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SEAWINES: Use of macroalgae as biostimulants against fungal diseases in grapevines

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Abstract. The outlook for climate change foresees major impacts on vineyards worldwide, shifting pathogens distribution and dynamics demanding more intense plant protection measures in certain regions, increasing viticulture's dependence on phytochemicals and pesticides. However, the European Commission is applying restrictions on their use, encouraging the development of more sustainable strategies efficient for disease control. Seaweeds represent an ecological alternative for a more sustainable production. Previous studies have shown that algae extracts contain compounds capable of reducing the abundance of plant fungal pathogens. Despite it, little is known about the molecular mechanism underlying this response.

SEAWINES project is evaluating the efficacy of the foliar application of *Ulva ohnoi* and *Rugulopteryx okamurae* extracts to control powdery and downy mildew, in addition to testing their effect on grape and wine quality. To our knowledge, this is the first study evaluating *R. okamurae* biostimulant capacity and fungicidal effect in viticulture. This macroalgae is relevant since it is an invasive species in our coasts, causing incalculable economic and environmental burdens. We aim to 1- Reduce the usage of chemicals in grapevines; 2- Reduce fungal diseases in viticulture; 3- Valorize polysaccharides from seaweeds; 4- Increase the added-value to wines (ecological and quality); and 5- Provide an alternative use to seaweed biomass, contributing to bio-circular economy and reducing its accumulation in our coasts.

1 Introduction

Current agriculture has a growing need to protect its crops in order to maintain its already low margins in certain productions, while preserving the quality of its products. Today, global agriculture is facing a triple challenge: (i) producing more, (ii) developing new crops, and above all, (iii) producing differently, to meet the expectations of an increasingly health and environmental risks conscious public. Reducing the dependence of the agricultural sector on chemical inputs is undoubtedly one of the most important challenges producers are facing today. This is especially important in the case of perennial high-value crops, such as grapevine, that undergo intensive antifungal spray programmes with no crop rotation. Moreover, under climate conditions, an increase on the risk of pests and diseases is expected, increasing further the need for phytosanitary treatments. The persistence of chemical pesticides in top-soils and leaching into groundwater besides their undesired effects on non-target organisms are of major environmental concern. Natural products are considered to be less harmful to the environment due to their higher biodegradability and influential biocidal activities. These

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natural compounds provide novel structures and mechanisms of action for the discovery of safer pesticides, as well as helping in development of organic agricultural products integrated with pest management.

Seaweeds (also called macroalgae) are often present in the estuary and coastal areas. Compounds extracted from seaweed (particularly polysaccharides, but also terpenes, phytohormones, aminoacids…) are believed to have promising prospects in agriculture [1] as they can promote the uptake of micro-macronutrients and are able to stimulate natural defences of plants. Plant biostimulants are defined by the European Commission as "products stimulating plant nutrition processes independently of the product's nutrient content, with the aim of improving the crop quality traits among other characteristics of the plant" (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32019R1009&from=EN). While studies are mounting on the seaweed effectiveness against fungal diseases, and promising results have been published for several crops, however, few studies address seaweed impact with an interdisciplinary approach. Studies usually focus on 1) the antifungal activity, mostly *in vitro*, or 2) the defence mechanisms activated in leaves, or 3) the impact on grape composition after several treatments in the field [2-3]. However, a comprehensive study addressing seaweed effects on vine health and wine quality through a holistic approach that integrates multiple scientific disciplines along the whole chain, from greenhouse to field to winery, is lacking.

In SEAWINES project we are studying the efficacy of *Ulva ohnoi* and *Rugulopteryx okamurae* seaweed extracts to reduce powdery and downy mildew diseases incidence/severity by enhancing grapevine resistance. The marine green algae *Ulva* sp. (Ulvales, Chlorophyta), also known as sea lettuce, is naturally present in most coastal ecosystems on the planet. These are fast-growth species that greedily capture CO_2. One of the main bioproducts of interest from *Ulva* is the sulphated polysaccharide known as ulvan. Ulvan has previously shown anti grapevine powdery mildew activity (by 90% reduction of symptom after foliar treatments) [4] and to confer protection against *Botrytis cinerea* [5]. The brown macroalgae *Rugulopteryx okamurae* (Dictyotales, Ochrophyta), is an exotic seaweed that has been recorded since 2002 in the north western zone, showing an aggressive invasion process in the Mediterranean and Atlantic. The ecological and landscape impacts provoked by this alga is unprecedented in European waters [6]. Few is known about this invasive seaweed but it has recently captured social and media interest due its environmental nuisance and socio-economic impact.

