklonia* sp. and *Halopithys* sp., the microalga *Chlorella* sp. and the cyanobacterium *Anabaena* sp. against the soil-borne pathogen *Rhizoctonia solani* was evaluated *in vitro* and *in vivo*. The extracts were tested on fungal colony growth and hyphal morphology at four concentrations, 2.5, 5.0, 10.0 and 20.0 mg of dry biomass per ml of water. Each extract was also tested against *R. solani* disease on tomato seedlings in vitro. In in vivo experiments, the extracts were applied at 2.5, 5.0 and 10.0 mg ml^{-1} on tomato seed and in growing substrate, under greenhouse conditions. Fungal colony growth was affected only by extract from *Anabaena* sp. Hyphal diameter was increased by *Anabaena* sp., and *Ecklonia* sp., and reduced by *Halopithys* sp. and *Jania* sp. Cytoplasm coagulation was observed for all extract except for that of *Chlorella* sp. All extracts increased the formation of monilioid cells. On tomato seedlings, extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. at all concentrations reduced *R. solani* disease severity and increased dry weight, except for 20.0 mg ml^{-1}. *Halopithys* sp. did not show any effects, while *Chlorella* sp. increased disease severity. In the absence of the pathogen, almost all concentrations of *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. increased seed germination and dry weight. In *in vivo* experiments, extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. applied both on seed and in soil, were effective against *R. solani* at all concentrations. Seed treatment increased seedling emergence, and plant calibre and dry weight, and chitinase activity in plant tissues. Soil irrigation with 2.5 and 5.0 mg ml^{-1} of *Ecklonia* sp. and *Jania* sp. and with *Anabaena* sp. at all concentrations, reduced disease severity and increased seedlings dry weight. This study suggests that algae and cyanobacteria water extracts applied on tomato may be considered in further experiments as useful preventative tool for the disease management in sustainable agriculture.

1. Introduction

Rhizoctonia solani J.G. Kühn (teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk.) is a cosmopolitan soil-borne fungal pathogen having a wide host range (Baker, 1970). The pathogen can survive in the soil in the form of mycelium or resistance structures (sclerotia) (Fig. 1, 2) during unfavourable environmental conditions for several years, and on debris from various plants such as tomato (Coa et al., 2004, Suwannarach et al., 2015), cucumber (Huang et al., 2012), turf grass (Adikaram and Yakandawala, 2017), fenugreek (Yadav and Tiwari, 2005) corn (Ogoshi, 1987), cotton (Howell et al., 2000), rice (Ou 1985; Groth and Bond, 2006), cucumber (Strashnov et al. 1985), strawberry (De Cal et al., 2004), French bean (Sinobas et al., 1994), soybean (Ploetz et al., 2003) and

several weeds (Suwannarach et al., 2015). The pathogen is disseminated by infested soil or movement of diseased plant tissues. It causes different symptoms on the same host depending on the time of infection. Juvenile tissues of nursery plants are especially vulnerable to pre- or post-emergence damping-off. Symptoms occurring after transplanting to a pathogen-infested soil, are root and neck rot, foliar blight, or fruit rot (Lewis and Lumsden, 2001).

This pathogen includes subspecific groups called anastomosis groups (AGs) created in relation to the hyphal anastomosis reactions. Many AGs include isolates that can differ on the base of their virulence, colony morphology, and other characteristics (Cubeta et al., 1997; Kuninaga et al., 1997; Carling et al., 2002 a, b). *Rhizoctonia solani* is divided into 14 AGs, from AG 1 to 13 and bridging isolate (BI) group (Carling, 1996; Carling et al., 2002b). Isolates belonging to AG BI can anastomose with isolates of both AG BI and with other AGs of *R. solani* such as AG 2, AG 3, AG 6, and AG 8 (Kuninaga et al., 1979; Sneh et al., 1991). On tomato, isolates of AGs 1, 2, 3, and 4 have been shown to be pathogenic and cause high yield losses every year worldwide (Misawa and Kuninaga, 2010; Kuramae et al., 2003).

Formation of basidiospores on diseased host plants in nature is rarely observed. In favourable environmental conditions, following infection of the host plant by *R. solani*, sexual spores are formed on specialized structures called basidia. Four spores are produced on each basidium. Basidia are formed when enough moisture is available and sufficient growth of the fungus has occurred. Basidiospores are wind dispersed and germinate in high moisture. Each basidiospore has a single nucleus. The hyphae produced by germinating spores will fuse or anastomose with each other to form new hyphae with a mixture of different types of nuclei (Webster and Weber, 2007).

In culture, colour of the fungal colony can vary from withe to dark brown and at the microscope, it is possible observe the septated hyphae, with lateral branching originating in right angles and constrained at branching points, and the presence of monilioid cells (Fig. 1).

One aspect that make the *R. solani* disease difficult to control is that sclerotia can survive in the soil for three years in a rotation system without hosts (Bell and Summer, 1987). Nowadays, the control of soil borne pathogens is problematic, because no highly effective strategies are available. Indeed, the 1107/2009 EU regulation prohibited synthetic pesticides for soil fungal pathogen control, because the side effects against non target organisms or the whole environment, such in the case of the methyl bromide/ozone layer issue (Bell, 1996; Ristaino and Thomas, 1997). Non chemical approaches include resistant plant, however, a few tomato cultivars partially resistant to *R. solani* are available with commercially acceptable horticultural traits (Nikraftar et al., 2013).

The development of bio-based strategies to control plant pathogens and enhance food safety has encouraged by the Directive 2009/128/EC and its implementation that promote specific actions to

support the establishment of sustainable agriculture. Several researches were focused on the activity against plant pathogens of extracts obtained from algae and cyanobacteria by using organic solvents (Rizvi and Shameel, 2004; Kumar et al., 2008; Arunkumar et al., 2010; Sivakumar, 2014; Jiménez et al., 2011), while few studies have examined the antifungal activity of water extracts (Roberti et al., 2015; Righini et al., 2018). Therefore, algae and cyanobacteria could be considered useful tools, alternative to synthetic products, for their role in plant disease control.

The objectives of the present work are to study the effect of water extracts from algae and the cyanobacterium *Anabaena* sp. on: (i) *R. solani* colony growth and hyphal morphology; (ii) possible phytotoxicity or plant biostimulation; (iii) induction of plant defence responses against the pathogen on tomato plants grown under laboratory and greenhouse conditions; (iv) root rot disease after transplant of tomato plants in growing substrate infected with *R. solani* under greenhouse conditions.

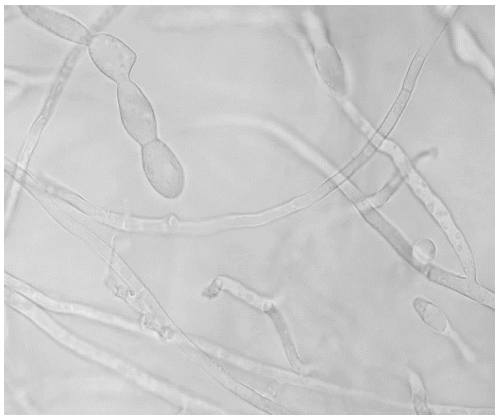

Fig. 1 Hyphae and monilioid cells of *Rhizoctonia solani*.

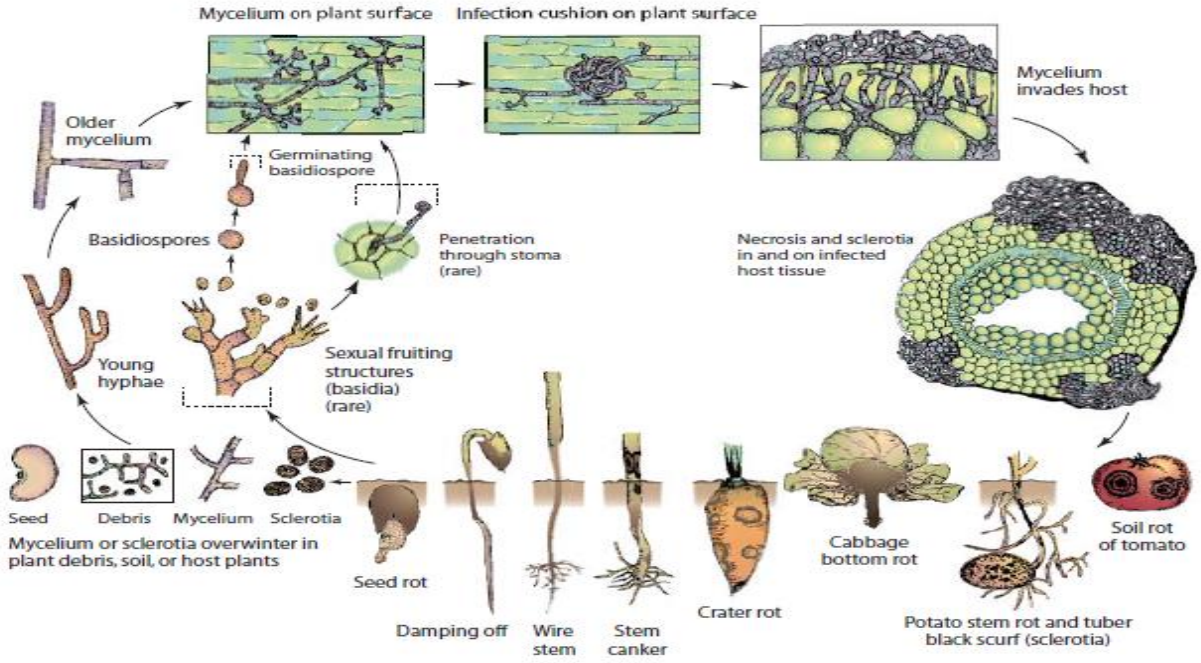

Fig. 2 Life cycle of *Rhizoctonia solani* (Agrios, 2005).

2. Materials and methods

2.1 Water extracts

Algae and cyanobacteria used in the experiments (Tab. 1) were provided by the Spanish Bank of Algae, Marine Biotechnology Center, University of Las Palmas de Gran Canaria. CHL and AN were provided as lyophilized biomass, while ECK, JAN and HAL as dry thallus. Dry thallus was grounded to fine powder with mortar and pestle. Water extracts were obtained by suspending each powder in sterile distilled water (0.5%) under continuous stirring at 50 °C for 12 h, then filtered (Roberti et al., 2015). Our previous experiments showed that concentration water extract used at the concentration 0.5%, (w: v, powder: water) was effective against *Podosphaera xanthii* on zucchini (Roberti et al., 2015). In the following experiments the concentration range was chosen considering the reference concentration and varied from 4-fold the reference concentration to 0.5 reference concentration.

Tab. 1 Generus, phylum (www.algaebase.org) and abbreviation of algae and cyanobacteria used for the experiments.

Genus	Phylum	Abbreviation
Anabaena sp.	*Cyanobacteria*	AN
Chlorella sp.	*Chlorophyta*	CHL
Ecklonia sp.	*Heterokontophyta*	ECK
Halopytis sp.	*Rhodophyta*	HAL

Jania sp.	*Rhodophyta*	JAN

2.2 Pathogen isolation and identification and plant material

The fungus *Rhizoctonia solani* 3001 belongs to DISTAL (Department of Agricultural and Food Sciences). The fungus was isolated from tissues of tomato plants showing symptoms of root and crown rot. Pieces of tissue were surface disinfected with 1.5% NaOCl for 2 min, rinsed two times in sterile distilled water and placed on Petri dishes containing potato dextrose agar (PDA 3.9%, Biolife S.r.l., MI, Italy) supplemented with 60 mg l^{-1} of streptomycin sulphate (Sigma - Aldrich Co.). The presence of RS from tomato tissues was examined after incubation at 25°C in the dark for 7 days, using a light microscope (Carl Zeiss mod. ZM, Germany) at ×500 magnification. The fungus was preliminary identified based on morphological features (biblio), then transferred in PDA medium. The fungus pathogenicity was verified through inoculation of 7-day-old colony portions on tomato root seedlings and waiting for the symptom appearance.

For all experiments, tomato seeds cv. Marmande (L'Ortolano, Savini Vivai, Italy) were used.

2.3 Effect of water extracts on colony growth, hyphal morphology and monilioid cells

Portions of 7 mm diameter were cut from 10-day-old fungal colony and transferred in test-tube containing a 600-µl aliquot of each extract at four concentrations (2.5, 5, 10 and 20 mg ml^{-1}). Sterile distilled water was used as untreated control. Three dishes (replicates) were used for each concentration, and for the control. After 6 h of treatment, colony portions were placed on PDA medium in Petri dish and incubated at 24 °C in the dark for 7 days. Colony diameters were measured daily along two perpendicular axes. The experiment was repeated twice.

The effect of water extracts on *R. solani* hyphae and monilioid cells was evaluated 10 days after treatment. Colony portions from treated and untreated colony were observed by using an Eclipse TE2000-E microscope (Nikon Instruments Europe BV, Amsterdam, The Netherlands) at ×600 magnification. Hyphal diameter was measured (4 replicates for each concentration and the control). Cytoplasm coagulation was evaluated in the same fungal portions by using the following scale: 1, 0-10% of coagulation; 2, 11-40% of coagulation; 3, 41-80% and 4, > 80% of coagulation. For monilioid cells three colony portions were evaluated through the following scale: 0, absence of monilioid cells; 1, 1-5 monilioid cells; 2, 6-15, 3, >16 monilioid cells. The assay was repeated once.

2.4 Effect of seed treatment with water extracts on seed germination and seedling dry weight, *in vitro*

Tomato seeds were sterilized according to Mbega et al. (2012) with modifications. Seeds were surface-disinfected in ethanol (70%) for 5 min, in 1.5% sodium hypochlorite (NaOCl) solution for three min and then rinsed three times in sterile distilled water. Seeds were treated by immersion in 600-µl aliquot of each extracts at 2.5, 5, 10 and 20 mg ml^{-1} overnight in the dark. Sterile distilled water was used as control. After treatment, seeds were dried on sterile paper in a laminar airflow cabinet for 10 min. Four seeds were sown on the edge of a half-cut PDA medium in Petri dishes. Dishes were incubated vertically in a growth chamber at 24-25 °C in the dark for 48 h and then shifted to light: dark cycle, 12h: 12h (Fig. 3 a, b). Ten replicates were considered for each treatment and the control. Seeds were considered germinated once the radicle protruded more than 2 mm (Hernández-Herrera et al., 2015). Three days after treatment, the percentage of germination was recorded daily for each Petri dishes over a period of 5 days. Twenty days after sowing seedlings were removed from the medium, dried in a hoven at 60 ± 5 °C for 48 h and dry weight was determined. The experiment was repeated once.

2.5 Effect of seed treatment with water extracts against *R. solani in vitro*

Tomato seeds were sterilized, treated with each water extract and sown as reported above. The control consisted in seeds immersed in water. After 12 days, *R. solani* plugs cut from 10-day-old colony were inoculated (2 plugs per dish) in the medium in all dishes at a distance of 5 mm from seedling roots (Fig. 3 a, b, c). Dishes were incubated vertically in a growth chamber at the same conditions above mentioned. Two weeks after inoculation, seedlings were removed and necrosis root symptoms were visually assessed using a six-point scale, where: 0, absence of necrosis (0% of symptoms); 1, very slight root necrosis (up to 3% of root with symptoms); 2, slight necrosis (4 - 30% of root with symptoms); 3, moderate root necrosis (31 - 70% of root with symptoms); 4, severe root necrosis 71 - 80% of root with symptoms); 5, very severe necrosis (81- 100% of root with symptoms). For dry weight, the infected seedlings were dried in a hoven at 60 ± 5 °C for 48 h. The experiment was repeated once.

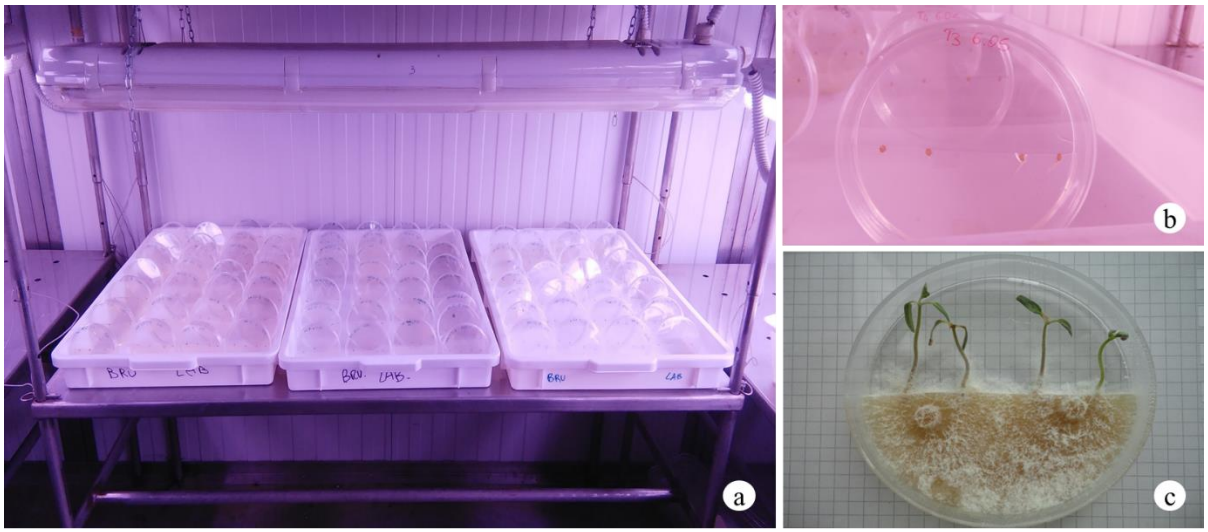

Fig. 3 Petri dishes with treated seeds sown in PDA under grown chamber conditions (a); tomato seeds sown on the edge of a half-cut PDA medium in Petri dishes (b); infected seedlings (c).

2.6 Effect of seed treatment with water extracts against *R. solani* under greenhouse conditions

Tomato seeds were treated as described above with water extracts from AN, ECK and JAN at the concentrations of 2.5, 5 and 10 mg ml^{-1}. Seeds were sown in plastic trays (11.5 × 14 × 9.5 cm) in a substrate consisting of a mixture of peat and sand (7: 3 w: w) inoculated with *R. solani*. For inoculation, 10-day-old colonies of *R. solani* (2% w/w, substrate/pathogen) grown on PDA medium were centrifuged with sterile distilled water and mixed with the substrate (2% w/w, pathogen/ substrate). The substrate was incubated for two days in a growth chamber at 25 °C in the dark before the sowing. Plants were grown at 24–26 °C (day), 20–22 °C (night) and 14 h photoperiod and 70% relative humidity under greenhouse conditions. Seeds treated with water and sown in growing substrate not infected was used as positive control. Seed treated with water and sown in infected substrated was used as negative control. One week after sowing, the seedling emergence was recorded and three weeks after sowing, stem calibre and plant dry weight were recorded.

2.7 Effect of substrate treatment with water extracts against *R. solani* under greenhouse conditions

Tomato seeds were sown in substrate, incubated at 24–26 °C (day), 20–22 °C (night), and 14 h photoperiod and 70% relative humidity under greenhouse conditions and regularly irrigated. Substrate was inoculated as described above. At the stage of second true leaf, one seedling per pot (4 replicates per treatment) was transplanted in infected substrate and irrigated with 50 ml of water extract at the concentrations of 10.0, 5.0 and 2.5 mg ml^{-1}. Irrigation with water was used as control.

Three weeks after inoculation, seedlings were removed and necrosis root symptoms were visually assessed by using a five-point scale, where: 0, absence of necrosis (0% of symptoms); 1, very slight root necrosis (up to 3% of root with symptoms); 2, slight necrosis (4 - 30% of root with symptoms); 3, moderate root necrosis (31 - 70% of root with symptoms); 4, severe root necrosis 71 - 80 % of root with symptoms); 5, very severe necrosis (81 - 100% of root with symptoms). To determine the dry weight, seedlings were dried in a hoven at 60 ± 5 °C for 48 h. The experiment was repeated once.

2.8 Chitinase activity of tomato seedlings treated with extracts

Tomato seed were treated with the extracts as reported above. Sixty seeds for treatment were sown on sterile paper in aluminium trays, and irrigated with 50 ml of sterile distilled water. Water control was considered. The trays were covered with transparent film and incubated at 24-25 °C in the dark for two days and then shifted to light: dark cycle, 12h: 12h. Twenty days after sowing, seedlings were harvested, weighed and snap frozen in liquid nitrogen. Frozen seedlings were grounded to fine powder by using a pre-chilled mortar and pestle, and total proteins were extracted by 20 mM sodium acetate buffer pH 5.2 (1 ml g^{-1} of fresh weight) containing 1% polyvinylpolypyrrolidone (Sigma–Aldrich Co.) (Roberti et al., 2008). After incubation at 4 °C for 90 min under continuous gentle stirring, the crude extracts were centrifuged twice at 12,000 rpm for 20 min at 4 °C. The supernatant was filtered using a GV Millex® Syringe Filter Unit (pore size 0.22 µm, Millipore Corporation, USA) to remove the solid particles. Protein concentration was determined by the protein–dye binding method of Bradford (1976) in a 96 wells microplate (Greiner CELLSTAR®), by using bovine serum albumin (Bio-Rad Laboratories, Inc.) as the standard.

Chitinase activity was assayed in triplicate following the procedure of plate assay (Bargabus et al., 2004). In agarose gel (1%) containing 0.01% glycol chitin in a glass Petri dish (17 cm diameter). Forty µg of proteins were added into wells for each sample. As chitinase standard *Streptomyces griseus* (Sigma) was used. After incubation at 37 °C for 24 h, 50 ml of 500 mM Tris–HCl (pH 8.9) containing 0.01% fluorescent brightener was added to each dish. Ten min later, the dishes were rinsed three times with distilled water, flooded with water, and maintained in the dark for 24 h. The non-fluorescent lytic zones on a fluorescent background were measured after exposure to 302nm UV light source. Images of gels were taken and the area (mm^2) corresponding to chitinase activity was calculated with the Quantity One program (Bio-Rad).

2.9 Statistical analysis

All experiments were arranged in a complete randomized design. Data obtained from *in vitro* and *in vivo* experiments were analyzed by factorial ANOVA, and means were separated by Fisher's least

significant difference (LSD) test ($P < 0.05$). Factors for ANOVA analyses were: extracts, their concentrations, methods of treatment (seed or substrate). All analyses were performed with GraphPad Prism software, version 4.03, 2005.

3. Results

3.1 Effect of water extracts on colony growth, hyphal morphology and monilioid cells

The colony growth of *R. solani* after treatment with the extracts with the submerged colony method is shown in Tab. 2. Two-way ANOVA indicated a significant interaction between extract and concentration factors. The growth was significantly reduced only by AN at 10.0 mg ml^{-1} (22.1.0%) and 20.0 mg ml^{-1} (27.9%) with respect to untreated control (0.0 mg ml^{-1}). These concentrations gave statistically similar values of growth reduction. On the contrary, ECK at 2.5 and 5 mg ml^{-1} increased colony growth. No concentration of CHL, JAN and HAL influenced fungal colony growth. No extracts influenced *R. solani* growth 3 d after treatment (data not showed). In Tab. 3, the effects of different concentrations of the extracts on *R. solani* hyphal diameter and cytoplasm coagulation are shown. Two-way ANOVA of both hyphal diameter and cytoplasm coagulation index indicated a significant interaction between extract and concentration factors. Extracts from AN and ECK significantly increased hyphal diameter at all concentrations with respect to untreated control, except for ECK at 2.5 mg ml^{-1}. For both treatments, the maximum increase was shown at 20.0 mg ml^{-1}, 37.0 and 23.9% respectively. On the contrary, JAN and HAL significantly reduced hyphal diameter at all concentration from 5.7 to 26.4.0% and from 12.7 to 45.5%, respectively, except for HAL at 20.0 mg ml^{-1}. The treatment with CHL did not show any effects with respect to the control. Extract from AN gave the highest values of hyphal diameter at 2.5 and 10.0 mg ml^{-1} (6.2 and 7.0 μm, respectively), while at 5.0 and 20.0 mg ml^{-1} the values were significant similar to those of ECK. Overall, the lowest values of hyphal diameter were obtained with extracts from HAL and JAN. Regarding cytoplasm coagulation, all treatments caused a significant increase of index values depending on the concentration (Fig. 4), except for CHL at all concentrations and JAN at 2.5 mg ml^{-1} that did not show any effect with respect to the control. Overall, the highest values of cytoplasm coagulation, were observed for ECK and HAL, except at 5.0 mg ml^{-1} for HAL. Particularly at 10.0 mg ml^{-1} and 20.0 mg ml^{-1} abundant presence of vacuoles in the cytoplasm was observed. In Fig. 4 the effect of the extracts on formation index of monilioid cells (FIMC) is shown. The index was influenced only by concentration factor. All concentrations significantly increased with similar values the formation of

monilioid cells with respect to the untreated control from 26.3 to 42.1% (Fig. 5). Microscopic observation of untreated mycelium showed regular growing hyphae and uniform cytoplasm (Figs. 4 and 6).

Tab. 2 Effect of different concentrations of water extracts on *Rhizoctonia solani* colony growth (mm) two days after treatment

Extract	Concentration (mg ml^{-1})				
	0.0	2.5	5.0	10.0	20.0
Anabaena sp.	33.0 ± 0.0 B	36.7 ± 6.4 aB	40.3 ± 3.2 abC	25.7 ± 3.2 aA	23.8 ± 3.2 aA
Chlorella sp.	33.0 ± 5.5	38.5 ± 5.5 a	33.0 ± 5.5 a	32.1 ± 1.6 ab	35.8 ± 2.8 b
Ecklonia sp.	34.8 ± 6.4 A	58.7 ± 6.4 bC	47.7 ± 6.4 bB	36.7 ± 4.2 abcA	43.1 ± 1.6 bAB
Halopithys sp.	31.2 ± 6.4	37.6 ± 5.7 a	44.9 ± 4.2 b	48.6 ± 13.6 c	39.4 ± 8.8 b
Jania sp.	34.8 ± 3.2	40.3 ± 6.3 a	38.5 ± 5.5 ab	43.1 ± 4.2 ab	44.0 ± 5.5 b

Extract, concentration and their interaction are significant according to factorial ANOVA ($P < 0.05$). F (4, 75) = 11.5, $P < 0.05$ (for extract factor), F (4, 75) = 5.9, $P < 0.05$ (for concentration factor), F (16, 75) = 3.4, $P < 0.05$ (for interaction). Means (± SD) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences, according to LSD test ($P < 0.05$).

Tab. 3 Effect of different concentration of water extracts on morphological characteristics of *Rhizoctonia solani*

Extract	Concentration (mg ml^{-1})				
	0.0	2.5	5.0	10.0	20.0
Hyphal Diameter (μm)					
Anabaena sp.	5.2 ± 0.5 A	6.2 ± 0.2 cB	6.2 ± 0.6 cB	7.0 ± 0.4 dC	7.4 ± 0.7 bC
Chlorella sp.	5.3 ± 0.1	5.5 ± 0.1 c	5.0 ± 0.2 ab	5.5 ± 0.5 c	5.1 ± 0.2 a
Ecklonia sp.	5.2 ± 0.2 A	5.2 ± 0.3 bA	6.6 ± 0.4 cB	6.2 ± 0.2 cB	7.1 ± 0.4 bC
Halopithys sp.	5.5 ± 0.1 C	4.8 ± 0.2 bB	4.7 ± 0.3 aB	3.0 ± 0.2 aA	5.4 ± 0.3 aC
Jania sp.	5.3 ± 0.1 C	3.9 ± 0.2 aA	4.8 ± 0.1 bB	4.8 ± 0.2 bB	5.0 ± 0.2 aB
Cytoplasm Coagulation Index					
Anabaena sp.	1.1 ± 0.2 A	2.5 ± 0.5 cB	2.2 ± 0.3 abB*	2.6 ± 0.5 abB*	2.1 ± 0.1 aB*
Chlorella sp.	1.2 ± 0.2	1.7 ± 0.2 b	1.5 ± 0.5 a	1.8 ± 0.3 a*	1.7 ± 0.4 a*
Ecklonia sp.	1.1 ± 0.1 A	2.2 ± 0.2 bcB	3.4 ± 0.5 cC*	3.5 ± 0.5 cC*	3.5 ± 0.5 bC*
Halopithys sp.	1.1 ± 0.2 A	2.1 ± 0.2 bcB*	1.8 ± 0.4 abB*	3.5 ± 0.5 cC*	3.3 ± 0.6 bC*
Jania sp.	1.1 ± 0.1 A	1.1 ± 0.1 aA	2.5 ± 0.5 bC	3.0 ± 0.2 bcC*	1.7 ± 0.3 aB*

*: abundant presence of vacuoles in the cytoplasm

For both hyphal diameter and cytoplasm coagulation index, extract and concentration factors and their interaction are significant according to factorial ANOVA ($P < 0.05$). For hyphal diameter, F (4,

100) = 128.5, $P < 0.05$ (for treatment factor), F (4, 100) = 23.0, $P < 0.05$ (for concentration factor), F (16, 100) = 23.2, $P < 0.05$ (for interaction). For cytoplasm coagulation index, F (4, 75) = 21.7, $P < 0.05$ (for extract factor), F (4, 75) = 48.5, $P < 0.05$ (for concentration factor), F (16, 75) = 6.3, $P < 0.05$ (for interaction). Means (± SD) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences.

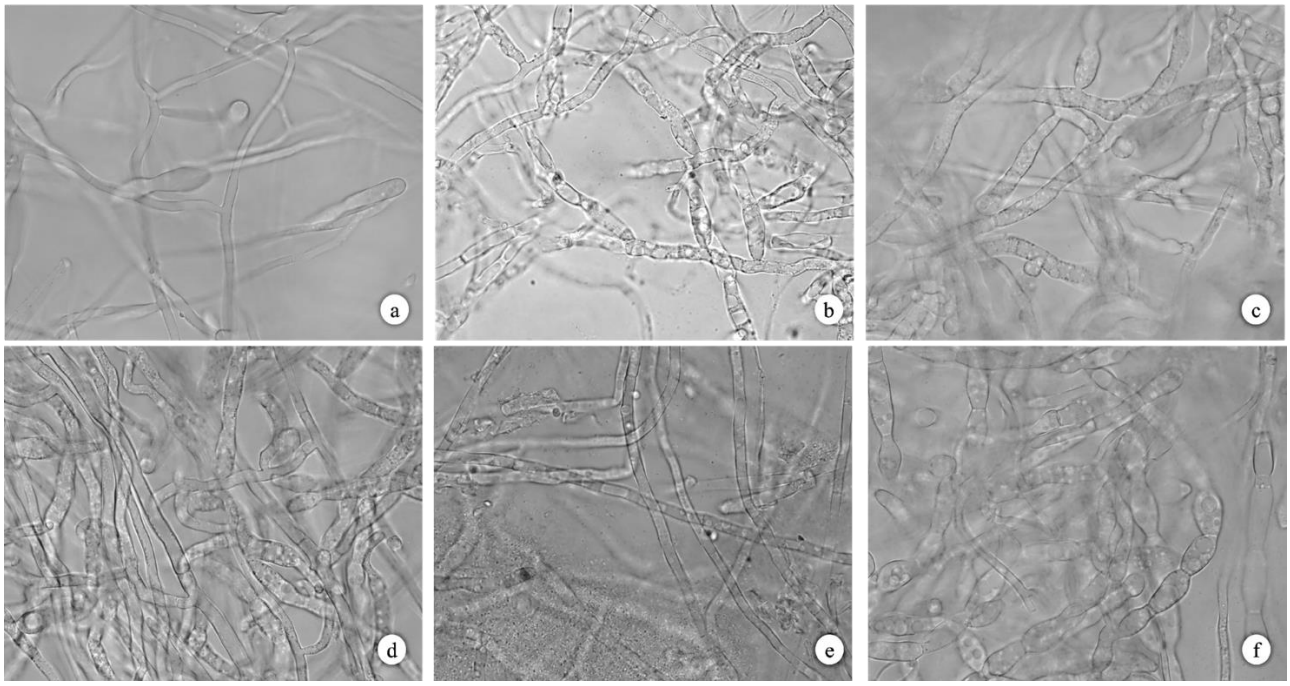

Fig. 4 Morphological alterations of *Rhizoctonia solani* mycelium grown on PDA after treatment with the extracts: untreated control (a); cytoplasm coagulation and presence of vacuoles caused by 10 mg ml^{-1} of *Anabaena* sp (b), *Chlorella* sp. (c), *Ecklonia* sp. (d), *Halopithys* sp. (e) and *Jania* sp. (f).

