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Antifungal activity of crude extracts from brown and red seaweeds by a supercritical carbon dioxide technique against fruit postharvest fungal diseases

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ABSTRACT

Fungal infections are the main cause of decay on fresh fruit during postharvest phase determining severe losses. Postharvest control is performed by fungicides, but their intense use have aroused issue relating to environmental protection and human health prompting to search alternative control means. The use of biofuel-used seaweed extracts by a supercritical carbon dioxide technique could be a valid alternative during postharvest handling of fresh fruit. The aim of this work was to assess the *in vitro* and *in vivo* activity of extracts from two brown seaweeds (*Laminaria digitata* and *Undaria pinnatifida*) and three red seaweeds (*Porphyra umbilicalis*, *Eucheuma denticulatum* and *Gelidium pusillum*) against three postharvest pathogens (*Botrytis cinerea*, *Monilinia laxa* and *Penicillium digitatum*) using three concentrations of extract (10, 20 and 30 g L^{-1}). The total content of fatty acids of the extracts was determined by CG-MS, those of polysaccharides by HIC, and phenolic compounds (phlorotannins) by HPLC-DAD. Twenty fatty acids were quantified in the extracts, while three polysaccharides categories and three phlorotannins classes were identified only in brown seaweed extracts. *L. digitata*, *U. pinnatifida* and *P. umbilicalis* showed the highest antifungal efficacy on *in vitro* cultures of the pathogens. *L. digitata* and *U. pinnatifida* completely inhibited mycelia growing and conidial germination of *B. cinerea* and *M. laxa* at the highest dose tested and strongly reduced those of *P. digitatum*. *P. umbilicalis* extract strongly inhibited mycelia and conidia growth on all the fungi. *E. denticulatum* and *G. pusillum* showed a lower but still significant reduction of mycelia growing and conidia germination on all the pathogens. In trials performed *in vivo* on wounded fruit, *L. digitata*, *U. pinnatifida* and *P. umbilicalis* extracts strongly suppressed grey mould on strawberries, brown rot on peaches, and green mould on lemons at 30 g L^{-1} dose both in preventive and curative treatments; *E. denticulatum* and *G. pusillum* poorly reduced disease development. In all cases, a dose-effect of the treatments was observed with an increase of fruit decay inhibition and reduction of disease severity as the dose of extract applied over the wound increased. Moreover, an increased peroxidase activity in the strawberries/*B. cinerea* and peaches/*M. laxa* systems by preventive treatment with 30 g L^{-1} extract was observed. The antifungal activity could be mainly ascribed to a direct toxicity of fatty acids found at the highest concentrations in *L. digitata*, *U. pinnatifida* and *P. umbilicalis* rather than to those of phenolic compounds and phlorotannins; but it could be related to possible peroxidase-mediated systemic resistance mechanisms elicited by the polysaccharides.

1. Introduction

Fungal infections are the main cause of decay on fresh fruit during transportation, commercialization and storage which determine severe economic losses. Postharvest pathogens limit shelf-life of fresh commodities contributing to deterioration of quality, reduction of nutrients, mycotoxin contamination and reduction of market value. Among the most important postharvest pathogen fungi on fruit, there are *Botrytis cinerea*, *Monilinia laxa* and *Penicillium digitatum*. Their control is efficiently performed by synthetic fungicides (Förster et al., 2007), however their intense use, reduced number of authorized active compounds, increased resistance of some postharvest fungal pathogens against the few authorized fungicides, and growing consumer demand for safer fruit and higher quality of these commodities, have aroused

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important issue relating to environmental protection and human health, prompting to search for safer and eco-friendly control means (Ippolito et al., 2005; Smilanick et al., 2008; Droby et al., 2009; Sanzani et al., 2009a; Sharma et al., 2009; Casals et al., 2010; Mari et al., 2010).

Plant extracts have traditionally gained great popularity and scientific interest for their antimicrobial activity (Lee et al., 2007; Verástegui et al., 2008; Santas et al., 2010). Many findings have been reported on the antimicrobial properties of plant extracts containing different classes of phenolic compounds (Gatto et al., 2011) that represent a rich source of biocides that have been widely explored as postharvest alternative control means (Schena et al., 2008). In particular, many studies have pointed out the antimicrobial efficacy of hydroxybenzoic acid derivatives (Veloz-García et al., 2010), coumaric and caffeic acid derivatives (Korukluoglu et al., 2008), flavonoids and coumarins (Sanzani et al., 2009b), catechin, epicatechin, proanthocyanidins and tannins (Engels et al., 2009; Parashar et al., 2009; Yoshida et al., 2009).

Seaweeds represent a profitable feedstock of bioactive compounds that have been studied as functional food, biocides and pharmaceutical drugs (Løvstad Holdt and Kraan, 2011). Seaweed extracts have good biostimulant properties of plant growth for crop development due to presence of a large number of plant growth-stimulating compounds (Khan et al., 2009; Tuhy et al., 2013). Seaweed extracts have antifungal properties against soil-borne plant pathogenic fungi. Raj et al. (2016) have tested crude extracts of brown seaweeds belonging to *Sargassum muticum*, *Dictyota bartyrensiana*, *Padina gymospora*, *Chnoospora implexa* and *S. wightii* for controlling sheath blight disease on rice in India caused by *Rhizoctonia solani*. Jayaraj et al. (2008) found that the foliar application of seaweed extract on carrot reduced leaf blights by *Alternaria* sp. and *Botrytis* sp. as well as the fungicide chlorothalonil. The commercial extract from the brown seaweed *Ascophyllum nodosum* reduce fungal diseases in greenhouse cucumber (Jayaraman et al., 2011). Ethanolic extracts of the brown alga *S. myricocystum* effectively *in vitro* inhibited the mycelial growth of *Colletotrichum falcatum*, the causal agent of red rot disease on sugarcane in India (Ambika and Sujatha, 2015). An increased attention of the seaweed extract use for avoiding postharvest losses of fruit has been given from the 2000s. Kamel (2014) has studied the impact of a commercial seaweed extract (Cytolan Star®) on keeping quality of orange fruit (cv. Valencia) during cold storage, revealing that fruit quality can be improved and maintained for longer time if used alone. Omar (2014) has investigated on the use of seaweed extract as a promising postharvest treatment on orange fruit recommending seaweed extract as a suitable mean for improving fruit quality and storability of Navel orange fruit if compared with chemicals that are of consumer concern. Current uses and novel application of seaweed extracts for controlling spoilage caused by microorganisms and plant pathogens have been found in literature. Khanzada et al. (2007) have screened various fractions of ethanolic seaweed extract of the red alga *Solieria robusta* for *in vitro* antifungal activity against five fruit spoiling fungi (*Aspergillus flavus, A. niger*, *A. ochraceus*, *P. funiculosum* and *Phytophthora infestans)*. *S. robusta* extract also inhibited growth of *Macrophomina phaseolina* and *R. solani* (Sultana et al., 2005) and *Fusarium solani* (Rizvi and Shameel, 2001). Seaweeds are also able to stimulate growth in strawberry fruit by protecting them from pathogens and physiological hazards either *in vivo* either under storage condition (Washington et al., 1999).

In general, brown seaweeds show high antifungal activity more than red algae. Brown seaweeds contain many lipophilic compounds (e.g. unsaturated- and saturated-fatty acids, hydroxylated un-satured fatty acids, glycolipids, terpenoids) more than red algae. They are soluble in hexane more than in chloroform and have a very strong antifungal property (Hanaa et al., 2008). The brown seaweeds contain phenolic compounds that could be the reason for their antifungal activity being solubilized in ethanol more than in water (Cowan, 1999). The laminarin, a storage polysaccharide (β-1,3-glucan) isolated for the first time from cell walls of the brown alga *Laminaria digitata*, is able to elicit host defence responses in tobacco (Klarzynski et al., 2000). Water-soluble laminarans can stimulate plant immunity mechanisms when applied as foliar sprays (Trouvelot et al., 2014). Other carbohydrates involved in plant immunity as elicitors of plant defences and/or as resistance inducers against pathogens are the fucoidans and alginates, the main extracellular matrix polysaccharides of the brown algae. On the other hand, bioactive compounds as amino-acids, peptides and proteins seems be not included in plant disease suppression (Løvstad Holdt and Kraan, 2011).

The aim of this work was to evaluate the *in vitro* and *in vivo* antifungal activity of five extracts from brown and red seaweeds against three fungal pathogens on fresh fruit under postharvest condition. Extraction of antifungal compounds was performed using an innovative and most selective technique that use carbon dioxide under supercritical condition (SC-CO_2) in place of the traditional methods with solvents. Analysis of the crude extract for detecting bioactive substances (fatty acids, polysaccharides, phenolic compounds and phlorotannins) were carried out. The antifungal activity of seaweed extract fractions was *in vitro* assessed on cultures of the pathogens, while those of crude extracts was *in vivo* tested on wounded fruit inoculated with the pathogens for to obtain preliminary data on their efficacy in preventive and curative treatments.

2. Material and methods

2.1. Starting material

A representative amount of biomass from healthy and matured marine macroalgae belonging to two brown seaweed species (*L. digitata* and *Undaria pinnatifida*) and three red seaweed species (*Porphyra umbilicalis*, *Eucheuma denticulatum* and *Gelidium pusillum*) was purchased from a marine biorefinery (about 100 kg of fresh weight for each species). Brown and red seaweeds were nowadays cultured on large scale into industrial photo-bioreactors for producing either third-generation biofuels either high-value added products for food industry and many other purposes (Table 1). Algal biomass was immediately refrigerated after harvesting, thoroughly washed with seawater, and washed with tap water to remove all the extraneous particles and epiphyte organisms. Fresh biomass was dried into an industrial drier, chopped, finely pulverized, heat-treated for 24 h with sodium hydroxide (1:10 w/w) for saponification of lipids (mainly triglycerides), weighed, and stored at 4 °C until extraction of bioactive compounds.

Seaweed extracts used in the experiments were obtained by a SC-CO_2 pilot-extractor located at ENEA Trisaia Research Center

Table 1
Seaweed species used in the experiments. They are industrially used for producing either third-generation biofuels either high-value added products into a marine biorefinery.

Scientific name	Family name	Common name	Colour	Country of origin	High-value added products and third-generation biofuels
Laminaria digitata	*Laminariaceae*	Oarweed	Brown	North-Western Europe	Organic fertilizer, laminarin, alginate, toothpaste, cosmetic, fatty acid, biodiesel.
Undaria pinnatifida	*Alariaceae*	Wakame	Brown	Japan	Functional food, sushi, laminarian, alginate, fatty acid, biodiesel.
Porphyra umbilicalis	*Porphyraceae*	Nori	Red	China	Functional food, sushi, porphyran, protein, peptides, fatty acid, biodiesel.
Eucheuma denticulatum	*Solieriaceae*	–	Red	Malaysia	Antimicrobial drug, insecticide against mosquitos, polysaccharide, fuel ethanol.
Gelidium pusillum	*Gelidiaceae*	–	Red	India	Functional food, agar, polysaccharide, fuel ethanol.

(Rotondella, Matera, Italy) equipped with two vessels of 9 L each and one separator vessel. In order to optimize extraction of lipophilic compounds, overall unsaturated and saturated fatty acids which could be involved in fungal suppression, about 2 kg of dry biomass was processed at the following parameters of the compressed CO_2 (pressure = 37.9 MPa, density = 0.701 g mL^{-1}, temperature = 50 °C and flow rate = 34 kg h^{-1}). Crude seaweed extract yield was calculated on three independent replicated samples of 50 g each after drying at 55 ± 5 °C for at least 2 h and expressed as percentage on a basis of biomass fresh weight. Stocks of crude seaweed extract were dried, weighed, and stored at − 20 °C in the dark till further uses.

