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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

Ugo De Corato[a,*], Rocco Salimbeni[b], Agostino De Pretis[b], Nicolla Avella[b], Giovanni Patruno[b]

[a] Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Department of Energetic Technologies, Division of Bioenergy, Biorefinery and Green Chemistry, Territorial Office of Bari – Uni. Versus Consortium, Viale Japigia 188, Bari 70126, Italy
[b] BIO-PLANTA Research Consortium, SS. 96, Bari 70100, Italy

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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

* Corresponding author.
E-mail address: ugo.decorato@enea.it (U. De Corato).

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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.