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An *Ulva armoricana* extract protects plants against three powdery mildew pathogens

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Abstract The protective activity of a crude extract prepared from the green macroalga, *Ulva armoricana*, previously shown to induce plant defence responses, was evaluated on three plant species, common bean, grapevine and cucumber, cultivated in the greenhouse and inoculated with three powdery mildew pathogens *Erysiphe polygoni*, *E. necator* and *Sphareotheca fuliginea* respectively. Chemical analyses showed that the extract was enriched in ulvans, which are green algae polysaccharides essentially composed of uronic acid and sulphated rhamnose. Weekly applications were performed by spraying of the green algal extract at various dilutions on bean, grapevine and cucumber leaves. A significant effect (50% protection) was observed using a dilution corresponding to about 3 gl^{-1} dry matter and up to 90% reduction of symptom severity was obtained for the highest concentration (1/9 dilution, 6 gl^{-1} dry matter) for the three plant species. To study the natural variability of the protective activity, five extracts prepared from algae batches harvested at different year periods were evaluated. Although polysaccharide composition varied among batches, all extracts elicit a reporter gene regulated by a defence-gene promoter in a transgenic tobacco line, and protect cucumber plants against powdery mildew infection. Together, these data demonstrate that *U. armoricana* is a reproducible source of active compounds which can be used to efficiently protect crop plants against powdery mildew diseases.

Keywords Plant immunity · Elicitor · Powdery mildew · Polysaccharides · Algae

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Introduction

Powdery mildew is a widespread disease affecting field and greenhouse grown plants, essentially controlled by chemical pesticides. In view of the public health concerns about the extensive use of these

compounds, alternative strategies are urgently needed and are the subject of intensive studies. One of these alternative strategies aims at stimulating plant defence reactions by the use of natural products and promising results have been obtained in the control of powdery mildew (Fofana et al. 2002; Renard-Merlier et al. 2007; Yang et al. 2009; Wurms et al. 1999). In this context, marine algae could be an interesting sources of active molecules since numerous algal elicitors have been identified, most notably polysaccharides such as linear β 1,3 glucans (laminarin), β 1,3 sulphated fucans, carrageenans and ulvans (Klarzynski et al. 2000; Mercier et al. 2001; Aziz et al. 2003, Klarzynski et al. 2003; Cluzet et al. 2004; Jaulneau et al. 2010). The structure and composition of algal oligosaccharides were shown to be crucial for their activities on signalling pathways regulating defence mechanism. For example, sulphated oligosaccharides obtained from fucan and carrageenans, were shown to induce the salicylic acid signalling pathway but not the non-sulphated molecule laminarin (Klarzynski et al. 2003; Mercier et al. 2001).

We have shown recently that active compounds can be obtained from green algae belonging to the genus *Ulva* sp., also known as sea-lettuce. *Ulva* sp. is one of the most common and abundant green macroalgae throughout the world and is frequently involved in ecological problems along coastlines since they proliferate as green-tides due to eutrophication (Lahaye et al. 1997; Lahaye and Robic 2007; Morand and Briand 1996). A *Ulva* extract induced expression of typical defence genes in the model legume *Medicago truncatula* (Cluzet et al. 2004). The active molecules present in this extract were subsequently purified and identified as ulvans (Jaulneau et al. 2010). Ulvans are complex sulphated heteropolysaccharides mainly composed of rhamnose, xylose and uronic acids (Jaulneau et al. 2010; Lahaye and Robic 2007; Robic et al. 2009a, b). The major repeating disaccharide sequence in ulvans, the aldobiuronic acid, is composed of rhamnose 3-sulphate and uronic acid. Rhamnose 3-sulphate can be linked to xylose and/or to xylose 2-sulphate. Additionally, rhamnose 3-sulphate can be partially branched at C-2 by single side-chains of glucuronic acid (Lahaye et al. 1997, 1999). Purified ulvan induced defence gene expression *via* the jasmonic acid pathway (Jaulneau et al. 2010). However, molecular mechanisms involved in ulvan perception by plant cells are still unknown.

