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Evaluation of an alternative mean for controlling postharvest *Rhizopus* rot of strawberries

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The authors declare no competing interests.

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Abstract: Crude extract of the brown seaweed *Laminaria digitata* was tested as an alternative mean to control postharvest *Rhizopus* rot of strawberries. The antifungal activity of four extracts (one un-fractionated and three soluble by hexane, ethanol, and water) was *in vitro* measured against one pathogenic isolate of *Rhizopus stolonifer* at a concentration range from 10 g L^{-1} to 30 g L^{-1}. The antifungal activity of the un-fractionated extract was *in vivo* measured into a climatic room at the same concentration range in comparison to fungicide Fenhexamid in preventive and curative treatments. The peroxidase activity in *L. digitata*-treated inoculated strawberries was performed. A significant inhibition of mycelia growth applying 30 g L^{-1} of un-fractionated extract (until 80% after 5 days) and suppression of sporangia germination (until 95% after 24 hours) were found by a dose-dependent manner of the treatment. Only the extracts fractionated by hexane and ethanol were likewise suppressive at the same concentrations against mycelia (until 71% and 66% respectively) and sporangia (until 82% and 69% respectively) such involving a direct toxicity induced by lipids and phenolic compounds to *R. stolonifer* suppression. Fruit decay inhibition of the *R. stolonifer*/strawberries pathosystem increased from 10 g L^{-1} to 30 g L^{-1} until 75% after 4 days only in preventive treatment. An increased peroxidase activity (4.84 Δ_{OD420} g^{-1} min^{-1}) seen in fruit tissue after one-day from the application of 30 g $^{-1}$ raw extract suggests that *in vivo* suppression could also be related to induced systemic resistance phenomena.

1. Introduction

Strawberry fruits are a perishable commodity due to industrial processing chain that occurs immediately after harvesting which causes mechanical injury, desiccation, physiological disorders, deterioration of quality and nutrient composition, decaying, abiotic stress, mycotoxin contamination, and reduction of their market value (De Cicco *et al*., 2008). The known fungi that are considered responsible of strawberry fruit

postharvest decay are *Botrytis cinerea* Pers. *ex* Fr. and *Colletotrichum* spp., the causal agents of gray mold and anthracnose respectively; *Penicillium* spp., the agent of green and bleu mold; *Rhizopus* spp., *Mucor* spp., and *Alternaria alternata* f. sp. *fragariae*, the main agents of rots (Husaini and Neri, 2016). Among the most important postharvest decay agents that occur in strawberries, the soft rot caused by the zygomycete fungus *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. induces economically severe losses both in the field and overall during long-distance transport, especially if storage temperature was more than 4-5°C and when strawberries are damaged during handling (Maas, 1998). Control of postharvest decay on strawberry (*Fragaria* × *ananassa* Duch.) fruit can be usually achieved by physical, chemical and biological methods (Ippolito *et al*., 1997; Droby *et al.,* 2009). Requirements from consumers for fruit free by chemical residues from synthetic fungicides have recently stimulated the researchers (Mari *et al.*, 2016) to find safer alternatives for environmental and human health based on the wide range of natural antifungal compounds, as phenolic substances given from wild edible herbs (Gatto *et al*., 2011, 2013), and terpenic compounds provided from aromatic plants (De Corato *et al*., 2010).

In this context, seaweed extract can stimulate growth in strawberries by protecting them from pathogens and physiological hazards under storage condition (Tuhy *et al*., 2013). Crude extract from brown seaweed contains a wide range of antifungal substances, mainly lipids (triglycerides), phenolic compounds (phlorotannins) and water-soluble polysaccharides (laminarans, fucoidans, and alginates), which were investigated for their antifungal properties in preventing postharvest fruit losses caused by plant pathogenic fungi (Washington *et al*., 1999). Recently, the use of crude extracts from brown and red seaweeds obtained by a supercritical carbon dioxide technique in preventing the severe fruit postharvest losses caused by several plant pathogenic fungi, has been investigated (De Corato *et al*., 2017). In addition to that, an excellent paper provides an overview of the most recent findings on the potential use of extracts from macroalgae for strawberry management, concerning both their biostimulant effects and antifungal properties against postharvest pathogens (Righini *et al.*, 2018). From this review, it is clear that very few papers about the practical use of algal extract as an effective alternative strategy to syntetic fungicides, such as Fenhexamid, for controlling postharvest strawberries rot by *R. stolonifer* are reported in literature, because this fungus is really most invasive and it shows a faster development of aerial mycelia in comparison to other fungi afore-mentioned, especially if storage temperature varies from 6°C to 10°C.