Within SEAWINES project we are developing and chemically characterizing *U. ohnoi* and *R. okamurae* seaweed extracts. We are conducting greenhouse experiments to select most efficient extracts against *Plasmopara viticola* and *Erysiphe necator*, and extrapolating to field conditions to determine extracts impact on grapevine resistance and on grape/wine quality. We are using a multidisciplinary approach by evaluating vines immune response, the physiological changes, the modifications in grapes physico-chemical composition, the shifts on microbial communities' diversity and composition, and finally, determining extracts impact on wine oenology.

2 SEAWINES framework

2.1 Development and chemical characterization of extracts

Four extracts were developed in the study: RU1 and RU2 from *Rugulopteryx okamurae* and UL1 and UL2 from *Ulva ohnoi*.

R. okamurae is collected in Algeciras (Cadiz, Spain, 5°25′34.75″W, 36°4′37.56″N) recurrently. After harvesting, the seaweed biomass is rinsed with tap water, freeze-dried and milled to a fine powder. RU1 extraction protocol was based on a serial extraction, with an initial extraction in hot water (70 °C, 2 hours shaking) and a water:ethanol (20:80) extraction after. RU2 just followed the first water extraction step.

U. ohnoi is provided by "La Huerta Marina" (Huelva, Spain, 7° 09′41.8″W, 37°15′20.9″N). The crude extract of this algae (UL1) was directly generated by the company, formulated without the addition of conserving agents to prevent any possible interference. UL2 was developed through hot aqueous extraction, same as RU2.

The composition of each algae extract was characterized for ash content, CNHS content, proteins, lipids, total carbohydrates, uronic acid sulfates, macroelements (Ca, K, Mg, P, Na), microelements (Fe, Mn, Cr, Mo, Cu, Zn, and Se) and heavy metals (Cd, Hg, Pb, and As) (Shelton, CT, USA). The variability of these compounds over seasons is also being addressed.

The crude extract UL1 showed a higher concentration of uronic acid and sulfates than UL2 (Table 1), which suggests a higher antimicrobial activity [7]. Opposite to *Ulva*, *Rugulopteryx* composition has not been yet described. The content of uronic and sulfates was considerably lower in *Rugulopteryx* extracts than in *Ulva* ones. Within *Rugulopteryx* extracts, both compounds were higher in RU1 than RU2, while fucose content was similar in both RU extracts. RU2 exhibited a particularly high C/N ratio (due to its low nitrogen content) and a low protein content.

Table 1. Biochemical composition of *Ulva ohnoi* and *Rugulopteryx okamurae* extracts.

	Ash (%)	Carbohydrates (%)	Proteins (%)	Lipids (%)	Sulfate (%)	Uronic (%)	Fucose (%)	C (%)	H (%)	N (%)	S (%)	C/N (%)
UL1	26.16 (0.74)	23.79 (0.04)	24.09 (1.38)	5.81 (0.41)	55.18 (0.14)	19.78 (1.52)	7.75 (0.50)	31.58	5.97	4.38	0.81	7.21
UL2	49.03 (0.84)	13.87 (0.01)	5.01 (1.49)	5.43 (0.33)	43.49 (0.03)	12.30 (1.09)	7.74 (0.28)	15.28	4.52	0.91	4.62	16.79
RU1	26.36 (1.43)	14.40 (0.04)	10.23 (1.55)	4.21 (0.26)	59.07 (0.11)	7.66 (2.85)	1.07 (0.20)	37.50	6.12	1.86	0.15	20.16
RU2	32.00 (0.64)	12.79 (0.02)	2.31 (0.21)	8.29 (0.79)	23.76 (0.07)	4.39 (1.00)	1.01 (0.08)	27.98	5.25	0.42	0.04	66.62

Results are referenced to extract dry weight and expressed in % as the means of samples analyzed in triplicate (n=3) but for CHNS. Standard deviation between brackets.