Plant protection upon treatments with *Ulva* extracts was mainly evaluated in laboratory conditions. It was found that a foliar treatment with a crude *Ulva* extract protected *M. truncatula* plants against *Colletotrichum trifolii,* a fungal pathogen causing alfalfa anthracnose (Cluzet et al. 2004). Similar results were obtained in the case of *Phaseolus vulgaris* against *C. lindemuthianum* (Paulert et al. 2009). Recently, treatment of wheat and barley with a partially purified ulvan preparation was found to be active against powdery mildew, particularly in barley (Paulert et al. 2010). These results indicated that *Ulva* preparations could be used in crop protection. The objective of the present work was to evaluate potential effects of an *Ulva* extract prepared through an industrial-compatible protocol against powdery mildew pathogens on three greenhouse grown crops, bean, grapevine and cucumber.

Materials and methods

Algae material and preparation of Ulva extracts

Green algae *Ulva armoricana* was harvested on the north Brittany coast (France) at Archipel de Brehat in June 2006 (0625001), July, September and October 2007 (0730001; 0738002, 0743003 respectively) and July 2008 (0830001). For each harvest, the water-washed algal material was incubated in water (100 g fresh algae/l) and the homogenate was heated for 2 h at 90°C. The resulting extracts were filtered and stored refrigerated.

Biochemical analyses of *U. armoricana* extracts

Total uronic acid content was determined after lyophilisation of the crude extract by using the procedure described by Blumenkrantz and Asboe-Hansen (1973) with glucuronic acid as a standard. Neutral sugars were released by hydrolysis in 2 M TFA (120°C, 1 h) and analysed through anion exchange chromatography (Dionex, CarboPac MA1) with suitable standards. Sulphur measurements were done using inductively coupled plasma atomic emission spectroscopy (ICP-AES; ULTIMA, HORIBA Jobin Yvon. Longjumeau, France). Infrared spectroscopy was carried out to identify chemical groups of compounds. Molecular weight of polysaccharides was determined by size exclusion chromatography on

Sepharose CL 6B column (25×2,6 cm) using 100 mM ammonium formiate as eluant. The column was calibrated with standard dextrans (10–1,500 kDa).

Plants

Pérola and Uirapuru cultivars of *P. vulgaris* were used in this study. The plants were cultivated on clay and organic matter in a mixed ratio of 3/1. Experiment began when the first trifoliated leaf was fully expanded. Grapevine plants were obtained in a growth chamber and used at five-leaf stage. Cabernet-Sauvignon and Merlot grapes cultivars were, respectively, used to perform tests on detached leaves or entire plants. Finally, cvs. Mercanter and Euphoria of *Cucumis sativus* at three expanded leaves and cultivated in compost were used.

Plant treatments

Plants were elicited by the liquid extract of green algae at various dilutions containing an adjuvant agent (Heliosol®, Action-Pin, France) at 0.1% (v/v). Control plants were treated with water containing the same concentration of adjuvant. Solutions were sprayed on the aerial part of plants until run-off. Green algal extracts were diluted at 1/18, 1/9, 1/2 in water and the number of elicitor treatments performed either once at 3 days or twice at 3 and 6 days before inoculation. Six replications (each with three plants) were done for each condition and plants sprayed with water served as control.

Pathogens, inoculation procedures and disease assessment

Phaseolus vulgaris plants were inoculated with a spore suspension of *E. polygoni* at 5×10^4 conidia ml^{-1}. Inoculated plants were placed in environmental conditions favouring fungal development and colonies on the leaf surface were counted. A monoconidial isolate of *E. necator* (isolate LAT 12 from Bordeaux region, France) was used to inoculate grape leaves (cv. Cabernet-Sauvignon) surface sterilised by immersion in 50 g l^{-1} calcium hypochlorite, rinsed with sterile water and dried between two autoclaved paper sheets (Délye and Corio-Costet 1998). Leaves were placed at the bottom of a Plexiglas settling tower and conidia were blown in at the top from sporulating leaves (500–600 conidia per cm² of leaf). Inoculated leaves were incubated for 12–14 days at 22°C with a 16/8 h photoperiod. Plants (cv. Merlot) were infected by *E. necator* by placing an infected plant nearby to mimic natural inoculation. Plants of *C. sativus* were inoculated with a spore suspension of *S. fuliginea*. They were also infected by spores dispersed from a naturally *S. fuliginea* infected plant, placed nearby.

