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In vitro suppression of *Sclerotinia minor* by a seaweed extract from *Durvillaea potatorum* and *Ascophyllum nodosum*

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Abstract Five in vitro bioassays demonstrated a capacity for an undiluted seaweed extract, made from a combination of *Durvillaea potatorum* and *Ascophyllum nodosum*, to suppress the growth of *Sclerotinia minor* by 18-100 %. Inundation in the seaweed extract significantly ($p \leq 0.05$) suppressed growth of sclerotia by 90 %, and reduced disease severity in lettuce seedlings. A control buffered to the same pH (8.8) as the seaweed extract also significantly ($p \leq 0.05$) suppressed the growth of sclerotia, but only by 22 %. This suggests that pH only partially explains the suppressive effect of the extract against *S. minor*, and undiscovered modes of action exist.

Keywords Kelp extract · Seasol® · Lettuce · Bioassay

Sclerotinia minor is a devastating necrotrophic pathogen of a number of horticultural crops, including lettuce, green bean, cabbage, and broccoli (Villalta et al. 2012). It survives in soil for many years as melanised sclerotia, which infect crops following their germination. The fungus generally does not produce any secondary inoculum within a season, and therefore the density of viable sclerotia in soil and their pathogenicity are the most crucial factors influencing disease epidemics (Wu et al. 2008). Currently, there are few fungicides or fumigants available for growers to control sclerotia and mycelia of *S. minor* in soil due to registration withdrawals (Villalta et al. 2012) and reduced efficacy with repeated use (Martin et al. 1990). Research has identified several alternatives that can reduce the viability of *S. minor* in soil, including biological controls (Villalta et al. 2012), biofumigation (Villalta et al. 2008), and raising soil pH (Wilson et al. 2005), but these are generally less effective and less reliable than chemical controls.

Since the inception of seaweed extracts as soil amendments in horticulture, authors have reported their potential benefits for crop health (Craigie 2010). Recent evidence has linked the effect of seaweed extracts on crop health to stimulation of resistance mechanisms in the host plant (e.g. Subramanian et al. 2011). Few published studies, however, have considered the direct role of seaweed extracts in reducing inoculum of fungal soil-borne pathogens (Kulik 1995). In laboratory bioassays, Ara et al. (2005) found that specific fractions from an extract of *Spatoglossum asperum* inhibited the growth of the soil-borne pathogens *Macrophomina phaseolina*, *Rhizoctonia solani* and *Fusarium solani*. They attributed the effect to various fatty acid esters identified in the fractions. Similarly, ether extracts from various species of brown, green and red algae showed fungicidal effects against two *Aspergillus* and *Penicillium* species grown in culture (Khaleafa et al. 1975). The authors postulated that the effect was due to the presence of halogenated phenolics in the extracts. Similarly, Chanthini et al. (2012) demonstrated that the direct antifungal activity of various species of red and green algae against the pathogen *Alternaria solani* correlated with their phenolic content.

We conducted four in vitro bioassays to test the hypothesis that a seaweed extract has the potential to directly suppress the growth of mycelia and sclerotia of *S. minor* (National Collection of Fungi, VPRI Herbarium, VPRI 41837, isolated from lettuce grown at Boneo, Victoria in 2008). This isolate was

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used in all experiments reported in this paper. Pre-testing with other isolates of *S. minor* from field-grown lettuce showed similar responses to treatments as the test isolate. The seaweed extract (Seasol Commercial®, Seasol International, Bayswater, Victoria) was made from *Durvillaea potatorum* and *Ascophyllum nodosum*, using a commercialised alkaline hydrolysis process. Prior to use, the extract was sterilised by exposure to UV for 2 h in a laminar flow cabinet. Pre-testing showed that this method successfully sterilised small volumes (15 ml) of the extract without affecting its bioactivity against *S. minor*. Distilled water formed the control and was also sterilised by exposure to UV. In all bioassays, agar plates containing the pathogen were incubated at 21ºC in the dark. Colony diameters (average of two perpendicular planes for each colony) of *S. minor* were recorded after 2 and 5 days incubation as a measure of fungal growth.