Currently we are deepening our understanding regarding the hormone content, lipids (fucoxantine, fucosterol, glucolipids, terpenes and lipids from betaine) and carbohydrates (laminarin, fucoidan, alginates) of the extracts under development.

2.2 Greenhouse experiments to evaluate the biostimulant capacity of the extracts

Tempranillo (*Vitis vinifera* L.) grapevine plants grown under greenhouse were treated either with one or two applications of *Ulva* and *Rugulopteryx* extracts (UL1, UL2, RU1 and RU2). The response of the plants was studied by collecting leaves at different time points (24, 48 and 144 hours after treatments, Fig. 1, [8]).

N Plants	FOLIAR TREATMENT DAY 0	TTO_1 Analysis 24h	TTO_1 Analysis 48h	FOLIAR TREATMENT DAY 5	TTO_2 Analysis 24h	TTO_2 Analysis 48h	TTO_2 Analysis 144h
X5	Water	○●□ N=5	○●				
X5	UL1	○●□ N=5	○●				
X5	UL2	○●□ N=5	○●				
X5	RU1	○●□ N=5	○●				
X5	RU2	○●□ N=5	○●				
X10	Water			Water	○● N=5	○● N=5	▽▲◎✗ N=5
X10	UL1			UL1	○● N=5	○● N=5	▽▲◎✗ N=5
X10	UL2			UL2	○● N=5	○● N=5	▽▲◎✗ N=5
X10	RU1			RU1	○● N=5	○● N=5	▽▲◎✗ N=5
X10	RU2			RU2	○● N=5	○● N=5	▽▲◎✗ N=5

Water: plants treated with water (Control), UL1: plants treated with UL1, UL2: plants treated with UL2, RU1: plants treated with RU1, RU2: plants treated with RU2. Symbols: ○, Defense genes; ●, Polyphenols; □, Hormones; ▽, photosynthetic pigments; ▲, Antioxidant enzymes; ◎, Plant physiology; ✗, Fungal community.

Figure 1. Experimental design for testing the extracts biostimulant capacity on Tempranillo greenhouse plants.

The biostimulant capacity was tested at multiple levels: 1) the expression changes of fifteen defense and stress-related genes were quantitatively determined by real-time quantitative PCR assay (RT-qPCR). 2) Polyphenolic compounds were determined by HPLC-DAD. 3) Endogenous plant hormones were measured: cytokinins, indole acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) by UHPLC/MS. 4) Vine development was monitored (number of leaves per plant, stem height etc.) and the concentration of chlorophylls and carotenoids was measured by spectrophotometry. 5) The activity of enzymes within the oxidative metabolism including the superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR) were measured by spectrophotometry. 6) The foliar fungal community diversity and structure was studied by Illumina amplicon sequencing.

UL1 and RU2 extracts stood out for their capacity to induce defence genes (PR10, PAL, STS48 and GST1 (Fig. 2). In particular, the upregulation of genes in RU2 treated samples was in general more evident after 24 hours, suggesting that this algal extract is triggering a fast response [8].

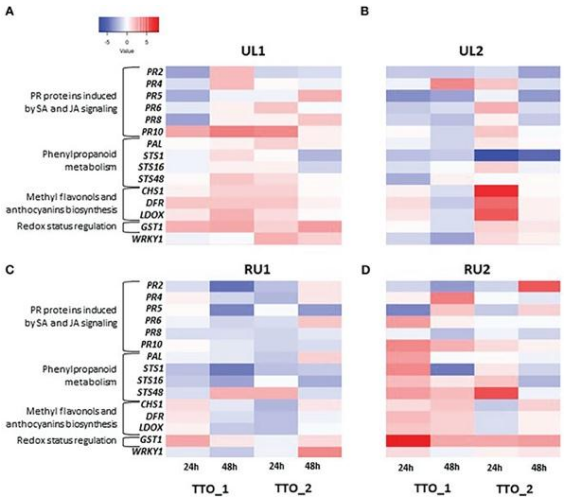


Figure 2. Transcript levels of defense-related genes in leaves induced by algae extract. A tree color scale is used to show fold induction of each gene (log transformed). The fold induction values are normalized to the reference genes PDC, GAPDH and COX and to water-treated leaves as the control samples.