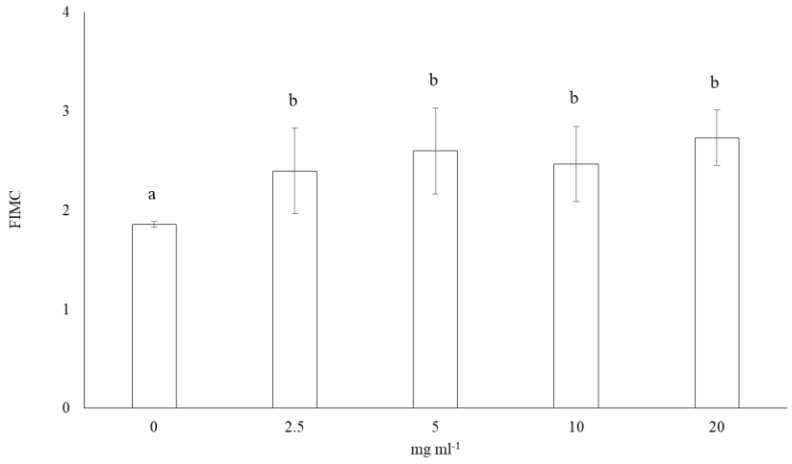

Fig. 5 Effect of concentrations of water extracts on the formation index of monilioid cells (FIMC). Extract factor and extract × concentration interaction are not significant according to factorial ANOVA ($P < 0.05$). F (3, 75) = 7.8, $P < 0.05$ (for concentration factor). Columns are mean values ± SD. Different letters indicate significant differences according to LSD test ($P < 0.05$).

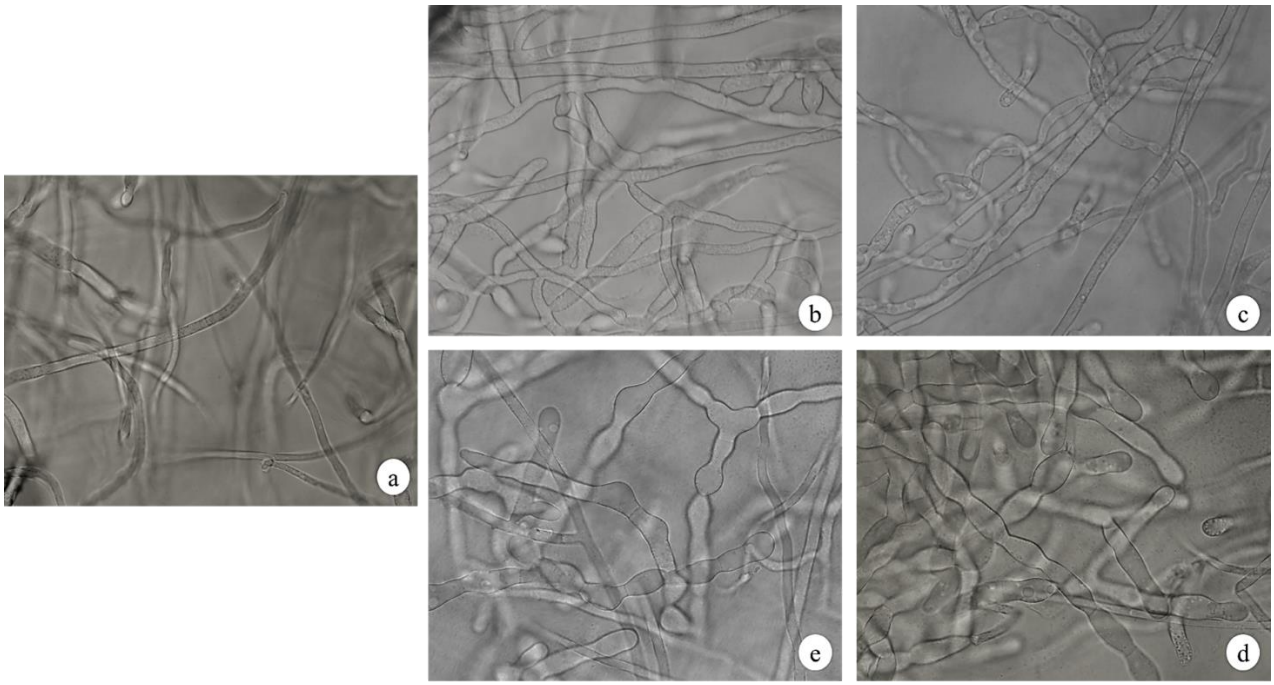

Fig. 6 Monilioid cells in *Rhizoctonia solani* mycelium in the untreated control (a) and after treatment with *Anabaena* sp. at 2.5 mg ml^{-1} (b), 5.0 mg ml^{-1} (c), 10.0 mg ml^{-1} (d) and 20.0 mg ml^{-1} (2).

3.2 Effect of seed treatment with water extracts on seed germination and seedling dry weight, *in vitro*

Table 4 shows the effect of seed treatment with different concentration of the extracts on tomato seed germination. Two-way ANOVA indicated a significant interaction between extract and concentration. The percentage of germination was significantly increased by AN, ECK and JAN at all concentrations, except for AN at 2.5 mg ml^{-1}. The maximum increase of AN was observed at 5.0 mg ml^{-1} by 18.1%, while for ECK, the percentage of seed germination was higher and with similar values at 5.0, 10.0 and 20.0 mg ml^{-1}. For JAN, the highest values were obtained at 5.0 and 10.0 mg ml^{-1}. At 5.0 and 10.0 mg ml^{-1}, AN, ECK and JAN increased seed germination in a similar way from 12.9 to 16.5% (5.0 mg ml^{-1}) and from 11.5 to 12.9% (10.0 mg ml^{-1}). At 20.0 mg ml^{-1}, the highest seed germination increase was obtained with ECK (14.3%).

Dry weight of seedlings grown from treated seeds with each extract is reported in Tab. 5. Two-way ANOVA indicated a significant interaction between extract and concentration factors. Dry weight was significantly increased by extracts from JAN and ECK at 2.5, 5.0 and 10.0 mg ml^{-1}, and by AN at 5.0 and 10.0 mg ml^{-1}. On the contrary, the treatment CHL at all concentrations significantly reduced dry weight, while for HAL no differences were observed between all concentration and the untreated control. Within concentration, at 2.5 and 10.0 mg ml^{-1} the highest dry weight increases were obtained by AN, ECK and JAN, at 5.0 mg ml^{-1} by JAN (61.4%).

Tab. 4 Effect of seed treatment with water extracts on tomato seed germination (%) *in vitro*

Extract	Concentration (mg ml^{-1})				
	0.0	2.5	5.0	10.0	20.0
Anabaena sp.	80.2 ± 2.3 A	81.7 ± 2.3 A	94.8 ± 5.5 bD	90.6 ± 1.7 bC	86.8 ± 2.8 aB
Chlorella sp.	79.7 ± 2.1	82.3 ± 5.2	82.2 ± 7.7 a	85.8 ± 4.0 a	83.4 ± 7.8 a
Ecklonia sp.	80.6 ± 2.2 A	83.4 ± 2.5 B	92.3 ± 3.0 bC	92.5 ± 2.8 bC	92.1 ± 2.6 bC
Halopithys sp.	80.0 ± 2.5	84.3 ± 7.2	86.5 ± 7.0 a	82.5 ± 7.0 a	84.3 ± 9.4 a
Jania sp.	80.4 ± 2.6 A	83.4 ± 2.5 B	93.6 ± 1.9 bC	91.7 ± 2.8 bC	83.6 ± 2.7 aB

Extract, concentration and their interaction are significant according to factorial ANOVA ($P < 0.05$). F (4, 250) = 13.0, $P < 0.05$ (for extract factor), F (4, 250) = 37.8, $P < 0.05$ (for concentration factor), F (16, 250) = 4.0, $P < 0.05$ (for interaction). Means (± SD) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences, according to LSD test ($P < 0.05$).

Tab. 5 Effect of seed treatment with water extracts on tomato seedling dry weight (g) *in vitro*

Extract	Concentration (mg ml^{-1})				
	0.0	2.5	5.0	10.0	20.0
Anabaena sp.	0.0071±0.0004 AB	0.0080±0.0007 bB	0.008±0.0003 bB	0.0092±0.0008 bC	0.0069±0.0009 bA
Chlorella sp.	0.0070±0.0009C	0.0059±0.0008 aB	0.0060±0.0007 aB	0.0050±0.0001 aAB	0.0045 ± 0.0004 aA
Ecklonia sp.	0.0070±0.0008 A	0.0083±0.0001 bB	0.0093±0.0015 cB	0.0097±0.0014 bB	0.0076±0.0005 bA
Halopithys sp.	0.0072±0.0006	0.0046±0.0004 a	0.0047±0.0005 a	0.0056±0.0003 a	0.0063±0.0023 ab
Jania sp.	0.0070±0.0007A	0.0087±0.0015 bB	0.0113±0.0008 dC	0.0107±0.0030 bBC	0.0072±0.0006 bA

Extract, concentration and their interaction are significant according to factorial ANOVA ($P < 0.05$).F (4, 100) = 38.8, $P < 0.05$ (for extract factor), F (4, 100) = 6.5, $P < 0.05$ (for concentration factor), F (16, 100) = 5.0, $P < 0.05$ (for interaction). Means (± SD) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences.

3.4 Effect of seed treatment with water extracts against *R. solani in vitro*

To further verify the effect of tomato seed treatment against *R. solani* on tomato seedlings *in vitro*, we inoculated seedlings with the pathogen, 15 days after sowing in PDA, corresponding to the stage of the first true leaf expanded. Two-way ANOVA indicated that the disease index was significantly influenced by extract, concentration and their interaction (Fig. 7). All concentrations of AN, JAN and ECK extracts reduced the disease index with a range of 47.9-56.8%, 47.3-62.2% and 14.0-60.0% respectively, towards to the untreated control. Figure 8 shows the effect of AN at all concentrations on root rot disease. All concentrations of CHL increased significantly disease index, while no

concentrations of HAL showed any significant effect. At 2.5, 5.0 and 10.0 mg ml^{-1} AN and JAN showed the lowest and similar disease index, and also ECK at 20.0 mg ml^{-1}.

Two-way ANOVA of dry weight values indicated that this parameter was influenced by extract, concentrations and their interaction (Tab. 6). Water extracts from AN, JAN and ECK significantly increased the dry weight at all concentrations, except for 20.0 mg ml^{-1}. Extracts from CHL and HAL did not influence dry weight, within each concentration, *Anabaena* sp. at 2.5, 5.0 and 10.0 mg ml^{-1} showed the highest increase by 187.7, 187.7 and 184.0%, respectively.

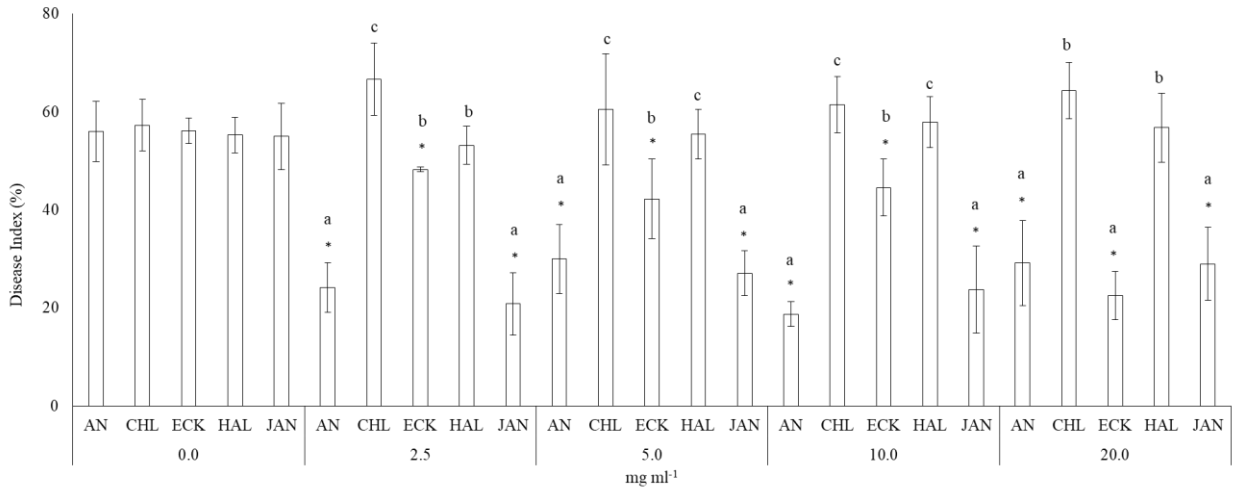

Fig. 7 Effects of tomato seed treatment with different concentrations of water extracts on disease index percentage of *R. solani* on seedling root rot, *in vitro*. AN, *Anabaena* sp.; CHL, *Chlorella* sp.; ECK, *Ecklonia* sp.; HAL, *Halopithys* sp.; JAN, *Jania* sp. Extract factor, concentration factor and treatment × concentration interaction are significant according to two way ANOVA. F (5, 96) = 109.9, $P < 0.05$ (for treatment factor), F (3, 96) = 0.54, $P < 0.05$ (for concentration factor), F (15, 96) = 3.61 $P < 0.05$ (for interaction). Columns are mean values ± SD. The asterisk indicates significant disease index reduction caused by each extract concentration towards the corresponding control (0.0 mg ml^{-1}) and different letters indicate significant differences within each concentration, according to LSD test ($P < 0.05$).

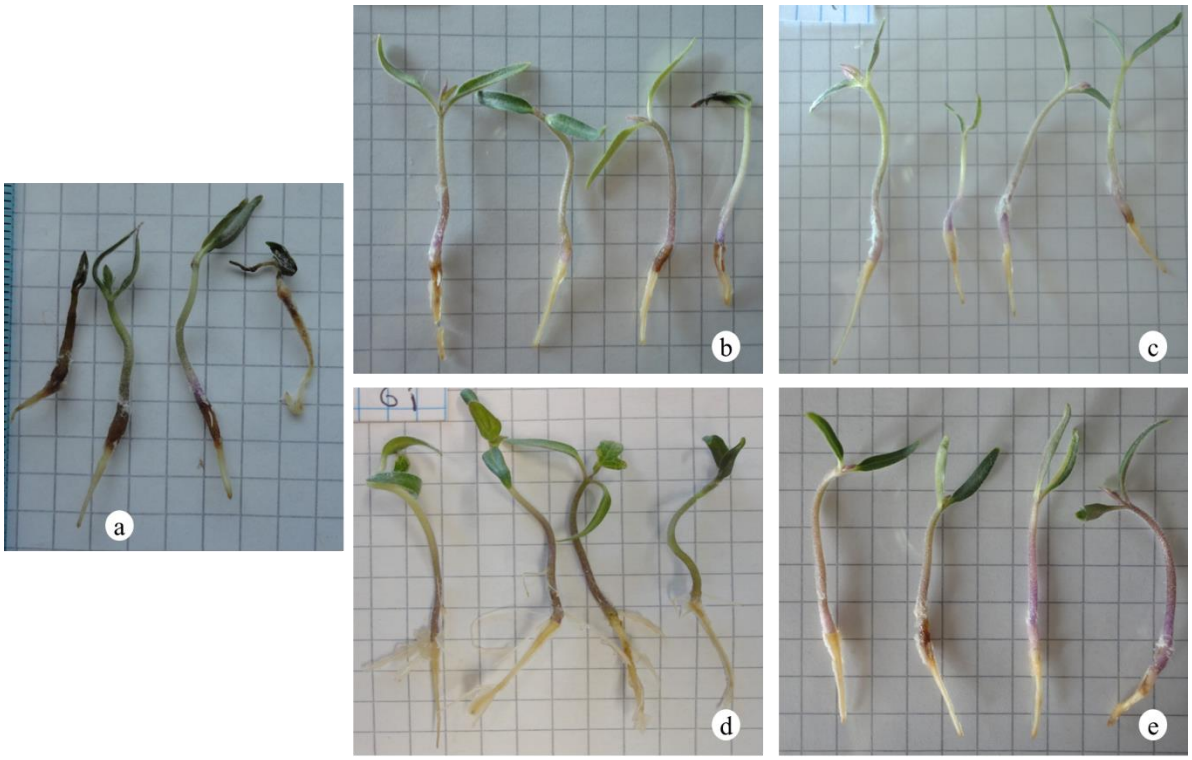

Fig. 8 Effect of seed treatment on tomato seedlings against root rot caused by *Rhizoctonia solani in vitro* infected control (a), treatment with extract from *Anabaena* sp. 2.5 mg ml^{-1} (b), 5.0 mg ml^{-1} (c), 10.0 mg ml^{-1} (d), and 20.0 mg ml^{-1} (e).

Tab. 6 Effects of tomato seed treatment with extracts on dry weight of seedlings (g) infected by *R. solani in vitro*

Extract	Concentration (mg ml^{-1})				
	0.0	2.5	5.0	10.0	20.0
Anabaena sp.	0.0081 ± 0.0010A	0.0233 ± 0.0025 cB	0.0233 ± 0.0017 cB	0.0230 ± 0.0033 cB	0.0079 ± 0.0018 A
Chlorella sp.	0.0081 ± 0.0009	0.0064 ± 0.0013 a	0.0071 ± 0.0010 a	0.0079 ± 0.0018 a	0.0063 ± 0.0018
Ecklonia sp.	0.0079 ± 0.0004 A	0.0105 ± 0.0002 bBC	0.0108 ± 0.0005 bBC	0.0121 ± 0.0037 bC	0.0089 ± 0.0003 AB
Halopithys sp.	0.0079 ± 0.0002	0.0064 ± 0.0025 a	0.0062 ± 0.0017 a	0.0070 ± 0.0021 a	0.0074 ± 0.0025
Jania sp.	0.0081 ± 0.0003 A	0.01110 ± 0.0012 bB	0.0107 ± 0.0015 bB	0.0105 ± 0.0008 abB	0.0080 ± 0.0007 A

Extract, concentration and their interaction are significantl according to factorial ANOVA. F (4, 100) = 116.4, P < 0.05 (for extract factor), F (4, 100) = 31.7, $P < 0.05$ (for concentration factor), F (16, 100) = 17.8, $P < 0.05$ (for interaction). Means (± SD) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences, according to LSD test ($P < 0.05$).

3.5 Effect of seed treatment with water extracts against *R. solani* under greenhouse conditions

Seed treatment with each extract enhanced seedling emergence by an average of 69.0% with respect to infected control without statistically differences among both concentration and extracts (data not

showed). Seed treatment significantly increased both calibre and dry weight of seedlings with respect to the infected control (Fig. 9). Two-way ANOVA indicated that for both the calibre and dry weight, no interaction there was between extract and concentration factors. No differences were observed among extracts for both calibre and dry weight (Fig. 9b, d). All concentrations of the extracts increased significantly the calibre by an average of 40.2% compared to infected control (Fig. 9a). Similarly, all concentrations increased significantly the dry weight by an average of 104.4% compared to infected control (Fig. 9c).

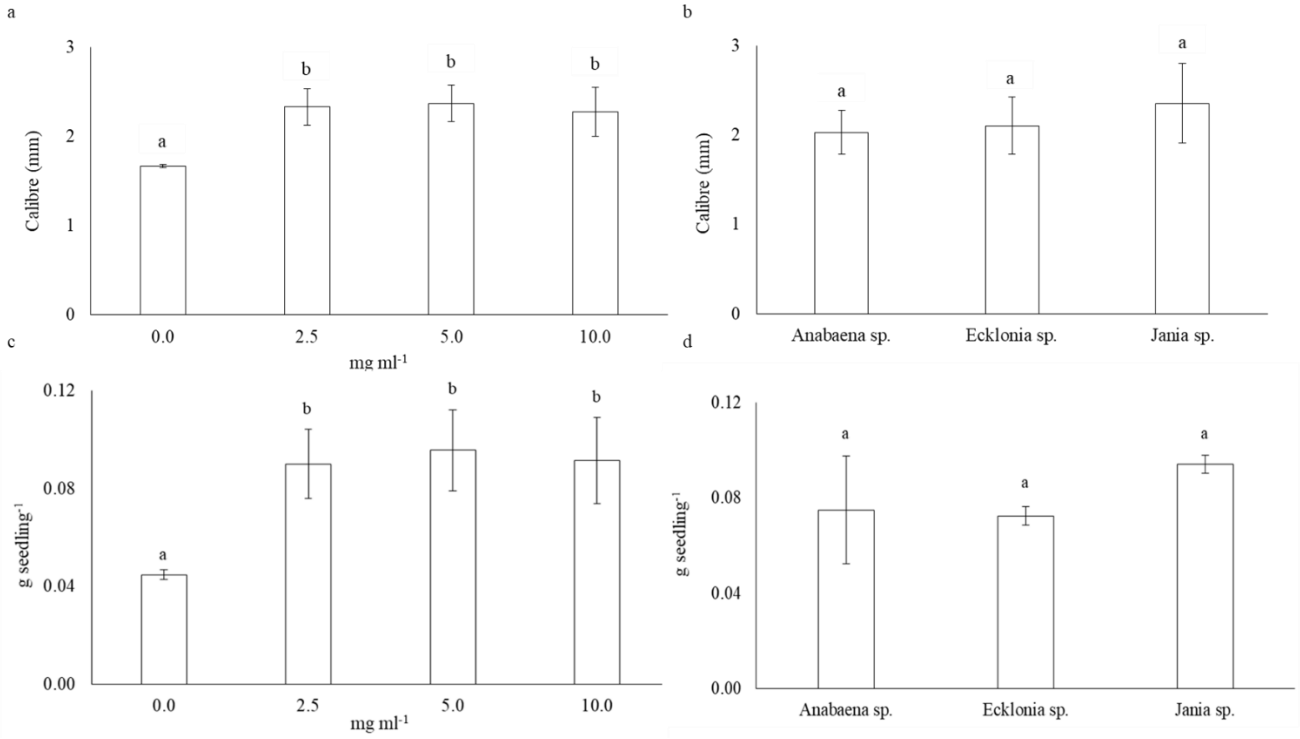

Fig. 9 Effect of tomato seed treatment with extracts on calibre (a, b) and dry weight (c, d) of seedlings grown in substrate infected with *Rhizoctonia solani*. For both calibre and dry weight extract × concentration interaction are not significant according to two way ANOVA ($P < 0.05$). For calibre, F (2, 36) = 13.6, $P < 0.05$ (for extract factor), F (3, 36) = 38.8, $P< 0.05$ (for concentration factor). Columns are mean values ± SD. Columns are mean values ± SD. Different letters indicate significant differences with respect to 0 mg ml^{-1}, according to LSD test ($P < 0.05$). For dry weight, F (2, 36) = 8.5, $P < 0.05$ (for extract factor), F (3, 36) = 26.0, $P< 0.05$ (for concentration factor). Columns are mean values ± SD. Different letters indicate significant differences with respect to the untreated control, according to LSD test ($P < 0.05$).

3.6 Effect of substrate treatment with water extracts against *R. solani* under greenhouse conditions

The effect of substrate treatment with the extracts of AN, JAN and ECK against *R. solani* root rot on tomato plants is shown in Fig. 10. Two-way ANOVA indicated that the disease index was significantly influenced by extract, concentration and their interaction. The three extracts at all

concentrations, except for JAN and ECK at 10.0 mg ml^{-1} significantly reduced disease index with respect to the infected control. Among concentrations, at 10.0 mg ml^{-1} AN showed the lowest disease index (20.2%), while at the other two concentrations, no differences between treatments were observed. Notably, AN at 10.0 mg ml^{-1} reduced disease index by 63.3% compared to infected control. Examining the effect of irrigation with the extracts on dry weight of tomato plants, two-way ANOVA indicated that the dry weight was significantly influenced by extract, concentration and their interaction (Tab. 7). Treatment with AN significantly increased dry weight at all concentrations, by 19.9, 23.2 and 41.1% at 2.5, 5.0 and 10.0 mg ml^{-1} respectively. Treatments with JAN and ECK increased dry weight only at 2.5 mg ml^{-1} by 55.6 and 43.8%, respectively. Within concentrations, the highest dry weight was observed at 2.5 mg ml^{-1} for JAN (2.38 ± 0.25 g) and at 5.0 and 10.0 mg ml^{-1} for AN 1.86 ± 0.15 g and 2.13 ± 0.34 g, respectively.

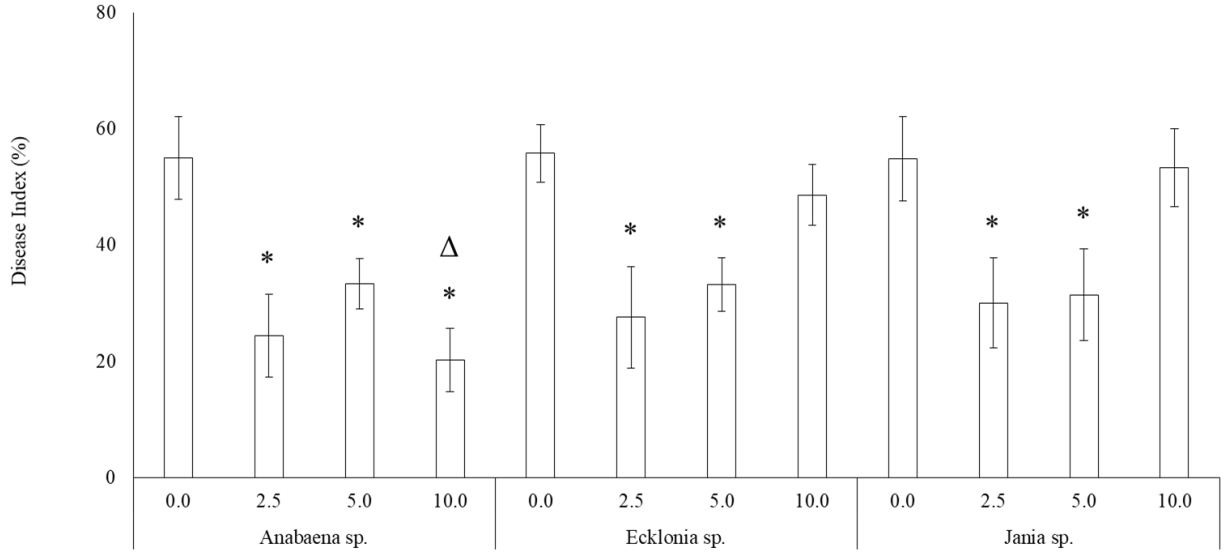

Fig. 10 Effect of substrate treatment with extracts on disease index caused by *Rhizoctonia solani* on tomato seedlings grown under greenhouse conditions. Extract and concnetration factor and treatment × concentrations interaction are significant according to two way ANOVA ($P < 0.05$). F (3, 48) = 40.6, $P < 0.05$ (for extract factor), F (2, 48) = 9.9, $P < 0.05$ (for concentration factor), F (6, 48) = 7.1, $P < 0.05$ (for interaction). Columns are mean values ± SD. The asterisk indicates significant disease index reduction of each treatment concentrations towards 0.0 mg ml^{-1}; Δ indicates significant differences within each concentration, according to LSD test ($P < 0.05$).

Tab. 7 Effect of substrate treatment with water extracts against *Rhizoctonia solani* on tomato seedlings dry weight (g) under greenhouse conditions

Extract	Concentration (mg ml^{-1})			
	0.0	2.5	5.0	10.0
Anabaena sp.	1.51 ± 0.06 A	1.81 ± 0.02 aB	1.86 ± 0.15 bBC	2.13 ± 0.34 bC
Ecklonia sp.	1.53 ± 0.09 A	2.20 ± 0.36 abB	1.46 ± 0.29 aA	1.22 ± 0.08 aA

Jania sp.	1.53 ± 0.06 A	2.38 ± 0.25 bB	1.39 ± 0.21 aA	1.29 ± 0.33 aA

Extract, concentration and their interaction are significant according to factorial ANOVA ($P < 0.05$). F (2, 48) = 4.7, $P < 0.05$ (for extract factor), F (3, 48) = 21.2 $P < 0.05$ (for concnetration factor), F (6, 6) = 9.8, $P < 0.05$ (for interaction). Means (± SD) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences, according to LSD test ($P < 0.05$).

3.7 Chitinase activity of tomato seedlings treated with extracts

As indicated by ANOVA, chitinase activity was significantly influenced by treatment, concentration and their interaction (Fig. 11). It was markedly increased at all concentrations by AN and JAN extracts and by ECK at 2.5 mg ml^{-1}. Within concentrations, the chitinase activity was similarly enhanced by AN and JAN at 5.0 and 10.0 mg ml^{-1}, while no differences were obtained among treatments at 2.5 mg ml^{-1}.

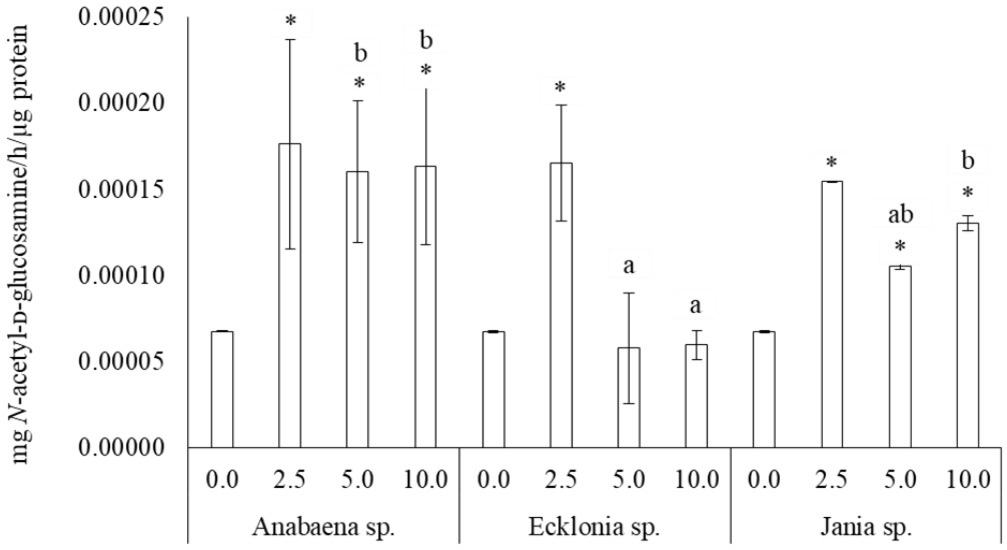

Fig. 11 *N*-acetyl-D-glucosamine activity determined in protein extract of tomato seedlings following seed treatment with water extracts at different concentrations. Extract, concentration and their interaction are significant according to factorial ANOVA ($P < 0.05$). F (2, 36) = 9.9, $P < 0.05$ (for extract factor), F (3, 36) = 16.3 $P < 0.05$ (for concentration factor), F (6, 36) = 2.9, $P < 0.05$ (for interaction). Enzyme activity was defined as the amount of enzyme that liberate 1.0 mg of *N*-acetyl-D-glucosamine from chitin per hour. Columns are mean values ± SD. The asterisk indicates significant differences towards untreated control (0.0 mg ml-1); different letters indicate significant differences within each concentration according to LSD test ($P < 0.05$).