1. *Disk diffusion bioassay*. Antimicrobial susceptibility disks (Oxoid, Basingstoke, UK) were individually soaked in 1 ml aliquots of the treatments for 1 h, and then allowed to dry in a laminar flow cabinet. Disks were placed in the centre of a Petri dish (84 mm diameter) containing potato dextrose agar (PDA, Oxoid). Three agar plugs (8 mm diameter) containing mycelia were cut from the edge of an actively growing culture of *S. minor* and placed equidistant (25 mm) from the disk on the PDA plates.
2. *Agar amendment bioassay*. Treatment solutions (1 ml) were spread evenly over the surface of PDA plates, and allowed to absorb into the media for 2 h in a laminar flow cabinet. An agar plug containing mycelium of *S. minor* was then placed in the centre of each of the plates.
3. *Mycelia inundation bioassay*. Agar plugs containing mycelia of *S. minor* were submerged in 1 ml of nutrient broth (Oxoid) in a Falcon tube. Treatment solutions (4 ml) were added to the tubes and mixed on a vortex shaker for 30 s. The tubes were then placed in a rotating incubator for 16 h. Agar plugs were retrieved from the tubes, rinsed in sterile distilled water, and briefly allowed to dry on sterile filter paper in a laminar flow cabinet. Individual plugs were then placed in the centre of a fresh PDA plate.
4. *Sclerotia inundation bioassay* (*a*). Laboratory-grown sclerotia were harvested from the edges of cultures of *S. minor* on PDA plates. Sclerotia were submerged in Falcon tubes containing 4 ml of the treatment solutions and 1 ml of nutrient broth, and placed in a rotating incubator for 16 h. Following this, sclerotia were rinsed in sterile distilled water and allowed to dry on sterile filter paper in a laminar flow cabinet, before transferring individually onto separate PDA plates (60 mm diameter).

Individual bioassays and the pathogenicity experiment (see below) were conducted as randomised complete block designs, with three blocks containing three replicate plates of each treatment. Where variation occurred across treatments, data were analysed by ANOVA using Genstat v. 12.1 (VSN International, Hemel Hempstead, UK), and Fisher's least significant difference test used to identify differences between treatment means (where $p \leq 0.05$). Homogeneity of variance was determined by examining plots of fitted values versus residuals, while histograms of residuals were examined for normality of distribution. Standard errors were calculated where variation occurred in one treatment only. Where no variation occurred in any treatment, there was no analysis of data.

At 2 days incubation, results from all bioassays demonstrated a capacity for the seaweed extract to significantly ($p \leq 0.05$) suppress the growth of mycelia and sclerotia of *S. minor* compared with the control, with the effect ranging from 18-100 % (Table 1). Overall, bioassays that involved inundation of *S. minor* in the seaweed extract (i.e. bioassays 3 and 4) showed greater inhibitory effects against fungal growth than bioassays conducted on agar surfaces (i.e. bioassays 1 and 2). This concurs with Hadacek and Greger (2000) who reported that inundating test pathogens in treatment solutions exposes them to a greater concentration of potential inhibitory compounds than bioassays conducted on agar surfaces.

By 5 days incubation, inundation of sclerotia in the seaweed extract had significantly ($p \leq 0.05$) suppressed the ensuing growth of mycelia by 50 % compared with the control (bioassay 4, Table 1). In contrast, inundation of mycelia in the seaweed extract killed the fungus, which was confirmed by sub-culturing on two separate occasions (bioassay 3, Table 1). These results are consistent with mycelia being more susceptible to inhibitory compounds and environmental factors than sclerotia, which have protective melanised rinds (Chet and Henis 1975). In the disk diffusion and agar amendment bioassays (bioassays 1 and 2, respectively), mycelia of the fungus had grown to the edge of the plate by 5 days of incubation, so the effect of treatment could no longer be determined (Table 1).

5. *Sclerotia inundation bioassay* (*b*). The alkaline hydrolysis process used to generate the seaweed extract gives the product a high pH (8–9). Optimal growth of *Sclerotinia* spp. in culture occurs at a low pH of 3–6 (Willetts and Wong 1980), and pH of>8.0 can suppress germination of their sclerotia (Wilson et al. 2005). For this reason we conducted a further sclerotia inundation bioassay, as described previously (bioassay 4), to test the hypothesis that the high pH of the seaweed extract suppresses the growth of *S. minor*. Treatments included: (a) undiluted extract from *D. potatorum* and *A. nodosum*, (b) water buffered with 1 M sodium borate to the same pH (8.8) as the seaweed extract, (c) a 1:500 dilution of seaweed extract, which is the lowest application concentration in the field and had an equivalent pH (7.5) to the water control, (d)