The purpose of this work was to assess a potential use of crude extract from the brown seaweed *Laminaria digitata* (Huds.) Lamouroux for effectively controlling strawberry fruit rot caused by *R. stolonifer* under postharvest condition. The first objective was to investigate the potential suppressive, under *in vitro* and *in vivo* conditions, of crude extracts of *L. digitata* in preventing postharvest losses of strawberries caused by infections of *R. stolonifer* which are of greater importance in the Italian markets. The second objective was to clarify the most probable hypothesis about the mechanisms explaining the suppressive effect observed. To reach these two objectives, the direct antifungal activity of four extracts (one un-fractionated and three fractionated by hexane, ethanol, and water) was *in vitro*, measured by a microbiological method within an increasing concentration range from 10 g L^{-1} to 30 g L^{-1}. The antifungal activity of the raw extract was *in vivo*, measured at the same concentration range by a phytopathological method into a climatic room in comparison to Fenhexamid during preventive and curative treatments. Finally, the peroxidase (POD) activity in *L. digitata*-treated inoculated strawberries was assessed by an enzymatic method to investigate an indirect antifungal activity of the raw extract by induced systemic resistance mechanisms.

2. Materials and Methods

Experimental trials

An amount of about 80 Kg of fresh algal biomass, collected from healthy and matured *L. digitata* cultures performed into a photo-bioreactor of 900 l capacity, was purchased from a marine biorefinery located near the coastal areas of Gibraltar and Morocco. Algal biomass was immediately refrigerated after harvesting thoroughly washed with seawater, and washed with tap water to remove all extraneous particles and epiphyte organisms. Fresh biomass was dried into an industrial drier located at ENEA - Trisaia Research Centre (Policoro, Matera, Italy) - chopped, finely pulverized, heat-treated for 24 hours with sodium hydroxide (1:10 w/w) for

triglycerides saponification, weighed, and stored at 4°C until extraction of biologically active substances. The extraction was carried out by using a mixture of un-polar and polar solvents in appropriate proportions (hexane:ethanol:water, 1:2:2 v/v) into a Clevenger apparatus. Extraction was repeated three times at room temperature, and total processing time was about 4-5 hours (Selvi *et al*., 2014). Stocks of raw crude extract were collected, suspended in methanol, collected into bottles, and stored at 4°C in the dark until tested. Crude seaweed extract yield was calculated on three independent replicated samples of 50 g each after drying at 55±5°C for 2 hours, and expressed as a percentage on the basis of fresh biomass weight. Stocks of crude seaweed extract were dried, weighed, and stored at -20°C in the dark till further uses. Aliquots of raw extract were fractionated into three fractions using three solvents with a different affinity towards fatty acids, phenolic compounds and water-soluble polysaccharides (Khanzada *et al*., 2007). Samples of 50 g of dry extract were suspended in n-hexane, or distilled water, or pure ethanol (1:5 w/v) in separating funnel for 20 days at room temperature to separate lipids, polysaccharides and phenolic substances, respectively from the crude extract. Suspensions were then filtered using Whatman filter paper and concentrated under reduced pressure at 35°C using a rotary evaporator (Strike 202, Steroglass, Perugia, Italy) till the extract become as a syrup. Three different fractions were finally separated from this residue. Each fraction was individually collected from the respective funnel, and each stock was air-dried, weighed, and stored at -20°C in the dark till *in vitro* and *in vivo* assayed by using a certified pathogenic strain of *R. stolonifer* (provided by the Collection of Microorganisms and Cell Cultures Institute, DSMZ, Braunschweig, Germany) causing severe rots on strawberries under postharvest condition.

Stocks of total extract, or fraction of it, suspended in sterile 0.1 M K-phosphate buffer were *in vitro* tested at the concentration range of 10 g l^{-1}, 20 g l^{-1} and 30 g l^{-1} for determining a minimum bioactive concentration by poison food technique (Shahi *et al*., 1999). Mycelia inhibition was quantitatively assessed measuring radial growth in Petri plates (100 mm diameter) containing Potato Dextrose Agar (PDA, Sigma-Aldrich, Milan, Italy) adding 18 ml PDA per plate. In treated plates, aliquots of 2 ml of sterile stock suspension containing extract (both raw and fractionated) were added to PDA at 42±3°C before solidification. In untreated plates (control), 2 ml of sterile buffer was added to PDA in place of the extract. Three mycelia plugs measuring 5 mm diameter each were cut out by the margin of 3-day-old fungal cultures actively growing, and then aseptically placed on the upper PDA surface. Treated and control plates were incubated in the dark at 22±1°C for 5 days. Mycelia growth inhibition was measured at three time set points (after 1 d, 3 d and 5 d of incubation) with respect to the control plates by the index