4. Discussion

Algae and cyanobacteria extracts are commonly used on several crops for their capacity to improve plant growth and crop yield (Pereira et al., 2009; Osman, 2010; Khan et al., 2009; Righini et al.,

2018). Moreover, the antifungal activity of the extracts obtained with organic solvents has been widely investigated (biblio), and few papers have examined the activity of water extracts (Roberti et al., 2015; Roberti et al., 2016). In this work, we present data of a first study on the effect of water extracts from species of macroalgae, *Jania*, *Ecklonia* and *Halopithys*, the microalga *Chlorella* and the cyanobacterium *Anabaena* against the soil-borne pathogen *Rhizoctonia solani*. Our experiments showed that some extracts depending on their concentration were active against *R. solani* growth and caused modifications of pathogen hyphal morphology. The extract from *Anabaena* sp., reduced the pathogen colony growth in accordance with Prasanna et al. (2008) on *Fusarium monoliforme*, *F. solani* and *Alternaria solani* following treatment with *A. variabilis* and *A. laxa*. The algal extracts did not reduce fungal colony growth, even if morphological changes, such as increase or reduction of hyphal diameter, presence of cytoplasm coagulation and high vacuolation, and monilioid cells, were observed. No correlation was found between the colony growth and morphological changes. Fungal morphological alterations were induced by the application of essential oils (Zambonelli et al., 1996; de Billerbeck et al., 2001; Romagnoli et al., 2005; Rasooli et al., 2006; Singh et al. 2008). According to what observed by Zambonelli et al. (1996) in *R. solani*, *Pythium ultimum* var. *ultimum*, *Fusarium solani*, *Colletotrichum lindemuthianum*, treatment with essential oils of *Thymus vulgaris*, *Lavandula* and *Mentha piperita*, caused hyphal diameter modifications. These essential oils also caused hyphal wall alterations leading to hyphal degeneration. Treatment with *Cymbopogon nardus* essential oil decreased hyphal diameter of *Aspergillus niger* and induced cytoplasmic granular aspect with vesicular structures and cell wall disruption (de Billerbeck et al., 2001). High presence of vesicles and big vacuoles and swollen hyphae were observed in *Botrytis cinerea* after treatment with the phenol pterostilbene (Xu et al., 2018). We also observed that treatment with water extracts increased the presence of monilioid cells, that fuse together to produce hard structures called sclerotia, which are resistant to environmental extremes, allowing the fungus to survive adverse conditions. The induction of monilioid cells could be indicative of the effect of bioactive compounds in algae and cyanobacteria extracts as observed by Nicoletti et al., (2004) for *R. solani* co-cultured with *Penicillium* isolates producing bioactive compounds.

Seed treatment is a technique ‘‘by which seeds can be led to absorb nutrients, protectants, growth regulators, etc. by immersing them in appropriate solutions for extended periods’’ (Scott, 1989). These substances can enhance several plants parameters, such as seed germination, root development and help seed and the future plant to overcome both abiotic and biotic stresses. (El-Mougy et al., 2012).

In this study, some water extracts of algae and the cyanobacteria *Anabaena* sp. applied as seed treatment reduced the disease index caused by *R. solani* and enhanced the chitinase activity on tomato

seedlings. The ability of the extracts to induce hyphal morphological alterations and to reduce the disease supports the hypothesis that water extracts contain bioactive metabolites that have a significant role in disease control. Moreover, seed treatment is a technique by which seeds can absorb highly substances (Scott, 1989). It is known that algae and cyanocabteria are interesting source of natural products with a broad spectrum of biological activities such as antimicrobial (Burja et al., 2001; Bouhlal et al., 2010), antiviral (Kim and Karadeniz, 2011; Patterson et al., 1996), antifungal (De Felício et al., 2010; Kim, 2006), antiallergic (Na et al., 2005), anticoagulant (Dayong et al., 2008), anticancer (Burja et al., 2001; Kim et al. 2011), antifouling and antioxidant activities (Devi et al. 2011; Ismaiel et al., 2013).

Cyanobacteria are considered one of the most promising source of bioactive compounds. As algae, cyanobacteria produce several molecules such as proteins, vitamins, carbohydrates, amino acids, polysaccharides and phytohormones that are the capacity to both promote plant growth and help them against plant pathogen (Singh, 2014). In addition, lipopeptides produced by cyanobacteria have demonstrated cytotoxic, antitumoral, antiviral, antibiotic activity and some effects as herbicides and antifungal against pathogens (Burja et al., 2000; Patterson et al., 1994).

Among the tested extracts, those from the red alga *Jania* sp., the brown alga *Ecklonia* sp. and *Anabaena* sp., were the most active against the *R. solan*i. The antifungal activity of the red algae *Corallina* sp. was showed against powdery mildew on zucchini cotyledons by preventive spray treatment (Roberti et al., 2016). Moreover, preventive and curative treatments on strawberry with extracts from *Porphyra umbilicalis*, strongly suppressed the disease severity caused by *B. cinerea* (De Corato et al., 2017). The antifungal activity showed by the extract from *Jania* sp., along with the increase of chitinase activity in tomato tissues, can be related to the presence of sulphated galactans, such as carrageenans, synthesized by red seaweeds, which are the major components of the extracellular matrix (Damonte et al., 2004; Aruna et al., 2010; Matsuhiro et al., 2005; Pujol et al., 2006; Souza et al., 2012). These polysaccharides are elicitors of plant defence responses against pathogens, such in case of *Phytophtora parasitica* var.*nicotianae* on tobacco plants (Mercier et al., 2001). On tomato seedlings infected by *Macrophomina phaseolina*, Agarwal et al. (2016) demonstrated that an extract from the red algae *Kappaphycus* a*lvarezii* increased the transcription of pathogenesis-related genes such as PR-1b1, PR-3, and PR-4 and the endogenous concentration of salicylic acid and the cytokinin zeatin. It is known that a high concentration of cytokinin leads to salicylic acid accumulation and activation of defence gene expression (Argueso et al., 2012).

Concerning the brown algae, some authors reported their antitumor, hypoglycaemic and anti-inflammatory activities (Koyanagi et al., 2003, Synytsya et al., 2010; Kang et al., 2011). Rekanović et al., (2010) showed the antifungal activity of *E. maxima* applied to the soil against *Verticillium* wilt

on pepper. *Ecklonia* sp. extract sprayed on zucchini cotyledons reduced the sporulation of *Podosphaera xanthii*, agent of powdery mildew (Roberti et al., 2016). As for red algae, also polysaccharides extracted from brown algae are known to be elicitors of plant responses against pathogens. For example, sulphated fucans, present in the cell walls of marine brown algae, applied on tobacco leaves induced locally accumulation of salicylic acid and of the phytoalexin scopoletin. Moreover, after the treatment, pathogenesis-related proteins were expressed in addition to systemic accumulation of SA (Klarzynski et al., 2003). Other brown alga extracts showed to induce resistance in plants by enhancing the activities of various defence-related enzymes and genes. For instance, extract from *Ascophyllum nodosum* applied as spray treatment and/or root drench on cucumber plants, enhanced activities of various defence-related enzymes such as chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, and lipoxygenase, enhanced levels of phenolic compounds, and reduced disease incidence caused by *F. oxysporum*, *Alternaria cucumerinum* and *B. cinerea* (Jayaraman et al., 2011). Among these enzymes, chitinases and β-1,3-glucanases are capable of degrade the fungal cell wall components and their increase is considered a molecular marker of induced resistance (Van Loon et al., 2006; Roberti et al., 2015).

As above mentioned, *Anabaena* sp. was effective in reducing disease index and increasing chitinase activity in tomato seedlings. Our results are in accordance with what we have previously observed in zucchini cotyledons after spray application of water extract from the same *Anabaena* strain (Roberti et al., 2015). Indeed, the treatment provoked an increase of chitinase, β-1,3-glucanase and peroxidase activities in cotyledon tissue. Moreover, this cyanobacterium showed a direct antifungal activity against *P. xanthii* sporulation. On tomato seedlings, *Anabaena variabilis* and *A. oscillarioides* applied by soil treatment decreased disease severity caused by *Pythium debaryanum, Fusarium oxysporum lycopersici*, *Fusarium moniliforme* and *Rhizoctonia solani* (Chaudhary et al., 2012). In this study, consistently with what obtained by these authors, the treatment with *Anabaena* sp. by both seed and substrate application increased plant weight. In addition, *Anabaena* sp., as well as the extracts from *Ecklonia* sp. and *Jania* sp., increased seed germination in infected substrate with *R. solani* and reduced disease symptoms, in accordance to what observed by Prasanna et al., (2013) on tomato with *A. variabilis* and *A. laxa* in soil inoculated with *Fusarium oxysporum* f.sp. *lycopersici*. These authors showed also an increase of defence enzymes activities such as PAL, PPO, chitosanase, and β-1,3 glucanase.

Algae and cyanobacteria extracts were extensively studied for their beneficial effects on plant (Craigie, 2011; Obana et al., 2007; Prasanna et al., 2013; Essa et al., 2015; Righini et al., 2018; Calvo et al., 2014). They can enhance several plants parameters, such as seed germination, root development and help seed and the future plant to overcome both abiotic and biotic stresses. (El- Mougy et al.,

2012). The application of extracts by seed treatment can led to optimal absorption of nutrients and growth regulators (Scott, 1989). *Ecklonia* sp., *Jania* sp. and *Anabaena* sp. extracts applied by seed soaking increased tomato seed germination. Similarly, Sivasankari et al. (2006) obtained a remarkable increase of black-eyed pea seed germination after treatment with extract from *Sargassum wightii*. Bean seed soaking in extract from *A. nodosum* gave high seedlings emergence (Carvalho et al., 2013). Indeed, extracts from brown algae are rich of many of the common higher plant hormones such as abscisic acid, auxins, cytokinins and other plant growth regulators such as brassinosteroids that can affect seed and plant metabolism, influencing their growth and development (Khan et al., 2009; Craigie, 2011; Stirk et al., 2014). Among hormones, gibberellins influence most plant growth and development including promoting seed germination, organ differentiation, shoot growth, stem elongation, leaf expansion, floral development and fruit set (Tanimoto, 2002; Yamaguchi, 2008). Concerning soil application, Crouch and Van Staden (1992) demonstrated on tomato plants that *E. maxima* increased fresh weight, leaf surface area and number of flowers more than spray treatment. To our knowledge, no study has been carried out on biostimulant effect of red algae applied by seed treatment. On tomato foliar application of *K.* a*lvarezii* improved growth and yield (Zodape et al., 2009; Zodape et al., 2011). Spray treatment with extract from *Jania rubens* increased shoot dry weight, shoot length, root dry weight and root length of chickpea plants (Latef et al., 2017). Moreover, the treatment enhanced photosynthetic pigments such chlorophyll *a, b* and carotenoids and increased enzymatic antioxidant activities in fresh leaves, decreasing H_2O_2 and malondialdehyde contents. Soil application of *Jania rubens* alone or mixed with other two red algae, *Laurencia obtusa* and *Corallina elongata* increased shoot and root length, leaves number, fresh and dry weight of maize *(*Safinaz and Ragaa, 2013). Biostimulant effect after soil application was also showed for *Gracilaria caudata* and *Palisada perforate* on *Pisum sativum* (Duarte et al., 2018). The effects of the red alga are related to their content in glycine betaine, plant growth regulators, carrageenans, phenolic compounds, micro nutrients that play a major role in improving crop productivity in addition to enhance abiotic stress tolerance (Prasad et al., 2010; Trivedi et al., 2018).

Concerning cyanobacteria, it is known that they are able to produce growth-promoting substances such as auxins, cytokinins, and gibberellins (Serdyuk et al., 1992; Stirk, et al., 1999; Singh et al., 2014). In particular, *Nostoc carneum* and *N. commune* produce the auxin indole-3-acetic acid (Chittapun et al., 2018), while *Anabena vaginicola* and *Nostoc calcicola* produce mainly the auxin indole-3-butyric acid (Hashtroudi et al., 2012). Indeed, in line with our results, some authors reported biostimulant effects on several crops. For example, *Anabaena variabilis* extract applied on seed of *Hordeum vulgare* L. and *Trigonella foenum-graecum* or in soil caused an increase of seed germination, as well as of shoot length, fresh and dry weight of seedlings (Ismail and Abo-Hamad,

2017). On wheat, *A. variabilis* applied on seed increased germination rate and stimulated the growth of the plants (Kumar and Kaur, 2014). Soaking seeds of *Sorghum durra* and *Helianthus annuus* with *Anabaena oryzae* extract increased seed germination, shoot and root length and fresh weight (Essa et al., 2015). One report on tomato, showed that soil application of *Anabaena* sp. and *Nostoc* sp. enhanced root length and fresh weight, plant height, root dry weight and both stem and leaf dry weight, and the presence of indole-3-acetic acid and indole-3-butyric acid in the cyanobacteria biomass (Shariatmadari et al., 2013).

In conclusion, this study shows that seed treatment with water extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. were active against R. solani root rot, by working both directly to the pathogen and indirectly through the involvement of plant defence responses helping plant to withstand the pathogen. Once these effects will be verified on tomato 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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3. Antifungal activity of water extracts from algae and cyanobacteria against *Fusarium oxysporum* f.sp. *lycopersici*

The effect of water extracts from the macroalgae *Jania* sp. and *Ecklonia* sp. and the cyanobacterium *Anabaena* sp. against the soil-borne pathogen *Fusarium oxysporum* f. sp. *lycopersici* was evaluated *in vitro* and *in vivo*. The extracts were tested on fungal colony growth and hyphal morphology at three concentrations, 2.5, 5.0 and 10.0 mg of dry biomass per ml of water. Each extract was also tested against *Fusarium oxysporum* f. sp. *lycopersici* disease on tomato seedlings by application on tomato seed and in growing substrate under greenhouse conditions. In plate assay, fungal colony growth was not affected by any extract, while in 96 plate wells assay, it was reduced by all extracts. Hyphal diameter was increased by *Jania* sp. at all concentrations, by *Ecklonia* sp. at 2.5 and 10.0 mg ml^{-1} and by *Anabaena* sp. at 10.0 mg ml^{-1}. Cytoplasm coagulation was observed for all extract at all concentrations. Increase or reduction of microspore dimensions were showed by *Anabaena* sp. and *Ecklonia* sp., depending on their concentration. On tomato seedlings, extract from *Ecklonia* sp. applied as seed treatment and seed treatment combined with substrate treatment reduced disease index at all concentrations. *Anabaena* sp. reduced the disease at 10.0 mg ml^{-1} in both treatments while extract from *Jania* sp. only by seed + substrate treatment at 10.0 mg ml^{-1}. In the absence of the pathogen, all concentrations of *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. increased tomato seedling emergence. Extract from *Jania* sp. at all concentrations significantly increased the dry weight, while *Anabaena* sp. increased dry weight only at 10.0 mg ml^{-1}. Treatment with *Ecklonia* sp. did not show any effect at all concentrations.

1. Introduction

Fusarium oxysporum f. sp. *lycopersici* ((Sacc.) W.C. Snyder and H.N. Hansen) is the causal agent of tomato vascular wilt and it has been known since the beginning of the twentieth century in many tomato growing regions (Walker, 1971). Authors reported that losses in yield can vary from 10 to 90% depending on the stage of the plant and on the environmental conditions (Singh and Kamal 2012). The present distribution of the pathogen in the world is showed in Fig. 1. Species of *Fusarium oxysporum* strains infect many others important horticultural crops such as peppers, watermelon, carnation and bananas (Takken and Rep, 2010; Velarde-Félix et al., 2018; Egel and Martin, 2007; Kyounge et al., 2001; Zheng et al., 2018). The pathogen is soil transmitted and plants can be infected in any growing stage. Infected seedlings wilt and die soon after symptoms appear. In older plants stunting, chlorosis and leaf epinasty are often followed by wilting of young leaves and defoliation of remaining leaves, leading to plant death. The roots can be infected directly through the root tips,

through wounds in the roots or at the formation point of lateral roots. Mycelium enters the xylem vessels, branches and produces micro-spores which are carried upward in the sap stream. Due to the growth of the fungus within the plant's vascular tissue, the plant's water supply is greatly affected. This lack of water induces the leaves stomata to close, the leaves wilt and the plant eventually dies. Infection usually occurred on plants in the form of chlorosis, leaf wilting and browning of the vascular system. Cutting longitudinal sections at the base of the stem reveals a dark-brown to red discoloration of xylem.

Nowadays, *F*. *oxysporum* f, sp. *lycopersici* race1, race 2 and race 3 are known on the basis of pathogen virulence to a set of differential cultivars within the same plant species (Armstrong and Armstrong, 1981). Race 1 was prevalent at the beginning of the last century. After the discovery of single-gene (*I*) resistance in *Lycopersicon* species (Bohn and Tucker, 1939) and its subsequent use in many tomato cultivars race 2 arose through mutation apparently selected by the extensive use of resistant cultivars of tomato (Alexander and Tucker, 1945). In tomato growing areas of Florida, race 2 has become widespread since 1961 (Jones and Litrell1965), and the resistance of race 3 was firstly reported (Volin and Jones, 1982). Race 3 is currently present in the United States, Australia, Mexico and Brazil (Bost, 2001; Chellemi and Dankers, 1992; Marlatt et al., 1996; Valenzuela-Ureta et al., 1996; Gale et al., 2003; Reis et al., 2005).

Colour of the fungal colony can vary from white to violet and red, as showed in Fig. 2. The fungus produces three types of asexual spores: macrospores, microspores and chlamydospores. Macrospores are thin-walled with a definite foot cell and a pointed apical cell produced mostly on branched conidiophores in sporodochia on the surface of infected parts of the plant, or produced singly in the aerial mycelium in culture. Microspores occur on short conidiophores in the aerial mycelium and are commonly produced in the xylem vessels during the host infection. Chlamydospores are thick-walled asexual spores that are usually produced singly or intercalary or terminal in the hyphae. These spores can survive for an extended time in plant debris and in soil. Life cycle of *F*. *oxysporum* f. sp. *lycopersici* is showed in Fig. 3.

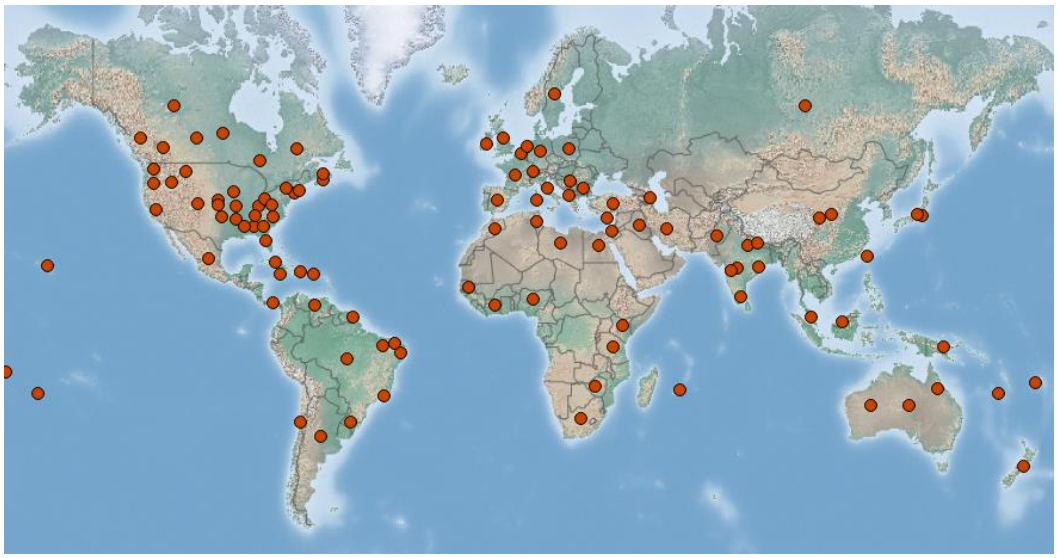

Fig. 1 World distribution map of *F. oxysporum* f. sp. *lycopersici*. (CABI, 2018).

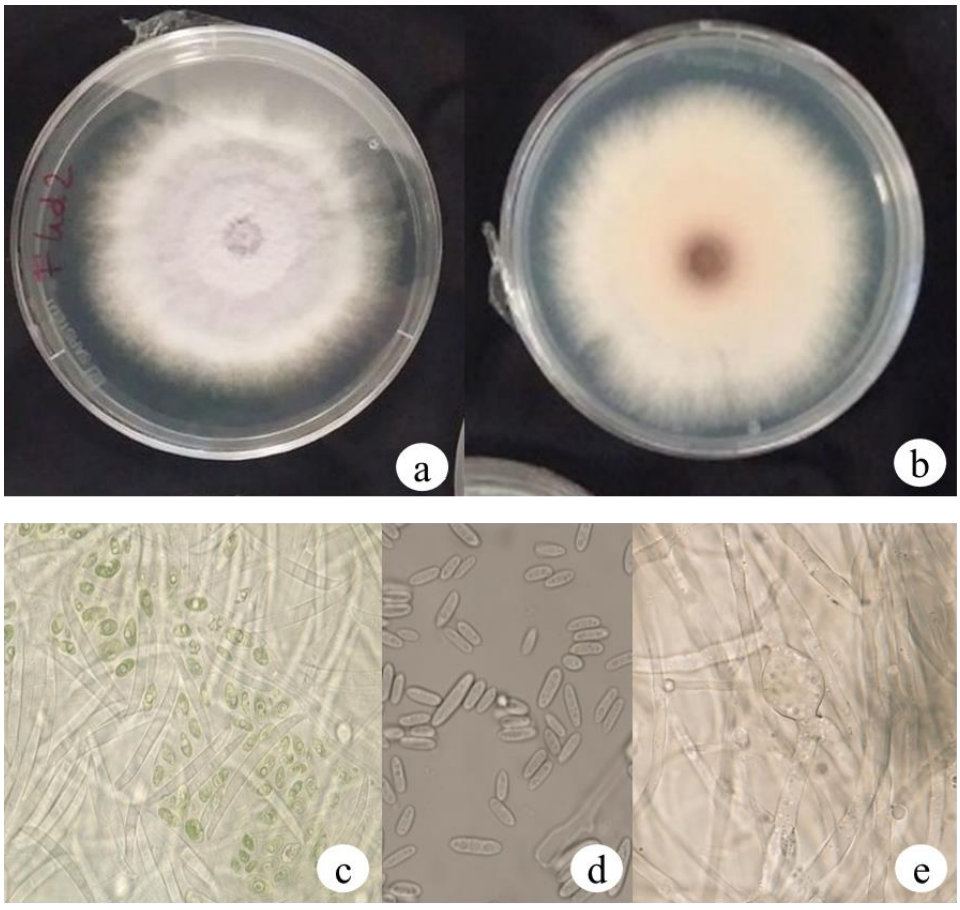

Fig. 2 *Fusarium oxysporum* f. sp. *lycopersici* colony, upper (a) and bottom side (b), and macrospores (c), microspores (d) and chlamydospores (e) at 600 magnification.

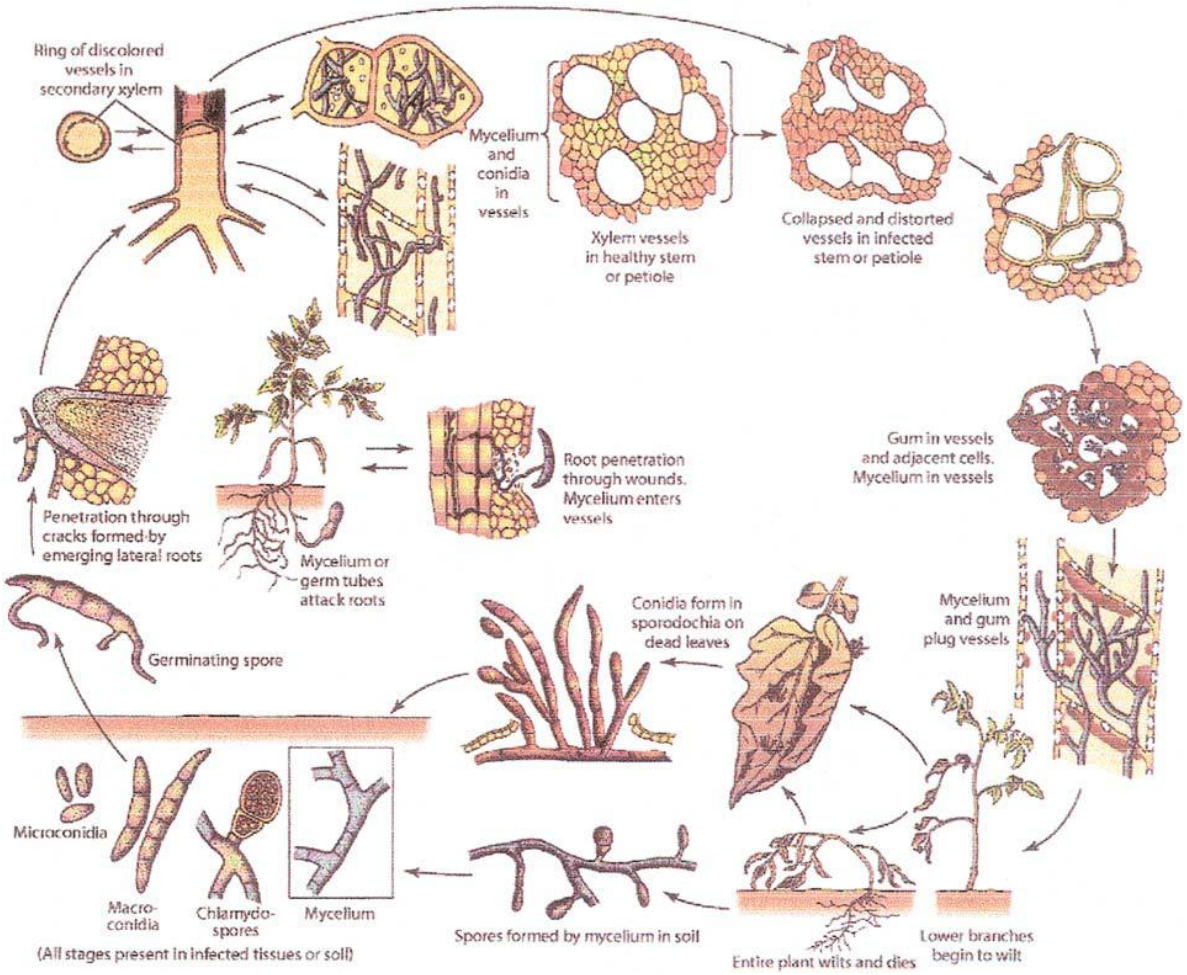

Fig. 3 Life cycle of *F. oxysporum* f, sp. *lycopersici* (Agrios, 2005).

The development of bio-based strategies to control plant pathogens and enhance food safety has encouraged by the Directive 2009/128/EC and its implementation that promote specific actions to support the establishment of sustainable agriculture. Several researches were focused on the activity against plant pathogens of extracts obtained from algae and cyanobacteria by using organic solvents (Rizvi and Shameel, 2004; Kumar et al., 2008; Arunkumar et al., 2010; Sivakumar, 2014; Jiménez et al., 2011), while few studies have examined the antifungal activity of water extracts (Roberti et al., 2015; Righini et al., 2018). Therefore, algae and cyanobacteria could be considered useful tools, alternative to synthetic products, for their role in plant disease control.

The objectives of the present work are to study the effect of water extracts from the algae *Ecklonia* sp. and *Jania* sp. and the cyanobacterium *Anabaena* sp. on: (i) *F. oxysporum* f. sp. *lycopersici* colony growth, hyphal and spore morphology; (ii) possible phytotoxicity or plant biostimulation; (iii) induction of plant defence responses against the pathogen on tomato plants grown under greenhouse conditions.

2. Materials and methods

2.1 Water extracts and pathogen

Lyophilized *Anabaena* sp. and dry thallus of *Ecklonia* sp. and *Jania* sp. were provided by the Spanish Bank of Algae, Marine Biotechnology Center, University of Las Palmas de Gran Canaria. Dry thallus was grounded to fine powder with mortar and pestle (Tab. 1). Water extracts were obtained by suspending each powder in sterile distilled water (0.5%) under continuous stirring at 50°C for 12 h, then filtered (Roberti et al., 2015). The concentrations considered were 2.5, 5 and 10 mg ml^{-1}.
The plant pathogenic fungus *F. oxysporum* f. sp. *lycopersici* race 1 (FOXL1) was used through the study, and was gently provided by CRA-PAV (Roma).

Tab. 1 Genus, phylum (www.algaebase.org) and abbreviation of algae and the cyanobacterium used in the experiments.

Genera	Phylum	Abbreviation
Anabaena sp.	*Cyanobacteria*	AN
Ecklonia sp.	*Heterokontophyta*	ECK
Jania sp.	*Rhodophyta*	JAN

2.2 Effects of water extracts on fungal growth, and on the morphology of hyphae and spores

Fungal growth was determined by plate assay and by spectrophotometric reading. For plate assay, portions of 7 mm diameter were cut from 10-day-old colony and transferred in test-tube containing a 600-µl aliquot of each extract at four concentrations (2.5, 5.0, 10.0 and 20.0 mg ml^{-1}). Sterile distilled water was used as control. Three dishes (replicates) were used for each concentration, and for the control. After 6 h of treatment, colony portions were placed on PDA medium in Petri dish and incubated at 24 °C in the dark for 7 days. Colony diameters were measured daily along two perpendicular axes. The effect of the extracts on FOXL1 hyphae and cytoplasm coagulation was evaluated 10 days after treatment. Colony portions from treated and untreated colony were observed by using an Eclipse TE2000-E microscope (Nikon Instruments Europe BV, Amsterdam, The Netherlands) at ×600 magnification. Hyphal diameter was measured (8 replicates for each concentration and the control). For cytoplasm coagulation three colony portions were evaluated by using the following scale: 1, 0-10% of coagulation; 2, 11-40% of coagulation; 3, 41-80% and 4, > 80% of coagulation. The assay was repeated once.

For spectrophotometric reading of fungal growth, the assay was performed in 96 well cell culture plate (Greiner bio-one Cell STAR®). A microspore suspension of FOXL1 was prepared from 15-day-old culture in 1.2 % malt extract (ME) (Difco, Laboratories, Detroit, USA). In each well a mixture of FOXL1 spore suspension (final concentration 2×10^3 microspores/ml) and each extracts (final concentration 2.5, 5.0, 10.0 and 20.0 mg ml^{-1}) were poured. Control consisted in ME + each extract concentration. Fungal growth was evaluated as increase of absorbance at 620 nm with a microplate reader (Tecan, NanoQuant, Infinite M200PRO, GmbH, Austria) at 0 h and after 24 and 48 h of incubation at 25 °C in the dark. Four wells (replicates) per treatment were considered. At the end of the incubation, fungal samples from each well were observed by using a Nikon ECLIPSE TE2000 E microscope (Nikon Instruments Europe BV, Amsterdam, The Netherlands) at 600 magnification, and length and width of non-germinated microspores were measured with the Nikon NIS Elements AR 2.20 software. Images were captured with a Nikon DXM1200F digital camera. Eight spores were considered for both measurements for each extract concentration and the untreated control. The experiment was repeated once.