GUS activity

Nicotiana tabacum P1Lox::GUS plants (Fammartino et al. 2010) were grown in soil in a growth chamber (16 h light at 25°C, 8 h dark at 22°C). Fully expanded leaves of 5-week old *N. tabacum P1Lox::GUS* were infiltrated with either water as control, an extract of *Phytophthora parasitica* cell wall (P2) at 30 µg ml^{-1} (Roux et al. 1994) or the green algal extracts at various dilultions. Infiltrated tissues from at least 5 plants per treatment were collected after 3 days. Glucuronidase activity was measured on protein extracts using a fluorometric assay (Jefferson et al. 1987). Briefly, 100 mg of ground tissues were mixed in 100 µl of GUS buffer (100 mM NaH_2PO4, pH 7.0, 10 mM EDTA) with 0.1% TritonX-100 and 10 mM β-mercaptoethanol. The fluorogenic reaction was carried out on 1 mM MUG (4-methyl umbelliferyl glucuronide, Sigma) in GUS buffer with 25 µl of protein extracts in a reaction volume of 200 µl. Fluorescence was measured every 5 min during 90 min, on a FL600 Microplate Fluorescence Reader (Bio-Tek Instruments) at 37°C with 360 nm excitation and 460 nm emission. The fluorometer was calibrated with freshly prepared MU (4-methylumbelliferone sodium salt, Sigma) standards in the same GUS buffer. Protein concentration was determined by the method of Bradford on 96 well plates. 200 µl of Bradford reagent (Bio-Rad Laboratories) were added to 10 µl of samples. Absorbance was measured at 565 nm on an ASYS Expert 96 (Isogen) spectrometer. Standard curve was done with 1–20 µg of BSA (Sigma). Glucuronidase activity was calculated from the linear part of the reaction (between 10 and 60 min) and expressed as nkatal mg^{-1} of protein.

Statistical analyses

The data from each experiment on more than 10 plants each time were used to calculate the mean and

the standard deviation. Data obtained on *P. vulgaris* came from a complete factorial treatment design involving two factors: the dilution of the green algal extract with three concentrations, and the number of plant elicitation (1 and 2). Statistical analyses were done using the Statistica 6.0 software (StatSoft), and analysis of variance was carried out to detect which factor significantly affects disease symptoms. Values of a significant factor were classed by using Tukey test ($p \leq 0.05$). Analyses of variance using the R software were also performed to test the effect of the extract origin on the protection level on cucumber against *S. fuliginea* and on the induction of the PR1-GUS gene expression. The protection level corresponds to the difference between infected surfaces on treated and control (not treated) leaves divided by the infected surface in control leaves. Treatment differences were evaluated using a Duncan test.

Results

Preparation and analysis of a crude soluble *U. amoricana* extract

Chemical characterization of the *Ulva* extract 0625001 showed that the soluble material contained mainly rhamnose (155 mg g^{-1}), uronic acid (59 mg g^{-1}) and sulphur (84 mg g^{-1}); xylose (16 mg g^{-1}), glucose (2 mg g^{-1}), and protein (15 mg g^{-1}) being present in lesser quantities. This composition is consistent with the presence of a heteropolysaccharide named ulvan, which represented more than 25% of the dry matter of *Ulva* sp. tissues. The presence of sulphate groups was confirmed by infrared analysis, which also showed the typical absorption spectra of molecular linkages present in uronic acids and sulfated rhamnose (Fig. 1a). Peaks observed at 840, 1100, 1622 and 3349 cm^{-1} are caused by the bending vibration of C-O-S in axial position, the stretching vibration of C-O, C=O and C-OH, respectively. Size-exclusion chromatography showed that the extract was mainly composed of two macromolecular populations of molecular weight around 500 kDa and 50 kDa, respectively (Fig. 1b). For elicitor experiments, the extract was used as a liquid solution either undiluted, or diluted as indicated (1/2 to 1/36).