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

where, Dco is the average of colony diameter (mm) in the control plates, and Dse is the average of colony diameter in the plates amended with seaweed extract at the three afore-mentioned concentrations. All experiments were carried out with three replications of 10 plates for each. Sporangia germination was evaluated on micro-cultures by a microassay on glass slides that allowed the quantitative analysis of sporangia suppression using an optical microscopy technique (Gatto *et al*., 2011; 2013). 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 sporangial suspension containing 10^8 CFU ml^{-1}, and 88 µl of extract (both raw 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 a control was filled with 10 µl PDB, 2 µl sporangial suspension, and 88 µl buffer. Each plate was incubated at 22±1°C for 24 hours. After incubation, aliquots of 5 µl sporangia cultures taken from each micro-well were sampled and mounted on the upper surface of glass slides sterilized with denatured ethanol. Number of the total, un-germinated, and collapsed sporangia were counted by a Burker's hemocytometer using a photomicroscope (40 × magnification) (BX60, Olympus, Milan, Italy). Sporangia germination suppression was measured at four time set points (after 2 h, 10 h, 18 h and 24 h of incubation) by the index

$$SG\% = Su/St \times 100$$

where, Su is the average of the sum of un-germinated + collapsed sporangia in un-supplemented cultures (control) or amended with the extracts at the three afore-mentioned concentrations, and St is the average of number of total sporangia in the same sample. All measurements were performed with three replications of 5 glass slides taken from each micro-well.

Preventive and curative treatments were *in vivo* evaluated on strawberries (cv. Camarosa) harvested from growers located in Basilicata (Policoro, Matera, Italy) under tunnel condition. 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 1 min in 2% sodium hypochlorite solution, rinsed with tap water and allowed to dry. Each fruit was 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 inoculated with a sporangial suspension of *R. stolonifer*. Trials treated with the stocks containing 10 g l^{-1}, 20 g l^{-1} and 30 g l^{-1} of raw 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 one commercial fungicide containing as active substances 50% Fenhexamid (1.2 g l^{-1}) were both included for preventive and curative treatments. Aliquots of 30 µl of the stock suspension of extract were dispensed over each wound of the treated fruit allowing the droplet to be absorbed into the fruit. Aliquots of 30 µl of sterile buffer alone, or fungicide, were dispensed over the wound of the control fruit. Each wound was inoculated with 10 µl of a sporangial suspension containing 10^6 CFU ml^{-1}. In preventive treatments, the pathogen was inoculated over the injured area 2 days later from the 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 8 hours later from inoculation of the pathogen allowing sporangia germination before treatment. Fruit were placed in trays, packaged in plastic bags and maintained into a climatic room for 4 days at the temperature of 20±2°C and relatively humidity (RH) of 96±2% in the dark. Trials were arranged in a completely randomized experimental design including six replicates per treatment, whenever thirty strawberry fruit with two wounds per fruit were considered per each replication. Disease incidence was assessed by counting the number of the infected wounds on each fruit. The disease incidence data were converted into the strawberries soft rot suppression data by the index

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

where, Nco is the average of number of infected wounds in the control plots treated with buffer alone, and Nse is the average of number of infected wounds in the plots treated with extract at the three afore-mentioned concentrations, or with the fungicide (Arras *et al*., 1999).

Trials were also arranged to assess the POD activity including thirty strawberry fruit treated with 30 g l^{-1} un-fractionated extract in preventive treatment, and sampled after one-day and five-days of incubation at temperature of 20±2°C and RH of 96±2% in the dark. Inoculated and healthy fruit (not injured) treated with only sterile 0.1 M K-phosphate buffer were both included as positive and negative control, respectively. Small pieces of tissue (diameter= 2-3 mm, deep= 3-4 mm) randomly collected from six points of each fruit were powdered with liquid nitrogen. Samples of one-gram of tissue were extracted with 2 ml of 0.1 M sodium-phosphate buffer (pH 7.0) at 4°C and used for assessing POD activity. All 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°C. Absorbance measured at a wavelength of 420 nm were recorded every 30 s for 3 minutes and the boiled enzyme preparation served as blank. POD activity was expressed as change in absorbance of the reaction mixture on the fresh weight basis (Δ_{OD420} g^{-1} min^{-1}).