2.3 Effect of seed and substrate treatment with water extracts under greenhouse conditions

The in vivo experiments were conducted on tomato plants (cv. Marmande), in a greenhouse at 26 ± 2 °C, 70% relative humidity, under daylight conditions (light: dark 16: 8). For the treatments, the extracts were prepared at the concentration of 10.0, 5.0 and 2.5 mg ml^{-1}. The extracts were applied both by seed treatment and by seed treatment + substrate treatment. For seed treatment, seeds were treated by immersion overnight in the extracts at each concentration, in sterile test-tubes. Water was used as control. After treatment, seeds were rinsed two times in sterile distilled water and sown in pots filled with a growing substrate composed by a mixture of peat moss and sand (7: 3 w: w). Fifteen days after treatment the percentage of seedling emergence was recorded and seedlings were maintained regularly irrigated until the stage of 2nd true leaf. At transplant, seedlings were divided in two batches: 1) seedlings transplanted in growing substrate inoculated with FOXL1; 2) seedlings transplanted in non-inoculated substrate. In both batches, transplanted seedlings were established in two groups: a) irrigated with the extracts (substrate treatment); b) irrigated with water only.

In the experiment 1, 15-day-old FOXL1 colonies were blended with sterile distilled water and mixed with the substrate. Seedlings were transplanted in pots (Ø 16 cm, h 15 cm; 3 seedlings per pot) filled with 600 g of substrate inoculated with FOXL1 (2.0 % w: w pathogen: substrate). Substrate treatment consisted in a first irrigation with 75 ml of each extract concentration (experiment 1a) or water (experiment 1b) per pot at the transplant. Controls consisted in tomato seedlings from untreated seeds,

transplanted in inoculated substrate and irrigated with water (75 ml per pot). Three pots (replicates) were used for both the treatments and the control. Forty days after transplant, yellowing and wilt symptoms of plants were visually assessed by using a five-point scale, where: 0, absence of yellow leaves; 1, one foliar branch with yellowing; 2, two foliar branches with yellowing and wilting; 3, three-four foliar branches with yellowing and wilting; 4, more than four foliar branches with yellowing and wilting.

In the experiment 2, seedlings were transplanted in glass tubes (Ø 4 cm, h 18 cm) each filled with 100 ml of growing substrate (one seedling per tube, three tubes per concentration) and irrigated with 12.5 ml of each concentration of extracts (experiment 2a) or water (experiment 2b) per tube. Controls consisted in tomato seedlings from untreated seeds, and irrigated with water (12.2 ml per tube). After two weeks, plants were harvested and washed under tap water. Plant height was measured, then plants were dried in a hoven at 60 °C for 72 h for dry weight determination.

Fig. 4 Tomato seedling transplanted in substrate inoculated with *F. oxysporum* f. sp. *lycopersici* (a) and in non-inoculated substrate (b).

2.4 Statistical analysis

All experiments were arranged in a complete randomized design. Data obtained from *in vitro* and *in vivo* experiments were analyzed by factorial ANOVA, and means were separated by Fisher's least significant difference (LSD) test ($P < 0.05$). Factors for ANOVA analyses were: extracts, their concentrations, methods of treatment (seed or seed+ substrate). All analyses were performed with GraphPad Prism software, version 4.03, 2005.

3. Results

3.1 Effects of water extracts on fungal growth, and on the morphology of hyphae and spores

The effects of the extracts on FOXL1 colony growth in plate assay after two days after treatments are shown in Fig. 5. Two-way ANOVA of colony growth indicated significant differences only among

the extract concentrations. Colony growth was increased by all concentrations with respect to untreated control (0 mg ml^{-1}) 2 days after treatment, while 3 days after no differences were found (data not showed). In Tab. 2, the effect of the extracts on fungal morphology is shown. Two-way ANOVA of hyphal diameter indicated a significant interaction between the two factors (treatment and concentration). *Ecklonia* sp. and *Jania* sp. significantly increased hyphal diameter at all concentrations with respect to the untreated control in a range of 20.6 - 42.4%, except for *Ecklonia* sp. at 2.5 mg ml^{-1}. *Anabaena* sp. significantly increased hyphal diameter only at 10.0 mg ml^{-1} (27.2 %). All treatments caused a significant increase of cytoplasm coagulation index depending on the concentration, with respect to the control. *Anabaena* sp. treatment showed the highest increases of cytoplasm coagulation at 2.5 and 5.0 mg ml^{-1} by 280.0 and 300.0%, respectively. At 10.0 mg ml^{-1} the index of AN was higher than the index of JAN and significantly similar to that of ECK.

In the Fig. 6 the mycelium of FOXL1 showed an homogeneous and clear cytoplasm in the untreated control, cytoplasm coagulation after treatment with the extracts, particularly induced by AN, and hyphal swelling induced by JAN.

Absorbance reading of fungal growth after 24 h of incubation with each extract at different concentrations showed that only by *Anabaena* significantly inhibited fungal growth sp. at 5.0 and 10.0 mg ml^{-1}, 8.2% and 11.6% respectively, whereas the other two extracts did not showed any effect. After 48 h, all the extracts except for ECK at 2.5 mg ml^{-1} reduced the absorbance (Fig. 7). Extract from AN reduced the absorbance at the three concentrations in the same way, and ECK reduced absorbance at 5.0 and 10.0 mg ml^{-1} with similar values. Extract from JAN progressively reduced the absorbance from 2.5 to 10.0 mg ml^{-1}. Within concentrations, at 10.0 mg ml^{-1} JAN reduced absorbance more than the other two extracts (53.0% of absorbance reduction with respect to the control).

The effect of extracts on FOXL1 microspores dimension after 48 of incubation is shown in Tab. 3. Two-way ANOVA of microspores length indicated a significant interaction between the two factors (extract and concentration). Extracts caused reduction, stimulation of microspores dimension, or no effect. *Anabaena* sp. increased the microspores length at all concentrations by an average of 51.1%. *Ecklonia* sp. reduced microspore length at 2.5 and 5.0 mg ml^{-1} (30.3 and 29.3%, respectively), while at 10.0 mg ml^{-1} the extract increased length by 24.2%. Within concentration, length was increased mostly by AN. With regard to microspores width determination, two extract showed an effect, AN and ECK that reduced width by 11.8% at 10.0 mg ml^{-1}, and by 29.4% at 2.5 mg ml^{-1}, respectively. *Ecklonia* sp. increased by 17.6% width value at 10.0 mg ml^{-1}. Within concentration, AN increased microspores width at 2.5 mg ml^{-1} both respect to ECK and JAN, while it reduced width at 10.0 mg ml^{-1} with respect to ECK.

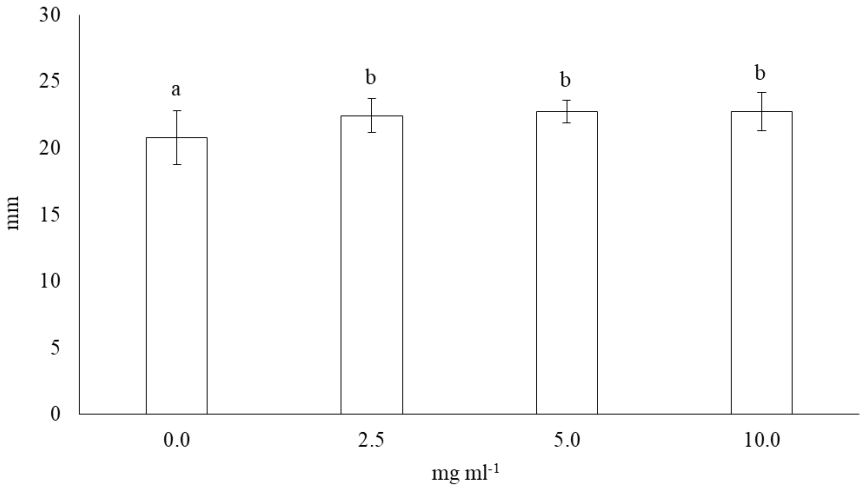

Fig. 5 Effect of different concentrations of extracts of *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. on colony growth of *Fusarium oxysporum* f. sp. *lycopersici*, 48 hours after incubation on PDA. Concentration factor is significant, whereas extract factor and extract × concentration interaction are not significant according to two way ANOVA ($P < 0.05$). F (3, 36) = 4.1, $P < 0.05$ for concentration factor. Columns are mean values ± SD. Different letters indicate significant differences according to LSD test ($P < 0.05$).

Tab. 2 Effect of different concentrations of extracts on hyphal diameter (µm) and cytoplasm coagulation index (0-4) of *Fusarium oxysporum* f. sp. *lycopersici* mycelium after incubation for 48 hours on PDA.

Extract	Concentration (mg ml^{-1})			
	0.0	2.5	5.0	10.0
Hyphal diameter				
Anabaena sp.	3.3 ± 0.4 A	3.5 ± 0.4 bA	3.6 ± 0.5 aA[a]	4.2 ± 0.7 B
Ecklonia sp.	3.4 ± 0.5 A	3.0 ± 0.3 aA	4.1 ± 0.6 abB	4.4 ± 0.5 B*
Jania sp.	3.3 ± 0.5 A	4.4 ± 0.5 cB	4.5 ± 0.7 bB	4.7 ± 0.8 B
Cytoplasm coagulation index				
Anabaena sp.	1.0 ± 0.0 A	3.8 ± 0.3 bB	4.0 ± 0.0 bB	4.0 ± 0.0 bB
Ecklonia sp.	1.2 ± 0.3 A	2.0 ± 0.0 aB	2.0 ± 0.0 aB	3.8 ± 0.3 bC
Jania sp.	1.0 ± 0.0 A	2.0 ± 0.0 aB	2.0 ± 0.0 aB	2.3 ± 0.6 aB

[a] presence of chlamydospores

For both hyphal diameter and cytoplasm coagulation, extract and concentration factors and their interaction are significant according to factorial ANOVA ($P < 0.05$). For hyphal diameter, F (2, 96) = 10.8, $P < 0.05$ (for extract factor), F (3, 96) =19.0, $P < 0.05$ (for concentration factor), F (6, 96) = 3.5, $P < 0.05$ (for interaction). For cytoplasm coagulation, F (2, 36) =122.7, $P < 0.05$ (for extract factor), F (3, 36) =178.8, $P < 0.05$ (for concentration factor), F (6, 36) =27.1, $P < 0.05$ (for interaction). Means (± SD) followed by different lower-case letters in a column and by different upper-case letters

in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences.

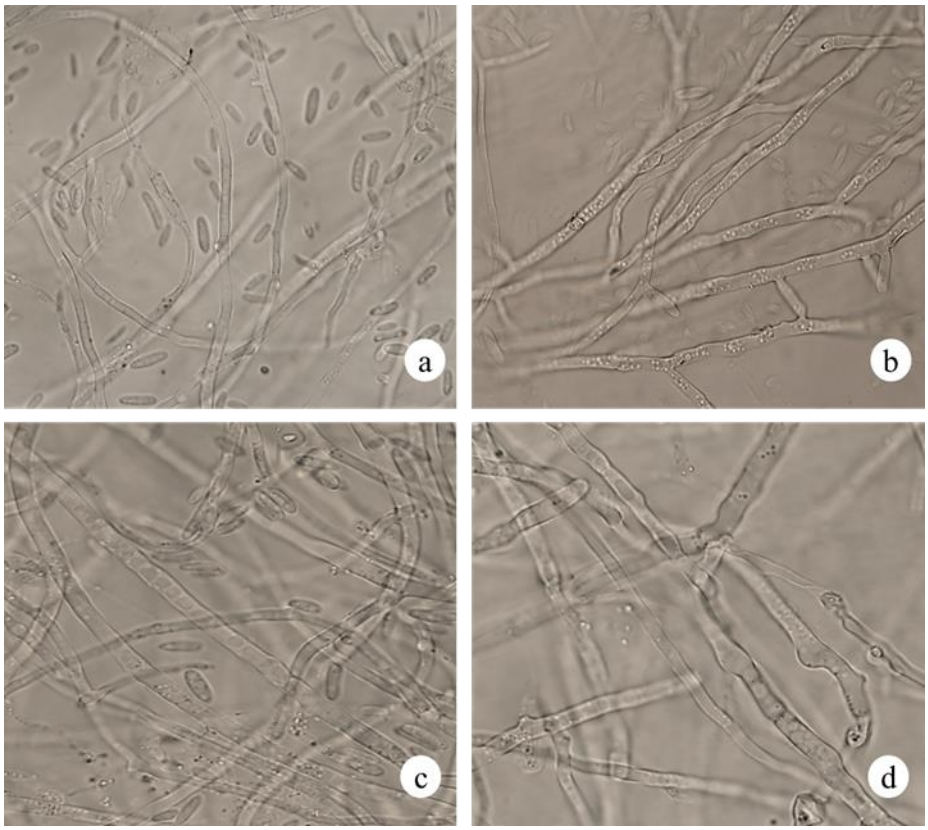

Fig. 6. Morphological alterations of *Fusarium oxysporum* f. sp.*lycopersici* mycelium grown on PDA for 48 hours after treatment with extracts: untreated control (a); cytoplasm coagulation and presence of vacuoles caused by 5.0 mg ml^{-1} of *Anabaena* sp. (b), *Ecklonia* sp. (c), and *Jania* sp. (d).

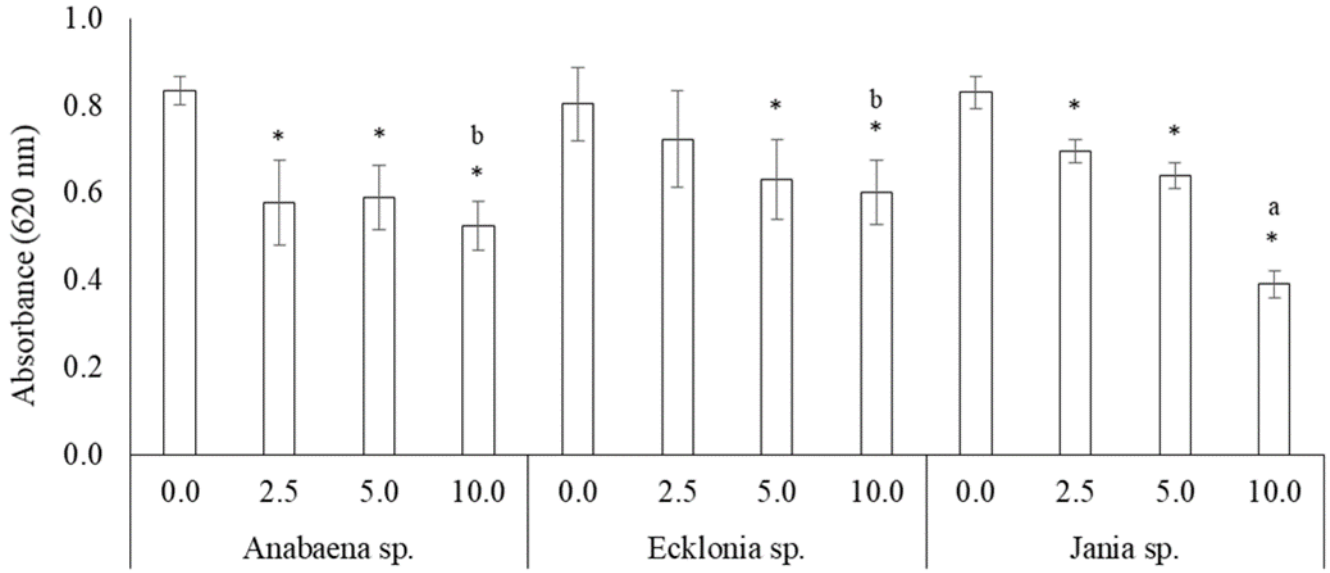

Fig. 7 Effect of different concentrations (mg ml^{-1}) of extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. on absorbance of *Fusarium oxysporum* f.sp. *lycopersici* growth , 48 hours after incubation.

Extract and concentration factors and their interaction are significant, according to factorial ANOVA ($P < 0.05$). F (2, 48) = 3.8, $P < 0.05$ (for extract factor), F (3, 48) = 31.6, $P < 0.001$ (for concentration factor); F (6, 48) = 8.5, $P < 0.001$ (extract × concentration). Columns are mean values ± SD. The asterisk within each extract indicates significant difference from the untreated control and, different letters within each concentration indicate significant differences, according to LSD test ($P < 0.05$).

Tab. 3 Effect of different concentrations of extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. on *Fusarium oxysporum* f.sp. *lycopersici* microspores dimensions after 48 h of incubation.

Extract	Concentrations (mg ml^{-1})			
	0.0	2.5	5.0	10.0
Length				
Anabaena sp.	10.1 ± 1.6 A	15.1 ± 2.1 cB	15.5 ± 2.3 cB	15.2 ± 2.7 bB
Ecklonia sp.	9.9 ± 1.7 B	6.9 ± 1.5 aA	7.0 ± 0.9 aA	12.3 ± 2.7 aC
Jania sp.	10.0 ± 1.5	9.9 ± 0.9 b	9.5 ± 1.3 b	11.1 ± 1.0 a
Width				
Anabaena sp.	3.4 ± 0.3 B	3.7 ± 0.3 cB	3.5 ± 0.4 B	3.0 ± 0.4 aA
Ecklonia sp.	3.4 ± 0.4 B	2.4 ± 0.4 aA	2.9 ± 0.6 AB	4.0 ± 0.9 bC
Jania sp.	3.5 ± 0.3	3.1 ± 0.6 b	3.1 ± 0.6	3.2 ± 0.2 a

For both length and width, extract and concentration factors and their interaction are significant according to factorial ANOVA ($P < 0.05$). For length, F (2, 96) = 62.6, $P < 0.05$ (for extract factor), F (3, 96) =10.9, $P < 0.05$ (for concentration factor), F (6, 96) = 9.9, $P < 0.05$ (for interaction). For width, F (2, 96) =1.8, $P < 0.05$ (for extract factor), F (3, 96) = 2.2, $P < 0.05$ (for concentration factor), F (6, 96) = 7.5, $P < 0.05$ (for interaction). Means (± SD) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences.

3.3 Greenhouse experiments

3.3.1 Effect of water extracts on seedling emergence

The effect of seed treatment with extracts on tomato seedling emergence is shown in Tab. 4. Two-way ANOVA of seed germination indicated a significant interaction between the two factors (treatment and concentration). The emergence was significantly increased by all treatments at all concentrations tested in a 28.0 to 59.2% range. At the concentrations of 2.5 and 10.0 mg ml^{-1}, the highest increases were showed by ECK (59.2% vs untreated control) and AN (56.0% vs untreated control), respectively.

Tab. 4 Effect of seed treatment with different concentrations of extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. at on seedling emergence percentage, 15 days after seed treatments

Extract	Concentration (mg ml^{-1})			
	0.0	2.5	5.0	10.0
Anabaena sp.	54.8 ± 3.0 A	75.8 ± 2.7 aB	87.3 ± 5.2 C	85.5 ± 4.8 bC
Ecklonia sp.	55.4 ± 4.0 A	88.2 ± 5.3 bC	80.0 ± 4.4 BC	70.9 ± 6.4 aB
Jania sp.	55.4 ± 3.8 A	76.6 ± 5.5 aB	76.3 ± 6.5 B	81.3 ± 5.3 abB

Treatment, concentration and their interaction are significant according to factorial ANOVA ($P < 0.05$). F (2, 36) = 1.5, $P < 0.05$ (for extract factor), F (3, 36) =59.3, $P < 0.05$ (for concentration factor), F (6, 36) = 5.2, $P < 0.05$ (for interaction). Means (± SD) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences.

3.3.2 Experiment 1: effect of water extracts against *Fusarium oxysporum* f. sp. *lycopersici* disease on plants

Multifactorial ANOVA on the effect of seed treatment and seed treatment combined with substrate treatment with the water extracts of AN, JAN and ECK against FOXL1 is shown in Tab. 5 and Tab. 6. Extract from ECK reduced disease index at all concentrations and in both treatments. Seed treatment combined with substrate treatment with ECK sp. at 5.0 and 10.0 mg ml^{-1} reduced the disease more than seed treatment by 50.7 and 60.0%, respectively. *Anabaena* sp. reduced the disease at 10.0 mg ml^{-1} in both treatments in a similar way by an average of 29.9% with respect to the untreated control. Extract from JAN applied by seed + substrate treatment at 10.0 mg ml^{-1} significantly reduced the disease by 28%. Within concentration 10.0 mg ml^{-1} ECK reduced disease index more than AN and JAN.

Tab. 5 Multifactorial ANOVA for *Fusarium oxysporum* f. sp. *lycopersici* disease index of tomato plants treated by seed treatment and by seed combined with substrate treatment with water extracts at different concentrations.

Source	Df	Sum of Squares	Mean Square	F-Ratio
A: Treatment	1	461.51	461.51	4.9*
B: Extract	2	7795.14	3897.57	41.4***
C: Concentration	3	6875.04	2291.68	24.3***
A × B	2	1744.15	872.07	9.3***
A × C	3	1263.65	421.22	4.5*
B × C	3	4089.79	681.63	7.2***
Error	54	05087.61	94.22	
Corr. total	71	27316.9		

* = $P < 0.001$
*** = $P < 0.05$

Tab. 6 Experiment 1: effect of treatment (seed or seed + substrate) with different concentrations of extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. on disease index of tomato plants transplanted in substrate infected with FOXL1.

Extract	Seed treatment				Seed + substrate treatment			
	Extract concentrations (mg ml^{-1})				Extract concentrations (mg ml^{-1})			
	0	2.5	5.0	10.0	0	2.5	5.0	10.0
Anabaena sp.	87.1±11.5 C	79.8±10.5 BCb	78.3±12.6 BC	60.0±10.0 A	87.4±10.1 C	84.3±5.1 Cb	83.3±8.3 Cb	62.2±11.7 ABb
Ecklonia sp.	85.8±9.2 D	35.0±5.0 ABa	72.2±4.8 C	70.0±5.0 C	84.3±7.8 D	39.4±5.9 Ba	35.6±5.9 ABa	28.0±5.2 Aa
Jania sp.	86.3±10.4 B	77.8±4.8 Bb	77.8±4.5 B	72.2±12.7 B	86.3±10.4 B	86.1±4.8 Bb	80.6±9.6 Bb	61.9±9.6 Ab

Treatment, extract, and concentration and treatment x extract, treatment x concentration and extract x concentration interactions are significant according to multifactorial ANOVA ($P < 0.05$). F (1, 72) = 4.9, $P < 0.05$ (for treatment); F (2, 72) = 41.4, $P < 0.001$ (for extract), F (3, 72) = 24.3, $P < 0.001$ (for concentration), F (2, 72) = 9.3, $P < 0.05$ (for treatment x extract); F (3, 72) = 4.5, $P < 0.05$ (treatment x concentration); F (6, 72) = 7.2, $P < 0.001$ (extract x concentration). Means (± SD) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences.

3.3.3 Experiment 2: biostimulant effect of water extracts on plant

Multifactorial ANOVA on the biostimulant effect of seed treatment and seed treatment combined with substrate treatment with the water extracts of AN, JAN and ECK is shown in Tab. 7 and Tab. 8. Extract factor and concentration factor, and their interaction were statistically significant. Since, the way of treatment (seed or seed + substrate treatment) was not significant ($P > 0.05$), in Tab. 8 means of the two way of treatment are reported. Extract from JAN at all concentrations significantly increased the dry weight by 55.4, 47.0 and 44.6% at 2.5, 5.0 and 10.0 mg ml^{-1}, respectively, while AN increased dry weight only at 10.0 mg ml^{-1} by 26.5%. Treatment with ECK did not show any effect at all concentrations.

Tab. 7 Multifactorial ANOVA for dry weight of tomato plants treated by seed treatment and by seed combined with substrate treatment with the extracts at different concentrations.

Source	Df	Sum of Squares	Mean Square	F-Ratio
A: Treatment	1	0.00737	0.00737	2.3
B: Extract	2	0.10125	0.05062	15.9***
C: Concentration	3	0.03565	0.11885	3.7*

A × B	2	0.02180	0.01090	3.4
A × C	3	0.11928	0.00397	1.3
B × C	6	0.21688	0.03614	11.4***
Error	51	0.16198	0.00317	
Corr. total	68	0.58611		

* = $P < 0.001$
*** = $P < 0.05$

Tab. 8 Experiment 2: effect of treatment (seed or seed + substrate) with different concentrations of extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. on dry weight of tomato plants.

Extract	Concentration (mg ml^{-1})			
	0.0	2.5	5.0	10.0
Anabaena sp.	0.256 ± 0.015 A	0.266 ± 0.033 aA	0.237 ± 0.047 bA	0.324 ± 0.079 bB
Ecklonia sp.	0.257 ± 0.009 A	0.254 ± 0.040 aA	0.249 ± 0.020 aA	0.252 ± 0.035 aA
Jania sp.	0.258 ± 0.018 A	0.401 ± 0.050 bB	0.379 ± 0.059 cB	0.373 ± 0.062 aB

Extract, concentration and their interaction are significant according to multifactorial ANOVA ($P < 0.05$). F (2, 69) = 15.9, $P < 0.05$ (for extract factor), F (3, 69) = 3.7, $P < 0.05$ (for concentration factor), F (6, 69) = 11.4, $P < 0.05$ (for interaction). Means (± SD) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences.

4. Discussion

Algae and cyanobacteria are an interesting source of bioactive compounds potentially useful for the control of plant pathogens. Most of the available literature regards the antifungal activity of extracts from algae and cyanobacteria with different solvents Several researches were focused on the activity against plant pathogens of extracts obtained from algae and cyanobacteria by using organic solvents (Rizvi and Shameel, 2004; Kumar et al., 2008; Arunkumar et al., 2010; Sivakumar, 2014; Jiménez et al., 2011), (Bouhlal et al. 2010). The activity against phytopathogens of water extracts obtained from algae and cyanobacteria is poorly investigated (Roberti et al., 2015; Roberti et al., 2016; De Corato et al., 2017). In this study, we present data of a first study on the effect of water extracts from species of macroalgae, *Jania* and *Ecklonia* and the cyanobacterium *Anabaena* against the soil-borne pathogen *Fusarium oxysporum* f. sp. *lycopersici* race 1. This study demonstrated that water extracts from the two algae *Ecklonia* sp. and *Jania* sp. and from the cyanobacterium *Anabaena* sp. caused a transient stimulation of *Fusarium oxysporum* f. sp. *lycopersici* colony growth in plates, whereas they affected fungal growth generated from spore, hyphal and microspore morphology. The absence of negative

effect on pathogen colony growth in plate assay is consistent with the findings of De Corato et al. (2017). These authors demonstrated that aqueous fraction from two brown algae, *Laminaria digitata* and *Undaria pinnatifida*, and three red algae, *Porphyra umbilicalis*, *Eucheuma denticulatum* and *Gelidium pusillum*, did not display any antifungal activity on mycelia growth of *Botrytis cinerea*, *Monilia laxa* and *Penicillium digitatum*. Similarly, Barreto et al. (1997) reported that ethanolic extract from the two red algae *Spyridia cupressina* and *Bekerella pinnatifada* did not exert any antifungal activity against *Rhizoctonia solani* and *Verticillium* sp. colony growth. Our experiments also demonstrated that water extracts induced hyphal morphological alteration and affected spores at microscopical level. Depending on the concentration, water extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. caused a significant increase of hyphal diameters and cytoplasm coagulation of *F. oxysporum* f. sp. *lycopersici* race 1 mycelium. To our knowledge, no other study is reported on the effects of extracts from algae and cyanobacteria on mycelium and spore morphology. We can suppose that these modifications are symptoms of fungal stress induced by the extracts as observed by other authors for different substances. Several authors showed that hyphae of *Aspergillus niger* exposed to different plant extracts were distorted and collapsed (Tolouee et al 2010; de Billerback et al., 2001; Sharma and Tripathi, 2008; Rasooli et al., 2006; dos Santos et al., 2012). Hyphal thinning, distortion and destruction and the loss of cytoplasmic material were also observed on *A. niger* and *Rhizopus stolonifera* exposed to different concentrations of chitosan and an essential oil from *Origanum vulgare* (dos Santos et al., 2012). On *Botrytis cinerea* essential oils from origanum, lavender and rosemary caused degenerations of the fungal hyphae such as cytoplasmic coagulation, vacuolation, hyphal shrivelling and protoplast leakage (Soylu et al., 2010). Concerning spore dimension, we observed a variable effect of the extracts depending on the concentrations, except for *Jania* sp. that did not influence both spore length and width. A sharp morphological changes was induced by essential oils on *A. niger* spores (dos Santos et al., 2012).

The mechanism by which the extracts affect both mycelia and spores may be the interaction between compounds contained in the extracts and fungal cell wall, as suggested by Sharma and Tripathi (2008) with *Citrus sinensis* essential oil on *A. niger*, by Ghfir et al. (1997) on *A. fumigatus* with oil of *Hyssopus officinalis* and by Giordani et al. (1996) on *Candida albicans* with sap of *Carica papaya*. The spore is as an important structure for the survival and spread of fungi (Rabea et al., 2009), and the application of extracts that inhibit spore germination or cause alterations of the fungus structures that lead to inhibition of spore production are potential candidates for the control of spore spread. It is known that algae and cyanobacteria produce many bioactive compounds. The antifungal activity we demonstrated for the extract from *Ecklonia* sp. could be related to secondary bioactive compounds, such as phenols, with antioxidant activities (Ahmed, 2016). Among phenols, dieckol

extracted from *E. cava*, depending on the concentration, inhibited spore germination of the *Trichophyton rubrum*, agent of dermatophytic nail infections in humans, after 48h of contact (Lee et al., 2010). Another phenol, eckol, caused a change in cell membrane integrity of the spores, while in hyphae the treatment lead to cell death.

Phenolic compounds such as phenol, tannin and flavonoids, are among the most effective antioxidants produced also by red algae, such as *Jania rubens*, *Corallina mediterranea* and *Pterocladia capillacea* (El-Din and El-Ahwany, 2015). Even cyanobacteria 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, in some case, effects as herbicides and antifungal against pathogens (Burja et al. 2001; Patterson et al. 1994). Frankmölle et al. (1992a) reported that extracts of *Anabaena laxa* inhibited the growth of fungi as *Aspergillus oryzae*, *Candida albicans, Penicillium notatum* and *Saccharomyces cerevisiae*. Moreover, Kellam et al. (1988) showed that *A. variabilis* extract inhibited the growth of the cellulolytic fungus *Chaetomium globosum*. This activity was attributed to the laxaficine (Frankmölle et al. 1992b). Recently, the potent antifungal macrolide scytophycin was detected in *Anabaena* sp., *Anabaena cf. cylindrica*, *Nostoc* sp. and *Scytonema* sp. (Shishido et al., 2015). Extracts from *A. solitaria, A. laxa*, *A. variabilis* and *A. anomala* inhibited the growth of several pathogens such as *F. oxysporum* (Kim, 2006; Prasanna et al., 2013; Shrivastava, 2018). El-Mougy and Abdel-Kader (2013) attribute to the presence of phenolic compounds, saponins, and alkaloids the inhibitory effects of extracts from *Spirulina platensis*, *Oscillatoria* sp. and *Nostoc muscorum* on spore germination of *Cercospora beticola*.

Concerning the biostimulant effect of the extracts on tomato plant, this study showed that seed treatment with extract from *Anabaena* sp, *Ecklonia* sp. and *Jania* sp. at all concentrations increased the seedling emergence. In transplanted plants, treatment with extract from the red alga *Jania* sp. at all concentration and the cyanobacterium *Anabaena* sp. at 10 mg ml-^{1} increased dry weight, but dry weight was not influenced by the two ways of treatments, on seed or on seed combined with substrate treatments. Red algae as well as brown algae are known to be rich in hormones such as the auxin indole-3-acetic acid, the cytokinins zeatin and zeatin riboside, and abscisic acid (Tarakhovskaya et al., 2007; Yokoya et al., 2010; Khan et al., 2009). In their extracts the presence of hormones in addition to mineral nutrient elements and polysaccharides are the responsible of the enhancement of plant growth (Blunden, 1991). On tomato, the brow alga *E. maxima* applied to soil increased fresh weight, leaf surface area and the number of flower (Crouch and van Staden, 1992), while foliar application of *Kappaphychus alvarezii* improved growth and yield (Zodape et al., 2011). Also

cyanobacteria are known for producing hormones such as auxins, gibberellins, cytokinins in addition to vitamins, polypeptides, aminoacids, and promote plant growth and development (de Caire et al., 1979; Stirk et al., 1999; Sergeeva et al., 2002). Cyanobacteria produce polysaccharides that can stimulate plants of tomato and pepper by 20% and 30%, respectively, in line to what we observed for *Anabaena* sp. that increased dry weight by 21 %. These biostimulant effects of polysaccharides depends also on the plant, as demonstrated for *N. commune* polysaccharides, that increase more roots weight in tomato than in pepper plants (Elarroussi et al., 2016). On tomato, soil application of *Nostoc* sp. and a strain of *Anabaena* sp., different from that used in our study, enhanced root length and fresh weight, plant height, root dry weight and both stem and leaf dry weight (Shariatmadari et al., 2013).