2.2. Characterization of crude seaweed extracts

2.2.1. Sample preparation

About 50 g of dry extract were suspended in *n*-hexane, or boiling distilled water, or 80% aqueous methanol (1:5 w/v) for 2 h for determining the content of fatty acids, water-soluble polysaccharides and phenolic compounds respectively. All the samples were replicated three times for each solvent used. Extracts were then filtered through Whatman filter paper and evaporated to dryness under reduced pressure at 35 °C using a rotatory evaporator (Strike 202, Steroglass, Perugia, Italy), according to Gatto et al. (2013). This residue was dissolved in 50 mL of 0.1 M potassium phosphate buffer (KH_2PO_4-K_2HPO_4, pH 5.5) to give a final stock solution containing 50 g of crude extract in 50 mL of buffer. This solution was centrifuged for few minutes at 10,000 × *g*, the supernatant was collected, filtered through a sterile 0.22 µm pore size membrane filter (Millipore, Bedford, MA, USA), and stored at −20 °C in the dark until use for analysis.

2.2.2. Analysis of total lipids and fatty acids

The total lipids content was determined on samples of 50 g of extract replicated three times after extraction with a mixture of chloroform–methanol in such proportions that a miscible system was formed among water and extract (Bligh and Dyer, 1959), and expressed as a percentage of crude extract dry weight.

Fatty acids analysis was carried out on each replicated sample suspended in *n*-hexane by the gas chromatography–mass spectroscopy (GC–MS) technique according to Raj et al. (2016). Three aliquots of 2 µL from each sample were filtered by 0.2 mm Millipore filters and injected in a split mode into a GC system composed of a gas-chromatograph (Varian CP 3800) equipped with a split-splitless injector (Varian CP-1177). The separation and quantification of the peaks were performed with a column turbo mass gold containing Elite-1 100% dimethylpolysiloxane VF–5 ms FactorFour Varian (30 m × 0.25 mm i.d., 0.25 mm film thickness). Column oven temperature program was between 110 °C (for 2 min) and 280 °C (for 9 min) with increasing temperature steps of 3 °C min^{-1}, followed by 30 min under isothermal conditions. The injector and detector temperatures were maintained at 250 °C and 300 °C respectively. The carrier gas was helium (flow rate = 1 mL min^{-1}). The fatty acids resulting from the GC–MS chromatogram were separated and identified by a computer-driven algorithm matching the mass spectrum with a database (NIST Version 2.0, 2005) given by the Turbo mass-5.1 software for spectra detection and data processing. The content of each fatty acid was expressed as a percentage of the total fatty acids content.

2.2.3. Analysis of water-soluble polysaccharides

The water-soluble polysaccharides content was determined on 50 g of extract replicated three times after extraction with a mixture of water–methanol in such proportions that a miscible system was formed among water and extract (Khanzada et al., 2007), and expressed as a percentage of crude extract dry weight.

Water-soluble polysaccharides analysis was carried out by a new and simple procedure based on a hydrophobic interaction chromatography (HIC) technique developed by Zvyagintseva et al. (1999) for brown seaweeds. Separation and quantification of three water-soluble polysaccharide categories (laminarans, fucoidans and alginates) from crude seaweed extract were carried out on each replicated sample suspended in boiling distilled water using three aliquots of 20 µL from each sample. Particularly, alginic acid is contained in cellular wall of seaweeds as insoluble alginates. The principle of extraction of them from crude seaweed extracts lies in modification of insoluble alginates (calcium or magnesium salts) into soluble ones (sodium or potassium salts) by a previous treatment of the stock solution composed by 50 g of crude extract in 50 mL of buffer adding calcium chloride before injection (Bashford et al., 1950). The content of laminarans, fucoidans and alginates was expressed as a percentage of the total water-soluble polysaccharides content.

2.2.4. Analysis of total phenolic compounds and phlorotannins

The total phenolic compounds content was determined on 50 g of extract replicated three times after extraction with a mixture of ethanol–water in such proportions that a miscible system was formed among water and extract (Khanzada et al., 2007), and expressed as a percentage of crude extract dry weight.

Phlorotannins, a specific class of phenolic compounds contained only in some marine algae species, was analyzed on each replicated sample suspended in 80% aqueous methanol by the high performance liquid chromatography-diode array detection (HPLC-DAD) technique using three aliquots of 20 µL from each sample, according to Martínez and Castañeda (2013). Owing to its ease of oxidation, phlorotannins have been previously extracted from the stock solution composed by 50 g of crude extract in 50 mL of buffer adding ascorbic acid under nitrogen before injection. Three aliquots of 20 µL each were filtered and injected into a HPLC-DAD system composed of a chromatograph (HP 1100, Series liquid) equipped with a binary gradient pump and a spectrophotometric photodiode array with DAD detector. Five phlorotannins categories were identified and separated on normal phase with a semi-preparative silica gel Lichrosorb Si 60 column (250 × 10 mm i.d., 5 µm film thickness) maintained at 35 °C in thermostatic oven with detection at 270 nm. A binary gradient elution program chloroform–ethanol (flow rate = 1 mL min^{-1}) with pure ethanol (solvent A) and 5% (v/v) chloroform in deionized water (solvent B) was used. The elution profile was the following: 0–25 min = 15–40% A in B; 25–30 min = isocratic 40% A in B; 30–45 min = 40–63% A in B; 45–47 min = isocratic 63% A in B; 47–52 min = 63–100% A in B; 52–56 min = isocratic 100% A, and then back to the equilibrium conditions (15% A in B). Phlorotannins were only qualitatively identified by a computer-driven algorithm matching the molecular weights with a database (Version A. 06.03) provided by the HP software for spectra detection.

2.3. Measurement of the in vitro inhibition of crude and fractionated seaweed extracts on fungal pathogens

2.3.1. Fractionation of crude seaweed extracts

Crude seaweed extracts were fractionated into three fractions using three solvents with a different affinity towards fatty acids, water-soluble polysaccharides and phenolic compounds according to Khanzada et al. (2007). Samples of 50 g of dry extract were suspended in *n*-hexane, or distilled water, or 100% ethanol (1:5 w/v) in separating funnel for 20 d at room temperature. Suspensions were then filtered using Whatman filter paper and concentrated under reduced pressure at 35 °C using a rotary evaporator till the extract become as a syrup. Three different fractions were then separated from this syrupy residue. Each fraction was individually collected from the respective funnel, and stocks of each fractionated extract were air-dried, weighed, and stored at −20 °C in the dark till use for *in vitro* activity assays.

2.3.2. Fungal pathogens

Crude and fractionated seaweed extracts were individually tested

against three isolates of fungal pathogens having origin at field conditions submitted to severe disease outbreaks in the past years: 1) *B. cinerea*, the causal agent of grey mould of strawberry fruit; 2) *M. laxa*, inducing brown rot of peach fruit; and 3) *P. digitatum*, causing green mould of lemon fruit. All the pathogens, isolated from naturally infected fruit coming from various Regions of Southern Italy (Apulia, Basilicata and Calabria), were cultured under purity condition in Petri plates on Potato Dextrose Agar (PDA, Sigma-Aldrich, Milan, Italy) at a 39 g L^{-1} dose in distilled water, and incubated at 25 ± 1 °C for 5 d. Conidia were collected from 2-week-old fungal cultures grown at 25 ± 1 °C adding few milliliters of distilled sterile water in each Petri dish. Conidial suspensions were filtered through two layers of cheesecloth and density of them was adjusted to 10^4 CFU mL^{-1}. The fungi were sub-cultured monthly throughout this study and maintained on PDA slants in tubes at 4 °C in the dark until use for *in vitro* and *in vivo* activity assays.

2.3.3. Mycelia growth inhibition

Stocks of total extract or each fraction of them suspended in sterile 0.1 M K-phosphate buffer were tested at the concentrations of 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} for determining minimum bioactive concentration (MBC) by poison food technique (Shahi et al., 1999). Mycelia inhibition was quantitatively assessed measuring radial growth in Petri plates (10 cm diameter) containing PDA (18 mL per plate). In treated plates, aliquots of 2 mL of sterile stock suspension containing extract (total and fractionated) were added to 18 mL PDA at 42 ± 3 °C before solidification. In untreated plates (control), 2 mL of sterile buffer was added to 18 mL PDA in place of the extracts. Three mycelia plugs measuring 5 mm diameter each were cut out of the margin of 7-day-old fungal cultures actively growing and aseptically placed on the upper PDA surface. Treated and control plates were incubated in the dark at 25 ± 1 °C. Mycelia growth inhibition percentage (MGI%) was measured when the control plates were totally covered by mycelium using the following index:

$$MGI\% = [(Dco - Dse)/Dco] \times 100$$

(Dco = average of colony diameter in the control plates, Dse = average of colony diameter in the plates amended with extract at the afore-mentioned concentrations). All the experiments were carried out with three replications of 10 plates for each.

Fungicide activity was assessed at the MBC value using standard methods (Grover and Moore, 1962; Hickey, 1986). Completely inhibited fungi (MGI = 100%) were re-plated on the upper PDA surface and qualitative observations were recorded from the 3th-5th day after incubation in the dark at 25 ± 1 °C. Fungal growth indicated a temporary block of mycelia growth, while lacking of growth indicated a fungicide effect.

2.3.4. Conidia germination suppression

Conidia germination was evaluated on micro-cultures by a micro-assay described by Gatto et al. (2013) on glass slides that allowed the quantitative analysis of conidial suppression by optical microscopy according to Gatto et al. (2011) with some modifications.

Assays on 96-microwell (100 µL volume) plates purchased from AES Laboratory (Milan, Italy) were performed. Each micro-well was set up with three replicates each containing 10 µL of Potato Dextrose Broth (PDB) provided from Sigma-Aldrich, 2 µL of conidial suspension 10^8 CFU mL^{-1}, and 88 µL of the extract (total and fractionated) suspended in sterile 0.1 M K-phosphate buffer to be tested at the concentrations of 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1}. One micro-well row used as control was filled with 10 µL of PDB, 2 µL of conidial suspension, and 88 µL of buffer alone. Each plate was incubated at 25 ± 1° C for 72 h. After incubation, aliquots of 5 µL conidia cultures taken from each micro-well were sampled and mounted on the upper surface of glass slides sterilized with denatured ethanol. Number of total conidia and germinated conidia was estimated by Burker's hemo-cytometer using a photomicroscope (40 × magnification) (BX60, Olympus, Milan, Italy). Conidia germination percentage (CGS%) was measured by the ratio:

$$CGS\% = C_g/C_t \times 100$$

(C_g = average of number of germinated conidia in cultures amended with extract at the afore-mentioned concentrations including control cultures, C_t = average of number of total conidia in the same sample). All the measurements were carried out on 10 glass slides taken from each micro-well.

2.4. Measurement of the in vivo suppression of crude seaweed extracts on inoculated fruit

Preventive and curative treatments were both evaluated on strawberries (*Fragaria* × *ananassa*) cv Camarosa, peaches (*Prunus persica*) cv Spring Crest, and lemons (*Citrus limon*) cv Sorrento, harvested from local growers located in Calabria (Rocca Imperiale, Cosenza, Southern Italy) and Basilicata (Policoro, Matera, Southern Italy). Healthy fruit untreated with synthetic fungicides and selected for uniform size, same ripening stage, and absence of visible defects and injuries, were washed under running tap water, surface-disinfected by dipping for 2 min in 2% sodium hypochlorite solution, rinsed with tap water and allowed to dry. Fruit were injured in the equatorial zone in two opposite points at the fixed dimensions (wide = 2 mm, deep = 2 mm), treated with the crude seaweed extract, and artificially inoculated with conidial suspension of *M. laxa* for peaches, *P. digitatum* for lemons, and *B. cinerea* for strawberries.