Statistical analyses

The suppressive effects of each *L. digitata* crude extract to mycelia growth and sporangia germination of *R. stolonifer* (using both raw extract and soluble extracts by hexane, ethanol and water), and *versus* strawberries soft rot and peroxidase activity (using only raw extract), were assessed by using variance analysis (ANOVA). In particular, two-way ANOVA was applied to test the interactions either among three extract concentrations (10 g l^{-1}, 20 g l^{-1} and 30 g l^{-1}) and three incubation times (1 d, 3 d and 5 d) on mycelia growth inhibition index (MGI%), either between three extract concentrations (10 g l^{-1}, 20 g l^{-1} and 30 g l^{-1}) and two treatment types (preventive and curative) on strawberries soft rot suppression index (DI%). For performing two-way ANOVA we have used the average data of MGI% and DI% assessed at the different extract concentrations, incubation times, and treatment types at *P*-value ≤0.05. Value percentage of the two indexed parameters was before transformed into arcsine for satisfying the assumption of normality, and then compared by applying the

Duncan Multiple Test Range (DMRT) whenever two-way ANOVA revealed a significant difference among the means at a $P \leq 0.05$ level. However, these data has been shown as un-transformed values. One-way ANOVA was instead used to test difference among four extract concentrations, including control (0 g l^{-1}), at the same incubation time (2 h, 10 h, 18 h and 24 h) on sporangia germination suppression index (SG%). SG% values were before transformed into arcsine and then compared by DMRT whenever one-way ANOVA revealed a significant difference among the means at $P \leq 0.05$. One-way ANOVA was also used to test difference among the three experimental trials at the same sampling time to assess peroxidase (POD) activity at the first and fifth day, and DMRT was used to compare data whenever one-way ANOVA revealed a significant difference among the means at $P \leq 0.05$. Regards to better clarify the effect of seaweed extract at the three considered concentrations including control on the un-germinated and collapsed sporangia vs. incubation time, the SG rates were submitted to regression analysis in order to obtain curves that were compared for slope and elevation for each set points. All statistics were managed by the 12.0 SPSS programme (Statistics Base™, Chicago, Illinois, USA).

3. Results

The seaweed extract yield obtained at the end of the extraction process was about 1.22±0.1 g on 100 g of fresh biomass. *R. stolonifer* mycelia growth was affected by extract concentration and incubation time showing a significant interaction between these two factors after supplementation to PDA with *L. digitata* crude extract (Table 1). The mycelia growth inhibition percentage by increasing doses of raw extract resulted of 10%, 31% and 48% after one-day of incubation; it increased until 22%, 45% and 58% after three-days; finally it reached 40%, 61% and 80% at the end of incubation time (after five-days) applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} extract at each sampling time (Fig. 1A). The mycelia growth inhibition percentage after supplementation to PDA with increasing doses of extract purified with hexane resulted of 4%, 15% and 22% after one-day of incubation; it increased until 16%, 31% and 53% after three-days, reaching 20%, 45% and 71% at the end of incubation time applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} extract at each sampling time (Fig. 1B). The mycelia growth inhibition percentage after amendment with increasing doses of extract soluble in ethanol resulted of 10%, 14% and 16% after one-day of incubation; it increased until 17%, 30% and 43% after three-days, it reached 19%, 43% and 66% after five-days applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} extract at each sampling time (Fig. 1C). No inhibition effect after amendment with extract purified by water was seen (data not shown). The un-germinated and collapsed sporangia percentage of *R. stolonifer* after supplementation to PDB with increasing doses of *L. digitata* un-fractionated extract resulted of 16%, 24% and 36% after 2 h of incubation, while it increased until 50%, 77% and 95% at the end of incubation time (after 24 h) applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} extract at each sampling time (Fig. 2A). The un-germinated and collapsed sporangia percentage following to supplementation with increasing doses of extract soluble in hexane resulted of 9%, 15% and 25% after 2 h of incubation, while it reached 40%, 65% and 82% after 24 h of incubation applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} extract (Fig. 2B). The un-germinated and collapsed sporangia percentage after adding of increasing doses of extract soluble in ethanol resulted of 5%, 13% and 14% after 2 h of incubation, while it reached 42%, 55% and 69% at the end of incubation applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} extract (Fig. 2C). No reduction of sporangia germination was found with respect to control after adding of extract soluble in water (data not shown).

Strawberry fruit soft rot suppression was affected by extract concentration and treatment type with a significant interaction between these two factors after adding of *L. digitata* raw extract over strawber-

Table 1 - Synthetic values to different two-way ANOVA analysis to *Rhizopus stolonifer* mycelia growth inhibition and strawberries *Rhizopus* soft rot suppression at P-value ≤ 0.05

Effect	df [c]	*F*	*P*-value
On mycelia growth inhibition [a]:			
1) - Extract concentration	2	44	0.02
2) - Incubation time	2	26.2	0.03
- Extract concentration × incubation time	4	2.8	0.02
On strawberries soft rot suppression [b]:			
3) - Extract concentration	2	31.6	<0.01
4) - Treatment type	1	6.6	0.01
- Extract concentration × treatment type	2	9.4	<0.01

[a] Extract concentration (10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1}) and incubation time (1 d, 3 d and 5 d) are the two factors considered on mycelia growth inhibition. [b] Extract concentration (10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1}) and treatment type (preventive and curative) are the two factors considered on strawberry fruit rot suppression. [c] Degree of freedom.