Concerning the effect of the extracts against *F. oxysporum* f. sp. *lycopersici* race 1 on tomato plant, this study showed that disease index was influenced by the two ways of treatments, on seed or on seed combined with substrate treatment. Treatments with extract from *Ecklonia* sp. were particularly effective. The reduction of the disease was higher in the combined treatment than in seed treatment only, suggesting that *Ecklonia* sp. may control the disease in two modes, against the pathogen and on plant by eliciting defence responses. The direct effect against the pathogen, already present in substrate before the treatment, can be related to the antifungal activity *Ecklonia* on the fungal morphological alterations demonstrated in *in vitro* experiments, while plant defence responses were elicitated by seed treatment. Disease control showed by *Jania* sp. was obtained only in combined treatment at the highest concentration, thus we hypothesize that its main mode of action is a direct effect against the pathogen. On the contrary, *Anabaena* sp. controlled the disease equally in both treatments, thus in this case we suppose a main action through plant defence responses that were induced by seed treatment. The induced resistance by algal and cyanobacterial extracts is widely documented against plant pathogens. Concerning brown algae, treatments of tomato plants with extracts from *Cystoseira myriophylloides*, *L. digitata*, and *F. spiralis* reduced disease severity caused by *V. dahliae*, and increased the activity of defence related enzymes such as polyphenol oxidase and peroxidase Esserti et al. (2017). Spray treatments and/or root drench with *Ascophyllum nodosum* on cucumber plants reduced disease caused by *F. oxysporum*, *Alternaria cucumerinum*, *B. cinerea* and *Phytophthora melonis* by enhancing activities of defence-related enzymes such as chitinase, *β*-1, 3-glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, and lipoxygenase and the levels of phenolic compounds (Jayaraman et al., 2011; Abkhoo and Sabbagh, 2016). With regard to red algae, on tomato, the extract from *K. alvarezii* increased the transcription of related pathogenesis genes such as PR1, PR3 and PR4 and the endogenous concentration of salycilic acid and zeatin (Agarwal et al., 2016). Extracts from *Corallina* sp. and *Halopithys* sp. applied before pathogen

inoculation decreased the infection by *Podosphaera xanthii* on zucchini cotyledons, presumably for their increase of plant defence response (Roberti et al., 2016).

The resistance in plants is induced by several compounds contained in algal extracts among them, polysaccharides are the most active. Brown algae are rich in polysaccharides such as fucoidan, laminarin and alginate (Rioux et al., 2007; Stadnik et al., 2014), and carrageenins are the most abundant polysaccharides in red algae (Mercier et al., 2001). Algae polysaccharides and derived oligosaccharides elicit the activation of salicylic, jasmonic acid and/or ethylene signalling pathways at systemic level. The activation of these signaling pathways leads to an increased expression of genes encoding those related to pathogenesis-related (PR) proteins with antifungal and antibacterial activities or those related to defence enzymes such as pheylalanine ammonia lyase and lipoxygenase which determine accumulation of phenylpropanoid compounds and oxylipins with antiviral, antifugal and antibacterial activities (Vera et al., 2011). Single compounds extracted from red algae, such as polysaccharides, are able to induce plant defence mechanisms that led to the plant pathogen resistance. The application of carrageenans in *Arabidopsis* induced resistance against *Sclerotinia sclerotiorum* (Sangha et al., 2010). This effect depended on the increasing in oxalate oxidase activity, related with the expression of jasmonic acid signaling that led to the expression of defence genes such PR3. Also Mercier et al. (2001) showed that carrageenans from red algae efficiently induced signalling and defence gene expression in tobacco leaves, unlike laminarin, from brown algae. The activity of polysaccharides depends on the carrageenan type, its degree of sulfation and on pathogen (Stadnik et al., 2014).

The plant resistance induction by cyanobacteria has recently demonstrated by *Anabaena* 0300B water extract on zucchini plants (Roberti et al., 2015). Powdery mildew control and systemic accumulation of endochitinase, *β-N*-acetylhexosaminidase, chitin 1,4-*β*-chitobiosidase, *β*-1,3-glucanase and peroxidases were showed and related to poly- and oligosaccharides, which are likely to be found in hot water extracts of *Anabaena* 0300B extract. On tomato, Prasanna et al. (2013) with soil treatment with *A. variabilis* and *A. laxa* obtained a disease reduction of symptoms by *F. oxysporum* f. sp. *lycopersici* and increasing of defence enzyme activities such as phenylalanine ammonia lyase, polyphenoloxidase and *β*-1,3-glucanase.

In conclusion, this study shows that seed treatment and substrate treatment with water extract from *Anabaena* sp., *Ecklonia* sp., *Jania* sp. were active against *Fusarium oxysporum* f. sp. *lycopersici* race 1 by working both directly to the pathogen and indirectly through the involvement of plant defence responses, helping the plant to withstand the pathogen. Considering that the control of the pathogen is currently problematic for the limited available pesticides, these extract may provide a useful tool

for the disease management in sustainable agriculture, once the antifungal effects will be verified on tomato plants in a larger scale experiment.

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4. Biocontrol activity of water extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. against cucumber powdery mildew

The direct effect and the induction of systemic defence responses of water extracts from the macroalgae *Jania* sp. and *Ecklonia* sp. and the cyanobacterium *Anabaena* sp. was evaluated *in vitro* and *in vivo* against *Podosphaera xanthii*, causal agent of cucurbit powdery mildew. For the direct effect of the extracts *in vitro* assay, detached cucumber cotyledons were sprayed with each extracts 2.5, 5.0 and 10.0 mg of dry biomass per ml of water, then inoculated with the pathogen (10^6 mg ml^{-1}). For the direct effect of the extracts in the *in vivo* assay, the same concentrations were applied preventatively on cucumber plants, under greenhouse conditions. In the *in vitro* experiment percentage of cotyledon infected area and fungal sporulation were inhibited by *Anabaena* sp. On cucumber plants, all the three extracts reduced both the infected area and fungal sporulation. The induction of systemic defence responses in cucumber plants by the extracts was studied under greenhouse conditions firstly, through a bioassay, secondly by evaluating enzyme activities correlated to induced systemic resistance and gene expression PR proteins 1, 2 and 3 days after treatment (DAT). For bioassay, one of the two cotyledons was treated with the extract and after two days the other cotyledon was inoculated with the pathogen. Extract from *Anabaena* sp. at 10 mg ml^{-1} and *Jania* sp. at all concentrations, reduced the infected area and provoked an increase of chitinase activity 1 and 3 DAT. All treatment did not show visible β-1,3-glucanase activity at any DAT. Gene expression of PR2, PR3 and PR4 was upregulated in relation to the treatment and to the DAT.

1. Introduction

Powdery mildew is one of the major worldwide fungal disease of cucurbits, both in open field and under greenhouse conditions (Cohen et al., 1996; Kobori et al., 2004; Brunelli and Gengotti, 2007; Davis et al., 2007; Pérez-García et al., 2009; Kousik et al., 2011; Lebeda et al., 2011; Fig. 1). The disease is caused by two obligate biotrophic ectoparasites, *Podosphaera xanthii* (Castagne) U. Braun and Shishkoff, and *Golovinomyces cichoracearum* (DC.) V.P. Heluta, and by *Leivellula taurica* which cause a less economically important disease (Vakalounakis and Klironomou, 2001; Lebeda et al., 2016). *Podosphaera xanthii* is considered to be the main causal agent of powdery mildew in many countries of Europe and around the world (Del Pino et al., 2002; Cohen et al., 2004; Brunelli and

Gengotti, 2007; Pérez-García et al., 2009; McCreight et al., 2012; Raymond and Ferguson, 2014). The distribution, pathogenicity and the virulence of *P. xanthii* change according to large number of races strictly connected to host-pathogen interactions (Lebeda et al., 2016; Lebeda et al., 2011). For example, Cohen et al. (2004) reported eight races identified across Europe, America and Africa and four races in Japan.

The disease is easily distinguishable by the characteristic white powdery (Fig. 2), consisting in the fungal structures, on leaf surface and stems even sometimes on fruits (Pérez-García et al., 2009). The white powdery mass is mainly composed of fungal mycelia and spores (Martínez-Cruz et al., 2014). When the environmental conditions are favourable, fungal colonies may join together and cover the entire leaf surface leading chlorosis and wrinkle and the death of the canopy (Keinath and Dubose, 2004). The infected cucumber fruits may be malformed, sunburned and sometimes may ripen prematurely, but the attack of powdery mildew on fruits of cucurbits is not common (Pérez-García et al., 2009). The disease thrives in moderate temperature and high humidity that usually make greenhouse atmosphere more prone to the disease (Huang et al., 2000).

The life cycle of powdery mildew (Fig. 3) includes agamic and gamic stages (Pérez-García et al., 2009; Martínez-Cruz et al., 2014). Agamic cycle is considered the main responsible of the disease spread. The typical spore of the pathogen is the conidium, which produces a short germ tube on host surface. The tip of germ tube swells and produces the primary appressorium to penetrate the host cuticle and cell wall. Then, the fungus produces primary haustorium inside a plant epidermal cell, which releases effectors and uptakes nutrients from plants (Martínez-Cruz et al., 2014). Then a succession of hypha production and conidiophores formation occurs. Five to ten ovoid shaped conidia are produced at the tip of each conidiophore in chains. Both the mycelium and conidia forms the white mycelium on the plant surface that looks like white powder, the typical symptom of powdery mildews (Pérez-García et al., 2009). *Podosphaera xanthii* is a heterothallic fungus. Chasmothecium (formerly cleistothecium), the ascocarp, is formed by gamic reproduction only after occurrence of two hyphae with opposite mating types. Chasmothecia overwinter and are considered source of primary inoculum. A single chasmothecium contains one ascus with eight ascospores. The formation of chasmothecia on cucurbits is rarely or was never observed in most of the important cucurbits growing areas, but it is assumed that ascospores have the ability to cause a disease outbreak similar to that of agamic spores (Pérez-García et al., 2009).

Nowadays, chemical control is needed considering that the disease can markedly limit cucurbit production, but the constant use of synthetic fungicides has led to the development of resistance of the pathogen to various pesticides, reducing the effectiveness of treatments (McGrath, 2001; Hollomon et al., 2002). The development of bio-based strategies to control plant pathogens and

enhance food safety has encouraged by the Regulation (EC) 1107/2009, and the Directive (EC) 128/2009 and its implementation that promote specific actions to support the establishment of sustainable agriculture. Among alternative methods for disease control, algae and cyanobacteria may be considered, because they are natural source of bioactive compounds against plant pathogens (Vera et al., 2011; Singh, 2014; Righini et al., 2018). To our knowledge, few studies have examined the protectant activity of extracts form cyanobacteria and algae against powdery mildew on different crops. The disease was controlled on turnip by a commercial seaweed extract (Stephenson, 1966), on common bean, grapevine and cucumber by *Ulva armoricana* extracts (Jaulneau et al., 2011), and on zucchini by the cyanobacterium *Anabaena* sp. and several algae extracts (Roberti et al., 2015; Righini et al., 2018).

The objective of the present work was to evaluate the preventative effects of extracts from the cyanobacterium *Anabaena* sp. and the algae *Ecklonia* sp. and *Jania* sp. against powdery mildew of cucumber by the evaluation: (i) of the direct antifungal activity on the pathogen on detached cotyledons and on plants under greenhouse conditions; (ii) the indirect activity through a bioassay and the accumulation of PR proteins in leaf tissue.

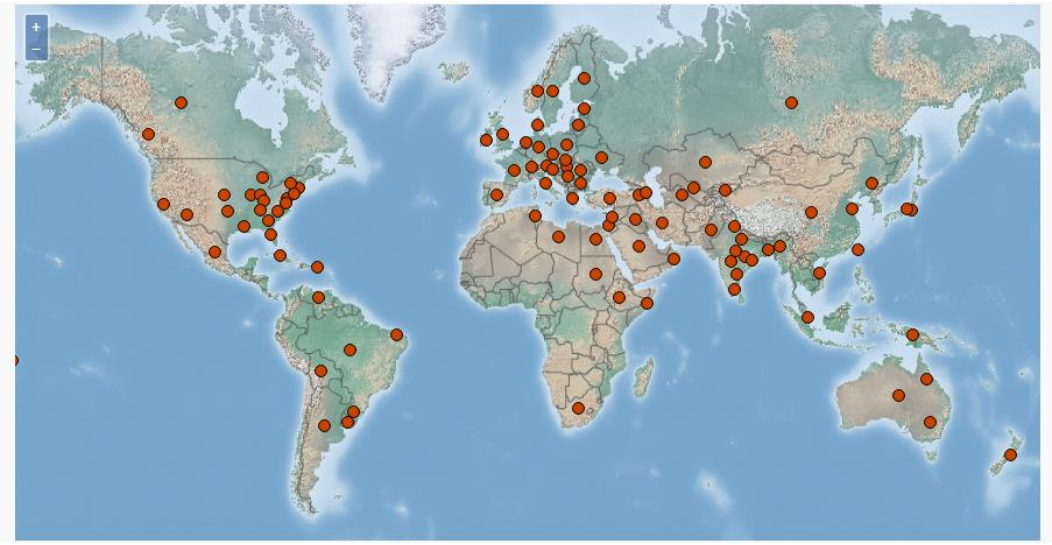

Fig. 1 World distribution map of *Podosphaera xanthii* (CABI, 2018).

Fig. 2 Symptoms of powdery mildew on cucumber leaves.

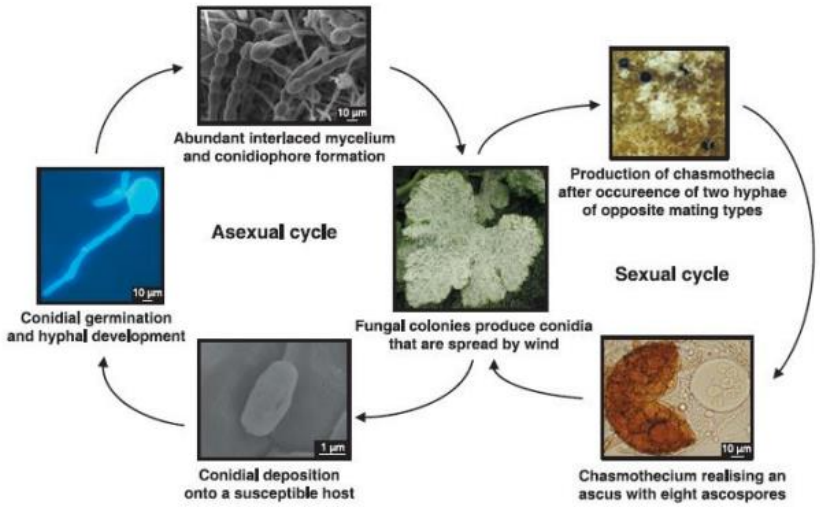

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Fig. 3 Life cycle of *Podosphaera xanthii* (Source: Pérez-García et al., 2009).

2. Materials and methods

2.1. Plant material, water extract, and pathogen

Cucumber plants var. Le Généreux (Vilmorin Jardin, St. Qentin Fallavier Cedex, France) 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 Ø). In all experiments, plants were grown at 24–26 °C (day), 20–22 °C (night) and 14 h photoperiod and 70% relative humidity under greenhouse conditions.

Lyophilized *Anabaena* sp. (AN) and dry thallus of *Ecklonia* sp. (ECK) and *Jania* sp. (JAN) were provided by the Spanish Bank of Algae, Marine Biotechnology Centre, University of Las Palmas de Gran Canaria. Dry thallus was grounded to fine powder with mortar and pestle. Water extracts were obtained by suspending each powder in sterile distilled water (0.5%) under continuous stirring at 50°C for 12 h, then filtered filtered through sterile filter paper before use (Roberti et al., 2015). The concentrations considered were 2.5, 5.0 and 10.0 mg ml^{-1}.

The pathogen *P. xanthii* was isolated from leaves of cucumber plants showing symptoms of powdery mildew located in the Po Valley, province of Bologna, Northern Italy, by vigorous shaking of infected leaves onto 15-day-old asymptomatic cucumber plants, maintained at 22–24 °C (day), 16–18 °C (night) 60–80% relative humidity and 14 h photoperiod under the greenhouse of the School of Agriculture, University of Bologna. The pathogen was identified on the basis of morphological features based on conidia germination (Zaracovitis, 1965) and the presence of fibrosin bodies (Lebeda, 1983). The pathogen was used for the antifungal activity and for induced resistance assays.

2.2. Direct activity of water extracts against *P. xanthii*

Assay on detached cotyledons

Cucumber cotyledons were randomly cut off from 15-day-old asymptomatic plants, sterilized with sodium chloride (1 % active Cl) for 3 minutes, washed three times with sterile distilled water and dried with adsorbent sterile paper under a sterile flow cabinet. Four cotyledons were placed an agarized medium containing Difco agar 1.5 %, glucose 10 g l^{-1}, benomyl 1 g l^{-1}, prochloraz 10 mg $l^{-1}$and streptomycin sulphate 0.5 g l^{-1} in Petri dish (13 cm Ø). Cotyledons were arranged with the abaxial surface in contact with the medium, then they were sprayed with 4 ml of each extract concentration of AN, ECK and JAN, separately. Control cotyledons were sprayed with 5 ml of sterile distilled water. When cotyledons were dry, they were inoculated on the adaxial surface with six 10-µl drops of a spore suspension of *P. xanthii* (1×10^6 spores ml^{-1}), according to Moret et al. (2009) with modifications (Fig. 4). Three replicates were considered for each extract concentration and for the untreated control. Cotyledons were incubated in a growth chamber at 24-25 °C in the dark for 48 h

and then shifted to light: dark cycle, 12 h: 12 h. Nine days after pathogen inoculation, the effect of the extracts on the disease was evaluated visually, as percentage of area showing symptoms of *P. xanthii* disease (white, powdery spots), and by a microscope (Zeiss, GmbH, Germany, × 300 magnification) 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 untreated cotyledons, were observed at the microscope and all spores were counted. The experiment was repeated once.

Fig. 4 Spore suspension preparation (a), treated and inoculated cotyledons in Petri dishes (b, c).

Assay on plants

Fifteen-day-old asymptomatic plants were treated with the extracts at each concentration (2 ml per plant). Two ml of water were sprayed on control plants. Once the cotyledonary leaves were dried, 2 ml of a conidial suspension of *P. xanthii* (1×10^6 spores ml^{-1}) were sprayed on treated and untreated plants. Six plants (replicates) for each treatment and for the infected control, were considered. The plants were randomly distributed on a shelf of the greenhouse and grown at 22–24 °C (day), 16–18 °C (night) 60–80% relative humidity and 14 h photoperiod under greenhouse conditions. For disease

evaluation percentage of area showing symptoms and pathogen sporulation were determined, 5-6 d after pathogen inoculation.

2.3. Systemic induced resistance assays

Bioassay on plants

For this assay, 15-day-old asymptomatic plants at the cotyledonary stage were considered (Roberti et al., 2015). Plant treatments with AN, ECK and JAN (2.5, 5.0 and 10.0 mg ml^{-1}) were performed on one of the two cotyledons (1 ml of extract per cotyledon), while the other cotyledon was left untreated by covering it with an aluminium sheet which was removed after 4 h (Fig. 5). Water (1 ml of extract per cotyledon) was sprayed on the controls, untreated not inoculated and untreated inoculated. One ml of conidial suspension of *P. xanthii* (1×10^6 spores ml^{-1}) was sprayed on each untreated cotyledon, 2 days after the treatment. Four plants (replicates) for each treatment the controls, were considered. They were randomly distributed on a shelf of the greenhouse at the same ambient conditions reported above. Disease was evaluated on untreated leaves as percentage of area showing symptom, 7–9 d after pathogen inoculation.

Detection of β-1,3-glucanase and chitinase activities

Untreated cotyledonary leaves of treated and untreated-not inoculated plants of the bioassay were harvested 1, 2 and 3 days after the treatment (DAT). In this case, the extracts were applied at the concentration of 5.0 mg ml^{-1}. Four plants (replicates) for each treatment and for control were considered. The leaf tissue was weighed and immediately frozen in liquid nitrogen. The leaves were then ground to fine powder using a pre-chilled mortar and pestle, and total proteins were extracted by 20 mM sodium acetate buffer pH 5.2 (1 ml g^{-1} of fresh weight) containing 1% polyvinylpolypyrrolidone (Sigma–Aldrich Co.) (Roberti et al., 2008). After incubation at 4 °C for 90 min under continuous gentle stirring, the crude extracts were centrifuged twice at 12,000 rpm for 20 min at 4 °C. The supernatant was filtered using a GV Millex® Syringe Filter Unit (Millipore Corporation, USA) to remove the solid particles, and samples were obtained. Protein concentration in samples was determined by the protein–dye binding method of Bradford (1976) in a 96 wells microplate (Greiner CELLSTAR®), using bovine serum albumin (BioRad Laboratories, Inc.) as the standard.

The activity of chitinase was assayed in triplicate by plate assay method of Bargabus et al. (2004) in agarose gel (1%) containing 0.01% glycol chitin in a glass Petri plate (17cm Ø) (Fig. 6). Two hundred of sample (40 µg of proteins), were added to wells (0.7 mm Ø) in the agar. As chitinase standard *Streptomyces griseus* (Sigma) was used. The same volume of water was added to wells. The plates were incubated at 37 °C for 24 h, then 50 ml of 500 mM Tris–HCl (pH 8.9) containing 0.01%

fluorescent brightener 28 was added. After 10 min, the plate was rinsed three times with distilled water, flooded with water, and maintained in the dark for 24 h. The non-fluorescent lytic zones on a fluorescent background were measured while the plate was on a 302 nm UV light source. Images of gels were taken the area (mm^2) corresponding to *β*-1,3-glucanase and chitinase activity was measured with Quantity One program 4.6.6, BioRad.

The activity of *β*-glucanase was assayed by a modified plate assay method of Bargabus et al. (2004) in agarose gel (1%) containing 0.5 mg/ml of laminarin (from *Laminaria digitata*, Sigma) in a glass Petri plate (17 cm Ø). Two hundred µl of a mixture containing water, sodium acetate buffer, sample (40 µg of protein) and *Aspergillus niger* (E.C. 3.2.1.4, Sigma-Aldrich Co) as standard were added to wells (0.7 mm Ø) in the agar (Fig. 6). The same volume of mixture without sample was added to wells. Three wells per treatment, standard, untreated control and water were considered. After incubation at 37 °C for 18-24 h, the gel were soaked in 0.15% (w: v) 2, 3, 5-triphenyl tetrazolium chloride in 1 M sodium hydroxide for 10 minutes at 37 °C. The plate was the rinsed three times with distilled water, then the pink lytic zones were measured on a white light source. Images of gels were taken the area (mm^2) corresponding to *β*-1,3-glucanase and chitinase activity was measured with Quantity One program 4.6.6, BioRad.

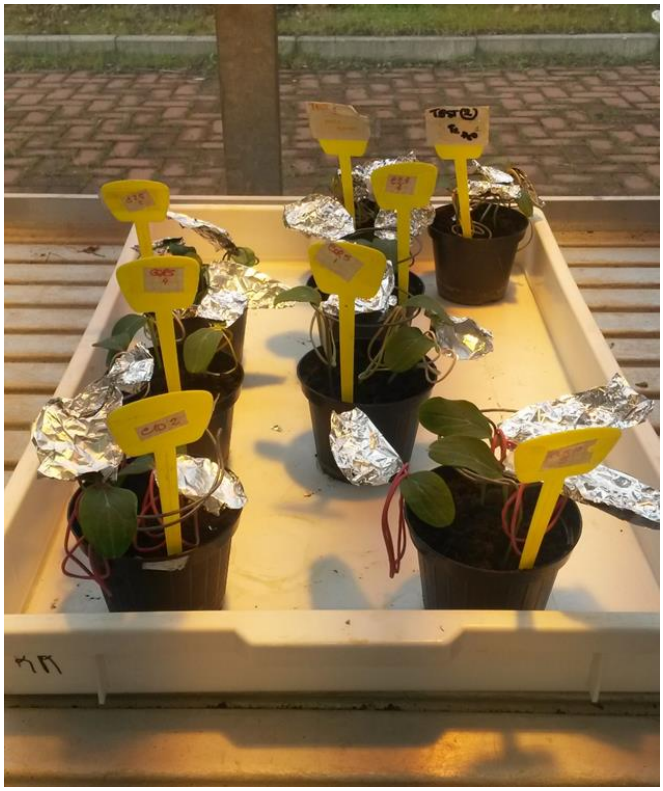

Fig. 5 Arrangement of cucumber cotyledons for treatment in the bioassay for resistance induction.

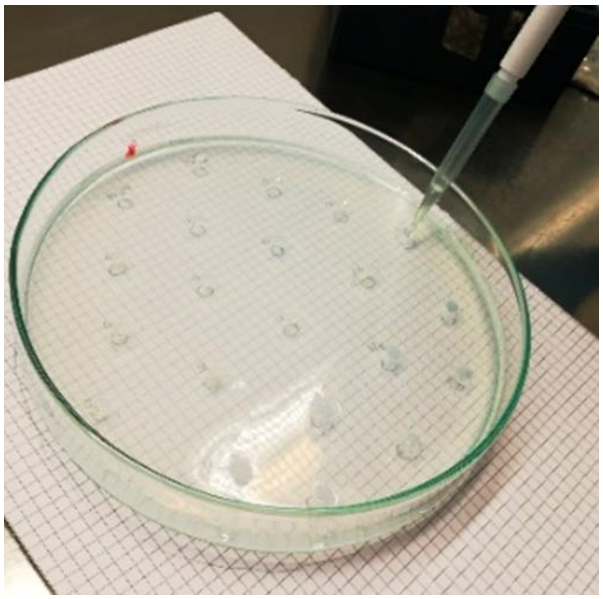

Fig. 6 Plate assay for enzymatic determination.

Gene expression of pathogenesis related proteins

Plant treatments were performed as reported above for the induced resistance assay. The extracts from AN and JAN were applied at the concentration of 5.0 mg ml^{-1}. Chitosan was used as a positive control, since it is a well-known potent elicitor of plant defence responses, and it was prepared from crab-shell chitosan (Sigma Chemical Co., St. Louis, Mo, USA) following the procedure of Romanazzi et al. (2002), and Benhamou et al. (1994). Total RNA was extracted from 2 g of cotyledon tissues as described above for enzyme activity determination. Cellular RNA was extracted with 700 µl TRIZOL (Invitrogen), according to manufacturer's instructions. The RNA pellets were treated with DNAse (DNA-free, Ambion, Austin, TX) and, after buffer exchange, were quantified by using RiboGreen RNA quantitation reagent (Molecular Probes, Eugene OR). The same quantity of total RNA (100 ng) was reverse transcribed by using random primers and the reagents provided with the Superscript VILO Master Mix for RT-qPCR (TermoFisher). The cDNA mixture (2 µl) was used in Real time PCR analysis in a LightCycler Instrument (Roche Molecular Biochemicals) by means of the QuantiTect SYBR Green PCR kit (TaKaRa, Japan) with the following protocol: initial activation of HotStart Taq DNA polymerase at 95°C for 10 min, followed by amplification (40 cycles: 95°C for 5 min followed by appropriate annealing temperature for each target as detailed below kept for 20 min). The protocol was concluded by melting curve analysis to check amplicon specificity. The gene names and primers sequences used in the experiment are given in Tab. 1. Primers were annealed at 59 °C, except GAPDH at 56°C. The amount of mRNA was normalized for GAPDH expression in each sample and referred to untreated, control sample. The experiment was repeated once.

Tab. 1 Genes name and primer sequences.

Gene name	Primer sequence (5'-3')
Actin-7	TCCACGAGACTACCTACAACTC GCTCATACGGTCAGCGAT
PR2 (*β*-1,3-glucanase)	TGTGGTTGGAGATTCGTGGG TCGCAACGTCCCGTTTAAGA
AePR3 (Acidic endochitinase)	TTATTCACTCTCCTCCGCCG CCTGAGCTAGTACGTCCCAG
BePR3 (Basic endochitinase)	GCGCCATTCGATGACGAAAA GATCCACATAACCCCGACCC
PR4 (Endochitinase)	GCCGACAAGCCTTTGGAATG TTCCGAAGCTCCCGTTTCAG

2.4 Statistical analysis

All experiments were arranged in a complete randomized design. Data obtained from *in vitro* and *in vivo* experiments were analyzed by factorial ANOVA, and means were separated by Fisher's least significant difference (LSD) test ($P < 0.05$). Factors for ANOVA analyses were: extracts, their concentrations and DAT. All analyses were performed with GraphPad Prism software, version 4.03, 2005.

3. Results

3.1 Antifungal activity on detached cotyledons

The extracts showed some antifungal activity by reducing the percentage of area with disease symptoms and the sporulation (Tab. 2). For both measures, two-way ANOVA indicated a significant interaction between extract and concentration factors. *Anabaena* sp. significantly reduced both infected area and sporulation at 5.0 (58.9% and 79.6%, respectively) and at 10.0 mg ml^{-1} (58.5% and 72.8%, respectively) towards the infected controls (0.0 mg ml^{-1}). The extract from AN inhibited also the sporulation at 2.5 mg ml^{-1} by 79.4% but not the infected area. Extract from ECK sp., did not reduce significantly the infected area at 5.0 and 10.0 mg ml^{-1} and it stimulated the percentage of disease symptoms at 2.5 mg ml^{-1}. This extract increased the pathogen sporulation at all tested doses. The extract from JAN sp. stimulated both the infected area and pathogen sporulation at all doses.

Tab. 2 Effect of treatment with extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. on infected area by *Podosphaera xanthii* and fungal sporulation on cucumber cotyledons *in vitro*

Extract	Concentration (mg ml^{-1})			
	0.0	2.5	5.0	10.0
Infected area (%)				
Anabaena sp.	56.4 ± 11.1 B	59.9 ± 12.1 aB	23.2 ± 2.4 aA	23.4 ± 2.3 aA
Ecklonia sp.	57.1 ± 10.5 A	126.5 ± 14.4 bB	59.0 ± 6.2 bA	64.8 ± 7.3 bA
Jania sp.	56.0 ± 9.5 A	141.7 ± 10.4 bB	143.0 ± 15.7 cB	137.9 ± 11.8 cB
Sporulation (spores mm^{-2})				
Anabaena sp.	2427.9 ± 180.4 B	498.0 ± 72.7 aA	494.2 ± 76.1 aA	660.0 ± 58.4 aA
Ecklonia sp.	2423.7 ± 175.5 A	4030.8 ± 670.3 aB	8004.9 ± 741.1 aC	15328.6 ± 750.7 bD
Jania sp.	2571.0 ± 457.7 A	68000.5 ± 4465.4 bB	71772.1 ± 6628.6 bC	18896.6 ± 1547.1 cC

For both infected area and sporulation, extract and concentration factors and their interaction are significant according to factorial ANOVA ($P < 0.05$). For infected area: F (2, 36) = 176.0 $P < 0.05$ (for extract factor), F (3, 36) = 41.1 $P < 0.05$ (for concentration factor), F (6, 36) = 26.3, $P < 0.05$ (for interaction). For sporulation: F (2, 36) = 2021.5 $P < 0.05$ (for extract factor), F (3, 36) = 687.1 $P < 0.05$ (for concentration factor), F (6, 36) = 600.7 $P < 0.05$ (for interaction). Means (± SEM) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences, according to LSD test ($P < 0.05$).