Trials treated with the stocks containing 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} of un-fractionated crude extract suspended in sterile 0.1 M K-phosphate buffer were set up. Two controls replacing the extracts, the first one with buffer alone, and the second one with two commercial fungicides containing as active substances 50% fenhexamid (1.2 g L^{-1}) for preventive and curative treatments of strawberries and peaches and 44.66% imazalil (2 mL L^{-1}) for preventive and curative treatment of lemons (Muccinelli, 2006), were also included. Aliquots of 30 µL of the stock suspension of extract were pipetted over each wound allowing the droplet to be absorbed into the fruit. Aliquots of 30 µL of sterile buffer, or fungicide, were pipetted over the wound. Each wound was inoculated with 10 µL of conidial suspension containing 10^5 CFU mL^{-1}. In preventive treatments, the pathogen was inoculated over the injured area two days later from application of the extract, or the fungicide, or the buffer alone, for enhancing plant defences before inoculation. Instead, in those curative, the extract, or the fungicide, or the buffer alone, were applied over the wound six hours later from inoculation of the pathogens for allowing development of germinative tube from conidia before treatment. Fruit were placed in trays, packaged in plastic bags and maintained at the temperature of 20 ± 2 °C for 6 d (peaches), 5 d (lemons) and 3 d (strawberries), and relatively humidity (RH) of 96 ± 2%.

Trials were arranged in a completely randomized experimental design, including six replicates per treatment. Thirty fruit per species with two wounds per fruit were considered per each replication. All the experiments were repeated twice. Two different disease parameters were evaluated, disease incidence and disease severity. The first one was evaluated by counting the number of the infected wounds. The percentage disease incidence data were converted into the percentage decay inhibition data (DI%) by the index (Arras et al., 1999):

$$DI\% = [(Nco - Nse)/Nco] \times 100$$

(Nco = average of number of infected wounds in the control plots treated with the buffer alone, Nse = average of number of infected wounds in the plots treated with the extracts at the afore-mentioned concentrations, or with the fungicide). Disease severity was assessed by measuring lesion diameter of the infected wounds.

Table 2
Content of fatty acids detected in crude extracts of two brown seaweeds and three red algae by a SC-CO_2 technique quantified by GC–MS analysis. The total lipids content was determined by extraction with a mixture of chloroform–methanol. Values are the pooled mean ± SD of three replicates.

Fatty acids	*Laminaria digitata*	*Undaria pinnatifida*	*Porphyra umbilicalis*	*Eucheuma denticulatum*	*Gelidium pusillum*
Lauric acid (C12:0)	0.08 ± 0.001[c]	n.d.	0.05 ± 0.001	n.d.	n.d.
Myristic acid (C14:0)	2.7 ± 0.01	2.1 ± 0.02	3.6 ± 0.09	0.3 ± 0.03	1.1 ± 0.05
Un-identified (C15:0)	0.4 ± 0.006	0.23 ± 0.002	0.41 ± 0.05	0.12 ± 0.08	0.06 ± 0.001
Palmitic acid (C16:0)	32.5 ± 0.6	13.2 ± 0.2	34.7 ± 0.6	1.2 ± 0.05	1.7 ± 0.06
Un-identified (C17:0)	0.15 ± 0.09	0.21 ± 0.002	0.19 ± 0.07	0.11 ± 0.09	0.16 ± 0.09
Stearic acid (C18:0)	2.1 ± 0.01	0.98 ± 0.08	1.1 ± 0.04	0.86 ± 0.09	0.92 ± 0.03
Un-identified (C20:0)	0.26 ± 0.003	0.35 ± 0.06	0.33 ± 0.1	0.32 ± 0.01	0.29 ± 0.07
Palmitoleic acid (C16:1, *n*-7)	1.8 ± 0.01	0.49 ± 0.08	2.2 ± 0.01	0.22 ± 0.01	0.32 ± 0.09
Un-identified (C17:1, *n*-7)	0.13 ± 0.09	0.12 ± 0.07	0.2 ± 0.05	n.d.	n.d.
Un-identified (C20:1, *n*-9)	1.6 ± 0.05	n.d.	1.6 ± 0.04	n.d.	n.d.
Un-identified (C22:1, *n*-11)	0.02 ± 0.001	n.d.	1.5 ± 0.05	n.d.	n.d.
Un-identified (C22:1, *n*-13)	0.96 ± 0.04	n.d.	0.56 ± 0.009	n.d.	n.d.
Oleic acid (C18:1, *n*-9cis)	5.5 ± 0.6	3.0 ± 0.1	3.8 ± 0.08	0.12 ± 0.09	0.48 ± 0.07
Linoleic acid (C18:2, *n*-6)	8.2 ± 0.1	7.2 ± 0.1	5.8 ± 0.1	0.31 ± 0.08	0.12 ± 0.05
Linolenic acid (C18:3, *n*-3)	3.9 ± 0.08	2.8 ± 0.1	3.3 ± 0.07	0.41 ± 0.05	0.5 ± 0.06
Octadecatetraenoic acid (C18:4, *n*-3)	2.9 ± 0.2	15.0 ± 0.2	3.1 ± 0.5	n.d.	0.23 ± 0.06
Un-identified (C20:3, *n*-6)	1.2 ± 0.06	0.57 ± 0.007	1.1 ± 0.05	n.d.	n.d.
Un-identified (C20:3, *n*-3)	0.01 ± 0.001	0.14 ± 0.09	0.1 ± 0.003	n.d.	n.d.
Un-identified (C20:4, *n*-3)	0.5 ± 0.06	0.8 ± 0.007	0.8 ± 0.009	0.21 ± 0.05	0.15 ± 0.09
Arachidonic acid (C20:4, *n*-6)	14.0 ± 0.2	18.2 ± 0.2	9.2 ± 0.1	0.22 ± 0.06	0.18 ± 0.06
Total lipids (%)[b]	48.6 ± 0.3	42.3 ± 0.2	42.8 ± 0.4	20.5 ± 0.5	25.6 ± 0.01
Dry crude extract yield (%)[a]	1.58 ± 0.01	1.66 ± 0.1	1.58 ± 0.1	1.37 ± 0.1	1.70 ± 0.1

[a] Value on a basis of biomass fresh weight.
[b] Value on a basis of crude extract dry weight.
[c] Percentage on a basis of total fatty acids. n.d. = not determined.

2.5. Measurement of enzymatic activity in inoculated seaweed-treated fruit

Trials were arranged including thirty fruit per specie treated with 30 g L^{-1} extract before inoculation of pathogen and sampled after one-day and five-days of incubation at RH of 96 ± 2% and temperature of 20 ± 2 °C. Untreated-inoculated fruit and untreated-healthy fruit (not injured) were both included as control. Small pieces of tissue (diameter = 2–3 mm, deep = 3–4 mm) randomly collected in six points from each fruit were powdered with liquid nitrogen. Samples of one-gram of powdered tissue were extracted with 2 mL of 0.1 M sodium-phosphate buffer (pH 7.0) at 4 °C and then used for assessing peroxidase (POD), polyphenol oxidase (PPO) and L-phenylalanine ammonia-lyase (PAL) activity. All the experiments were performed with three replications of 10 fruit for each.

POD activity was assayed according to Hammerschmidt et al. (1982). The reaction mixture consisted of one-gram of tissue, 1.5 mL of 0.05 M pyrogallol, 0.5 mL of enzyme (1.5 U mL^{-1}) and 0.5 mL of 1% H_2O_2 that it was incubated at 28 1 C. Absorbance measured at a wavelength of 420 nm were recorded at interval of 30 s for 3 min and the boiled enzyme preparation served as blank. POD activity was expressed as change in absorbance of the reaction mixture on a fresh weight basis (Δ_{OD420} g^{-1} min^{-1}). PPO activity was assayed according to Mayer et al. (1965). The reaction mixture consisted of one-gram of tissue, 1.5 mL of 0.1 M Na-phosphate buffer and 200 µL of enzyme (1.8 U mL^{-1}). To start the reaction, 200 µL of 0.01 M catechol was added. PPO activity was expressed as change in absorbance of the reaction mixture measured at 495 nm on a fresh weight basis (Δ_{OD495} g^{-1} min^{-1}). PAL activity was assessed according to Ross and Sederoff (1992). The assay mixture containing one-gram of tissue, 100 µL of enzyme (2.0 U mL^{-1}), 500 µL of 50 mM Tris-HCl (pH 8.8) and 600 µL of 1 mM L-phenylalanine was incubated for 60 min. The reaction was arrested by adding 2 N HCl. Later, 1.5 mL of toluene was added and mixed for 30 s. The centrifuged (1,000*g* × 5 min) toluene fraction containing *trans*-cinnamic acid was separated. The toluene phase was measured at 290 nm against the blank of toluene. Standard curve was drawn with graded amounts of cinnamic acid in toluene as described earlier. PAL activity was expressed as moles of released cinnamic acid on a fresh weight basis (mol g^{-1} min^{-1}).

2.6. Statistical analysis

The data were before averaged and then subjected to analysis of variance (ANOVA) by a statistical software-pack (SPSS program, version 12.0 Statistics Base™, Chicago, Illinois, USA). Data expressed as percentage were transformed into arcsin square root values to normalize distributions before ANOVA. The percentage values are however shown as untransformed values.

Duncan's multiple range test (DMRT) at probability $P \leq 0.01$ levels was used to compare and separate the means (Gómez and Gómez, 1984).

Averaged data regarding to decay inhibition percentage (DI%) and disease severity (lesion diameter, mm) obtained from the two experiments were pooled and treated as a mean alone whenever a preliminary statistical analysis determined homogeneity of variances without significant interactions among them accordingly with to Bartlett's test. Because the experiments regarding to measurement of the *in vivo* suppressive effect included a factorial scheme with two variables, the first 'qualitative' (extract obtained from five seaweed) and the second 'quantitative' (three levels of extract dose), experimental design have been done in two steps. The first one in order to evaluate the relation between crude seaweed extracts tested at 30 g L^{-1} including the fungicides and response to suppression; and the second one in order to study the relation dose-effect only in those cases where a significant suppression was found.

3. Results

3.1. Content of fatty acids, polysaccharides and phenolic compounds in seaweed extracts

On a basis of GC–MS performance, analysis revealed that a total of twenty fatty acids, most of them un-identified, were found in all the seaweed extracts (Table 2). The molecular weights, chemical names, retention times and peak areas percentage were given from the chromatogram of each seaweed extract (data not shown). Among the fatty acids detected, the palmitic acid (or *n*-hexadecanoic acid), the linoleic acid (or 9,12-octadecadienoic acid), and the arachidonic acid

Table 3
Content of the main water-soluble polysaccharides detected in crude extracts of two brown seaweeds and three red algae by a SC-CO_2 technique quantified by HIC analysis. The total water-soluble polysaccharides content was determined by extraction with a mixture of water–methanol. Values are the pooled mean ± SD of three replicates.