The most induced gene was the one coding for Ribonuclease-like PR10 protein, mainly in UL1 and RU2, which has been shown to be induced by pathogen attack in a wide variety of plant species [8], suggesting that grapevines could recognize the algae extract compounds as an elicitor of plant defence [9].

The increased expression level of these defence genes agreed with an increase in jasmonic acid and decrease in salicylic acid, RU2 and UL1 being the extracts that showed a higher hormonal response (Fig. 3). Importantly these extracts were also the ones with highest capacity for phytoalexin production, inducing stilbene biosynthesis (data not shown) [8].

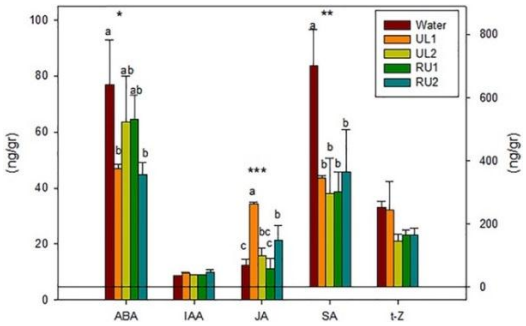


Figure 3. Leaves phytohormones content 24 hours after first treatment (TTO_1, 24h) (ng/g DW).

An induction of the activity of the antioxidant enzymes was also observed with an increase in SOD and CAT in the RU2-treated leaves in particular (data not shown). Previous studies demonstrated an increase of SOD in grapevine leaves after fungal infection [11], and

therefore our results point again to the eliciting potential of RU2 seaweed extract to enhance protection due possibly to the structural similarity with pathogen-derived molecules. Interestingly, while foliar fungal diversity was not influenced by the treatments, alga extract amendment modified fungal community composition, RU2 application resulting in the enrichment of various groups known for their biocontrol activity (Table 2). Sporobolomyces, known for their antifungal activity [11], were particularly abundant in the RU2 samples compared with the water-treated leaves. It was also noteworthy that *Debaryomyces hansenii* was significantly enriched in UL2 (0.68% relative abundance), and particularly in RU2 leaves (2%), while it was almost absent in the remaining treatments [8]. Several strains within this genus exhibit antagonistic activity against fungal phytopathogens through diverse mechanisms, such as competition for nutrients and space, mycoparasitism, the secretion of antifungal substances (e.g. volatile organic compounds, glucanases, and killer toxins) or the induction of plants' immune response to pathogens [12].

Table 2. Mean relative abundance (%) and standard deviation of beneficial genera known to have antifungal or antagonistic activity.

	Trichoderma	Aspergillus	Penicillium	Fusarium	Aureobasidium	Candida	Rhodotorula	Debaryomyces	Sporobolomyces	Saccharomyces
Water	0.04 (0.06)	0.12 (0.04)	0.56 (0.46)	0.14 (0.24)	0.84 (0.96)	1.11 (1.93)	0.09 (0.16) a	0.00 (0.00) ab	0.62 (1.07) a	2.26 (1.97) a
UL1	0.00 (0.00)	0.11 (0.11)	0.19 (0.07)	0.00 (0.00)	0.24 (0.41)	0.52 (1.02)	8.09 (1.07) b	0.00 (0.00) a	0.62 (0.47) a	0.35 (0.45) a
UL2	0.02 (0.05)	0.12 (0.13)	0.70 (0.33)	0.00 (0.00)	0.18 (0.34)	1.72 (1.81)	0.97 (0.82) a	0.68 (0.69) ab	0.82 (0.78) a	18.71 (4.72) b
RU1	0.00 (0.00)	0.13 (0.15)	0.52 (0.39)	0.06 (0.08)	0.40 (0.30)	0.60 (0.58)	0.60 (0.43) a	0.00 (0.00) a	1.03 (0.59) a	6.21 (5.51) ac
RU2	0.03 (0.05)	0.05 (0.04)	0.37 (0.14)	0.00 (0.00)	0.75 (0.87)	0.87 (0.96)	0.26 (0.30) a	2.32 (2.28) b	2.80 (1.29) b	13.03 (6.61) bc