3.2. Antifungal activity on plants

The effect of the extracts against the pathogen on plants, is shown in Tab. 3. Two-way ANOVA indicated a significant interaction between extract and concentration factors. Extract from AN significantly reduced at all doses both the infected area by an average of 23.9%, and pathogen sporulation by an average of 44.1% with respect to the control. *Ecklonia* sp. extract significantly reduced both the infected area by an average of 38.2% and pathogen sporulation by an average of 83.2% with respect to the control. Extract from JAN inhibited the infected area at all tested doses by 10.9%, 6.9% and 38.5% for 2.5, 5.0 and 10.0 mg ml^{-1}, respectively. Sporulation was reduced at 5 and 10 mg ml^{-1}, by 55.8% and 83.4%. Within concentration, sporulation was reduced mostly by ECK at all concentrations.

Tab. 3 Effect of treatment with extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. on infected area by *Podosphaera xanthii* and fungal sporulation on cucumber plants under greenhouse conditions.

Extract	Concentration (mg ml^{-1})			
	0.0	2.5	5.0	10.0
Infected area (%)				
Anabaena sp.	85.0 ± 5.2 C	69.2 ± 5.4 B	66.5 ± 6.4 bB	58.3 ±7.7 bA
Ecklonia sp.	85.0 ± 6.8 D	63.3 ± 9.5 C	52.8 ± 8.9 aB	41.6 ± 3.5 aA
Jania sp.	80.0 ± 5.7 C	71.3 ± 7.7 B	74.5 ± 4.2 cB	49.2 ± 8.6 aA

Sporulation (spores mm^{-2})				
Anabaena sp.	43955.3 ± 6440.2 C	31111.1 ± 1618.2 bB	19611.1 ± 2544.4 bA	23055.6 ± 2939.5 cA
Ecklonia sp.	42472.2 ± 4074.4 C	11814.2 ± 1735.3 aB	4781.8 ± 595.4 aA	4839.1 ± 397.8 aA
Jania sp.	46987.0 ± 5080.3 C	56128.8 ± 9461.5 cD	20765.1 ± 1938.6 bB	7822.1 ± 642.7 bA

For both infected area and sporulation, extract and concentration factors and their interaction are significant according to factorial ANOVA ($P < 0.05$). For infected area: F (2, 72) = 13.4 $P < 0.05$ (for extract factor), F (3, 72) = 71.3 $P < 0.05$ (for concentration factor), F (6, 72) = 6.0, $P < 0.05$ (for interaction). For sporulation: F (2, 72) = 116.0 $P < 0.05$ (for extract factor), F (3, 72) = 256.7 $P < 0.05$ (for concentration factor), F (6, 72) = 42.7 $P < 0.05$ (for interaction). Means (± SEM) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences, according to LSD test ($P < 0.05$).

3.3. Systemic induced resistance

Bioassay on plants

The bioassay results showed reduction of the percentage of infected area by AN and JAN (Tab. 4). Two-way ANOVA indicated a significant interaction between the two factors (treatment and dose). The extract from AN caused a 28.4% reduction of the infected area at 10.0 mg ml^{-1} with respect to the control (0.0 mg ml^{-1}). Extract from JAN sp. decreased the infected area by 40.7%, 33.3% and 33.3%, at 2.5, 5 and 10.0 mg ml^{-1}, respectively. No inhibitory effect was observed by ECK sp. extract at any tested doses. At 10.0 mg ml^{-1}, AN and JAN showed statistically similar values.

Tab. 4 Systemic induced resistance of treatment with water extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. on cotyledons evaluated as percentage of *Podosphaera xanthii* infected area. Treatments were applied on one cotyledonary leaf, and the pathogen was inoculated on the other leaves 2 days later.

Extract	Concentration (mg ml^{-1})			
	0.0	2.5	5.0	10.0
Anabaena sp.	73.3 ± 4.7 B	75.0 ± 5.8 bB	65.3 ± 10.8 bB	52.5 ± 9.6 aA
Ecklonia sp.	77.5 ± 6.5	80.0 ± 8.2 b	82.5 ± 9.6 c	72.5 ± 5.0 b
Jania sp.	67.5 ± 9.6 B	40.0 ± 8.2 aA	45.0 ± 10.0 aA	45.0 ± 5.8 aA

Extract, concentration and their interaction are significant according to factorial ANOVA ($P < 0.05$). F (2, 48) = 51.4 $P < 0.05$ (for extract factor), F (3, 48) = 8.0 $P < 0.05$ (for concentration factor), F (6, 48) = 4.5, $P < 0.05$ (for interaction). Means (± SEM) followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($P < 0.05$). The absence of lower- or upper-case letters indicates no significantly differences, according to LSD test ($P < 0.05$).

Enzymatic activities

Chitinase activity was increased at 1 DAT by AN and ECK with respect to the control (11.3% and 28.8%), while at 3 DAT it was increase by JAN only (16.5%) (Fig. 7). The extract from AN showed a progressive chitinase activity decrease from 2 DAT to 3 DAT. No significant increasing was observed at 2 DAT with respect to the control. Enzymatic activity decrease was also showed by JAN at 1 DAT.

On the contrary, the extract treatment, did not show visible β-1,3-glucanase activity at any DAT.

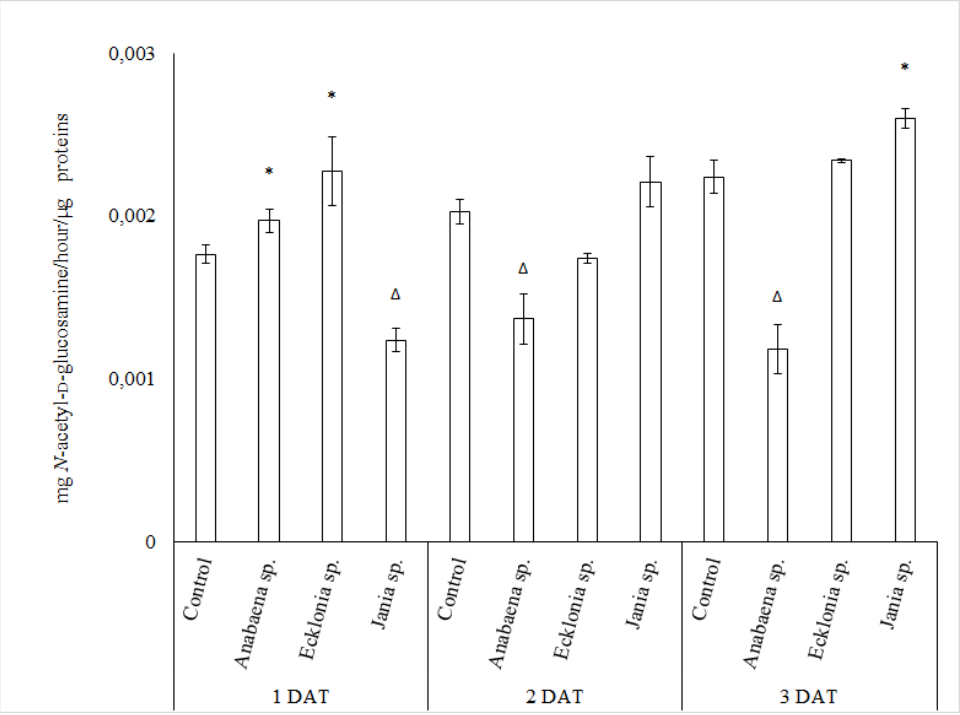

Fig. 7 Chitinase activity determined in protein extract of cotyledons following treatment with water (Control), *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. at 5 mg ml^{-1}extract at 1, 2 and 3 DAT (day after treatment). Extract, DAT and their interaction are significant according to factorial ANOVA ($P < 0.05$). F (3, 36) = 58.2 $P < 0.05$ (for extract factor), F (2, 36) = 22.4 $P < 0.05$ (for DAT factor), F (6, 36) = 54.1, $P < 0.05$ (for interaction). Enzyme activity was defined as the amount of enzyme that liberate 1.0 mg of *N*-acetyl-D-glucosamine from chitin per hour. Columns are mean values ± SD. The asterisk and the triangle indicate respectively significant increasing and reduction of chitinase activity within each DAT towards the control, according to LSD test ($P < 0.05$).

Gene expression of pathogenesis related proteins

Gene expression of *PR2*, *PR3* and *PR4* was upregulated in relation to the extract and to the DAT (Fig. 8). Level of transcript expression of *PR2* was almost two-fold higher with JAN 1 DAT with respect to the control (water). Two DAT chitosan, AN and JAN significantly increased the expression of *PR2*. Level of transcript expression of *AePR3* was significantly increased by chitosan and AN 1 DAT, by chitosan, AN and JAN both 2 and 3 DAT. Level of transcript expression of *BePR3* was increased only 2 DAT by chitosan, AN and JAN. Level of transcript expression of *PR4* was highly increased 1 DAT with chitosan, 2 DAT with Chitosan, AN and JAN and 3 DAT with chitosan and JAN.

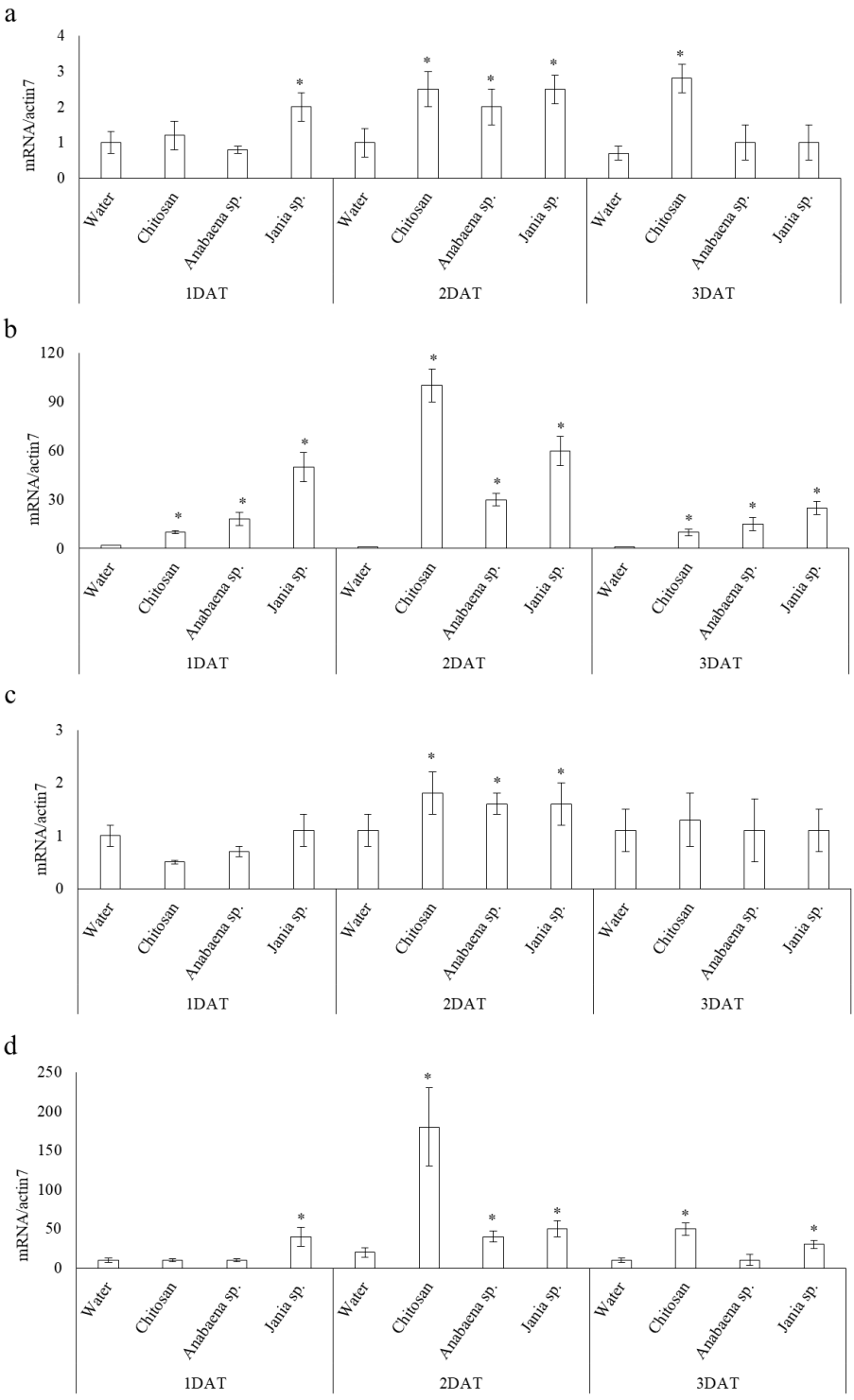

Fig. 8 Quantitative gene expression of PR2 (a), AePR3 (b), BePR3 (c) and PR4 (d) in cotyledons. Data are normalized to actin-7 reference gene expression. Columns are mean values ± SD. The asterisk indicates significant increases of level of gene expression induced by each extract towards the corresponding control (water) within each DAT, according to Student's t-test ($P < 0.05$).

4. Discussion

Algae and cyanobacteria are promising candidates to be used as biological control agents of plant pathogens, for their antimicrobial properties, as already shown for turnip (Stephenson, 1966), tomato

(Jiménez et al. 2011; Chaudhary et al., 2012; Prasanna et al., 2013), strawberry (Righini et al., 2018), cucumber (Jaulneau et al., 2011; Jayaraman et al. 2011), zucchini (Roberti et al. 2015; Roberti et al. 2016) and common bean and grapevine (Jaulneau et al., 2011).

The majority of available studies both *in vitro* and *in vivo* are focused on extracts obtained with organic solvents such as methanol, ethanol and acetone (Kulik, 1995; Arunkumar et al., 2010; Righini et al., 2018). On the contrary, water extracts have not been thoroughly investigated, even though they may be more sustainable for plant disease control, because they do not contain solvent residues. This study demonstrated that water extracts of the cyanobacterium *Anabaena* sp. and from the algae *Ecklonia* sp. and *Jania* sp. were able to control powdery mildew disease on cucumber by reducing both infected area and the sporulation of *P. xanthii* on cucumber cotyledons, depending on their concentration. In previous experiments on zucchini, *Anabaena* sp. extract, showed to reduce the disease caused by *P. xanthii* cotyledons (Roberti et al., 2016). Extracts of other *Anabaena* species, *A. variabilis* and *A. oscillarioides* obtained with organic solvents decreased disease severity caused by *Pythium debaryanum, Fusarium oxysporum* f. sp. *lycopersici, Fusarium moniliforme* and *Rhizoctonia solani* on tomato seedlings (Chaudhary et al., 2012). Reduction of *Fusarium oxysporum* f.sp. *lycopersici* disease was also showed by Prasanna et al. (2013) with application of filtrates of *A. variabilis* and *A. laxa* fresh cultures. Moreover, we showed an increase of chitinase enzyme activity, and the expression of the genes PR2, PR3 and PR4. The induction of plant resistance by *Anabaena* sp. extracts are in accordance with Prasanna et al., (2013) which demonstrated the increase of defence enzymes activities such as phenylalanine ammonia-lyase, polyphenol oxidase, chitosanase, and *β*-1,3 glucanase in tomato plants grown in substrate treated with filtrates of *A. variabilis* and *A. laxa* fresh cultures. Increases of enzyme activities correlated to induced systemic resistance, such as endochitinase, *β-N*-acetylhexosaminidase, chitin 1,4-*β*-chitobiosidase, *β*-1,3-glucanase and peroxidases were showed with *Anabaena* sp. spray treatment on zucchini cotyledonary leaves (Roberti et al., 2015).

The high antifungal activity showed by the extract from *Jania* sp., along with the increase of chitinase activity and the expression of *PR2, PR3* and *PR4* can be related to the presence of sulphated galactans, such as carrageenans, synthesized by red seaweeds, which are the major components of the extracellular matrix (Damonte et al. 2004; Matsuhiro et al. 2005; Pujol et al. 2006; Aruna et al. 2010; Souza et al. 2012). These polysaccharides are elicitors of plant defence responses against pathogens, such in case of *Phytophtora parasitica* var.*nicotianae* on tobacco plants (Mercier et al., 2001). On tomato seedlings infected by *Macrophomina phaseolina*, Agarwal et al. (2016) demonstrated that an extract from the red algae *Kappaphycus* a*lvarezii* increased the transcription of pathogenesis-related genes such as PR-1b1, PR-3, and PR-4 and the endogenous concentration of salicylic acid and the

cytokinin zeatin. It is known that a high concentration of cytokinin leads to salicylic acid accumulation and activation of defence gene expression (Argueso et al., 2012). Extract from *Ecklonia* sp. controlled the disease on plants when applied preventatively just before inoculation with the pathogen. In the induced resistance bioassay this extracts did not reduced the disease and the chitinase activity was not determined two days after treatment as well as the glucanase activity. This is in contrast with studies by Cluzet et al. (2004) and Walters et al. (2005) which demonstrated that several compounds in brown algae extracts can be involved in plant defence response. We suppose that the disease control by the *Ecklonia* extract may be due to its direct antifungal activity in our experimental conditions. Indeed, as other brown algae, *Ecklonia* sp. extract could contain secondary bioactive compounds (e.g., phenols) with antioxidant activities as demonstrated against both food-borne and human pathogens (Nagayama et al., 2002; Athukorala et al., 2006; Lee et al., 2010). Rekanović et al. (2010) showed antifungal activity of *E. maxima* applied to the soil against *Verticillium* wilt on pepper and Roberti et al. (2016) showed that *Ecklonia* sp. extract sprayed on zucchini cotyledons reduced the sporulation of *Podosphaera xanthii*, agent of powdery mildew. As for red algae, also polysaccharides extracted from brown algae are known to be elicitors of plant responses against pathogens. For example, sulphated fucans, present in the cell walls of marine brown algae, applied on tobacco leaves induced locally accumulation of salicylic acid and of the phytoalexin scopoletin. Moreover, after the treatment, pathogenesis-related proteins were expressed in addition to systemic accumulation of salicylic acid (Klarzynski et al., 2003). Other brown alga extracts showed to induce resistance in plants by enhancing the activities of various defence-related enzymes and genes. For instance, extract from *Ascophyllum nodosum* applied as spray treatment and/or root drench on cucumber plants, enhanced activities of various defence-related enzymes such as chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, and lipoxygenase, enhanced levels of phenolic compounds, and reduced disease incidence caused by *F. oxysporum*, *Alternaria cucumerinum* and *B. cinerea* (Jayaraman et al., 2011). Among these enzymes, chitinases and *β*-1,3-glucanases are capable of degrade the fungal cell wall components and their increase is considered a molecular marker of induced resistance (Van Loon et al., 2006; Roberti et al., 2015).

In conclusion, this study shows that treatment with water extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. were effective against *P. xanthii* on cucumber, by working both directly to the pathogen and indirectly through the involvement of plant defence responses helping plant to withstand the pathogen. The extracts obtained with water may be important sustainable tools to apply in environmentally- friendly disease management, reducing the adverse environmental effects of pesticides, once their biocontrol activity will be verified in a larger scale experiment.

5. References:

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5. Quantification of carbohydrates, proteins, chlorophyll, carotenoids, phycobiliproteins and antioxidant activity of water extracts

The objective of the present work was to quantify carbohydrates, proteins, chlorophyll, carotenoids, phycobiliproteins and antioxidant activity of water extracts from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp.

This study was carried out at BEA (Banco Español de Algas), Muelle de Taliarte, Gran Canaria, Spain.

1. Materials and methods

1.1 Water extracts

The *Anabaena* sp. strain BEA 0300B was isolated from a coastal humid basaltic wall in Ajuy, Gran Canaria, Spain. The strain was cultivated in a low cost bioreactor (Almeida et al., 2009) for 20 days, then collected after centrifugation (12,000 rpm) and finally lyophilised and maintained at 4 °C. The cyanobacteria *Anabaena* sp. strain BEA 0300B together with the red alga *Jania* sp. and the brown one *Ecklonia* sp. belong to the Spanish Bank of Algae (BEA), Marine Biotechnology Centre, University of Las Palmas de Gran Canaria, Spain. Algae and cyanobacteria used in the experiments were provided by the Spanish Bank of Algae, Marine Biotechnology Center, University of Las Palmas de Gran Canaria. *Anabaena* sp. were provided as lyophilized biomass, while *Ecklonia* sp. and *Jania* sp. as dry thallus. Dry thallus was grounded to fine powder with mortar and pestle. Water extracts were obtained by suspending each powder in sterile distilled water (0.5%) under continuous stirring at 50°C for 12 h, then filtered (Roberti et al., 2015). Extracts were frozen at -20 °C and lyophilized (Freezone 6L, Labconco).

1.2 Yield determination

The yield related to the biomass extracted from water extracts was calculated as reported by Álvarez-Gómez et al. (2016): (solubilized (obtained) / biomass (provided)) × 100. Solubilized (obtained) is the biomass (g) obtained after lyophilisation of water extract and biomass (provided) is the biomass (g) used to make the extraction.

1.3 Carbohydrates determination

Carbohydrates concentration was assayed in triplicate by following the procedure of Dubois et al. (1956). For carbohydrates digestion 10 mg of lyophilized extract were mixed with 2 ml of trichloroacetic acid (TCA, 10% w: v) in glass tube. After incubation for 60 min in a water bath (Buchi B-491 Heating Bath) at 90 °C they were cooled, transferred in test tubes and centrifuged at 1.000 g for 5 min. For carbohydrates determination, 0.5 ml of the surnatant, 0.5 ml of phenol (5%) and 3 ml of H_2SO_4 (95-97%) were added in the cuvettes. After 30 min, absorbance was measured at 480 nm (Perkin Elmer, Lambda 25 UV/VIS) at room temperature. D-(+)-Glucose (Sigma-Aldrich) was used as standard and, as blank, phenol in addition to H_2SO_4 and TCA.

1.4 Protein determination

Protein concentrations were determined by Lowry method (1951) in triplicate. Protein determination were based on colorimetric determination using bovine serum albumin (BioRad Laboratories, Inc.) as the standard. Ten milligrams of lyophilized water extracts were dissolved in 2 ml of NaOH (0.1M) in test tubes and mixed briefly. Tubes were incubated for 1 h in a thermomixer at 42 °C, samples were mixed and centrifuged for 10 min at 10.000g and surnatants were used for protein determination at the spectrophotometer. In cuvettes, 0.5 ml of each surnatant in addition to 0.980 ml of Lowry solution were mixed together and incubated for 20 min in the dark at room temperature. For colorimetric determination, 140 µl of Folin (0.83%, 2N, Sigma) reagent were added and mixed and the cuvettes were incubated in the dark for 30 min. The absorbance were measure at 750 nm. As blank 0,980 ml of Lowry solution was mixed with 0.5ml NaOH and 0.140 ml of Folin reagent. Lowry solution was prepared as follows:

-solution A, NaOH (0.57%) + Na2CO3 (2.86%) in distilled water under stirring until they were dissolved and stored at 4 °C until use;

-solution B, $CuSO_4$.5 (H_2O) 1.42 % in distilled water and stored at 4 °C until use;

-solution C, Na_2tartrate.2 (H_2O) 2.85 % in distilled water and stored at 4 °C until use;

-mixed for use with following alphabetical order sol.A + sol. B + sol.C (ratio 100:1:1).

1.5 Chlorophyll and carotenoids determination

In 2 ml test tube, 10 mg of lyophilized biomass of extract were dissolved in 0.5 MeOH (100%) and mixed (Retsch MM400) for 10 min with 8-10 glass beads at 30 Hz. Then, 0.5 ml of MeOH (100%) were added and test tubes were incubated in the dark for 3 hours in a Thermomixer (Eppendorf Thermomixer R Mixer), at 23 °C at 450 rpm. Surnatants were used to measure the absorbance at 665,

652 and 470 nm. Methanol (100%) was used as blank. Chlorophyll content of the lyophilized extracts was calculated using the following equations (Wellburn 1994; Hartmut et al. 2001):

chlorophyll *a* ($\mu g\ ml^{-1}$) = 16.72 $A_{665.2}$ – 9.16 $A_{652.4}$

chlorophyll *b* ($\mu g\ ml^{-1}$) = 34.09 $A_{652.4}$ – 15.28 $A_{665.2}$

carotenoids ($\mu g\ ml^{-1}$) = (1000 A_{470} - 1.63 Cl *a* - 104.9 Cl *b*) / 221

1.6 Phycobiliproteins determination

From lyophilized biomass, 10 mg was mixed with 8-10 glass beads (perline) for 5 minutes in 2ml test tubes. One ml of phosphate buffer was added and mixed (Retsch MM400) with the powder for 5 min at 30 Hz. The tubes were left for 3 hours in a Thermomixer at 23 °C at 450 rpm and then they were centrifuged for 15-20 min at 10 000 g. The surnatant were transferred in the cuvettes and adjusted with phosphate buffer. Phosphate buffer was used as blank. The absorbance was measured at 652, 615 and 562 nm. Phycobiliproteins content of the lyophilized extracts was calculated using the following equations (Bennett and Bogorad, 1973):

phycocyanin (PC) ($mg\ ml^{-1}$) = [A_{615} – (0.474 × A_{652})] / 5.34

alophycocyanin (APC) ($mg\ ml^{-1}$) = [A_{652} – (0.208 × A_{615})]/5.09

phycoerythrin ($mg\ ml^{-1}$) = [A_{562} – (2.41 × PC) – (0.849*APC)] / 9.62

1.7 Antioxidant activity

The free-radical scavenging activity of lyophilized water extracts was analysed using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Güenaga, 2011). In test tubes, 10 mg of lyophilized extracts were mixed with 0.5 ml of MeOH (100%). After and vortex for 5 min. Then, 0.5 mL of MeOH were added and the test tubes were incubated in the dark for 3 hours in a Thermomixer at 23 °C at 450 rpm. In cuvettes, 150 µl of DPPH (1.27 mM in MeOH 90%) were mixed with 1.35 ml of MeOH (90%) and 150 µl of surnatant. The solution was kept at room temperature for 30 min, and the absorbance at 517 nm (A517) was measured. As control MeOH (90%) + DPPH was used, and MeOH (90%) only, as blank. The DPPH scavenging effect was calculated as follows:

% scavenging activity = (A_{cotrol} - (A_{sample} - A_{blank}) / A_{cotrol}) × 100.

2. Results

The yield was significantly different depending on the extract (Fig. 1). The highest yield has been obtained from *Anabaena* sp. while the lowest from *Jania* sp.

Carbohydrate concentration of the lyophilised water extracts is shown in Fig 2.1. Lyophilised extract from *Anabaena* sp. contains significantly more carbohydrates (46.2%) than the extracts from *Ecklonia* sp. and *Jania* sp. (23.6 and 22.6 %, respectively). *Anabena* sp. showed the highest protein

content, 80.7% more than *Ecklonia* sp. and 46.5 % more than *Jania* sp (Fig. 2.2). Extract from *Anabaena* sp. showed also the highest content in chlorophyll *a* and carotenoids (Fig. 3.1), and in phycobiliproteins (Fig. 3.2). The cyanobacterial biomass exhibited the highest scavenging effect (Fig. 4) in comparison to *Ecklonia* sp. and *Jania* sp.

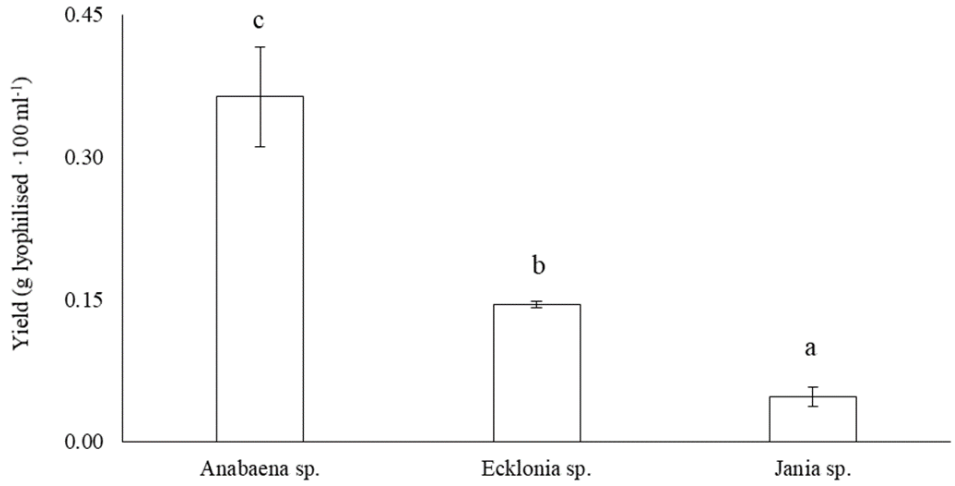

Fig. 1 Yield of the extracts from *Anabaena* sp., *Ecklonia* sp., *Jania* sp. after lyophilisation. Columns are mean values + SD. Different letters indicate significant differences within each extracts, according to LSD test ($P < 0.05$).

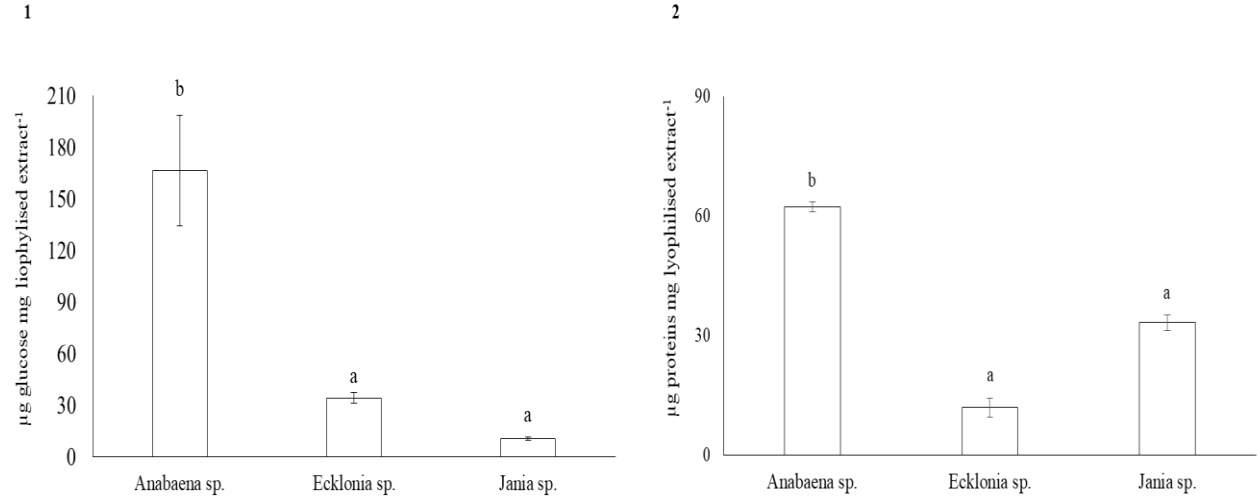

Fig. 2 Content in carbohydrates (1) and proteins (2) content of lyophilised extracts of *Anabaena* sp., *Ecklonia* sp., *Jania* sp. Columns are mean values + SD. Different letters indicate significant differences within each extracts, according to LSD test ($P < 0.05$).