Water-soluble polysaccharides	*Laminaria digitata*	*Undaria pinnatifida*	*Porphyra umbilicalis*	*Eucheuma denticulatum*	*Gelidium pusillum*
Laminarans	14.4 ± 0.2[c]	3.4 ± 0.5	–	–	–
Fucoidans	5.5 ± 0.1	1.5 ± 0.1	–	–	–
Alginates	32.2 ± 0.7	26.6 ± 0.4	–	–	–
Total water-soluble polysaccharides (%)[b]	23 ± 0.8	22 ± 0.8	15 ± 0.6	16 ± 0.6	14.5 ± 0.7
Dry crude extract yield (%)[a]	1.58 ± 0.1	1.66 ± 0.1	1.58 ± 0.1	1.37 ± 0.1	1.70 ± 0.1

[a] Value on a basis of biomass fresh weight.
[b] Value on a basis of crude extract dry weight.
[c] Percentage on a basis of total water-soluble polysaccharides. – absence.

were found at the highest concentrations in extracts of *L. digitata* (32.5% for palmitic acid, 8.2% for linoleic acid and 14.0% for arachidonic acid), *U. pinnatifida* (13.2% for the palmitic, 7.2% for the linoleic and 18.2% for the arachidonic) and *P. umbilicalis* (34.7% for the palmitic, 5.8% for the linoleic and 9.2% for the arachidonic). While, they were scarcely represented (less than 1.8%) in extracts of *E. denticulatum* and *G. pusillum*. The total lipids content ranged from 48.6% in *L. digitata* extract to 20.5% in those of *E. denticulatum*.

On a basis of HIC performance, laminarans, fucoidans and alginates were found only in brown seaweeds. In *L. digitata* extract the content of laminarans was 14.4%, those of fucoidans was 5.5% and alginates was 32.2%. In *U. pinnatifida* extract the content of laminarans was 3.4%, those of fucoidans was 1.5% and alginates was 26.6%. The total water-soluble polysaccharides content varied between 23% in *L. digitata* extract to 14.5% in those of *G. pusillum* (Table 3).

On a basis of HPLC-DAD performance, phlorotannins were found only in brown seaweeds. Phlorethols and fucophloretols were both detected in *L. digitata* extract, while eckols in those of *U. pinnatifida*. The total phenolic compounds content ranged from 2.3% in *L. digitata* extract to 0.15% in those of *G. pusillum* (Table 4).

3.2. Inhibitory effect of seaweed extract on in vitro fungi growth

The inhibitory effect of crude seaweed extracts tested at 10 g L^{-1}, 20 g L^{-1}, and 30 g L^{-1} on mycelia growth and conidial germination of *B. cinerea*, *M. laxa* and *P. digitatum* are reported in Table 5. In general, *L. digitata*, *U. pinnatifida* and *P. umbilicalis* extracts were similarly effective in reducing mycelia growth and inhibiting conidial germination of all the pathogens. In particular, *L. digitata* and *U. pinnatifida* extracts tested at 30 g L^{-1} dose showed the best results, completely inhibiting *B. cinerea* and *M. laxa* ($P \leq 0.01$) exerting a fungicide activity only on *B. cinerea* (by *L. digitata*) and a fungistatic effect towards *B. cinerea* (by *U. pinnatifida*) and *M. laxa* (by *L. digitata* and *U. pinnatifida*); and completely suppressing conidial germination of *B. cinerea* and *M. laxa* ($P \leq 0.01$). Extract of *P. umbilicalis* induced inhibition of mycelia growth of 88% and 71%, and a conidial germination ratio of 12% and 15%, on cultures of *B. cinerea* and *M. laxa* respectively. Contrarily to this, extracts of *E. denticulatum* and *G. pusillum* showed a significant lower activity against *B. cinerea* and *M. laxa* towards mycelia growth inhibition (less than 49%) and conidia suppression ratio (more than 38%). Whereas, *P. digitatum* was the less sensitive pathogen to antifungal effect of the seaweed extracts if compared to *B. cinerea* and *M. laxa*. Extracts of *L. digitata* and *U. pinnatifida*, followed by *P. umbilicalis*, showed a reduced but still significant inhibition on cultures of *B. cinerea* and *M. laxa*, followed by *P. digitatum*, using a dosage of 20 g L^{-1}. The lowest inhibition was instead seen for all fungi with 10 g L^{-1} dose of extract. Therefore a 'dose-effect' was evident, where fungal inhibition increases as the dose of extract added to growing media increased. The control did not show suppression of conidial germination after incubation.

The inhibitory effect of the hexane-soluble extract fraction tested at 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} on mycelia growth and conidial germination of *B. cinerea*, *M. laxa* and *P. digitatum* is reported in Table 6. The strongest inhibitor effect was seen testing this fraction in comparison to those of the crude extracts (Table 5). Instead, only a reduced antifungal activity against *B. cinerea* and *M. laxa* with 30 g L^{-1} extract was found on the mycelia growth (less than 25%) and conidial germination (more than 70%) using the ethanol-soluble extract fraction of *L. digitata*, *U. pinnatifida* and *P. umbilicalis* (Table 7). Finally, any antifungal activity was seen testing the aqueous fraction at the same conditions (Table 7).

3.3. Suppressive effect of seaweed extract on inoculated fruit

Crude seaweed extracts tested at 30 g L^{-1} showed different activity in inhibiting fruit decay and reducing disease severity in inoculated strawberries (Figs. 1 and 2), peaches (Figs. 3 and 4) and lemons (Figs. 5 and 6). The efficacy of the different extracts in increasing decay inhibition is shown in Figs. 1, 3 and 5, while those in reducing disease severity measuring lesion diameter on fruit epicarp is shown in Figs. 2, 4 and 6.

In general, decay inhibition of grey mould in strawberries caused by *B. cinerea*, brown rot in peaches due to *M. laxa*, and green mould in lemons induced by *P. digitatum*, significantly increased ($P \leq 0.01$) by use of *L. digitata*, *U. pinnatifida* and *P. umbilicalis*, but no by *E. denticulatum* and *G. pusillum* in preventive and curative treatments.

Table 4
Detection of phlorotannins in crude extracts of two brown seaweeds and three red algae by a SC-CO_2 technique determined by HPLC-DAD analysis. The total phenolic compounds content was determined by extraction with a mixture of water–ethanol. Values are the pooled mean ± SD of three replicates.

Phlorotannins	*Laminaria digitata*	*Undaria pinnatifida*	*Porphyra umbilicalis*	*Eucheuma denticulatum*	*Gelidium pusillum*
Fucols	–	–	–	–	–
Phlorethols	+	–	–	–	–
Fucophloretols	+	–	–	–	–
Fuhalols	–	–	–	–	–
Eckols	–	+	–	–	–
Total phenolic compounds (%)[b]	2.3 ± 0.4	0.4 ± 0.001	0.3 ± 0.001	0.2 ± 0.001	0.15 ± 0.001
Dry crude extract yield (%)[a]	1.58 ± 0.1	1.66 ± 0.1	1.58 ± 0.1	1.37 ± 0.1	1.70 ± 0.1

[a] Value on a basis of biomass fresh weight.
[b] Value on a basis of crude extract dry weight. + presence – absence.

Table 5
Effect of crude total extract of five seaweed species on mycelia growth inhibition (MGI) and conidia germination suppression ratio (CGS) of *B. cinerea*, *M. laxa* and *P. digitatum*. Activity of extracts applied at three decreasing concentrations was tested by evaluation of MGI after incubation on PDA into Petri plates at 25 ± 1 °C and by assessment of CGS after 72 h of incubation in PDB into micro-well plates at 25 ± 1 °C. In each column, values followed by different letters are significantly different according to DMRT at $P \leq 0.01$.

Seaweed species	Concentration of crude total extract																	
	30 g L^{-1}						20 g L^{-1}						10 g L^{-1}					
	B. cinerea		*M. laxa*		*P. digitatum*		*B. cinerea*		*M. laxa*		*P. digitatum*		*B. cinerea*		*M. laxa*		*P. digitatum*	
	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS
Laminaria digitata	100A[a]	0E	100A[b]	0E	64A	45C	88A	28D	73A	37D	45A	73C	55A	34C	51AB	43C	29A	81B
Undaria pinnatifida	100A[b]	0E	100A[b]	0E	58AB	43C	85A	21D	78A	34D	46A	69CD	58A	39C	57A	41C	25A	80B
Porphyra umbilicalis	88B	12D	71B	15D	50B	47C	62B	24D	64B	38D	34B	71C	30B	37C	32C	49C	22A	83B
Eucheuma denticulatum	38CD	39C	48C	42C	27C	75B	36C	51C	38C	63C	0C	83B	0C	78B	0D	87AB	0B	95A
Gelidium pusillum	47C	59B	39D	68B	21C	79B	38C	71B	32C	73B	0C	82B	0C	80B	0D	85B	0B	93A
Control	–	98A	–	94A	–	95A	–	98A	–	95A	–	96A	–	94A	–	97A	–	98A

[a] Fungicide action.
[b] Fungistatic effect. MGI values ranging from 0% (no inhibition) to 100% (total inhibition); CGS ratio ranging from 0% (total inhibition) to 100% (no inhibition). Values of MGI and CGS are the mean of three replicates.

Lesion diameter (mm) on fruit skin caused by grey mould, brown rot and green mould were significantly reduced ($P \leq 0.01$) by use of *L. digitata*, *U. pinnatifida* and *P. umbilicalis*, but no by *E. denticulatum* and *G. pusillum* in both treatments.

In particular, application of 30 g L^{-1} extract of *L. digitata*, *U. pinnatifida* and *P. umbilicalis* in preventive treatments respectively induced 85%, 79% and 73% decay inhibition in strawberries (Fig. 1); 82%, 66% and 69% inhibition in peaches (Fig. 3); and 60%, 58% and 51% disease suppression in lemons (Fig. 5). Moreover, application of 30 g L^{-1} extract of *L. digitata*, *U. pinnatifida* and *P. umbilicalis* respectively caused lesion diameter of 2 mm, 3 mm and 4 mm in strawberries (Fig. 2); lesion diameter of 5 mm, 9 mm and 13 mm in peaches (Fig. 4); and lesion diameter of 10 mm, 18 mm and 15 mm in lemons (Fig. 6). Particularly, the efficacy of *L. digitata* extract applied in preventive treatment in *L. digitata*-treated peaches remained statistically unchanged from 6th to 10th day of incubation (data not shown) either in decay inhibition (DI = 80%) either in lesion diameter (5.5 mm). Instead, extracts of *E. denticulatum* and *G. pusillum* both resulted less suppressive than those afore-mentioned either in increasing decay inhibition (Figs. 1, 3 and 5) either in reducing lesion diameter (Figs. 2, 4 and 6). At the same conditions, fenhexamid applied in preventive treatments on strawberries and peaches (Figs. 1–4) and imazalil on lemons (Figs. 5 and 6) almost completely inhibited infection development either in increasing decay inhibition either in reducing lesion diameter. Curative applications of 30 g L^{-1} extract of *L. digitata*, *U. pinnatifida* and *P. umbilicalis* in curative treatments including the fungicides were efficacy less than those preventive in suppressing all diseases, but maintaining the same suppression patterns of them.

Basing on the relation dose-effect in suppressing fruit decay (Fig. 7)

Table 7
Effect of the ethanol-soluble and water-soluble extract fractions applied at 30 g L^{-1} on mycelia growth inhibition (MGI) and conidia germination suppression ratio (CGS) of *B. cinerea* and *M. laxa*. Activity was tested by evaluation of MGI after incubation on PDA into Petri plates at 25 ± 1 °C and by assessment of CGS after 72 h of incubation in PDB into micro-well plates at 25 ± 1 °C. In each column, values followed by different letters are significantly different according to DMRT at $P \leq 0.01$.