3 Greenhouse experiments to evaluate the fungicide effect against downy mildew

3.1 *In vitro*

The effectiveness of UL1, UL2, RU1 and RU2 against *Plasmopara viticola* was evaluated in detached leaves. Grapevine plants of the Tempranillo variety were grown in greenhouse at the University of Bordeaux. Plants were foliar treated with the algae extracts at different concentrations ranging from 1 to 8 g/L. Foliar disks were inoculated with *P. viticola* at 10.000 sporangia/mL and the percentage of sporulation was determined by visual scoring 6 days post-inoculation.

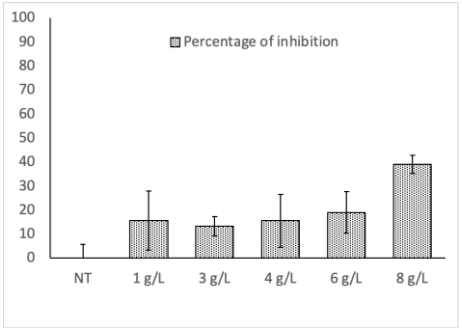


Figure 4. Percentage of *Plasmopara viticola* sporulation inhibition of RU2 extract. NT: water treated plants.

Promising results were found for RU2 extract, with the most concentrated dosage being able to reduce the oomycetes sporulation by 39.1+/-3.7% (Fig. 4).

3.2 *In planta*

Grapevine plants of *cv*. Tempranillo plants were grown in greenhouse (Neiker-Tecnalia, Arkaute). Plants were treated three times with water, copper or *R. okamurae* aqueous extract (RU2), and after, plants were infected with *P. viticola* by foliar application. Leaves samples were collected prior and after fungal inoculation to examine gene expression (using the NeoViGen96 chip), hormone and polyphenols induction, changes on the photosynthetic pigments, oxidative enzymes activity and soil microbiological characterization. In addition, disease incidence/severity was monitored for 2 weeks, visually and by microscopy (Fig. 5).

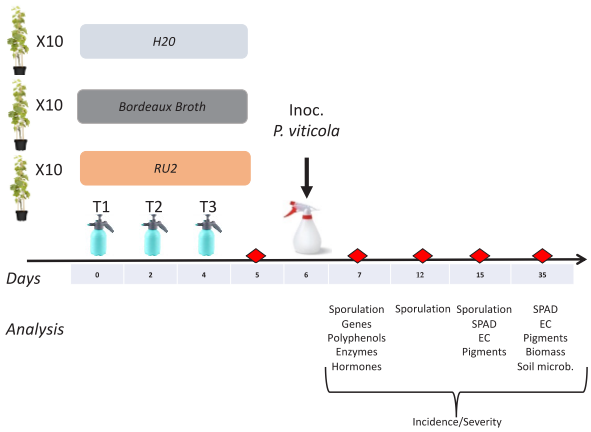


Figure 5. Experimental greenhouse design to test for RU2 anti-oomycete capacity. EC, electrolytic conductivity.

The processing of these samples is ongoing, but the data obtained so far validated our previous results regarding the biostimulant capacity of RU2. A significant induction of stilbenes (*trans*-piceid, *trans*-resveratrol, ε-viniferin and ω-viniferin) was observed in leaves after receiving 3 applications of RU2 (prior to the pathogen inoculation). In addition, preliminary analysis of the monitoring both the disease incidence and severity after *P. viticola* inoculation suggested that while the algae extract had not a significant influence on the disease incidence, RU2 reduced the severity of the disease (Fig. 6).