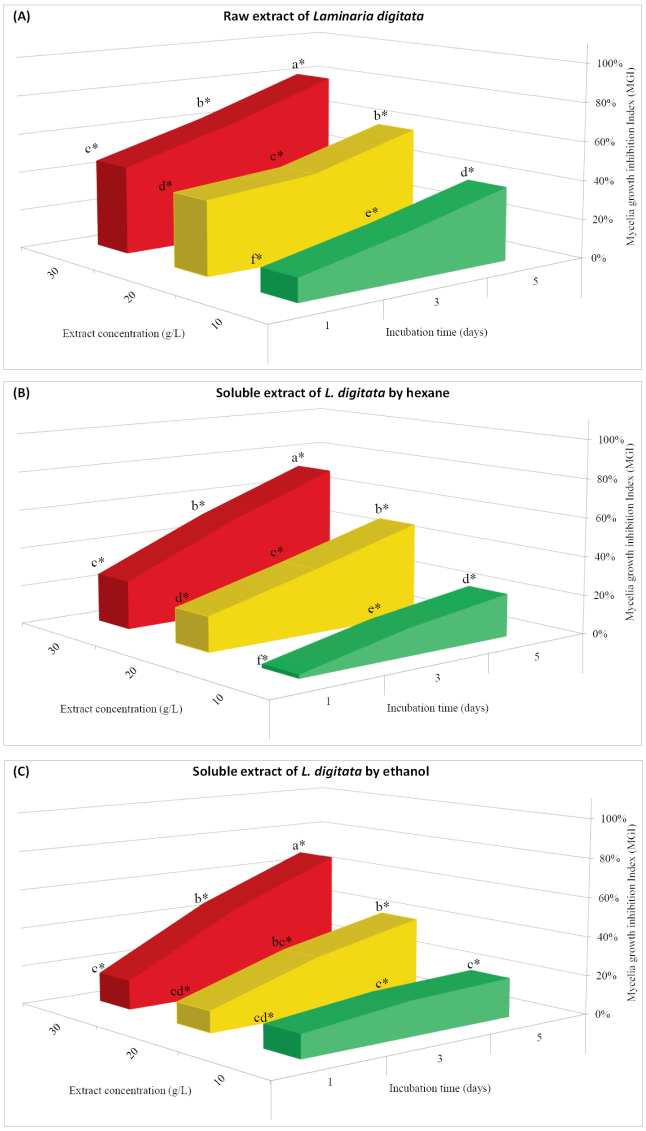

Fig. 1 - Suppressive effect of *Laminaria digitata* raw extract (A), and soluble extracts by hexane (B) and ethanol (C), on *Rhizopus stolonifer* mycelia growth inhibition index (MGI%). The suppressive activity of extracts applied at three different concentrations was tested after incubation at 22±1°C for 5 days into Petri plates on PDA medium. Each value, ranging from 0% (no inhibition) to 100% (total inhibition), represents the pooled mean of three replicates with 10 plates for each. MGI% values were collected after 1 d, 3 d and 5 d and analysed by two-way ANOVA. Asterisk indicates P-value ≤0.05 according to table 1. Histogram points with different letters are significantly different according to Duncan Multiple Test Range (DMRT) at a probability P≤0.05 level.