Seaweed species	Ethanol-soluble fraction				Water-soluble fraction			
	B. cinerea		*M. laxa*		*B. cinerea*		*M. laxa*	
	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS
Laminaria digitata	24A	71C	23A	71C	0A	98A	0A	88A
Undaria pinnatifida	8B	81B	9B	87B	0A	93A	0A	97A
Porphyra umbilicalis	6B	85B	7B	83B	0A	92A	0A	96A
Control	–	98A	–	94A	–	95A	–	98A

MGI values ranging from 0% (no inhibition) to 100% (total inhibition); CGS ratio ranging from 0% (total inhibition) to 100% (no inhibition). Values of MGI and CGS are the mean of three replicates.

Table 6
Effect of the hexane-soluble extract fraction on mycelia growth inhibition (MGI) and conidia germination suppression ratio (CGS) of *B. cinerea*, *M. laxa* and *P. digitatum*. Activity of extracts applied at three decreasing concentrations was tested by evaluation of MGI after incubation on PDA into Petri plates at 25 ± 1 °C and by assessment of CGS after 72 h of incubation in PDB into micro-well plates at 25 ± 1 °C. In each column, values followed by different letters are significantly different according to DMRT at $P \leq 0.01$.

Seaweed species	Concentration of the hexane-soluble fraction																	
	30 g L^{-1}						20 g L^{-1}						10 g L^{-1}					
	B. cinerea		*M. laxa*		*P. digitatum*		*B. cinerea*		*M. laxa*		*P. digitatum*		*B. cinerea*		*M. laxa*		*P. digitatum*	
	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS	MGI	CGS
Laminaria digitata	100A[a]	0D	100A[a]	0E	70A	38C	91A	18D	78AB	27D	55A	66C	67A	24C	61AB	31D	34A	71B
Undaria pinnatifida	100A[a]	0D	100A[b]	0E	68AB	33D	88B	19D	88A	24D	53A	60D	63AB	29C	67A	33D	29B	69C
Porphyra umbilicalis	100A[b]	0D	97B	5DE	58B	38C	72C	16DE	74B	28D	44B	63CD	40C	27C	39C	40C	27B	73B
Eucheuma denticulatum	49C	29C	58C	38C	28C	60BC	41DE	45C	45C	56C	5C	73B	10D	70B	2D	78B	0C	85A
Gelidium pusillum	52B	51B	44D	58B	23CD	64B	48D	68B	39D	65B	0CD	72B	2DE	72B	0D	77B	0C	83A
Control	–	98A	–	94A	–	95A	–	98A	–	95A	–	96A	–	94A	–	97A	–	98A

[a] Fungicide action.
[b] Fungistatic effect. MGI values ranging from 0% (no inhibition) to 100% (total inhibition); CGS ratio ranging from 0% (total inhibition) to 100% (no inhibition). Values of MGI and CGS are the mean of three replicates.

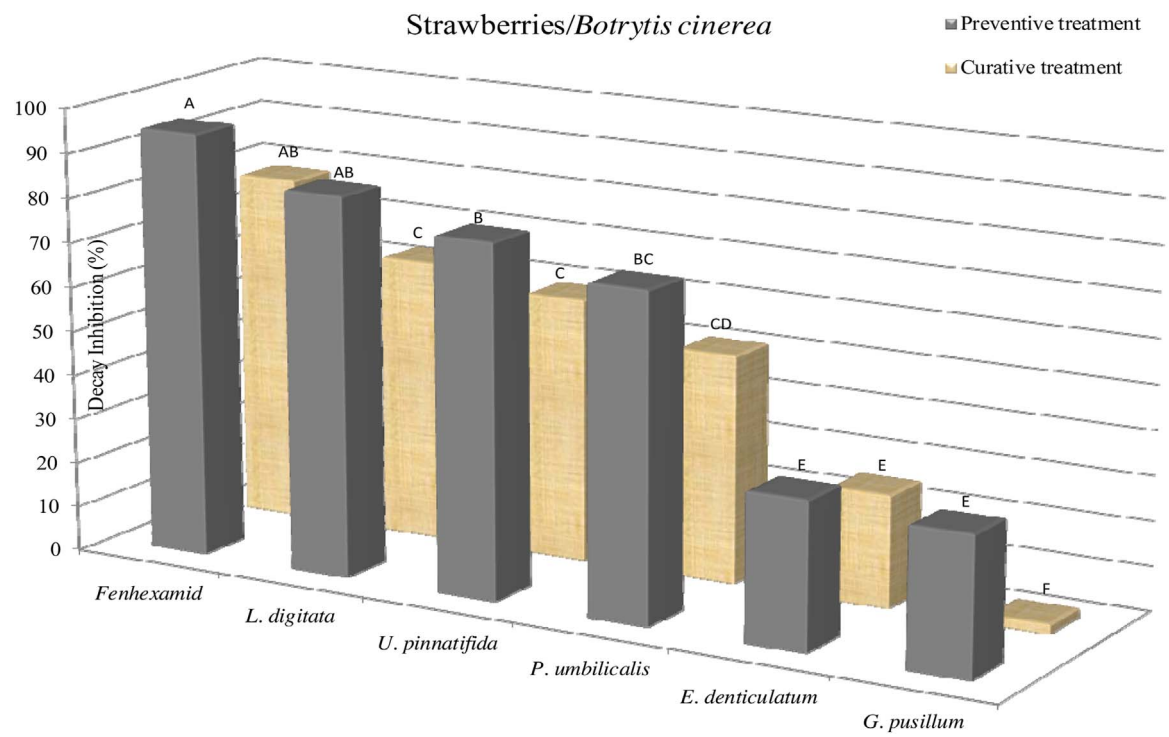

Fig. 1. Suppression of grey mould on strawberries by *B. cinerea* after 3 d of incubation at 20 ± 2 °C and 98% RH following to preventive and curative application of 30 g L^{-1} crude extract of five seaweed species compared to fenhexamid. Values ranging from 0% (no inhibition) to 100% (total inhibition) are the pooled mean of two experiments. Histograms surrounded by different letters are significantly different according to DMRT at $P \leq 0.01$.

and reducing lesion diameter on fruit skin (Fig. 8) by preventive application of three decreasing doses of crude extract of *L. digitata*, *U. pinnatifida* and *P. umbilicalis* in grey mould on strawberries, brown rot on peaches and green mould on lemons whenever a significant response in suppression was found, *L. digitata* and *U. pinnatifida*, followed by *P. umbilicalis*, showed still a significant suppression with 20 g L^{-1} extract. Whereas, the lowest disease suppression was seen using 10 g L^{-1} extract. A remarkable dose-effect was evident because decay inhibition induced by seaweed-treatment decreases as the dose of extract applied over the fruit wound decreased, and lesion diameter increases as the dose of extract decreased (Figs. 7 and 8).

3.4. Enzymatic activity in inoculated seaweed-treated fruit

Enzymatic activity of peroxidase, polyphenol oxidase and L-phenylalanine ammonia-lyase in seaweed-treated-inoculated fruit were shown in Table 8. POD activity was significantly increased on the strawberries/*B. cinerea* and peaches/*M. laxa* systems in treated-inocu-

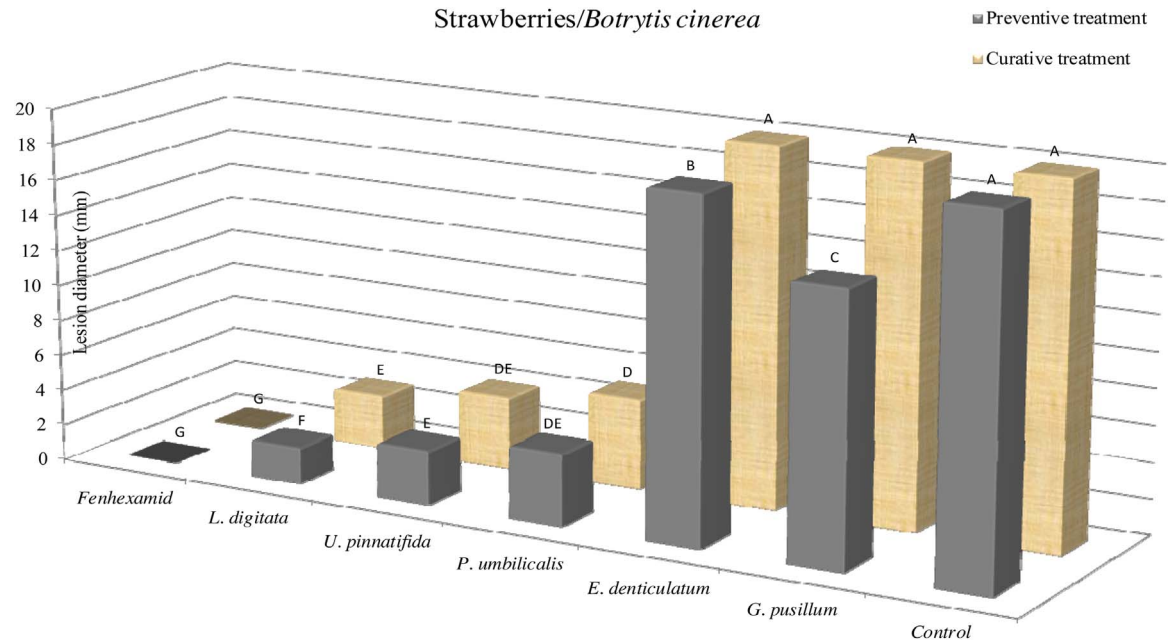

Fig. 2. Reduction of lesion diameter on strawberries inoculated with *B. cinerea* following to preventive and curative application of 30 g L^{-1} seaweed extract after 3 d of incubation at 20 ± 2 °C and 98% RH compared to fenhexamid and buffer (control). Values are the pooled mean of two experiments. Histograms surrounded by different letters are significantly different according to DMRT at $P \leq 0.01$.

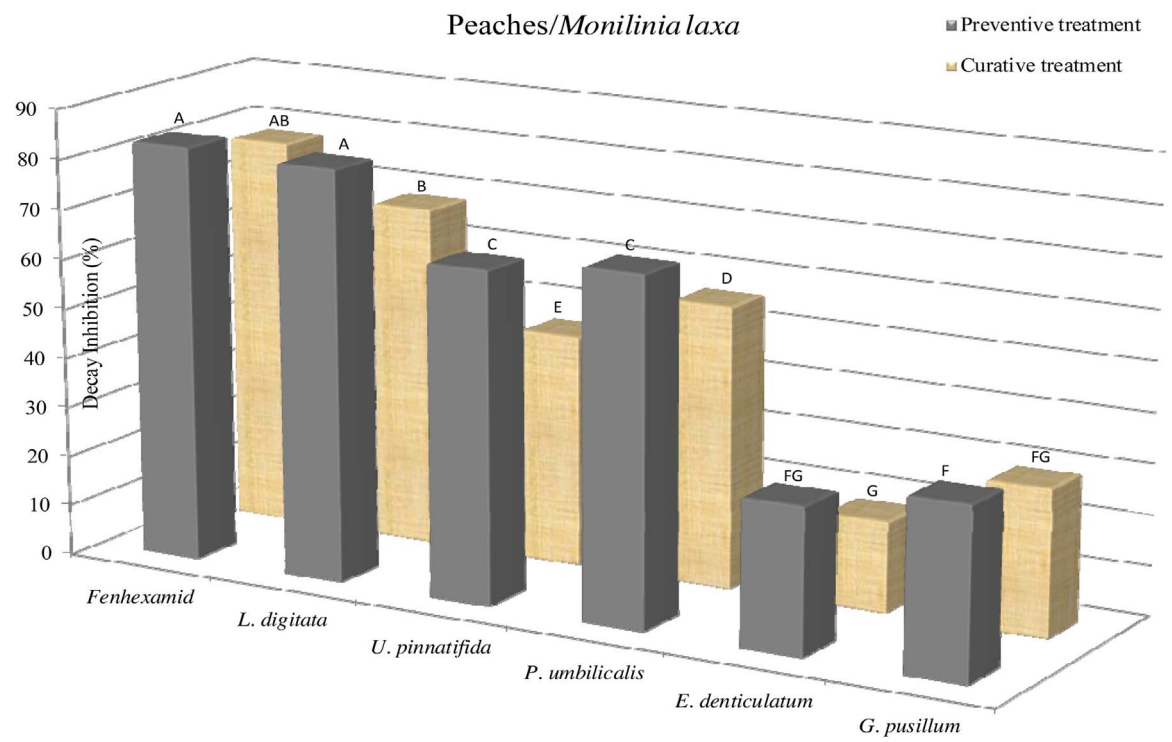

Fig. 3. Suppression of brown rot on peaches by *M. laxa* after 6 d of incubation at 20 ± 2 °C and 98% RH following to preventive and curative application of 30 g L^{-1} crude extract of five seaweed species compared to fenhexamid. Values ranging from 0% (no inhibition) to 100% (total inhibition) are the pooled mean of two experiments. Histograms surrounded by different letters are significantly different according to DMRT at $P \leq 0.01$.

lated fruit after only one-day from inoculation if compared to untreated-inoculated fruit. PPO activity also increased on the strawberries/*B. cinerea* and peaches/*M. laxa* systems after one-day from inoculation when compared to untreated-healthy fruit, but differences between treated-inoculated fruit and untreated-inoculated fruit were not found. PAL activity increased on the strawberries/*B. cinerea* and peaches/*M. laxa* systems after five-days from inoculation following the same pattern of PPO. Instead, any increasing of enzymatic activities was found on the lemons/*P. digitatum* system.