Effect of the ulva extract on bean, grapevine and cucumber powdery mildew infections

P. vulgaris plants were treated by spraying either undiluted or diluted extract (0625001) onto well-expanded leaves. Inoculations with *E. polygoni* spores were artificially performed three days after the first or the second application of the extract. The extract reduced disease symptoms induced by *E. Polygoni,* a significant effect being still observable at the lowest concentration used (Fig. 2). Protection level increased up to 90%. Two successive applications did not increase the activity, irrespective of the concentration used.

On grapevine, entire plants were initially treated three days before inoculation of detached leaves with *E. necator* spores. Quantification of powdery mildew symptoms (Table 1) showed a strong reduction (77%) of pathogen development when the extract was applied at a dilution of 1/9. To confirm the protective effect of the *Ulva* extract, plantlets treated with the extract were placed in close contact of a heavily contaminated plant. This was efficient leading to symptom development on nearly all leaves 15 days after contamination (Fig. 3). The extract treatment of plants with the extract led to a significant reduction of symptom appearance (50%), and importantly, the symptom severity decreased dramatically: only a few percent of the total leaf area had symptoms (Fig. 3).

Activity of the *Ulva* extract was also tested on cucumber against *S. fuliginea*. Infected leaf surface area was measured over one month after inoculation. The total surface of infected leaves was much reduced by the treatment as compared to the controls (Fig. 4 a, b): at 30 dpi, about 0 to 10% of the leaf area were colonised by the pathogen (Fig. 4c). Interestingly, the protection level was more than 80% even when a severe mildew was observed on the control plants (Fig. 4d).

Seasonal variability of *Ulva* extracts composition and biological activity

To study seasonal variability of *Ulva* sp. preparations, extracts were prepared from five green algae samples harvested at different period of the year from June to October. The dry matter part of the liquid extract varied from 4.7 g l^{-1} to 7 g l^{-1} (Table 2)

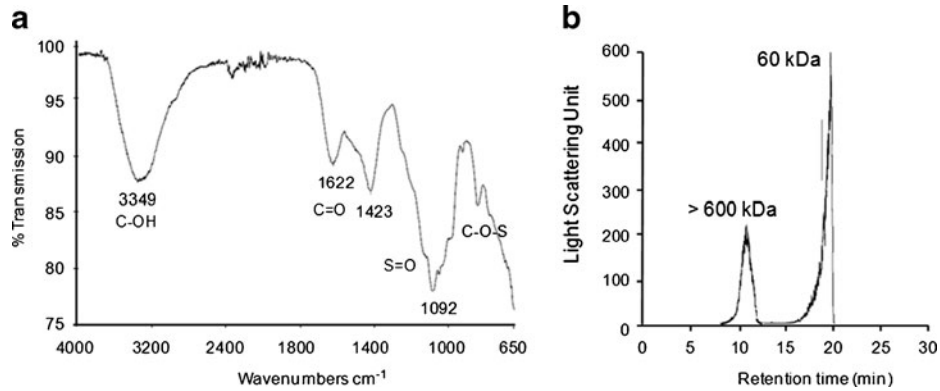

Fig. 1 Biochemical characterization of the green algal extract 0625001. **a** Infrared spectra, **b** Size-exclusion chromatography

and the uronic acid concentration ranged from 25 to 100 mg g^{-1} dry matter indicating variability of the ulvan concentration.

Infiltration of *U. armoricana* extracts (dilution: 1/9) strongly induced expression of the β-glucuronidase gene, ranging from 3 to 8 fold, compared to the water-control (Fig. 5). All extracts were able to protect cucumber against *E. fuliginea*, the protective activity ranging from 75 to 90%.

Discussion

Marine algae represent an abundant and inexpensive source of bioactive compounds which can be used in agriculture but their biological activity was mainly studied on model plants in laboratory conditions.