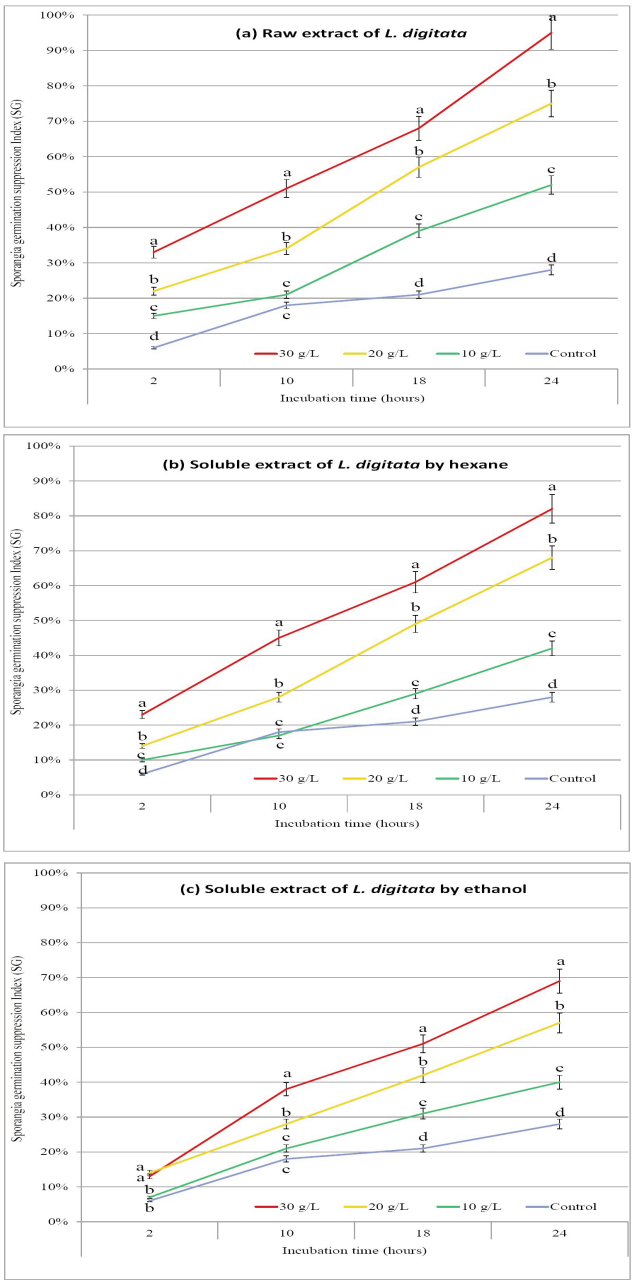

Fig. 2 - Suppressive effect of *Laminaria digitata* raw extract (A), and soluble extracts by hexane (B) and ethanol (C), on *Rhizopus stolonifer* sporangia germination suppression index (SG%). The suppressive activity of extracts applied at four different concentrations (including control) was tested after incubation at 22±1°C for 24 hours into micro-well plates filled with PDB medium. Each value, ranging from 0% (no suppression) to 100% (total suppression), is the pooled mean±SD (bars) of three replicates of 5 glass slides taken from each micro-well. SG% values were collected after 2 h, 10 h, 18 h and 24 h and submitted to regression analysis. For each incubation time, SG% values analysed by one-way ANOVA with different letters are significantly different according to DMRT (P≤0.05).

ries wound (Table 1). The fruit decay inhibition percentage of the *R. stolonifer*/strawberries system after preventive treatment with *L. digitata* was of 22%, 49% and 75%; but it remained of 2%, 11% and 21% in curative treatment applying respectively 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} raw extract (Figs. 3, 4). The chemical treatment with Fenhexamid suppressed the strawberries rot until 100% in those preventive and 70% in those curative (Figs. 3, 4).

Finally, as regards to measurement of peroxidase activity in strawberries (Table 2), a significant increment of absorbance in plots treated with *L. digitata* raw extract was seen yet after one-day of incubation (6.84 Δ_{OD420} g^{-1} min^{-1}) if compared to those of the untreated-inoculated strawberries (5.01 Δ_{OD420} g^{-1} min^{-1}). Moreover, the untreated-healthy fruit shows a significant lower POD (1.12 Δ_{OD420} g^{-1} min^{-1}) with respect to untreated-inoculated strawberry fruit at the same sampling time.

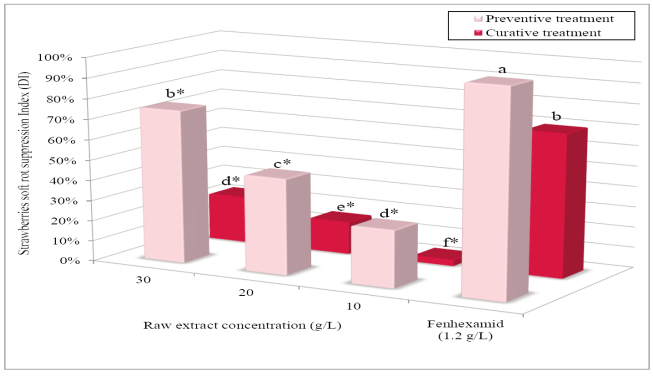

Fig. 3 - Strawberries *Rhizopus* soft rot suppression index (DI%) after 4 days of incubation at 20±2°C and 96±2% RH in the dark following to preventive and curative applications of 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} *Laminaria digitata* raw extract, with respect to trial treated with Fenhexamid. Values ranging from 0% (no suppression) to 100% (total suppression) are the pooled mean of six replicates per treatment, each one carried out with thirty strawberries with two wounds per fruit. DI% values were analysed by two-way ANOVA. Asterisk indicates P-value ≤0.05 according to table 1. Histograms with different letters are significantly different according to DMRT (P≤0.05).