Table 1 Colony diameters (mm) of *Sclerotinia minor* treated with a commercial extract from *Durvillaea potatorum* and *Ascophyllum nodosum* in various in vitro bioassays. LSD is the least significant difference where p=0.05. Values in square parentheses are standard errors

Bioassay method	2 Days incubation			5 Days incubation		
	Seaweed extract	Control (water)	LSD (p=0.05)	Seaweed extract	Control (water)	LSD (p=0.05)
1. Disk diffusion	32.9	44.1	4.7	60.0†	60.0†	–
2. Agar Amendment	37.7	46.0	3.2	84.0†	84.0†	–
3. Mycelia inundation	0.0	44.0 [0.9]	–	0.0	84.0†	–
4. Sclerotia inundation	0.0	8.7 [0.5]	–	31.1 [0.9]	60.0†	–

† Colonies had reached the edge of the agar plate

distilled water as a control, (e) freshly harvested sclerotia from actively growing cultures of *S. minor* as a laboratory control, and (f) allyl isothiocyanate (Sigma-Aldrich, St Louis, USA), which is a proven biofumigant treatment for control of sclerotia (Villalta et al. 2008). Colony growth (diameter) of *S. minor* was measured after 2, 3, 4, and 5 days incubation.

Inundation of sclerotia in undiluted seaweed extract significantly ($p \leq 0.05$) delayed their germination by 3 days and reduced the ensuing growth of mycelia by 90 % (at 5 days of incubation), compared with the water control (Table 2). By comparison, water buffered to the same pH as undiluted seaweed extract also significantly ($p \leq 0.05$) suppressed the growth of mycelia from germinated sclerotia, but only by 22 % (significantly, $p \leq 0.05$, less than the effect of the undiluted seaweed extract). Diluting the seaweed extract to a low concentration significantly ($p \leq 0.05$) suppressed the growth of mycelia from germinated sclerotia by 30 % compared with the water control (which was at an equivalent pH). There was no difference ($p > 0.05$) in the growth of mycelia from germinated sclerotia in the water and laboratory controls, but allyl isothiocyanate killed sclerotia.

Table 2 Colony diameters (mm) of *Sclerotinia minor* treated with a commercial extract from *Durvillaea potatorum* and *Ascophyllum nodosum* and various controls, in a sclerotia inundation bioassay (bioassay 5). LSD is the least significant difference where p=0.05

Treatment	Incubation period			
	2 days	3 days	4 days	5 days
Seaweed extract (undiluted), pH 8.8	0.0	0.0	0.0	5.3
Buffered water, pH 8.8	0.0	4.0	15.8	41.2
Seaweed extract (1:500), pH 7.5	0.0	4.7	16.7	37.7
Water (negative control), pH 7.4	4.9	11.1	24.0	53.2
Laboratory control	5.4	12.7	26.6	53.3
Allyl isothiocyanate (positive control), pH ND	0.0	0.0	0.0	0.0
LSD (p=0.05)	–	3.2	4.2	6.4

This bioassay demonstrated that the high pH of undiluted seaweed extract only partially explains its suppressive effect against *S. minor*. Furthermore, there was no evidence that pH contributed to the action of the extract when it was diluted to the lowest concentrations applied in the field. Previous research has attributed the direct suppressive effects of extracts from other species of marine algae to their content of fatty acid esters (Ara et al. 2005), phenolics (Khaleafa et al. 1975, Chanthini et al. 2012), and betaines (Wu et al., 1998). Koivistoinen et al. (1959) found that indole acetic acid, which is one of several plant growth regulators contained in the seaweed extract used in the current experiments (Seasol International 2013), inhibits the growth of *Sclerotinia trifoliorum* in culture. This suggests that the mechanism of extract from *D. potatorum* and *A. nodosum* in suppressing *S. minor* may involve a complex mixture of organic compounds, in addition to high pH. Further research is proposed to understand the mode of suppressive action, and to fractionate and characterise the active components of the seaweed extract against *S. minor*, and other soil-borne pathogens.