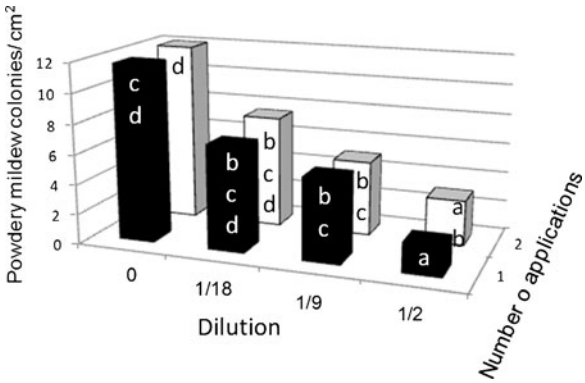

Fig. 2 Protection induced by the *Ulva* extract (0625001) on *Phaseolus vulgaris* against powdery mildew. Protection was evaluated by quantifying powdery mildew colonies at 10 dpi on inoculated leaves treated once or twice with various dilutions of ulvan extract. Columns with the same letter are not significantly different (Tukey test; $P \leq 0.05$)

The initial *U. armoricana* extract was obtained by using about 100 g of fresh algae per litre of hot (90°C) water. Chemical analyses showed that it contained two major sugars, rhamnose and uronic acid, and sulphate. This composition was consistent with the presence of ulvan, a typical sulfated polysaccharide that accumulates in *Ulva* spp. The presence of ulvan was confirmed by the infrared spectra which revealed C-O-S linkages typical of ulvan within polymers ranging from high (1200–300 kDa) to medium (180–85 kDa) molecular mass population (Robic et al. 2009a, b; Jaulneau et al. 2010). The protocol used in this work is compatible with an industrial-scale production of the extract and does not release any chemical wastes which could be harmful for the environment.

When applied on plants before pathogen inoculation, the extract protected effectively *P. vulgaris*, *V. vinifera* and *C. sativus* plants against powdery mildew pathogens. A concentration corresponding to about 6 g l^{-1} dry matter (1/9 dilution of the extract) was found to be sufficient to obtain more than 80% of protection. Since significant accumulations of sea-

Table 1 Protection induced by *Ulva* sp. extract (sample 0625001) on grapevine leaves 12 dpi after infection by *E. necator*

Dilution	% infection/control[a]
1/36	63.4±18.2
1/18	47.4±21.9
1/15	33.7±10.6
1/12	23.8±10.7
1/9	23.4±10.5

[a] % of disease expansion compared to control plants; ± Standard deviation

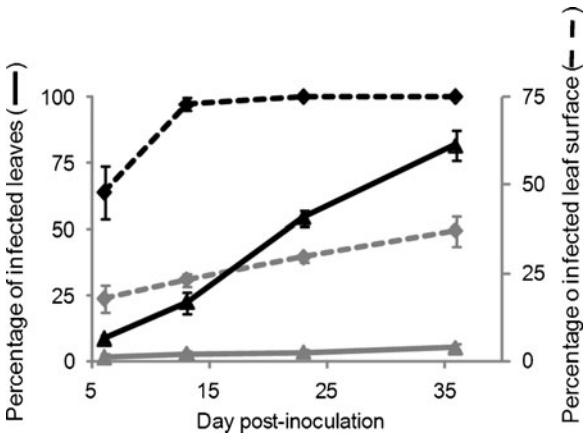

Fig. 3 Protection induced by the *Ulva* extract (0625001) against natural infection by *Erysiphe necator* on grapevine. *Ulva* extract at a dilution of 1/9 was sprayed onto grapevine leaves and mildew infection resulted from placing an infected plant nearby. The percentage of infected leaves (◆: control, ◆: treated) and the total infected tissue surface (▲: control, ▲: treated) were quantified daily

weeds causing "green-tides" are observed annually (more than 100 000 T annually in France; Morand and Briand 1996) the primary source of the extract is not limited and can be adapted to an industrial production.

Protection against powdery mildews by natural extracts has been scarcely reported. However, treatment of cucumber with extracts of the giant knotweed (*Reynoutria sachalinensis* L., also known under the commercial name of Milsana®) was found to have a protective effect (Daayf et al. 1995), acting indirectly by inducing cytological and molecular defense responses (Fofana et al. 2002; Wurms et al. 1999). It