4. Discussion

Brown and red seaweeds being considered as among the most significant varied feedstock for making third-generation biofuels (Sikes et al., 2010; Milledge et al., 2014), are nowadays a potential and easily available sunlight-driven cell biorefinery for carbon dioxide conversion

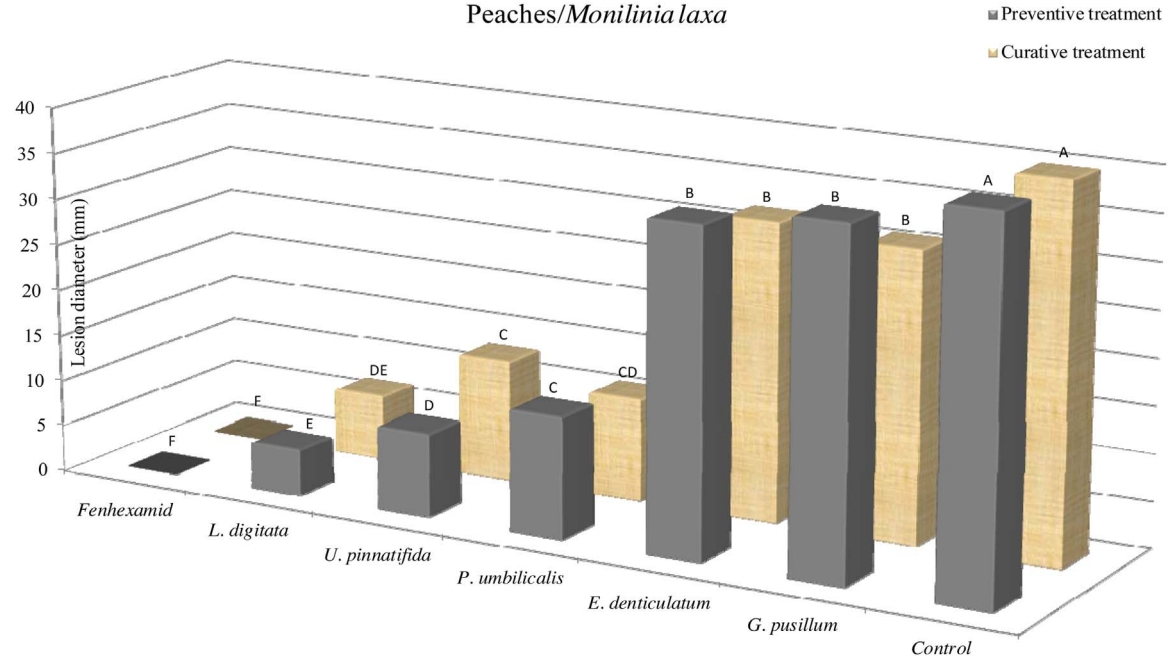

Fig. 4. Reduction of lesion diameter on peaches inoculated with *M. laxa* by preventive and curative application of 30 g L^{-1} seaweed extract after 6 d of incubation at 20 ± 2 °C and 98% RH compared to fenhexamid and buffer (control). Values are the pooled mean of two experiments. Histograms surrounded by different letters are significantly different according to DMRT at $P \leq 0.01$.

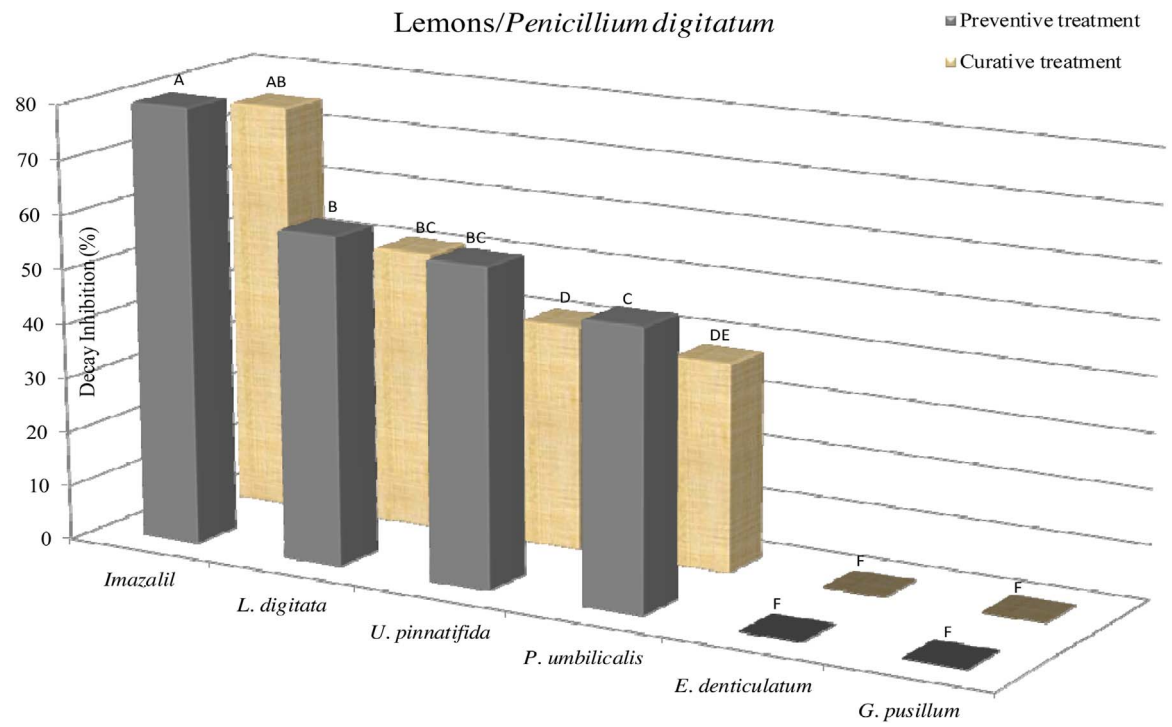

Fig. 5. Suppression of green mould on lemons by *P. digitatum* after 5 d of incubation at 20 ± 2 °C and 98% RH by preventive and curative application of 30 g L^{-1} crude extract of five seaweed species compared to imazalil. Values ranging from 0% (no inhibition) to 100% (total inhibition) are the pooled mean of two experiments. Histograms surrounded by different letters are significantly different according to DMRT at $P \leq 0.01$.

into a wide range of bioactive compounds and various green chemicals most of them used in food industry and other industrial fields as high-value added products (Table 1). The SC-CO_2 is considered as a suitable and eco-friendly extractive technique from plant biomass (Reverchon, 1997) if compared to the traditional methods that use organic solvents less selective and more pollutant for environment. SC-CO_2 allows to reach more purity, efficiency and selectivity in extracting bioactive compounds belonging to specific chemical groups from plant biomass including marine algae opportunely selecting the process parameters. SC-CO_2 being resulted promising for extracting terpenes and other volatile antifungal compounds from laurel leaves in postharvest applications (De Corato et al., 2010), has been similarly used in this work for extracting certain specific classes of antifungal substances from brown and red seaweeds. The chosen SC-CO_2 processing parameters were able to maximize extraction of lipids and fatty acids (between 48.6% in *L. digitata* and 20.5% in *E. denticulatum*) accordingly with Cheung et al.

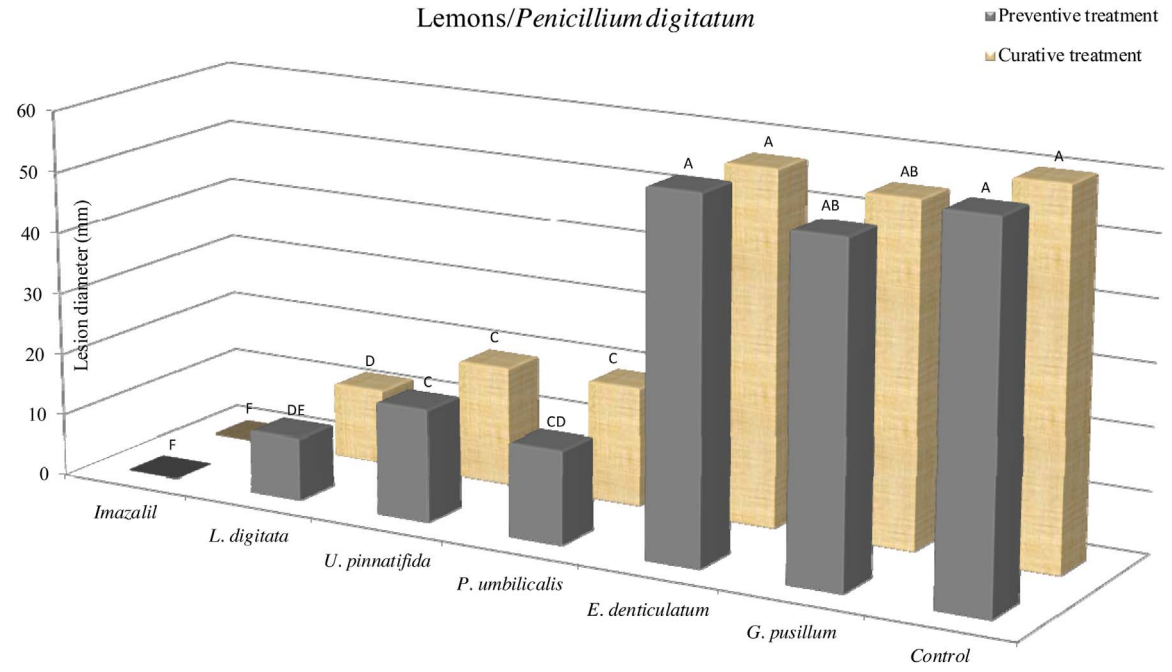

Fig. 6. Reduction of lesion diameter on lemons inoculated with *P. digitatum* by preventive and curative application of 30 g L^{-1} seaweed extract after 5 d of incubation at 20 ± 2 °C and 98% RH compared to imazalil and buffer (control). Values are the pooled mean of two experiments. Histograms surrounded by different letters are significantly different according to DMRT at $P \leq 0.01$.