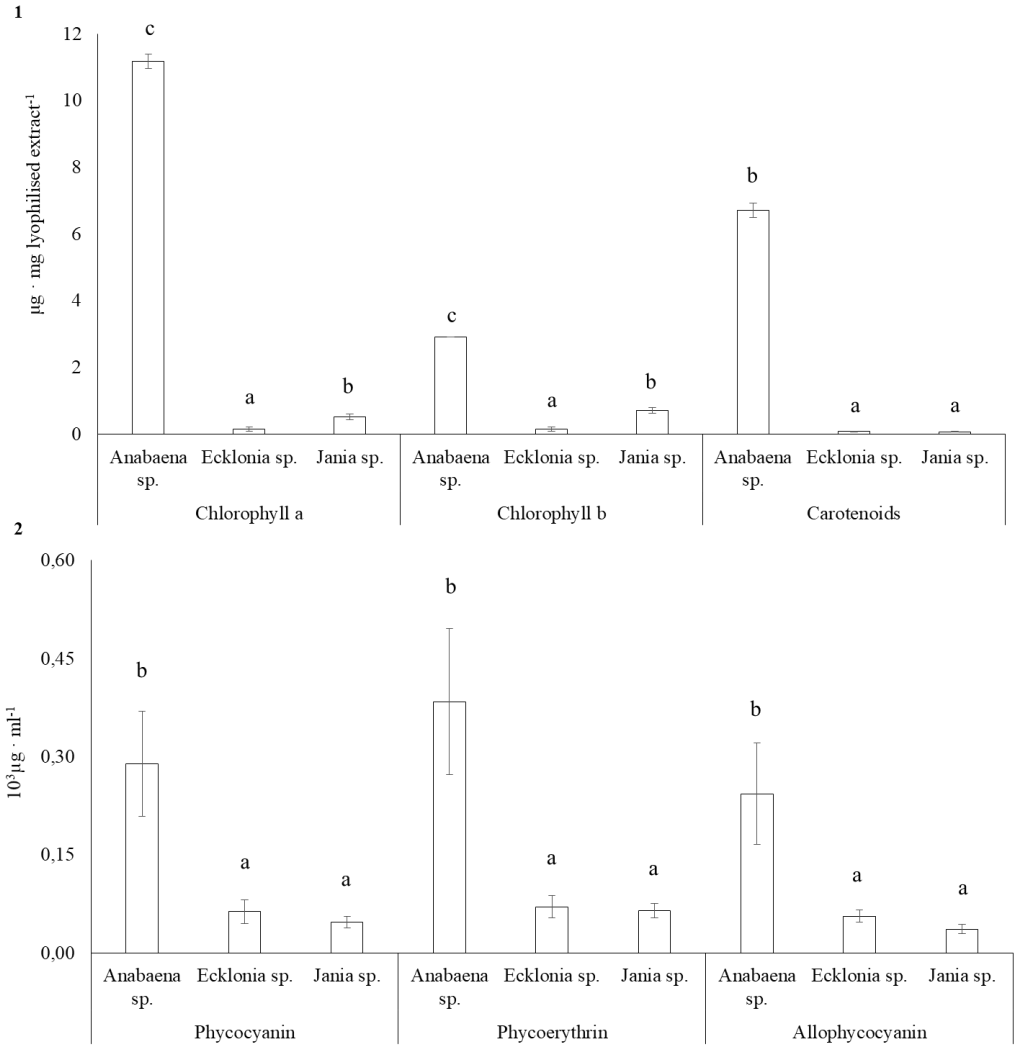

Fig. 3 Content in chlorophyll *a* and *b* and carotenoids (1) and phycobiliproteins (2) content of lyophilised extracts of *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. Columns are mean values + SD. Different letters indicate significant differences within each extracts, according to LSD test ($P < 0.05$).

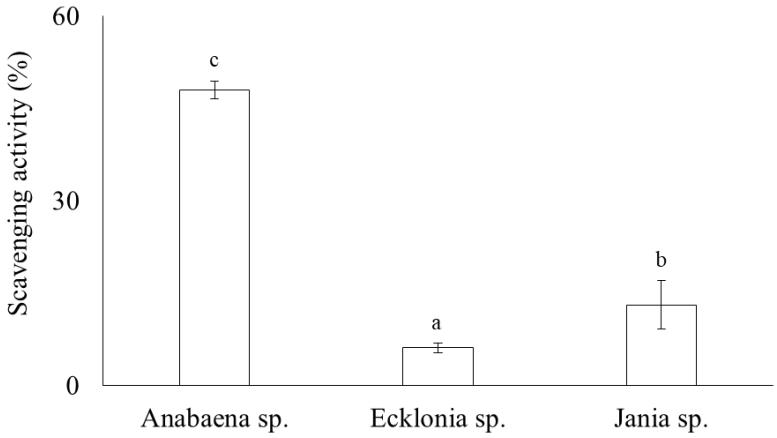

Fig. 4 Scavenging activity of lyophilised extracts from *Anabaena* sp., *Ecklonia* sp., *Jania* sp. Columns are mean values + SD. Different letters indicate significant differences within each extracts, according to LSD test ($P < 0.05$).

3. Discussion

Algae and cyanobacteria are an interesting source of bioactive compounds potentially useful for the control of plant pathogens. Most of the available literature regards the quantification of extracts from algae and cyanobacteria with different solvents. To our knowledge there are no studies on the quantification of compounds contained in water extracts.

Among the lyophilised biomass used in this study, that from the cyanobacterium *Anabaena* sp. showed the highest amount of carbohydrates and proteins content with respect to *Ecklonia* and *Jania* sp. extracts. The content of carbohydrates in *Anabaena* sp. was 16.6 %. Möllers et al. (2014) observed that in *Synechococcus* sp. the total carbohydrate content per dry weight was about between 20 and 35% (w: w) depending on the growth conditions. López et al. (2010) found that protein content of the cyanobacteria *Synechocystis aquatilis* and *Arthrospira platensis* ranged by 15-30% and 50-55%, respectively, while our data showed that *Anabaena* content was lower (6.2%). About *Jania* sp., carbohydrates represent 1.1 % and proteins 3.3 % of its lyophilised biomass. On *Jania rubens* Ahmed et al. (2011) demonstrated that the total hydrolysable carbohydrates was 23.3% on dry weight, while the content of proteins was 9.6%. About *Ecklonia* sp., we found that carbohydrates represent 3.4 % of the lyophilised biomass. In other brown algae such as *Laminaria digitata* harvested in the North Sea in August, glucose represented 51 % w/w of the dry matter (Manns et al., 2014). It is also reported that *Laminaria digitata* and *Saccharina latissima*, differ widely in laminaran content (0–33 %) the principal carbohydrate reserve of brown seaweeds, due to large seasonal variations (Adams et al., 2011). Differences among algae in compounds content is highlights by Lourenço et al. (2002). The same authors showed that protein of the brown and red algae can varies from 10.8% to 23.1%, as in case of the brown alga *Chnoospora minima*, and of the red alga *Aglaothamnion uru-guayense*, respectively. In our experiment, protein content in the red alga *Jania* sp. was higher than that in the brown *Ecklonia* sp., in line with what Lourenço et al. (2002) observed.

Chlorophylls, carotenoids and phycobiliproteins are the classes of pigments that play an important role in the photosynthetic and pigmentation metabolism of algae and cyanobacteria. Carotenoids are accessory pigments acting as light energy harvesters and antioxidants that inactivate reactive oxygen species formed by the exposure of cells to environmental stress (Ioannou and Roussis, 2009). Our results on *Ecklonia* sp. showed similar values as reported by Gao et al. (2016) in juvenile *Ecklonia* sporophytes using acetone as solvent. A study on the three brown algae species *Sirophysalis trinodis*, *Polycladia myrica* and *Colpomenia sinuosa* showed that the amount of chlorophyll *a* and *b* and carotenoid vary in relation to the solvent and the alga species (Etemadian et al., 2017). Comparing *Ecklonia* sp. data to these algae, emerge that *Ecklonia* sp. has lower amount of chlorophyll and

carotenoid. Concerning *Jania* sp., chlorophyll *a* content is lower than chlorophyll *b*, in contrast to what El-Din and El-Ahwany (2016) reported for *Jania rubens*. The authors also showed that among the red algae examined, *Pterocladia capillacea* has the maximum chlorophyll *a* chlorophyll *b* and carotenoid content, followed by *Jania rubens* and *Corallina mediterranea.* In line with our data, the same authors showed that carotenoid content was lower than chlorophyll *a* and *b*. From our results, comparing the three extracts, the amount of chlorophyll *a* and *b* and carotenoid in *Anabaena* is highest than the amount in *Ecklonia* sp and *Jania* sp. Two *Anabaena* sp. strains showed higher value of chlorophyll *a* and carotenoid (average of 16.6 and 2.7 μg mL-1, respectively) with respect to two strains of *Nostoc* sp. (2.5 and 1.7 μg mL^{-1}, respectively).

Concerning phycobiliproteins, they are coloured and water-soluble protein pigments organized in complexes called phycobilisomes (Glazer, 1994; Hemlata and Fareha, 2011). They are present in red algae, cyanobacteria and cryptomonads (biflagellate unicellular eukaryotic algae) (MacColl, 1998). The main classes of phycobiliproteins based on their colour and adsorption properties are allophycocyanin, (for bluish green, 650–655nm), phycocyanin (for blue, 610–620), phycoerythrin (for purple, 540–570nm), (Samsonoff and MacColl, 2001; Pandey et al., 2013). Phycobiliproteins are water soluble and stable at physiological pH. To understand better the contribution of each proteins to the efficiency of light harvest, some authors have studied the so-called 'delayed fluorescence', also termed delayed luminescence or delayed light emission. It is a long-lived light emission by plants, algae and cyanobacteria after being illuminated with light and placed in darkness (Strehler and Arnold 1951; Berden-Zrimec et al., 2010). It can last from milliseconds to several minutes, which is by itself an odd phenomenon in an otherwise nanosecond world of classical fluorescence. Delayed fluorescence excitation spectroscopy is also a fast in vivo method to determine phytoplankton composition in freshwaters using the signature of photosynthetically active pigments that is characteristic in each cyanobacteria. A study conducted on the several cyanobacteria species grown in different light conditions, showed a high species-specific variability of the ratio phycocyanin: phycoerithrin: allophycocyanin derived from delayed fluorescence excitation (Bodemer, 2004). In particular the cyanobacterium *Pseudoanabaena* spp. showed pronounced higher delayed fluorescence excitation efficiency in the wavelength range 400-700 nm where phycoerythrinis absorbing light. Its ratio content in phycobiliprotein was 1.6: 3.1: 1, phycocyanin: phycoerithrin: allophycocyanin (Bodemer, 2004). Concernig red algae, Lage-Yusty et al. (2013) reported that in *Porphyra umbilicalis* and Palmaria palmate the phycocyanin is the most abundant phycobiliprotein, followed by Phycoerythrin while allophycocyanin has been no detectable. The same authors supposed that this variability might depends on the time harvest. In our study *Anabaena* sp. showed the highest content

of phycocyanin, phycoerythrin and allophycyanin compared to *Ecklonia* sp. and *Jania* sp. Among the phycobiliproteins, phyerythrin was the most abundant in *Anabaena* sp.

In general, cyanobacteria and algae have been known for their content in antioxidants compounds such carotenoids, phycobilin pigments, catechin, flavonols, glycosides, sulphated polysaccharides, vitamins, phlorotannins and phenolic compounds (Cornish and Garbary, 2010; Kepekçi and Saygideger, 2012). In this experiment, the lyophilised biomass of extract from *Anabaena* sp. showed the highest antioxidant activity, followed by *Jania* sp. and *Ecklonia* sp. Many authors reports the potential of other cyanobacteria species as source of antioxidants (Kepekçi and Saygideger, 2012; Hassouani et al., 2017; Hossain et al., 2016; Nagasathya and Thajuddin, 2008; Ismaiel et al., 2013; Pant et al., 2011). A study conducted on the antioxidant activity of several cyanobacteria species showed that extracts of *Anabaena doliolum* and *Anabaena constricta*, were among those that exhibited high free radical scavenging activity in terms of DPPH (Singh et al., 2017). For *Arthrospira* sp. authors reported production of antioxidants including the pigment C-phycocyanin, carotenoids, polyunsaturated fatty acids (Kepekçi and Saygideger, 2012), and phenolic acids (Miranda et al., 1998; Abd El-Baky et al. 2009; Shalaby and Shanab 2013). Indeed, Ismaiel et al. (2013) reported that several *S. platensis* has the highest radical scavenging activity (524%) and the highest chlorophyll *a* and carotenoids contents (10.6 ± 0.6 and 2.4 ± 0.02 mg $g^{-}1$ of dry weight, respectively) with respect to several *Anabaena* and *Nostoc* species. On the contrary, Blagojević et al. (2018) reported high antioxidants activity by several *Nostoc* species, in comparison to those of *Anabaena* and of *Arthrospira*. There are numerous publications that highlight the potential of algae as source of natural antioxidants. Khairy and El-Sheikh (2015) showed as antioxidant scavenging activity of the red algae *J. rubens* and *Pterocladia capillacea* and the green alga *Ulva lactuca* increase along with algae concentration and change along with season of harvest. In fact *U. lactuca* and *J. rubens* showed the maximum antioxidant scavenging activity especially during summer. Also their content in natural antioxidants such phenols and b-carotene changed among species and in relation to the harvest. *J. rubens* showed highest content in phenolic compounds while *P. capillacea* in *ß*-carotene during summer. In contrast to what we have observed, several authors reported the antioxidant activity of extracts from *Ecklonia* sp. and of compounds or fraction extracted from the alga (Lee et al., 1996; Nakamura et al., 1996; Heo et al., 2009; Kang et al., 2011) For example, Athukorala et al (2006) showed that extract crude polyphenolic fraction from *Ecklonia cava* ml scavenged 70% of DPPH radical and, the same fraction also showed interesting antiradical properties, expressed by its capacity to scavenge superoxide anion (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). Among several fractions obtained from *E. cava*, the diethyl ether fraction displayed noticeable DPPH radical scavenging activity (Kang et al., 2011). From this fraction the authors isolated the active compound

pyrogallol-phloroglucinol-6,6'-bieckol (PPB), that showed antioxidant activities higher than that the commercial antioxidant ascorbic acid. Furthermore, PPB effectively inhibited DNA damage induced by H_2O_2. Phlorotannins, polyphenols from brown algae, have demonstrated several biological activitities such as anti-plasmin inhibitor, anti-allergic, antibacterial and antioxidant (Nakamura et al., 1996; Nagayama et al., 2002; Sugiura et al., 2006). The phlorotannins phloroglucinol, eckol and dieckol extracted from *E. cava* reduced the intracellular ROS induced by UV-B radiation on human fibroblast. Among the three phlorotannins tested, dieckol demonstrated strong protective properties against UV-B radiation-induced DNA damage Heo et al. (2009). As for brown algae, antioxidant activity of red algae is widely reported (Mellouk et al., 2017; Bartolomeu et al., 2011; El-Din et al.2016; Rode and Sabala, 2018; Ganesan et al., 2008).

Among several fractions obtaind with different solvent from the red algae *Jania rubens, Corallina mediterranea* and *Pterocladia capillacea* the methanolic one was found to have the highest reducing power and total antioxidant capacity hydrogen peroxide scavengingactivity, reducing power and total antioxidant activity of various extracts of selected seaweeds. Methanolic in addition to the aqueous extract from *Asparagopsis taxiformis* exhibited higher inhibition against superoxide and nitric oxide radicals and radical scavenging activity compared with standard ascorbic acid (Mellouk et al., 2017). As phlorotannins for brown algae, polysaccharides from red algae showed antioxidant activity. Sulfated polysaccharides extracted from *Corallina officinalis* showed considerable antioxidant properties and had more excellent abilities than de-sulphated polysaccharides (Yang et al., 2011). A screening of the antioxidant activity of extracts from several algae belonging to Phaophyceae, Rhodophyta and Chlorophyta algae harvested around Hawaii islands, revealed significantly variability among algae (Kelman et al., 2012). Brown algae had the highest mean antioxidant activity, followed by the green and the red.

The data obtained from this study showed significant variation in carbohydrates, protein chlorophyll, carotenoids and phycobiliproteins content and antioxidant activity of *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. lyophilised extracts. This variation depends to the fact that *Anabaena* sp. is a cyanobacterium and *Ecklonia* sp. and *Jania* sp. are two macroalgae belonging to different phylum. Moreover, the environmental condition growth and season of harvest can change their composition and property. It must also be said that in natural conditions also climatic changes of the last years such as nutrient availability, pH, temperature, light and precipitation may affects algae and cyanobacteria composition and their property (Ansari and Ghanem, 2017). Nonetheless, different methodologies; extraction processes can lead to different final results (Lourenço et al., 2002).

Anabaena sp. showed the high content in carbohydrates, proteins, chlorophyll and carotenoids and phycobiliproteins. Moreover, it had the highest antioxidant activity. In light of these results, *Anabaena* sp. can be considered a promising source of bioactive substances that might have several applications against fungal plant pathogens.

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6. Antifungal activity of polysaccharides extracted from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. against *Botrytis cinerea*

The effect of polysaccharides extracted from water extracts of *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. against *Botrytis cinerea* was evaluated *in vitro* and *in vivo*. Polysaccharide concentrations used were: 0.86, 1.72 and 3.45 mg ml^{-1} for *Anabaena* sp.; 0.41, 0.82 and 1.64 mg ml^{-1} for *Ecklonia* sp.; 0.045, 0.09 and 0.18 mg ml^{-1} for *Jania* sp. These concentrations were tested *in vitro*, on fungal colony growth, spore germination and on colony forming units (CFUs), and on strawberry fruits against *B. cinerea* with pre- and post-harvest application. In *in vitro* experiments, polysaccharides from *Anabaena* sp. at 1.72 and 3.45 mg ml^{-1} and from *Ecklonia* sp. at 0.82 and 1.64 mg ml^{-1} inhibited pathogen colony growth. CFUs were inhibited by all concentrations of *Anabaena* sp. and *Ecklonia* sp. polysaccharides and *Anabaena* sp. polysaccharides inhibited spore germination at all concentrations after 4 and 24 h of incubation. In *in vivo* experiments, the pre-harvest treatment on strawberry fruits with polysaccharides from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. showed to reduce both fruit infected area and pathogen sporulation at all concentrations, while post-harvest treatment did not show any effect against *B.cinerea*.

1. Introduction

Botrytis cinerea is a fungal pathogen agent of fruits rotting worldwide (Fig. 1), both in field and during storage after harvesting. The classic symptom on fruits is the grey mould. The fungus can affect plant leading severe defoliation, flower death, reduction in the fruit market value; stem lesions and, consequently, plant death (Dik et al., 1999; Borges et al., 2014). Under favourable conditions both in field and during the storage, the pathogen can cause heavy fruit losses. *Botrytis cinerea* can infect a large host range of fruits such as pea (Dallagnol et al., 2014), zucchini (Cheon et al., 2013), tomato (Rodríguez et al., 2014), red chicory (Bertolini et al., 2003), kiwi (Michailides and Elmer, 2000), grape (Ciliberti et al., 2015), strawberry (Blanco et al., 2006) and golden berry (Erper et al., 2015). On strawberry under favourable conditions during flowering and harvest, yield loss can exceed 50%. Symptoms can become evident in the field on fruits, flowers, leaves and petioles when high humidity conditions occur, and are widely found in harvested fruits (Fig. 2). At the beginning, the infection on fruits appears as soft, light brown area that rapidly enlarges. If infected fruits remain on the plant, they usually dries up and starts to be covered by the grey mould. Fruit infection is most severe in well-protected areas of the plant, where the humidity is high and air movement is poor. The

infection can start on young green fruits, but fruits become more susceptible during ripening and once they are mature. After the harvesting, mature fruits are extremely susceptible to *B. cinerea* and the handling of infected fruit will spread the fungus to healthy ones. The fungus overwinters as black bodies, called sclerotia, or as mycelium in strawberry plant debris, such as fruits or leaves (Sutton, 1995). In early spring, the mycelium produces large numbers of spores on the surface of both plant and fruit debris. Spores are spread by wind throughout the planting where they are deposited on blossoms and fruits. They germinate when a film of moisture is present and infection can start within a few hours. Temperatures between 20 to 26 °C and free moisture on the foliage from rain, dew, fog, or irrigation water are ideal conditions for disease development. The disease can develop also at lower temperatures if foliage remains wet for long periods. Strawberries are susceptible to *B. cinerea* during bloom and fruit ripening. During bloom, the fungus colonizes healthy or senescing flower parts, often turning the blossoms brown. These blossom infections establish the fungus within the receptacle of the young fruit as a "latent" or "quiescent" infection. The fungus generally remains latent in developing (green) fruit until the fruit starts to mature, at which time the fungus becomes active and symptoms such as rotting appear. Thus, the most critical period for applying fungicides to control grey mould is during bloom. This consideration is an important point to remember when considering fungicide applications for controlling this disease.

The development of bio-based strategies to control plant pathogens and enhance food safety has encouraged by the Directive 2009/128/EC and its implementation that promote specific actions to support the establishment of sustainable agriculture. Several researches were focused on the activity against plant pathogens of extracts obtained from algae and cyanobacteria by using organic solvents (Rizvi and Shameel, 2004; Kumar et al., 2008; Arunkumar et al., 2010; Sivakumar, 2014; Jiménez et al., 2011), while few studies have examined the antifungal activity of water extracts (Roberti et al., 2015; Righini et al., 2018). Therefore, algae and cyanobacteria could be considered useful tools, alternative to synthetic products, for their role in plant disease control.

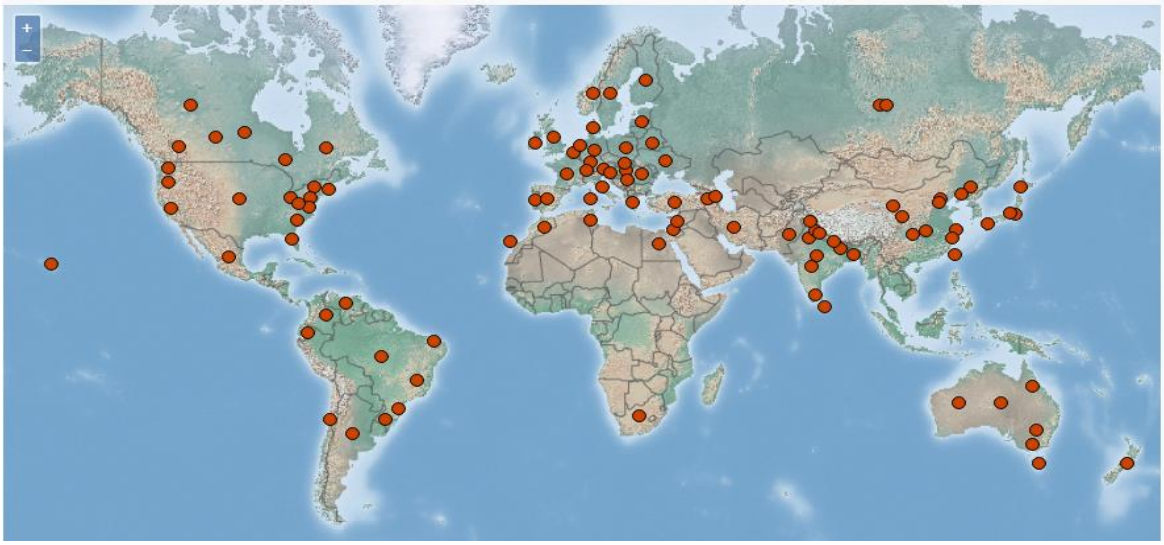

Fig. 1 World map distribution of *Botrytis cinerea* (CABI source).

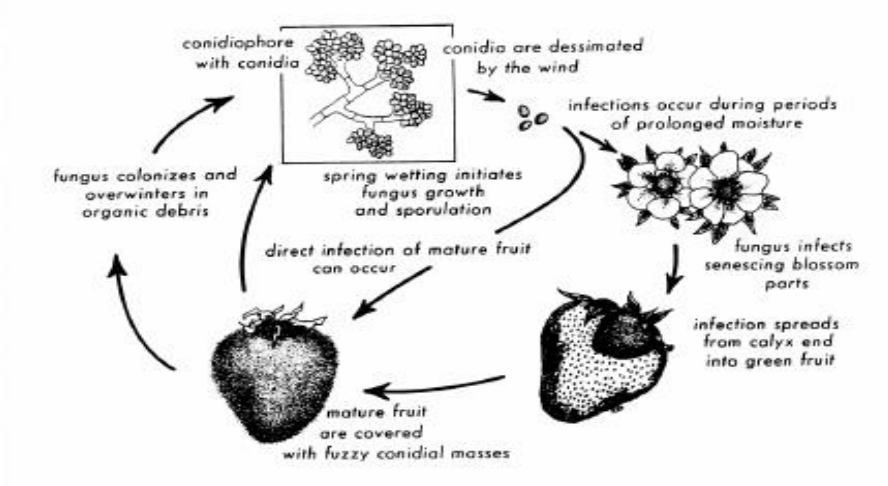

Fig. 2 Life cycle of *Botrytis cinerea* on strawberry (Small Fruit IPM Disease Identification Sheet N. 1).

The objectives of the present work are to: (i) extract the polysaccharides from the water extracts; (ii) test the antifungal activity of polysaccharides against *Botrytis cinerea* both *in vitro* and *in vivo*.

This study was carried out at BEA (Banco Español de Algas), Muelle de Taliarte, Gran Canaria, Spain.

2. Materials and methods

2.1 Polysaccharides extraction

Anabaena sp., (AN) *Ecklonia* (ECK) and *Jania* sp. (JAN) were provided by the Spanish Bank of Algae, Marine Biotechnology Center, University of Las Palmas de Gran Canaria. *Anabaena* sp. was provided as lyophilized biomass, while *Ecklonia* sp.and *Jania* sp. as dry thallus. Dry thallus of Ecklonia sp. and Jania sp.was grounded to fine powder with mortar and pestle. Water extracts were

obtained by suspending each powder in sterile distilled water (0.5%) under continuous stirring at 50°C for 12 h, then filtered (Roberti et al., 2015). Polysaccharides were extracted by following the method of Abdala Díaz et al. (2010). After filtration of water extracts, polysaccharides extraction were carried out by selective precipitation with 2% (w: v) O-*N*-cetylpyridinium bromide (Cetavlon; Morris Quevedo et al. 2000). The precipitated acidic polysaccharides were purified with 4 M NaCl, flocculated again with 96% (v/v) ethanol and centrifuged (10,000×g for 10 min; Beckman Coulter Avanti J26XP). Then they were dialyzed against 2 M NaCl, and lyophilized (Fig. 3).

The yield related to the polysaccharides biomass extracted from water extracts was calculated as reported by Álvarez-Gómez et al. (2016) with modifications: (solubilized (obtained) / biomass (provided)) × 100. Solubilized (obtained) is the lyophilised polysaccharides biomass (g) obtained from water extract and biomass (provided) is the biomass (g) used to make the water extract.

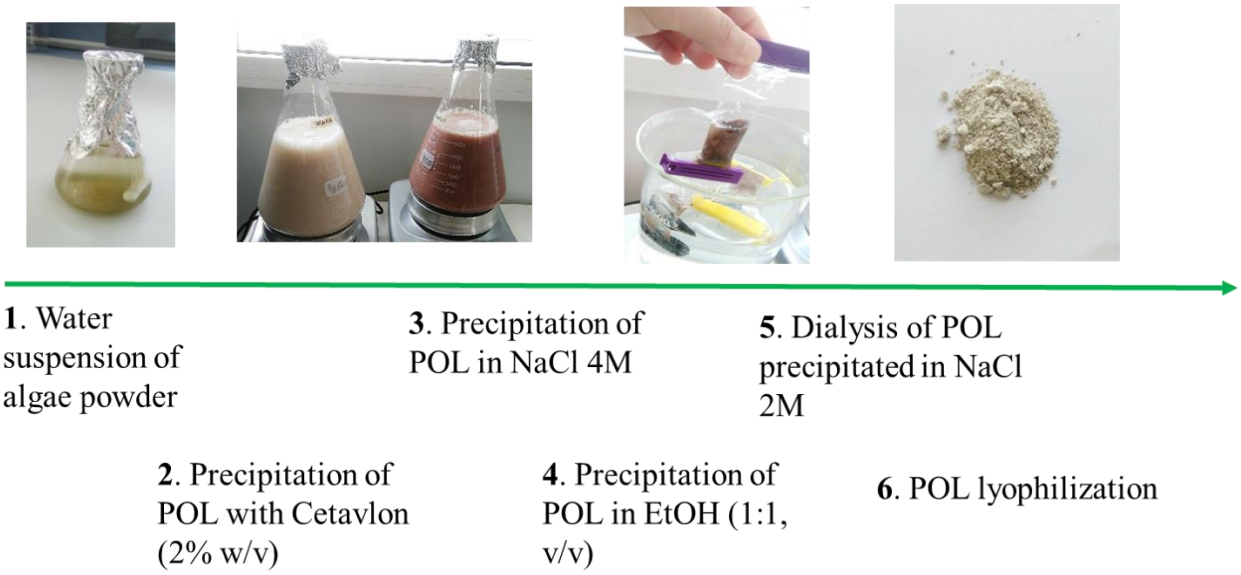

Fig. 3 Main phases of polysaccharides extraction.

2.2 Pathogen and polysaccharides

Botrytis cinerea strain B05.10 was isolated from strawberry fruits showing symptoms as grey mould (Fig. 4 a, b). Fruit surface was gently scratched with a sterile needle and slithered on PDA supplemented with 60 mg of streptomycin sulphate (Sigma-Aldrich Co.) in Petri dishes. The fungus pathogenicity was verified through inoculation of spore suspension obtained from 7-day-old colonies on strawberry fruits and waiting for the symptom appearance.

Polysaccharides concentration was calculated considering their amount extracted and the volume to make the extraction. In Table 1 polysaccharides concentration used in the experiment are reported.

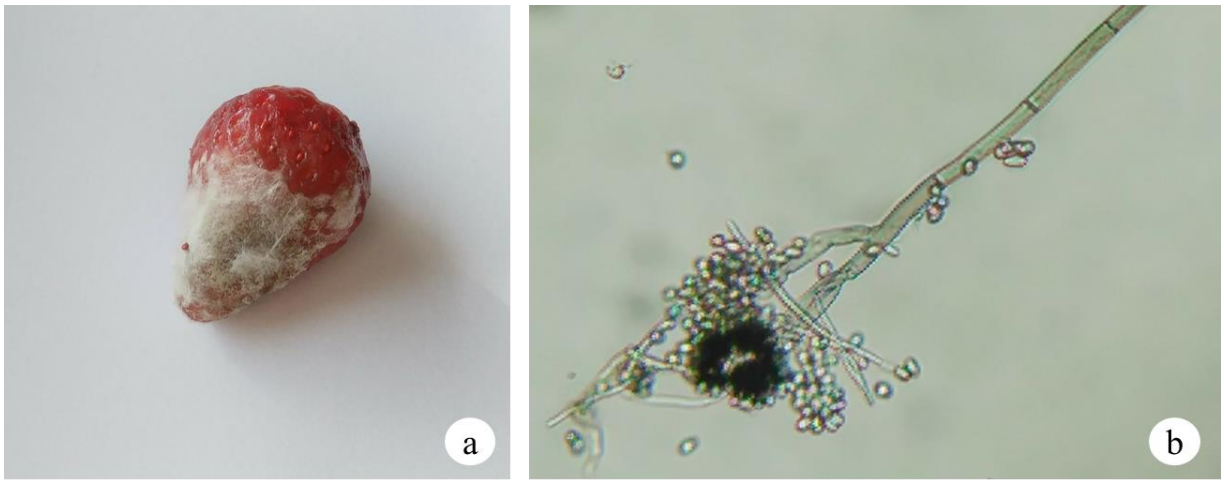

Fig. 4 Strawberry fruit infected by *Botrytis cinerea* (a), *B. cinerea* spores and hyphae at the microscope (400× magnitude).