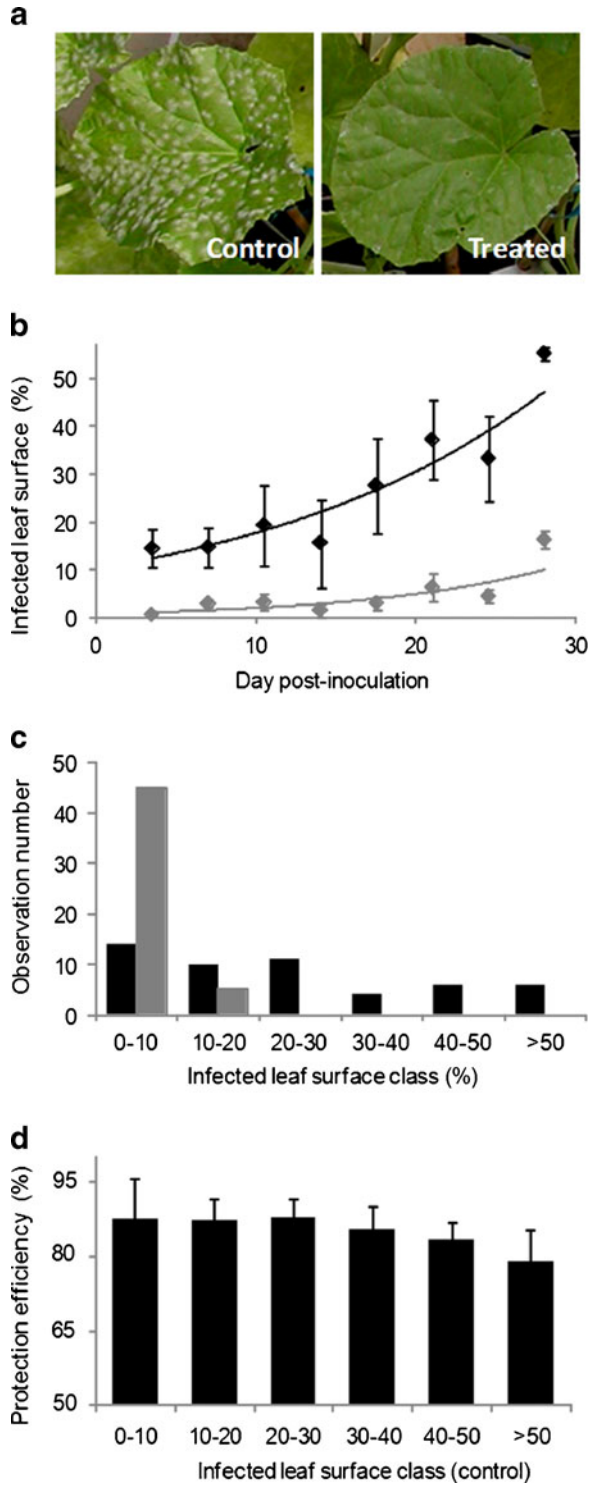

Fig. 4 Protection induced by the extract (0625001) against natural *S. fuliginea* cucumber infection. **a** Macroscopical symptoms of control and treated leaves (1/9 dilution) inoculated with *S. fuliginea* conidies at 30 dpi. **b** Quantification of powdery mildew symptom development on control (*black line*) and treated (*grey line*) cucumber leaves. Infected surface was assessed at the time of appearance of symptoms at 3 dpi and measured every 3 days. The results were from 17 independent experiments. **c** Evaluation of symptom severity upon treatment with the *Ulva* extract. Data (infected leaf surface) were organized in 6 classes and the number of observations falling in each class was determined for control (*black bars*) and treated (*grey bars*) plants. **d** Protection activity according to the pathogenic constraint. For each biological repeat, symptoms observed on control leaves were classified according to the total infected leaf surface and for each class, protection efficiency was reported for the corresponding treated plants

Table 2 Seasonal variation of uronic acid content and dry matter of *Ulva* sp. extracts

Year	Month	Sample	Dry matter g l^{-1}	Uronic acid mg g^{-1}
2006	June	0625001	4.7	100
2007	July	0730001	5.5	129
2007	September	0738002	4.9	49
2007	October	0743003	5.0	94
2008	July	0830001	7.0	25

was recently reported that foliar treatment of barley seedlings with a partially purified ulvan preparation led to protection against powdery mildew (Paulert et al. 2010), confirming the potential of *Ulva* preparations to control powdery mildew. The molecular mechanisms underlying the ulvan-induced defence to powdery mildew pathogens are still not known. It was