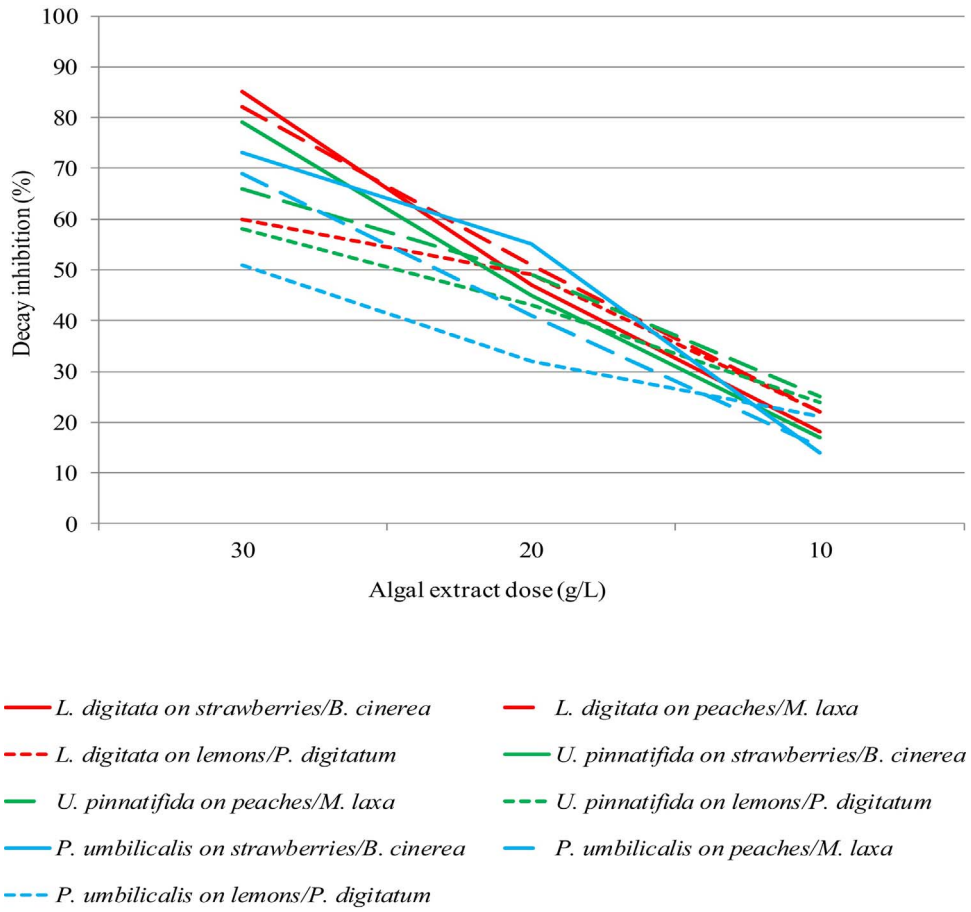

Fig. 7. Relation dose-effect in suppressing grey mould on strawberries by *B. cinerea*, brown rot on peaches by *M. laxa* and green mould on lemons by *P. digitatum* following to preventive application of three decreasing doses of crude extract of *L. digitata*, *U. pinnatifida* and *P. umbilicalis*. Values are the pooled mean of two experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(1998). These authors describe that processing fresh biomass from the brown seaweed *S. hemiphyllum* using compressed CO_2 at the conditions of 37.9 MPa at 40 °C or 37.9 MPa at 50 °C, the highest lipids and fatty acids yield was obtained (more than 20% on a basis of extract dry weight) if compared to efficiency obtained with the Soxhlet. In fact, SC-CO_2 is used in the marine biorefinery platforms for to maximize bioconversion of lipids (main triglycerides) from seaweeds into third-generation biodiesel (Milledge et al., 2014).

The *in vitro* experiments performed with the hexane-soluble seaweed extract fraction suggest that inhibition on mycelia growth and conidial germination could be due to direct toxicity of the fatty acids found at the highest concentration in extracts of *L. digitata*, *U. pinnatifida* and *P. umbilicalis* rather than in those of *E. denticulatum* and *G. pusillum* (Table 2). In supporting this hypothesis, only the hexane-soluble extract fraction exerted a similar strong antifungal effect (Table 6) in comparison to those seen with the un-fractionated crude extract (Table 5). Moreover, the inhibition patterns of both extracts were closely related to concentration of extract added into growing media (PDA or PDB) with an increase of antifungal activity as the dose of extract added increased. A dose-dependent manner of the treatments with seaweed extracts was found at least in the concentration range considered here. The *in vivo* experiments performed with crude extract at 30 g L^{-1} dose showed a strong efficacy by use of *L. digitata* and *U. pinnatifida*, followed by *P. umbilicalis*, in both treatments (preventive and curative) in increasing fruit decay inhibition (Figs. 1, 3 and 5) and reducing disease severity (Figs. 2, 4 and 6) on infected fruit by *B. cinerea* (in strawberries), *M. laxa* (in peaches) and *P. digitatum* (in lemons). Application of fenhexamid and imazalil during the postharvest phase of stone fruit is actually not authorized by Italian and European legislation, however they were used in this work only as a reference control. Findings coming from the *in vivo* experiments highlighted an interesting and very competitive antifungal activity of *L. digitata*, *U. pinnatifida* and *P. umbilicalis* extracts against *B. cinerea*, *M. laxa* and *P. digitatum* on strawberries, peaches and lemons when compared to action of the two synthetic fungicides in both treatments. Particularly, brown rot development in *L. digitata*-treated peaches was statistically comparable to that found in fenhexamid-treated peaches until 10th-day of incubation from infection (data not shown). Fruit decay inhibition and disease severity were both related to dose of extract showing an evident increase of suppressive activity and a remarkable reduction of lesion diameter on fruit surface as the dose of extract applied over the wound increased (Figs. 7 and 8). Therefore a dose-effect of the treatment was observed at least in the concentration range considered here.

Although many findings about the relationships between antifungal activity of brown and red seaweed extracts and specific bioactive compounds are available in literature, antifungal potential of seaweed extract has not been still fully exploited due to lack of data on inhibition factors present in seaweeds and their modality of action in suppressing fungi development. In this work three chemicals able to suppress postharvest fungal diseases were found in crude seaweed extract (lipids, phenolic compounds and water-soluble polysaccharides) that together represented about the 74% (*L. digitata*), 65% (*U. pinnatifida*), 58% (*P. umbilicalis*), 40.3% (*G. pusillum*) and 36.7% (*E. denticulatum*) of crude extract dry weight (Tables 2, 3 and 4). Some hypothesis about the relationships between antifungal activity and specific chemical compounds are given.

Firstly, a direct antifungal activity of the crude seaweed extracts

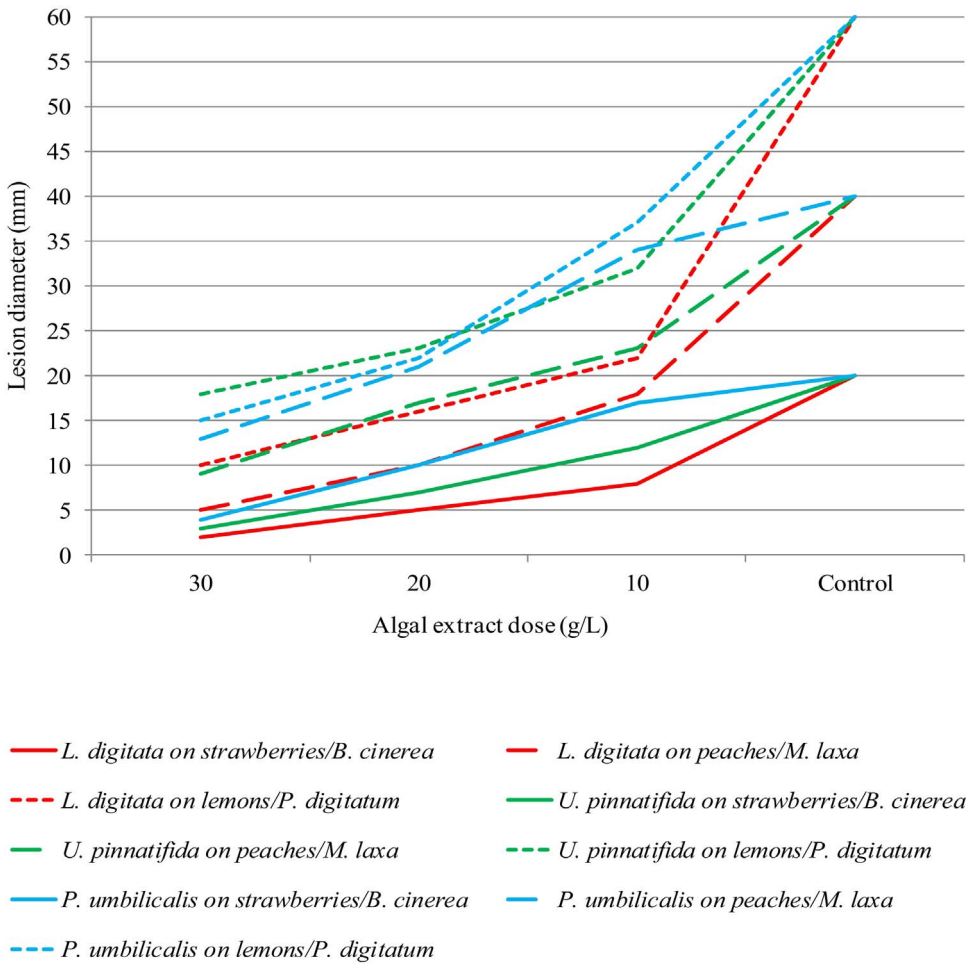

Fig. 8. Relation dose-response in reducing lesion diameter by grey mould on strawberries, brown rot on peaches and green mould on lemons by preventive application of three decreasing doses of crude extract of *L. digitata*, *U. pinnatifida* and *P. umbilicalis*. Values are the pooled mean of two experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tested here could be mainly attributed to content of fatty acids (or lipids) that could be considered as the most probable candidates able to suppress mycelia growth and conidial germination of the pathogens accordingly with the *in vitro* tests. Several authors report many findings of this topic. Some of them reports that certain unsaturated fatty acids are known to have antimicrobial application in pharmaceutical drug industry. Antibacterial and antifungal activity of fatty acid methyl esters extract from leaves of mangrove were found against bacteria of

Table 8
Enzymatic activity of peroxidase (POD), polyphenol oxidase (PPO) and L-phenylalanine ammonia-lyase (PAL) in strawberries, peaches and lemons respectively inoculated with *B. cinerea*, *M. laxa* and *P. digitatum* and preventively treated with 30 g L^{-1} crude seaweed extract. Assessment was carried out after one-day and five-days of incubation at 20 ± 2 °C and 98% RH. Values are the mean of three replicates. In each column, values followed by the same letters are not significantly different according to DMRT at $P \leq 0.01$.