4. Discussion and Conclusions

In vitro experiments performed with the *L. digitata* hexane-soluble extract fraction suggests that inhibition on mycelia growth and sporangial germination of *R. stolonifer* could be due to direct toxicity of the fatty acids found in extracts of *L. digitata* purified with chloroform (Løvstad Holdt and Kraan, 2011). By supporting this hypothesis, in ours finding the hexane-soluble extract fraction exerted a similar antifungal effect in comparison to those seen with the total extract. A very similar effect was also found testing the ethanol-soluble extract, suggesting that suppression could be due to direct toxicity exerted by the phenolic substances found in ethanolic extracts of *L. digitata* (Løvstad Holdt and Kraan, 2011). In fact, the ethanol-soluble extract fraction incited a similar antifungal effect in comparison to those seen either with the hexane-soluble fraction either with the un-fractionated extract. Instead, no suppressive effect on mycelia and sporangia was seen applying the water-soluble extract fraction, suggesting that the water-soluble polysaccharides (laminarans, fucoidans and

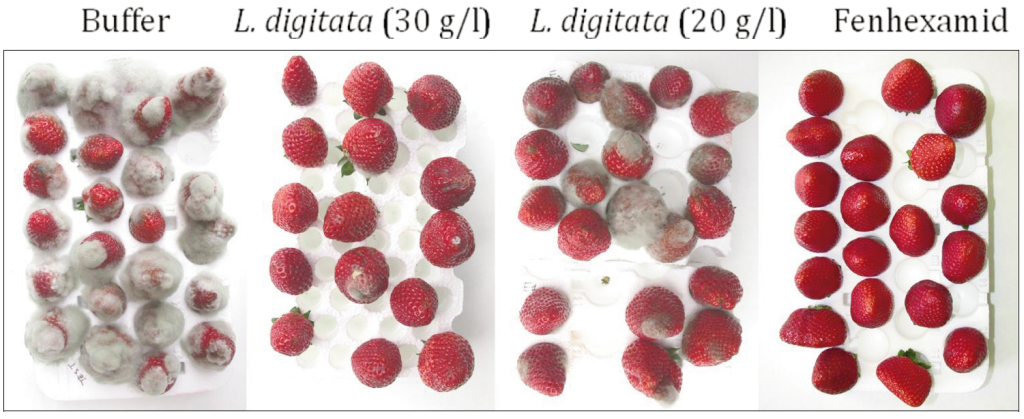

Fig. 4 - *Rhizopus* soft rot symptom on strawberries cv. Camarosa after 4 days of incubation at 20±2°C and 96±2% RH in the dark after a preventive application of 20 g L^{-1} and 30 g L^{-1} *Laminaria digitata* raw extract with respect to the control trial treated with buffer alone and Fenhexamid (1.2 g L^{-1}).

Table 2 - Peroxidase (POD) activity in strawberries inoculated with *R. stolonifer* and preventively treated with 30 g L^{-1} *Laminaria digitata* un-fractionated extract. POD assessment was carried out after one-day and five-days of incubation at 20±2°C and 98% RH in the dark

Experimental trial	One-day	Five-days
L. digitata-treated inoculated strawberries	6.84±0.3 a	53.15± 1.5 a
Untreated-inoculated strawberries	5.01±0.2 b	20.38±0.9 b
Untreated-healthy strawberries (not injured)	1.12±0.06 c	5.27±0.2 c

Values are the pooled mean±SD of three replicates of 10 fruit for each analyzed by one-way ANOVA. In each column, values followed by different letters are significantly different according to Duncan Multiple Test Range (DMRT) at a probability P≤0.05 level. Change in absorbance on the fresh weight basis (Δ_{OD420} g^{-1} min^{-1}).