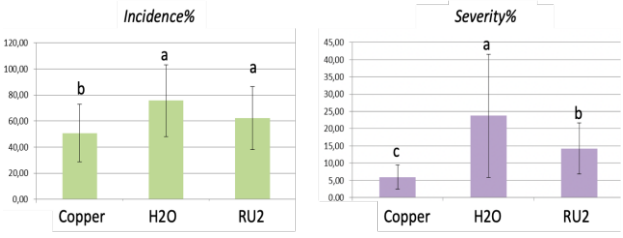


Figure 6. Downy mildew incidence and severity values (%) in treated samples. Values represent the mean and standard deviation of the measurements collected 1 day, 7 and 15 days post-pathogen inoculation (day 7, 15 and 35 in Fig. 5).

We are currently applying a similar greenhouse experimental approach (by developing *in vitro* and in planta antimicrobial assays tests) to examine the effectiveness of *Ulva* and *Rugulopteryx* extracts as potential control agents for *Erysiphe necator*.

Extrapolating greenhouse data to field conditions: 4. Impact on grapevine resistance, and, 5. Wine quality

Currently we are validating the greenhouse results in field trials in two locations: Jerez de la Frontera, where powdery mildew is endemic, and Logroño (Qualified Designation of Origin, D.O. Rioja), where downy mildew is very frequent.

In line of the objectives of the European Commission that is aiming to reduce the use and risk of pesticides by 50% by 2030, in SEAWINES a conventional treatment based on sulphur and copper applications (T_{conv}) will be compared to treatments where sulphur and copper amendments will be reduced to half the dosage (Figure 7). For instance, in T_{eco} treatment we will alternate sulphur/copper with natural products commonly used in organic agriculture (e.g orange oil, formulates based on *Equisetum arvense*, etc.), in T_{RU2} and T_{UL1} treatments we will alternate them with the algae extracts RU2 and UL1, respectively, and with water in T_{H2O}.

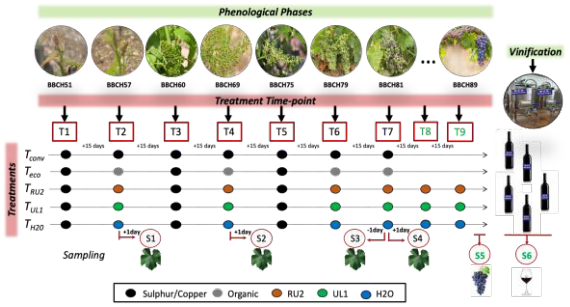


Figure 7. Experimental field design. Tempranillo plants defence response will be evaluated (in S1 and S2 samplings), while plants nutritional status (in S3 and S4). The antimicrobial efficiency of treatments will be followed *in vivo* through all the experiment (S1-S5) by measuring disease incidence/severity, as well *in vitro* (in S3 and S4). Moreover, the shifts on the native microbiota will be studied (in S3, S4 and S5). Grapevine yield and grape quality will be evaluated (in S5), and must and wine composition will be assessed (in S6).

Aside from evaluating the biostimulating and fungicide effects of the algae extracts, RU2 and UL1 will be foliar applied from véraison to harvest to evaluate their impact on productivity and grape composition and quality (by studying sugar content, pH, organic acids, amino acids, polysaccharides, phenolic and aroma compounds) and microbial changes. In addition, the must collected from grapes receiving each of the treatments will be further processed in the winery and extracts effect on must and wine composition and quality will be evaluated, including sugars/alcohol, total acidity and pH, organic acids, metals, anthocyanins and tannins, among others.

6 Conclusion

Greenhouse trials evidenced the capacity of UL1, and particularly RU2, for grapevine biostimulation. In addition, while the extracts antifungal activity against downy and powdery mildew is still under evaluation, preliminary results suggest RU2 extract being a good candidate. While further studies are needed to unravel the bioactive compound(s) involved, and other aspects such as extracts effect on grape and wine quality are to be still determined, the current findings are the first steps towards the inclusion of *Rugulopteryx okamurae* in a circular scheme that would reduce its accumulation on the coast, and therefore, also reducing its high environmental burden.

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