Seaweed species	Strawberries/*Botrytis cinerea*						Peaches/*Monilinia laxa*						Lemons/*Penicillium digitatum*					
	POD [a]		PPO [b]		PAL [c]		POD		PPO		PAL		POD		PPO		PAL	
	1 d	5 d	1 d	5 d	1 d	5 d	1 d	5 d	1 d	5 d	1 d	5 d	1 d	5 d	1 d	5 d	1 d	5 d
Laminaria digitata	7.04 a	33.14 a	0.26 a	2.86 a	0.46 a	2.41 a	5.41 a	30.04 a	0.36 a	3.86 a	0.66 a	5.41 a	1.51 a	10.04 a	0.16 a	0.86 a	0.36 a	0.91 a
Undaria pinnatifida	6.58 a	34.65 a	0.21 a	2.72 a	0.42 a	2.33 a	5.38 a	31.05 a	0.31 a	3.72 a	0.62 a	5.33 a	1.38 a	11.05 a	0.11 a	0.72 a	0.32 a	0.83 a
Porphyra umbilicalis	6.03 a	33.51 a	0.29 a	2.69 a	0.40 a	2.29 a	5.23 a	29.71 a	0.39 a	3.69 a	0.60 a	5.29 a	1.73 a	12.71 a	0.19 a	0.69 a	0.30 a	0.79 a
Eucheuma denticulatum	6.98 a	31.49 a	0.25 a	2.61 a	0.38 a	2.27 a	6.18 a	30.23 a	0.35 a	3.61 a	0.58 a	5.27 a	1.26 a	10.23 a	0.15 a	0.61 a	0.28 a	0.77 a
Gelidium pusillum	6.88 a	31.43 a	0.23 a	2.56 a	0.35 a	2.21 a	5.98 a	33.48 a	0.33 a	3.56 a	0.55 a	5.21 a	1.38 a	13.48 a	0.13 a	0.56 a	0.25 a	0.81 a
Untreated-inoculated fruit	3.21 b	9.31 b	0.26 a	2.78 a	0.44 a	2.35 a	3.28 b	8.67 b	0.36 a	3.78 a	0.64 a	5.35 a	1.28 a	9.67 a	0.16 a	0.78 a	0.34 a	0.75 a
Untreated-healthy fruit	1.32 c	5.38 c	0.10 b	0.55 b	0.43 a	0.80 b	1.43 c	4.51 c	0.20 b	0.65 b	0.63 a	1.98 b	1.42 a	10.51 a	0.12 a	0.57 a	0.33 a	0.78 a

[a] Change in absorbance on a fresh weight basis (Δ_{OD420} g^{-1} min^{-1}).
[b] Change in absorbance on a fresh weight basis (Δ_{OD495} g^{-1} min^{-1}).
[c] Moles of released cinnamic acid on a fresh weight basis (mol g^{-1} min^{-1}).

genera *Bacillus*, *Micrococcus*, *Staphylococcus*, *Pseudomonas* and *Klebsiella* (Vallinayagam et al., 2009). Lauric, palmitic, linolenic, linoleic, oleic, stearic, arachidonic and myristic acids isolated from different plant sources are known to have a potential antibacterial and antifungal activity (McGaw et al., 2002; Seidel and Taylor, 2004) and, among them, the palmitic and linoleic acids result closely correlated to antimicrobial effects toward yeast species of genus *Candida* (Agoramoorthy et al., 2007). Authors have found antibacterial activity of extracts obtained from *Rhodophyceae* samples collected from Gibraltar and the Moroccan Mediterranean coast (*Ceramiales*, *Gelidiales*, *Gigartinales*, *Bonnemaisoniales* and *Rhodymeniales*). Their bioactivity has been investigated using methanolic extracts against three gram-positive bacteria and two gram-negative bacteria showing that certain seaweed species exhibit the highest antibacterial activity against a broad spectrum of human pathogen bacteria including *Staphylococcus aureus* (Bouhlal et al., 2010). On the other hand, the antimicrobial activity of *E. denticulatum* extract that in ours findings showed a lower antifungal efficacy, in other study shows a very strong antibacterial inhibitor effect on gram-positive bacteria (Al-Haj et al., 2009). Ours findings accord with other works that report as certain fatty acids are known to have antifungal application in crop protection. Raj et al. (2016) have found that antifungal activity of brown seaweed extracts toward *R. solani* on rice might be due either to toxic levels of *n*-hexadecanoic acid (or palmitic acid) and 9,12-octadecadienoic acid (or linoleic acid), either to accumulation of phenolic compounds (phytoalexins) in rice tissues as response to infection. In ours findings about the potential control of strawberry fruit rot by *Rhizopus stolonifer* with extract of *L. digitata* under postharvest condition, we have seen by scanning electron microscopy that sporangia and hyphae exposed to 20 g L^{-1} for 3 d of extract have a reduction of sporangia number, swelling of sporangia, collapse of sporangia wall, blowing of hyphae and alteration of hyphae wall. Several damaging mechanisms induced by botanical organic compounds including fatty acids were reported in literature, such as a partition of lipid layer of the cell membrane due to their hydrophobic nature, and affection of permeability of the cell membrane that cause leakage of cell components (Soylu et al., 2006; Laird and Phillips, 2011; Da Cruz et al., 2013; Shao et al., 2013). Cell ultrastructural changes caused to fungal cell wall were also reported as a result of the direct interaction of certain botanical bioactive metabolites with the enzymes responsible for cell wall synthesis (Rasooli et al., 2006). Reasonably these complex mechanisms could include the fatty acids detected in seaweed extract, nevertheless more studies in depth are needed for to confirm these hypothesis.

Secondly, the role of phenolic compounds including phlorotannins could be no significant in reducing fungal growth and disease development. Supporting this hypothesis, the *in vitro* inhibition effects seen with the ethanol-soluble extract fraction of *L. digitata*, *U. pinnatifida* and *P. umbilicalis* were really inconsistent on the mycelia growth and conidia suppression of *B. cinerea* and *M. laxa* (Table 7) if compared to those of the hexane-soluble extract fraction (Table 6). On the other hand, phlorotannins were found only in extracts of *L. digitata* and *U. pinnatifida* (Table 4). Several authors report many findings of this topic. Red seaweeds have generally lower concentrations of phenols if compared to brown seaweeds (Løvstad Holdt and Kraan, 2011). Among brown algae, *Ecklonia* sp., *Eisenia bicyclis*, *Ishige okamurae*, *S. thunbergii, Hizikia fusiformis*, *U. pinnatifida*, and *Laminaria* sp. have been reported for the highest content of phlorotannins. Instead, any red algae has been highlighted for the content of phlorotannins. Numerous biological activities and many potential benefits of phlorotannins were reported in literature, either in enhancing human health (antioxidant activity, enzyme inhibitory effect, anti-viral activity, anticancer action, radioprotective and antiallergic effect, and other beneficial biological actions) either in reducing growth of certain human pathogens bacteria (Li et al., 2011). Although Liu et al. (2015) have investigated about the effects of phlorotannins from brown seaweed on the quality of harvested nectarine fruit, nevertheless any finding regarding to their direct antifungal effect on postharvest pathogens was found in literature.

Thirdly, but no less important, fruit disease suppression could also be related to a decreased hydrogen peroxide production after inoculation of the pathogens induced probably by the water-soluble polysaccharides. This study shows that an increased POD activity could be related to activation of mechanisms of induced systemic resistance (ISR) on the strawberries/*B. cinerea* and peaches/*M. laxa* systems after only one-day from inoculation (Table 8). Peroxidases usually employ hydrogen peroxide as a substrate causing defence reactions which earlier occur in the fruit tissue after infection. Hydrogen peroxide has antimicrobial properties due to its strong oxidizing power and its capacity to generate other oxidizing species (hydroxyl radicals, singlet oxygen species and hydrogen peroxides), well known as reactive oxygen species (ROS), which are toxic to living cells. Inactivation of membrane respiratory chain enzymes and damage to DNA are the probable mechanisms of action for hydrogen peroxide and ROS (Imlay and Linn, 1988; Tatsuzawa et al., 1998). Laminarans, fucoidans and alginates were found in crude extract of brown algae, but many other water-soluble polysaccharides were detected in red seaweeds (Table 3) accordingly with the literature. However the *in vitro* inhibition effects against mycelia development and conidia germination were not found using the aqueous fraction of crude extract (Table 7) revealing that the water-soluble polysaccharides not exert a direct toxicity at least in these experimental conditions. Therefore is reasonable affirm that laminarans, fucoidans and alginates found in *L. digitata* and *U. pinnatifida* could be involved in increasing POD activity and suppressing grey mould on strawberries and brown rot on peaches working most as resistance inducers rather than as toxic chemicals. Probably the porphyrans, a group of polysaccharides present in *P. umbilicalis* at a concentration of 47.8% (MacArtain et al., 2007) could justify an increased activity of POD, as well as the total polysaccharides present in *E. denticulatum* and *G. pusillum*. Instead, an increased activity of PPO and PAL in the strawberries/*B. cinerea* and peaches/*M. laxa* systems was found either in treated-inoculated fruit either in untreated-inoculated fruit if compared to untreated-healthy fruit. *B. cinerea* and *M. laxa* could have induced mechanisms of ISR *in planta* increasing the PPO and PAL activities due most to unspecific reactions induced by the artificial wounds rather than to specific reactions elicited by the seaweed extracts. Several authors report many findings of this topic. Laminarin is known as an efficient resistance inducer against *B. cinerea* and *Plasmopara viticola* (Aziz et al., 2003) being authorized in France as 'Iodus 40′ (Stähler Suisse, SA) for controlling powdery mildew on wheat (De Miccolis et al., 2009). Foliar application of seaweed liquid fertilizer (SLF) from brown algae enhanced defence in carrot and tomato in relation to various defence-related enzymes including POD, PPO, PAL, chitinase and β-1,3-glucanase (Jayaraj et al., 2008; Solanki et al., 2012). Similar results were found in cucumber that show increased activities of chitinase, 3-glucanase, POD, PPO, PAL and lipoxygenase following to foliar application of SLF (Jayaraman et al., 2011). Also certain unsaturated fatty acids including linoleic and arachidonic acids can play the crucial role of elicitor to induce resistance against *P. infestans* in tomato and potato (Cohen et al., 1991).

5. Conclusions

Few referred papers on the bioactivity of crude seaweed extracts obtained by means of a SC−CO_2 technique against postharvest fungal diseases on fresh fruit have been found in literature which have not allowed adequate comparisons with the existing References

The *in vitro* antifungal activity of crude seaweed extracts and those of three purified extract fractions with hexane, ethanol and water, as well as the *in vivo* antifungal efficacy of the crude extracts, were both evaluated. Purity of the extract has been the main drive of this work for to exclude possible compounds which would have antimicrobial activity other than fatty acids, lipids, polysaccharides, polyphenols

and phlorotannins. The findings coming from the *in vivo* experiments indicate that crude seaweed extracts had a variable degree of antifungal activity in relation to the different fungi/host systems tested here. Extracts of *L. digitata* and *U. pinnatifida*, followed by *P. umbilicalis*, showed the highest antifungal activity against *B. cinerea* on strawberries and *M. laxa* on peaches, followed by *P. digitatum* on lemons. However, *L. digitata* extract should be selected as the best antifungal tool among those tested here due to its higher competitiveness with respect to fenhexamid and imazalil. The antifungal activity of the crude seaweed extracts could be mainly attributed to their content of fatty acids rather than to those of phenolic compounds including phlorotannins, but also an increased peroxidase activity probably elicited by the polysaccharides could be related to activation of ISR mechanisms able to suppress grey mould on strawberries by *B. cinerea* and brown rot on peaches by *M. laxa*.

Numerous factors really can affect the biological activity of antifungal compounds when they interact with fruit tissue. In the complex host/antimicrobial compound/pathogen system, many biochemical processes can occur with different effects on biological activity of the antimicrobial substances. It could be hypothesized that the molecules of the applied antimicrobial compounds can undergo some structural changes (degradation, hydrolysis and polymerization) causing modifications of their biological activity during infective process. The fatty acids and other substances present in the seaweed extracts may reasonably act as probable inducers of resistance through different mechanisms mediated by the host tissue (Ippolito et al., 2000; Lattanzio, 2003).

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