We conducted an in vitro pathogenicity experiment using the method described by Christensen et al. (1988) to test the hypothesis that treatment of sclerotia of *S. minor* with the seaweed extract reduces its ability to cause disease in a host. One hundred seeds of lettuce var. Great Lakes (New Gippsland Seeds and Bulbs, Silvan, Victoria) were surface

Table 3 Disease score† of lettuce seedlings infected with *Sclerotinia minor* treated with commercial extracts from *Durvillaea potatorum* and *Ascophyllum nodosum*. LSD is the least significant difference where p=0.05

Treatment	Disease score† (0–3)
Seaweed extract (undiluted)	0.97
Seaweed extract (1:500)	1.53
Water (negative control)	2.58
Laboratory control	2.56
LSD (p=0.05)	0.31

† 0=healthy green shoots, 1=< 50 % of shoot system wilted, 2 =>50 % of shoot system wilted, 3=shoots rotted

sterilised for 3 min in a 70 % ethanol/10 % sodium hypochlorite solution, followed by three rinses in sterile distilled water, and dried in a laminar flow cabinet on sterile filter paper. Seed was then placed in a Falcon tube and soaked in sterile distilled water overnight (12 h) at 21ºC. Four seeds were placed in a line (20 mm apart) across a water agar (Sigma-Aldrich) plate (84 mm diameter). After 3 days germination, when roots had extended half way across the plate, a treated sclerotium of *S. minor* was placed 10 mm from each of the seedlings. Sclerotia were treated as described previously with: (a) undiluted extract from *D. potatorum* and *A. nodosum*, (b) a 1:500 dilution of the seaweed extract, (c) distilled water as a control, or (d) were freshly harvested from actively growing cultures as a laboratory control. Plates were incubated at 21ºC in the light for a further 7 days, and the severity of shoot disease scored using the scale described in Table 3.

Inundating sclerotia in undiluted seaweed extract significantly ($p \leq 0.05$) reduced the severity of disease they caused in lettuce seedlings (Table 3). *S. minor* produces a range of pectolytic and cellulolytic enzymes, and oxalic acid that degrade host tissue. Production of these enzymes is optimal under low pH (5.0), and acidic conditions enhance the aggressiveness of the pathogen (Willetts and Wong 1980). For example, Wilson et al. (2005) found that raising soil pH with calcium hydroxide reduced disease incidence caused by *S. minor* in lettuce by 58 %. Therefore it is possible that the reduction in disease severity caused by the undiluted seaweed extract in the current experiment is due to the high pH of the product, even though its pH only played a partial role in the direct suppression of the pathogen (see previously). Further controlled trials, however, are needed to confirm this. Other researchers have demonstrated that extracts from *A. nodosum* can stimulate host resistance against *Sclerotinia* pathogens (Subramanian et al. 2011), and this may also explain the ability of the extract to reduce disease severity in the current experiment. Inundation of sclerotia in seaweed extract diluted to the lowest concentrations applied in the field also significantly ($p \leq 0.05$) reduced disease severity in lettuce seedlings, but not to the same degree as undiluted extract (Table 3). This suggests that dilutions of the seaweed extract used in the field to maximise crop growth may differ from those needed to maximise suppression of disease caused by *S. minor*, and potentially other soil-borne pathogens.

It is important to note that sclerotia used in the current experiments were laboratory-grown and potentially less tolerant of inhibitory compounds and environmental factors than field-hardened sclerotia. Also concentrations of the seaweed extract used in the experiments were generally higher and exposure of *S. minor* to the extract longer and more controlled than what would normally occur in the field. This was done to determine the maximum potential for the seaweed extract to suppress *S. minor*. Consequently, further pot and field trials are needed to expand the results from the current bioassays to farm applications. Nonetheless, previous studies have shown that other commercial extracts from *A. nodosum* have the capacity to reduce disease incidence caused by *Sclerotinia* spp. in the field and glasshouse (Zhang et al. 2003; Guinan et al. 2013). This combined with a potential of extracts from *D. potatorum* and *A. nodosum* to directly suppress the growth and pathogenicity of *S. minor*, shows that further field research is warranted.

We conclude that a seaweed extract from *D. potatorum* and *A. nodosum* has a potential to directly suppress the growth of *S. minor*, and reduce its ability to cause disease. This effect is only partially due to the alkaline pH of the undiluted extract, and other organic compounds in the extract are likely to be involved.

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