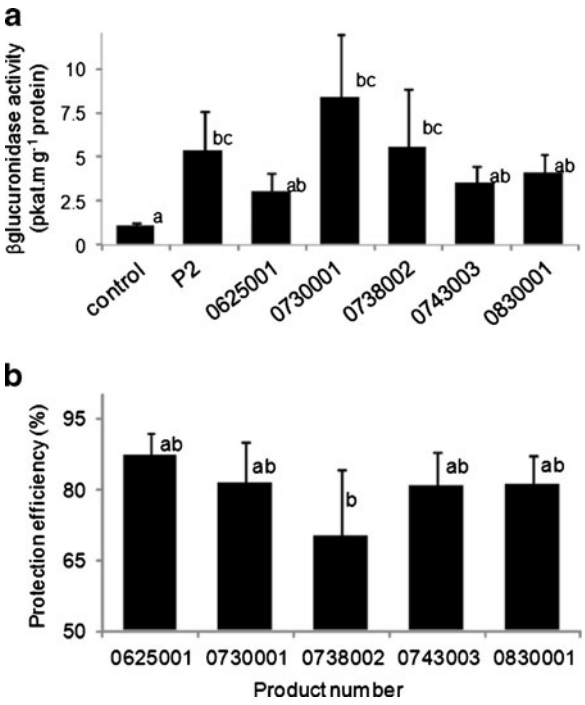

Fig. 5 Biological activities of *Ulva* extracts obtained from five independent green algae harvests. **a** Glucuronidase activity of tobacco leaves expressing the reporter construct *P1Lox::GUS* three days after infiltration of *Ulva* extracts at a 1/9 dilution. **b** Protection induced by *Ulva* extracts on cucumber leaves against *S. fuliginea*

previously shown that ulvans induced defence responses controlled by jasmonic acid (Jaulneau et al. 2010). In the model plant *Arabidopsis thaliana*, defence reactions regulated by the jasmonic acid signalling pathway are usually associated to resistance towards necrotroph pathogens whereas salicylic acid pathway is involved in resistance to biotrophs (Glazebrook 2005; Bari and Jones 2009). However, it is probably more complex in the case of resistance to powdery mildew pathogens, which are typical biotrophs. It has been shown that the *A. thaliana* mutant *Cev1*, which has a constitutive jasmonic acid signalling, displayed an increase resistance to the powdery mildew pathogen *E. cichoracearum* (Ellis et al. 2002; Ellis and Turner 2001). Treatment of grapevine and barley with jasmonic acid or methyl jasmonate induced protection against powdery mildews (Belhadj et al. 2006; Walters et al. 2002).

The protection induced by natural elicitors could be also affected by others factors such as the time interval between elicitation and pathogen attack, the number of elicitor treatments and the environmental conditions. Plant elicitation with the green algal extract 3 days before artificial inoculation was sufficient to protect against powdery mildew pathogens. We also showed that weekly application of the extract on cucumber and grapevine plants could protect against natural inoculation over 4 weeks, despite the presence of a heavily infected plant. While this application frequency was similar to those actually used with fungicide products for grapevine or market gardening plants, it would be important to determine the maximum time interval that it is still able to control powdery mildew effectively.

The quality and content of active compounds extracted from natural sources can vary according to environmental conditions. It has been shown that the ulvan composition can slightly vary in samples harvested at different location or during various time periods (Lahaye et al. 1999; Robic et al. 2009a, b). Consequently, significant variations in term of uronic acid contents were observed in the samples that we prepared. However, elicitor activity tested on a reporter transgenic plant did not vary significantly, suggesting that the amount of active ulvan in the sample is not a limiting factor. Interestingly, their activity against powdery mildew was

almost identical, suggesting that variation of ulvans present in *Ulva* sp. tissues obtained at different locations and at different times did not affect its efficacy against powdery mildew.

We conclude that the profuse green macroalga, *U. armoricana*, from the green tides is a non-expensive source of bioactive compounds able to protect plants against fungal infections. The reproducible and highly effective activity of ulvans against pathogens illustrates the potential applications of defence stimulators for the management of plant disease in agriculture.

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