alginates) present in aqueous extracts of *L. digitata* (Løvstad Holdt and Kraan, 2011) could not be involved in mycelia and sporangia suppression under *in vitro* condition. Moreover, mycelia inhibition was closely related to extracts concentration added into growing media, showing an increasing of antifungal activity as the dose of extract increased. A dose-dependent manner of the treatments with *L. digitata* extracts was therefore found at least in the concentration range considered here. Finally, our findings showed that mycelia inhibition increased from the first to the last day of incubation in a time-dependent manner, showing that the extracts (both un-fractionated and fractionated by hexane and ethanol) were really efficacy in suppressing mycelia growth during all incubation time. *In vivo* experiments performed with crude extract employed at 30 g l^{-1} dose showed a stronger efficacy of *L. digitata* in suppressing decay on infected strawberries by *R. stolonifer* in preventive treatments with respect to those curative. Findings coming from *in vivo* experiments highlighted an interesting and very competitive antifungal efficacy of *L. digitata* raw extracts against *R. stolonifer* on strawberries when compared to action of Fenhexamid in preventive treatments. Our findings show that strawberries soft rot suppression was closely related either to extract dose or treatment type, since a remarkable increasing of suppressivity was seen as the concentration of extract applied over the wounds increased, as well as passing from the curative treatment into those preventive. Therefore, an evident dose-effect of the preventive treatment was observed at least in the concentration range here considered. Finally, a significant POD activity in *L. digitata*-treated inoculated strawberries was early found with respect to untreated-inoculated fruit. Moreover, healthy strawberries treated with only sterile buffer showed a significant POD decreasing with respect to untreated-inoculated fruit. Increments of POD activity early found after one-day of incubation after treatment with 30 g L^{-1} crude extract, and confirmed after five-days of incubation, could be related to activation of induced systemic resistance mechanisms into the *R. stolonifer*/strawberries system. In fact, the artificial inoculation with the pathogen without extract application induced a lower absorbance change with respect to *L. digitata*-treated inoculated strawberries, while not injured fruit showed a lower absorbance change in comparison to untreated-inoculated strawberries.

Our findings show that a direct antifungal activity exerted by crude extract of *L. digitata* could be attributed to its content of fatty acids, accordingly with De Corato *et al.* (2017), which have investigated on the antifungal properties of five crude seaweed extracts obtained by a supercritical carbon dioxide technique, including those derived from *L. digitata*, on three fruit/pathogen pathosystems in preventing postharvest losses caused by *B. cinerea*, *Monilinia laxa* (Aderh. & Ruhland) Honey and *Penicillium digitatum* (Pers.) Sacc. on strawberries, peaches and lemons, respectively. In our findings, phenolic compounds could be considered as good candidates able to suppress mycelia growth and sporangia germination of the pathogen together to fatty acids accordingly with the *in vitro* tests. This finding is nevertheless divergent if compared to the observations of De Corato *et al.* (2017), whenever ethanolic extracts of *L. digitata* were weakly suppressive against *B. cinerea* and *M. laxa* when applied at 30 g L^{-1} dose with respect to extracts purified by hexane. The discordance between these two experimental evidences probably is due to the two different extractive techniques used, being employed a supercritical carbon dioxide technique to prevalently extract lipids rather than phenolic substances and water-soluble polysaccharides, while extraction by mixtures of polar and un-polar solvents in appropriate rates are generally less selective and more suitable to extract lipids, phenolic substances, and polysaccharides without preferences among them (Selvi *et al*., 2014). Several damaging mechanisms induced by exposure to vapour of essential oils derived from various aromatic plants, as well as after longer treatments with fatty acids and various phenolic substances 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 (Rasooli *et al*., 2006; Soylu *et al*., 2006; Laird and Phillips, 2011; da Cruz *et al*., 2013; Shao *et al*., 2013). In our findings, *in vitro* inhibition were not found by using aqueous extract revealing that the water-soluble polysaccharides fraction does not exert direct toxicity against mycelia development and sporangia germination. Therefore, is reasonable affirm that laminarans, fucoidans and alginates present in aqueous extracts of *L. digitata* (Løvstad Holdt and Kraan, 2011) could be involved in POD increasing by working most as resistance inducers (or elicitors) rather than as toxic chemicals. Peroxidases usually employ hydrogen peroxide as a substrate causing defence reactions which earlier occur in the fruit tissue after infection. Hydrogen peroxide has an antimicrobial properties due to its

strong oxidizing power and its capacity to generate other oxidizing species (hydroxyl radicals, singlet oxygen species and hydrogen peroxides), on the whole well known as ROS ('reactive oxygen species'), 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 related ROS (Imlay and Linn, 1988; Tatsuzawa *et al*., 1998). The laminarin, a storage polysaccharide (β-1,3-glucan) isolated for the first time from cell walls of *L. digitata*, elicits host defence responses in grapevine against *B. cinerea* (Aziz *et al*., 2003), and the use of various chemical resistance inducers, including laminarin, for controlling postharvest gray mold and *Rhizopus* rot in strawberry fruit, was studied in the past years (De Miccolis *et al*., 2009; Santini *et al*., 2009).

We can conclude that the antifungal activity showed by crude extract from *L. digitata* could be mainly attributed to content of fatty acids and phenolic compounds extracted from this profitable algal biomass source by appropriate mixtures of polar and un-polar solvents; but, also an increased peroxidase activity probably elicited by the water-soluble polysaccharides content, as laminarin, could be related to activation of an induced systemic resistance mechanism able to suppress postharvest *Rhizopus* soft rot of strawberries under *in vivo* condition.

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