Tab. 1. Polysaccharides concentration used in the experiments

Polysaccharides	mg ml^{-1}		
	0.5×	1*	2×
Anabaena sp.	0.86	1.72	3.45
Ecklonia sp.	0.41	0.82	1.64
Jania sp.	0.045	0.09	0.18

*polysaccharides concentration calculated considering the concentration 0.5% (w: v, powder: water).

2.3 Antifungal activity of polysaccharides against *B. cinerea in vitro*

Portions of 7 mm diameter were cut from 10-day-old fungal colony and transferred in test-tube containing a 600-µl aliquot of each polysaccharides concentration (Fig. 5). After 6 h, colony portions were placed on PDA medium in Petri dish and incubated at 24 °C in the dark. Colony growth was measured daily in two directions, along two mutually perpendicular diameters until growth of the untreated control reached the edge of the Petri dish. Three dishes were considered for each treatment and water control was considered. The experiment was repeated twice. To evaluate the antifungal effect of polysaccharides on *B. cinerea* spores, PDA medium amended with the three polysaccharides concentrations was prepared. In a half Petri dish 50 µl of spore suspension at 5×10^2 spores/ml were spread. Three Petri dishes were used for each treatment and for the untreated control. Inoculated Petri dishes were incubated at 25 °C in the dark After 48 h the number of colony forming units (CFUs) was counted, and the percentage of CFU compared to the total spores inoculated (i) was determined as follows: CFU (%) = number CFUt/number CFUi × 100%, where CFUt is the number of CFU obtained from treated spores and CFUi is the number of inoculated spores (25 spores per each half dish). The experiment was repeated twice. The diameter of three CFUs per dish were recorded by

measuring along two mutually perpendicular diameters. Polysaccharides that showed highest growth inhibition were considered for testing their effect also on spore germination. On microscope glass slides, equal volumes of spore suspension at final concentration of 5×10^5 spores/ml and polysaccharides solution were mixed. The glass slides were incubated in Petri dishes placed on wet paper to keep the humidity. After 4h and 24h germinate and no germinated spores were counted in a total of 100 spores at the microscope (×400 magnification).

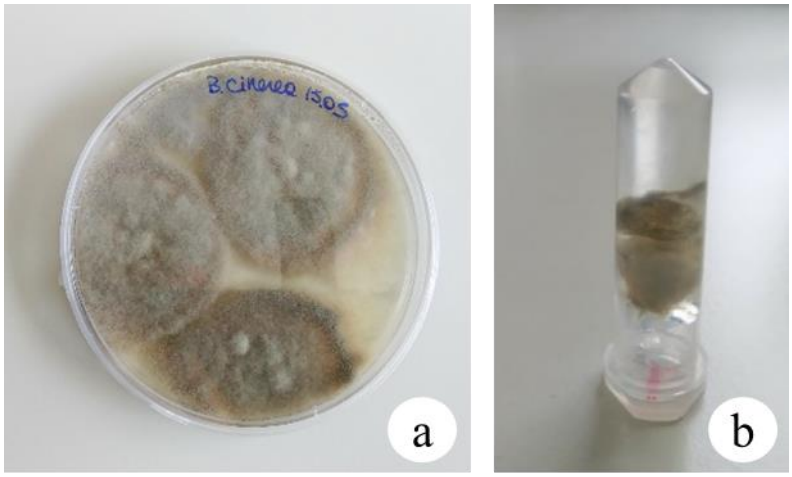

Fig. 5 Colonies of *Botrytis cinerea* (a) and colony portions treatment with polysaccharides (b).

2.4 Effect of polysaccharides against *B. cinerea* on strawberry fruits

Strawberry plants cv. Cristal were maintained in greenhouse at BEA (Banco Español de Algas), Muelle de Taliarte, Gran Canaria, Spain. Plants were irrigated and fertilise regularly (N+P+K, 12+12+17). About twenty days after blossom, ripened fruits were used for two experiments:

- the first to verify the effect of pre-harvest treatment: strawberry fruits on plant were immerged in polysaccharides solutions (Fig. 6a). After 24 hours fruits were harvested and inoculated by spraying a spore suspension of B. cinerea (1×10^5 spores/ml) (Fig. 6 c).
- the second to verify the effect of post harvest treatment: strawberry fruit were treated by immersion after harvesting. After 24 hours fruits were inoculated by spraying a spore suspension of *B. cinerea* (1×10^5 spores/ml) (Fig. 6 b, c).

In both cases, disease symptoms were evaluated as percentage of fruit area showing symptoms of grey mould (infected area) over the total area inoculated and as fruit sporulation.

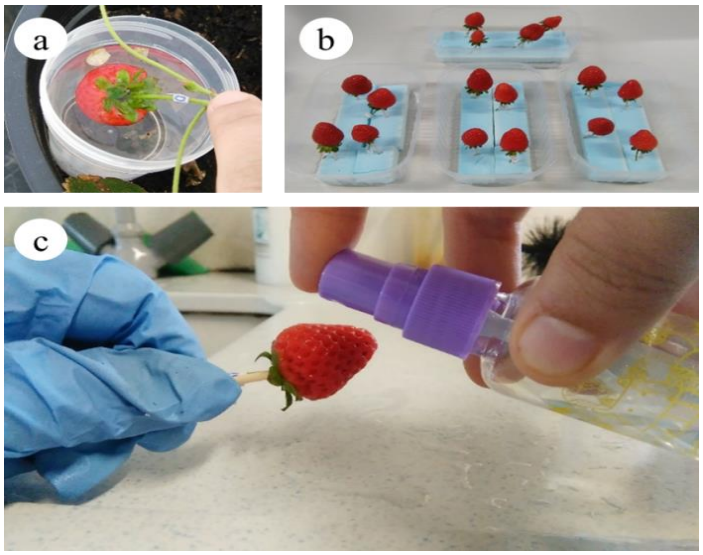

Fig. 6 Treatment of strawberry fruits with polysaccharides (a), fruits after treatment (b), inoculation of *Botrytis cinerea* on fruits (c).

3. Results

The yield extraction of polysaccharides was significantly different depending on the extract. The highest yield has been obtained from AN (58.7%) followed by ECK (40.6 %) and JAN (13.4 %).

3.1 Antifungal activity against *B. cinerea* of polysaccharides *in vitro*

Polysaccharides from AN at 1.72 and 3.45 mg ml^{-1} and ECK at 0.82 and 1.64 mg ml^{-1} significantly inhibited *B. cinerea* colony growth every DAT until 4 DAT (Fig. 7). *Anabaena* sp. polysaccharides at 0.86 mg ml^{-1} inhibited *B. cinerea* growth only 1 DAT. *Jania* sp. polysaccharides did never inhibit fungal colony growth. In Fig. 8-9 effect of *Ecklonia* sp. and *Anabaena* sp. polysaccharides on *Botrytis cinerea* colony growth three and four days after treatment are showed. Spore germination after AN treatment was significantly inhibited at all concentrations after 4 and 24h. Development of CFUs were significantly decreased when *B. cinerea* spores were exposed to the three concentration of AN and ECK polysaccharides after 48 h (Fig. 10a), except for ECK at 0.41 mg ml^{-1}. Polysaccharides from Jania did not show any effect. Development of colonies where significantly inhibited only by AN polysaccharides by 57, 253.3 and 292.6 % at 0.86, 1.72 and 3.44 mg ml^{-1} respectively (Fig. 10b). After 4 and 24 h of exposure to AN polysaccharide *B. cinerea* spore germination was significantly inhibited. Especially after 48 hours only few not germinated spores were found (Tab. 2).

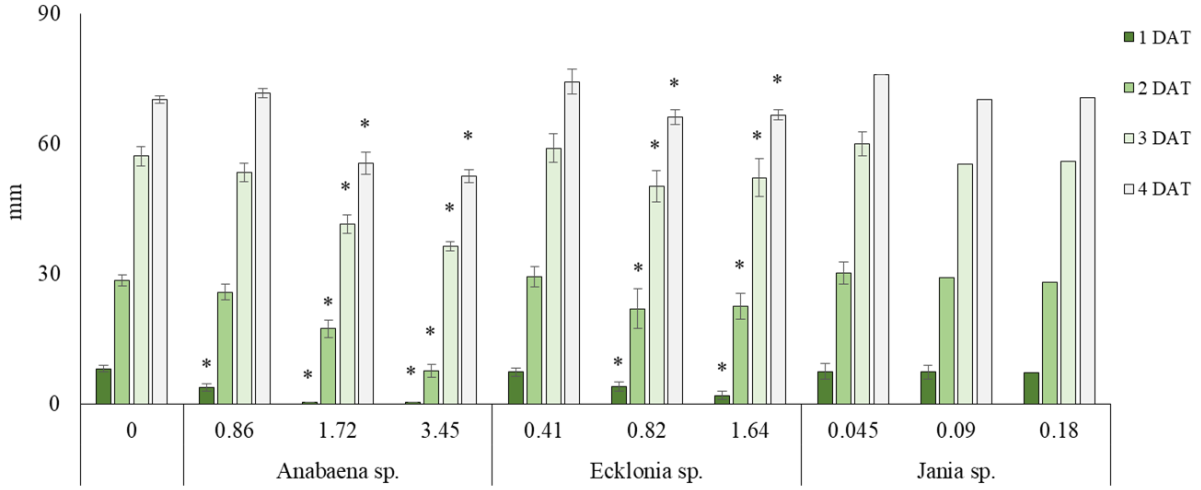

Fig. 7 Effect of different concentrations (mg ml^{-1}) of *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. polysaccharides on daily growth of *Botrytis cinera*. Columns are mean values + SD. Asterisk indicates significant differences with respect to the untreated control (0 mg ml^{-1}), according to LSD test ($P < 0.05$).

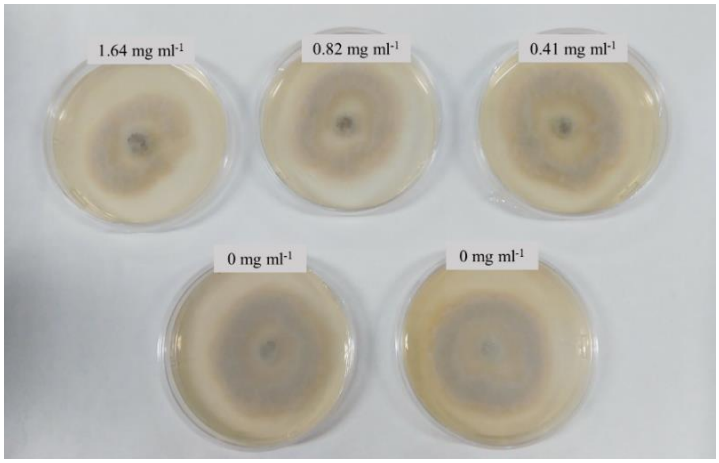

Fig. 8 Effect of *Ecklonia* sp. polysaccharides on *Botrytis cinerea* colony growth three days after treatment.

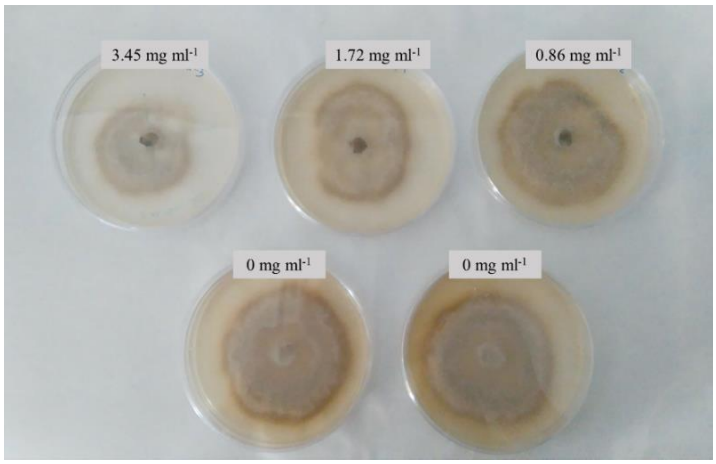

Fig. 9 Effect of *Anabaena* sp. polysaccharides on *Botrytis cinerea* colony growth four days after treatment.

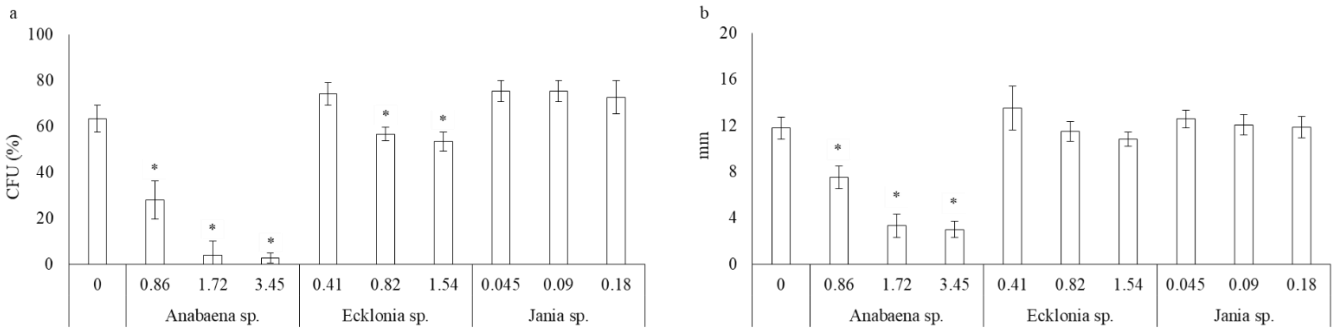

Fig. 10 Effect of different concentrations (mg ml^{-1}) of *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. polysaccharides on percentage of CFUs (a) and on colony development (b). Columns are mean values + SD. Asterisk indicates significant differences with respect to the untreated control (0 mg ml^{-1}), according to LSD test ($P < 0.05$).

Tab. 2 Percentage of germinated spore 4 and 24 h after treatment with polysaccharides extracted from *Aabaena* sp.

mg ml^{-1}	% germinated spores 4 h after treatment	% germinated spores 24 h after treatment
0	71.3 ± 4.0	88.3 ± 2.9
0.86	1.7 ± 1.5	0.0 ± 0.0*
1.72	0.0 ± 0.0	0.0 ± 0.0*
3.45	0.0 ± 0.0	0.0 ± 0.0*

*: few no germinated spores detected

3.2 Effect of polysaccharides against *B. cinerea* on strawberry fruits

The pre-harvest treatment on strawberry fruits with polysaccharides from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. showed to reduce both fruit infected area and pathogen sporulation at all concentrations (Tab. 13). Fruit infected area was reduced by 28.6-50.6%, 12.3-12.3% and 42.0-93.7% range by *Anabaena* sp., *Ecklonia* sp. and *Jania* sp., respectively. In Fig. 11 effect of pre-harvest treatment against *B. cinerea* with *Jania* sp. polysaccharides at 0.045 and 0.09 mg ml^{-1} are shown. Pathogen sporulation was reduced by36.8-67.3%, 49.3-78.9%, and 34.4-97.3% by *Anabaena* sp., *Ecklonia* sp. and *Jania* sp., respectively. Post-harvest treatment performed on harvested fruits did not inhibit neither fruit infected area nor pathogen sporulation (Tab. 14). In Fig. 12 effect of post-harvest treatment against *B. cinerea* with *Ecklonia* sp. polysaccharides at are shown.

Tab. 13 Infected area after pre-harvest treatment of strawberry fruits with different concentrations of polysaccharides from *Anabaena* sp. (AN), *Ecklonia* sp. (ECK) and *Jania* sp. (JAN).

Polysaccharides	mg ml^{-1}	% fruit infect area	Fruit sporulation (10^4 spores ml^{-1})
	0	70.0 ± 6.2 f	609.4 ± 73.1 f
Anabaena sp.	0.86	50.0 ± 9.6 d	385.0 ± 65.2 e
	1.72	35.0 ± 8.8 bc	218.1 ± 53.6 c
	3.44	34.6 ± 7.7 b	199.2 ± 38.8 c
Ecklonia sp.	0.41	61.4 ± 3.6 e	308.8 ± 63.5 d
	0.82	58.6 ± 8.6 e	299.9 ± 55.2 d
	1.64	57.9 ± 7.8 e	128.7 ± 38.8 b
Jania sp.	0.0045	40.7 ± 12.5 c	401.9 ± 86.1 e
	0.009	4.6 ± 4.1 a	18.8 ± 7.9 a
	0.018	4.4 ± 3.4 a	16.3 ± 5.8 a

Means ± SD followed by different lower-case letters in a column are significantly different according to LSD test ($P < 0.05$).

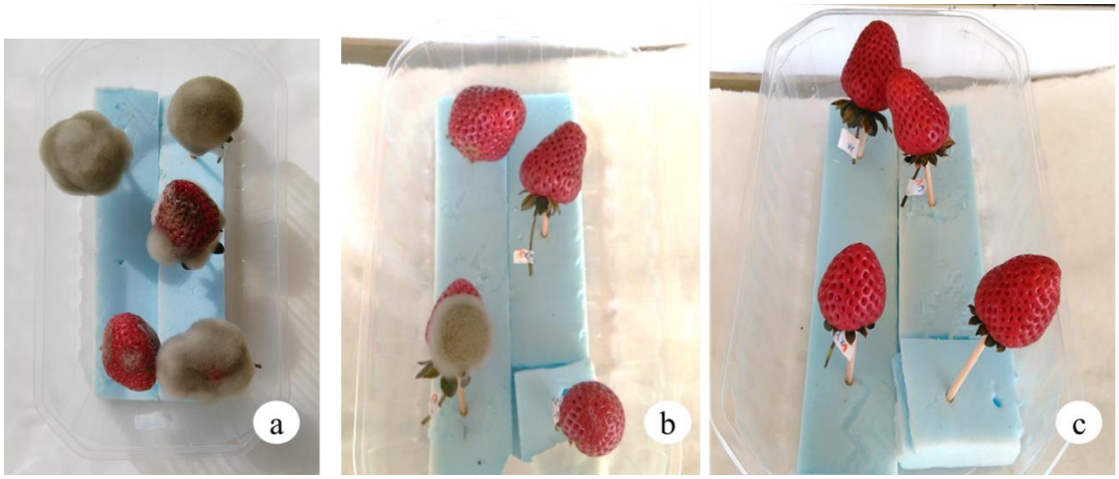

Fig. 11 Effect of pre-harvest treatment against *B. cinerea* with water (a) and with *Jania* sp. polysaccharides at 0.045 (b) and 0.09 mg ml^{-1} seven days after fruit inoculation.

Tab. 14 Infected area after post-harvest treatment of strawberry fruits with different concentrations of polysaccharides from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp.

Polysaccharides	mg ml^{-1}	% fruit infect area	Fruit sporulation (10^4 spores ml^{-1})
	0	71.4 ± 19.1	367.5 ± 76.9
Anabaena sp.	0.86	72.9 ± 15.4	403.8 ± 62.4
	1.72	77.9 ± 5.4	437.5 ± 61.2
	3.44	76.4 ± 4.6	400.6 ± 46.6
Ecklonia sp.	0.41	78.6 ± 19.6	353.8 ± 47.1
	0.82	77.1 ± 9.1	390.0 ± 61.2
	1.64	75.4 ± 8.0	390.0 ± 65.7
Jania sp.	0.0045	65.7 ± 10.9	362.9 ± 19.9
	0.009	74.3 ± 23.4	428.5 ± 65.2
	0.018	72.5 ± 21.6	377.5 ± 66.3

Means ± SD followed by different lower-case letters in a column are significantly different according to LSD test ($P < 0.05$).

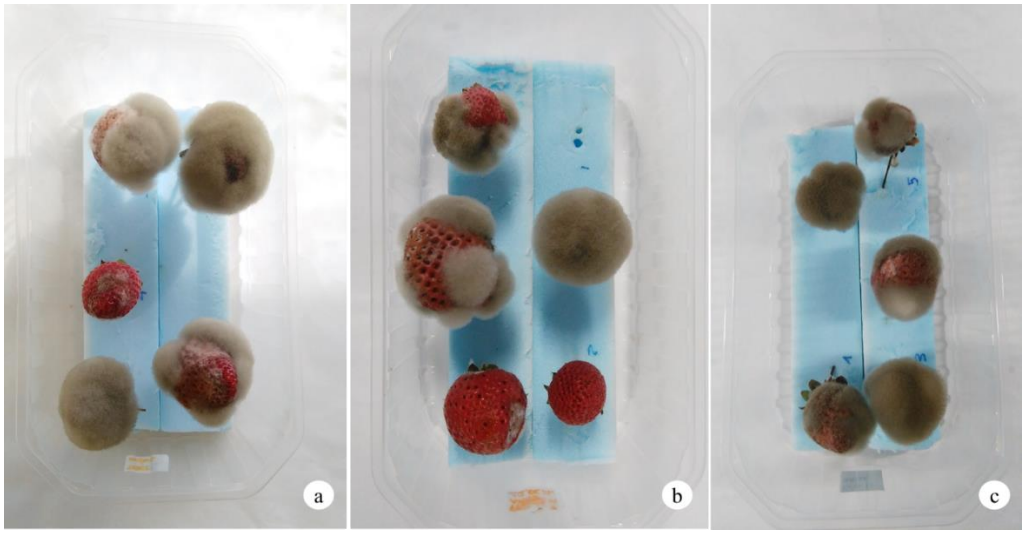

Fig. 12 Effect of post-harvest treatment against *B. cinerea* with water (a) and with *Ecklonia* sp. polysaccharides at 0.045 (b) and 0.09 mg ml^{-1} seven days after inoculation.

4. Discussion

The use of algal polysaccharides for plant disease control as an alternative to synthetic products has been fully explored (Hahn et al., 1981; Vera et al., 2011; Stadnik and Freitas, 2014). Indeed,

polysaccharides are well known inducers of plant resistance (Vera et al., 2011), acting as pathogen-associated molecular patterns that activate a series of plant reactions. These succession of reactions include rapid changes in intracellular calcium concentration, an oxidative burst and the activation of salicylic and jasmonic acid and ethylene signalling pathways (Jaulneau et al., 2010; Zhao et al., 2012). In addition they increased expression of defence enzymes and genes (Vera et al., 2011). Ulvans, alginates, fucans, laminarin and carrageenans are cell wall and storage polysaccharides of green, brown and red seaweeds, showing to increase plant protection against pathogens (Vera et al., 2011). Nonetheless, the antifungal activity of polysaccharides from *Ecklonia* sp. and *Jania* sp. have never been explored as well as the antifungal activity of polysaccharides extracted from cyanobacteria. This study showed that polysaccharides extracted from *Anabaena* sp. *Ecklonia* sp. and *Jania* sp. were effective in controlling *B. cinerea*, agent of grey mould in strawberry.

The antifungal effect of polysaccharides from the cyanobacterium *Anabaena* sp. *in vitro* was higher than that of the two macroalgae. Cyanobacteria has considered one of the most promising source of natural bioactive compounds and several studied have demonstrated their antifungal activity against fungal plant pathogens (Moon et al., 1992; Prasanna et al., 2008; Radhakrishnan et al., 2009; Manjunath et al., 2010; Roberti et al., 2016). Cyanobacteria belonging to Nostocales order, such as *Anabaena* sp., are able to produce more than one hundred compounds that display antifungal, anticancer, antiviral and antimicrobial activities (Singh et al., 2005). Most of the studies regarded the antifungal activity of their extracts obtained with different organic solvent. Frankmolle et al. (1992a, b) reported that crude ethanolic extracts from *Anabaena laxa* inhibited the growth of different fungus, plant pathogens: included *Aspergillus oryzae*, *Candida albicans*, *Penicillium notatum*, *Saccharomyces cerevisiae* and *Trichophyton mentagrophytes*. Many study have demonstrated the inhibitory effect by different Anabaena species on the growth *Pythium* sp., *Fusarium* sp. and *Rhizoctonia* sp. (Moon et al., 1992; Prasanna et al., 2008; Radhakrishnan et al., 2009; Manjunath et al., 2010). Among all the compounds produced by these microorganisms, hydrolytic enzymes and the homologues for chitosanase, endoglucanase and benzoic acid were identified and correlated to their activity against fungus (Gupta et al., 2010, 2011; Natarajan et al., 2013; Prasanna et al., 2008). In addition cyanobacteria are also able to produce phenolic compounds that was demonstrated to inhibit *Candida albicans* growth (de Cano et al., 1990). Moreover, the terpenoid noscomin, extracted *N. commune* showed antibacterial activity against *Bacillus cereus*, *Staphylococcus epidermidis*, and *Escherichia coli (*Jaki et al., 2001). Again from *Nostoc* sp., two cyclic peptides from the cyanobacterium *f*, tolybyssidin A and B, showed antifungal activity against the human pathogen *Candida albiacans* (Jaki et al., 2001). Cryptophycin, a depsipeptide first isolated from Nostoc sp. showed antifungal activity against filamentous fungi and certain species of yeast (Hirsch et al., 1990).

Among the macroalgae, the brown algae *Ecklonia* to inhibit *B. cinerea* growth and development of CFUs. Antifungal activity of extracts from brown algae is widely reported (Righini et al., 2018; Khallil et al., 2015; Ibraheem et al., 2017; De Corato et al., 2017; Roberti et al., 2016). Crude and hexan extract from *Laminaria digitata* reduced *B. cinerea* growth and spore germination up to 100 % (De Corato et al. 2017). Methanolic extract from *Sargassum latifolium* and *Padina gymnospora* inhibited the growth of *Fusarium solani* and *Rhizoctonia solani* up to 84% (Ibraheem et al, 2017). Aqueous and cyclohexanic extracts from *Sargassum* sp. inhibited mycelial growth of *Aspergillus* spp. by 37% and 54.5%, respectively (Mabrouk et al. 1985; Khallil et al., 2015). The cyclohexanic extract also inhibited the growth of *Fusarium oxysporum* and *Penicillium* spp. (Khallil et al., 2015). Some authors related the presence of certain compounds in brown algae to their antifungal activity. For example, Fernandes Peres et al. (2012) related the phenols and terpens found in extract from *Sargassum muticum*, *Ascophyllum nodosum*, *Fucus spiralis*, *Stypopodium zonale*, and *Pelvetia canaliculata* to the inhibition of *Colletotrichum lagenarium* growth showed by the extracts. Brown algae contain phlorotannins, phenolic compounds that have been reported to have antifungal, antibacterial, antioxidant, and anti-HIV activities (Eom et al., 2012). Dieckol, a phlorotannin isolate from *Ecklonia* sp.showed antifungal activity against *Trichophyton rubrum*, a dermatophytic fungus (Lee et al., 2010). An antifungal substance, namely bromophenol bis (2,3-dibromo-4,5-dihydroxybenzyl) ether (BDDE), extracted from both the red alga *Rhodomela confervoides* and from the brown algae *Leathesia nana*, inhibited the mycelial growth, spore germination and the germ tube elongation of *B. cinerea* (Liu et al. 2014). The same substance was also capable to inhibit mycelial growth of *Coleltotrichum gloeosporioides*. Concerning the effect of polysaccharides against *B. cinerea* on strawberry fruits, it should be considered that (i) generally, pre-harvest treatment with polysaccharides from Anabaena sp., Ecklonia sp. and Jania sp. decreased disease severity while post-harvest treatment did not showed any effect; (ii) polysaccharides extracted from *Jania* sp. showed the highest decreasing of disease severity and sporulation of the pathogen, even if no direct effects were observed on the pathogen. As regards to the type of treatment, as mentioned above, algal polysaccharides elicit plant defence responses, activating signalling pathways, among which salicylic acid, jasmonic acid and ethylene either alone or in combination, play major roles in local and systemic induction of defence responses (Hammond-Kosack and Jones, 1996; Reymond and Farmer, 1998; Paulert et al., 2009; Sharma et al., 2014). We supposed that treatment of strawberry fruits with polysaccharides in plant probably activated these responses in the plant/fruit, unlike in detached fruits. Polysaccharides and derived oligosaccharides extracted from algae, such as agarans and carrageenans in red algae and alginates, fucans and laminarin in brown can increase the expression of defence genes and enzymes such as chitinase and glucanase, involved in plant defence response. For example,

laminarin from the brown alga *Laminaria digitata* induced the release of hydrogen peroxide in tobacco cells (Klarzynski et al, 2000). In addition, it induced a transient increase in pheylalanine ammonia lyase activity with a maximal levelat 4 h, a sustained increase in lipoxygenase activity up to 20 hours and the accumulation of PR-1, PR-2 (glucanase), PR-3 (chitinase) and PR-5 at 48 h of treatment (Klarzynski et al., 2000). Furthermore, laminarin injected in tobacco plants induced protection against the bacteria *Erwinia carotovora* infection by reducing the diameter of the necrotic lesion. Application of laminarin and alginate reduced the development of wilt symptoms caused by *Verticillium dahliae* on olive twigs, stimulating the phenolic metabolism (Salah et al., 2018). Moreover, alginates, reduced the pathogen growth *in vitro*. Concerning *Jania* sp., carrageenans showed to protect the plant against tobacco mosaic virus, *B. cinerera* and *Erwinia*. *carotovora* infections indicating that the polysaccharide treatment induced protection against a broad range of pathogens (Vera et al., 2011). Again on tobacco, carrageenan infiltrated in tobacco leaves increased the expression of genes coding for a sequiterpene cyclase involved in the synthesis of the antimicrobial terpenoid capsidiol, of PR-3 proteins and proteinase inhibitor with antipathogenic activity. Ghannam et al. (2013) showed that a carrageenan from red alga *Hypnea musciformis* activated the mechanisms of strengthening cell walls and producing phenolic compounds that led to a reduced number of TMV spots on plants (Ghannam et al., 2013).

Reports on the use of cyanobacterial polysaccharide are very limited, despite of the fact that they are an abundant source of variable polysaccharides that could have the potential to elicit several responses in plants. Cyanobacterial polysaccharides are present as a mucilaginous external layer around the cell, either being organized into a well defined sheath or a capsule that is intimately associated with the cell surface, or as slime that is only loosely associated with the cell surface or as soluble polysaccharides released into the environment during cell growth.

In conclusion, this study shows that polysaccharides extracted from *Anabaena* sp., *Ecklonia* sp. and *Jania* sp. were active against *B. cinerea*, by working both directly to the pathogen and indirectly through the involvement of fruit/plant defence responses as pre-harvest treatment helping plant to withstand the pathogen. Once these effects will be verified on a larger scale experiment, the polysaccharides 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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Acknowledgements

Firstly, I would like to express my sincere gratitude to my tutors, Professor Roberti and Professor Baraldi, for their continuous support during my Ph.D for their patience, motivation and knowledge, their guidance helped me all the time. I would like to thank also Professor Chilosi and Professor Martel Quintanafor their useful comments in the thesis review.

A special thank to my mother, father and grandparents to support me in all situations to allow me to be the person I am.

My sincere thanks also goes again to Professor Martel Quintana and Professor Gómez_Pinchetti who gave me opportunity to join their team, special people in a special place that I consider my second family and home. I think it's only fair to appoint each one: Moneiba (mas Evolet), Yolanda, Noelia, Emilio, Mireia, Fernando, Juan, Loly, Agueda, Carlos, Virginia, Elena, Edu, Judith and Ella!

I thank my fellow labmates (who still work here and don't still) for all the fun and supports we have had in these years: Pamela, Sandra, Lorenzo, Angela, Federica, Selene, Agnese and Michela. A special thank to Francesca and Michela for sharing with me not only the office!

I am also so grateful to my dearest friends: first Michela, Musso, Zava, Michele, Ylenia, Dario, Eleonora, Milena, Marco, Susanna, Riccardo and Lorenzo. Without you, my life would have been totally empty.