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Rapid bioassays to evaluate the plant growth promoting activity of *Ascophyllum nodosum* (L.) Le Jol. using a model plant, *Arabidopsis thaliana* (L.) Heynh

Prasanth Rayorath · Mundaya N. Jithesh · Amir Farid · Wajahatullah Khan · Ravishankar Palanisamy · Simon D. Hankins · Alan T. Critchley · Balakrishnan Prithiviraj

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Abstract *Ascophyllum nodosum* extract products are used commercially in the form of liquid concentrate and soluble powder. These formulations are manufactured from seaweeds that are harvested from natural habitats with inherent environmental variability. The seaweeds by themselves are at different stages of their development life-cycle. Owing to these differences, there could be variability in chemical composition that could in turn affect product consistency and performance. Here, we have tested the applicability of using *Arabidopsis thaliana* as a model to study the activity of two different extracts from *A. nodosum*. Three different bioassays: *Arabidopsis* root-tip elongation bioassay, *Arabidopsis* liquid growth bioassay and greenhouse growth bioassay were evaluated as growth assays. Our results indicate that both extracts promoted root and shoot growth in comparison to controls. Further, using *Arabidopsis* plants with a DR5:GUS reporter gene construct, we provide evidence that components of the commercial *A. nodosum* extracts modulates the concentration and localisation of auxins which could account, at least in part, for the enhanced plant growth. The results suggest that *A. thaliana* could be used effectively as a rapid means to test the bioactivity of seaweed extracts and fractions.

Keywords *Asophyllum nodosum* · Bioassays · Plant growth · Bioactivity

P. Rayorath · M. N. Jithesh · A. Farid · W. Khan · R. Palanisamy · B. Prithiviraj (✉)
Department of Plant and Animal Sciences,
Nova Scotia Agriculture College,
PO Box 550, Truro, NS B2N 5E3, Canada
e-mail: bprithiviraj@nsac.ca

S. D. Hankins · A. T. Critchley
Acadian Seaplants Ltd,
Dartmouth, NS B3B 1X8, Canada

Introduction

Ascophyllum nodosum (L.) Le Jol. is a brown marine alga that grows along shallow coasts and backwaters and dominates the rocky intertidal zones of the Atlantic shores of Nova Scotia and New Brunswick, Canada (Ugarte et al. 2006). For centuries, this seaweed species has been used in agriculture as a source of nutrients and as a soil amendment/conditioner, predominantly along the coastal agricultural zones where it is found in abundance (Temple and Bomke 1989). However, inherent factors such as high salinity, excessive sand content and low rate of decomposition have hindered the widespread use of this important resource. This has resulted in the development of formulations of seaweed suitable for use in agriculture such as liquid concentrates, soluble powders and dry meals.

Blunden (1991) reviewed the numerous beneficial effects of both extracts and suspensions of marine brown algae used in agriculture and horticulture. These beneficial effects included increased seed germination, increased crop yield and performance, improved resistance to stress factors like frost and salinity, and also reduced incidence of fungal diseases and insect infestation (Moller and Smith 1999; Nabati 1991). Biochemical studies conducted to assess the reported physiological effects of *A. nodosum* extracts have

shown that some of the beneficial effects could be due to the presence of laminaran and other complex oligosaccharides. This study showed that laminaran elicited endogenous β-D-(1,3) glucanases, important regulators of plant defence and development (Patier et al. 1993). Other studies have demonstrated that the use of seaweed extracts derived from *A. nodosum* led to enhanced leaf chlorophyll levels in treated plants (Blunden et al. 1997). This latter study suggested that the reported beneficial effects are due to betaines present in extracts of *A. nodosum*. It is important to isolate and characterise bioactive compounds of seaweeds that elicit beneficial properties. There are some reports on the characterisation of bioactives from algal sources that show antifungal or antioxidant properties (Tang et al. 2002; Shanab 2007). These studies confirm the presence of a wide range of bioactive compounds such as sterols, phenols and fatty acids (Tang et al. 2002; Shanab 2007).

Products such as liquid concentrates and soluble powders are manufactured from seaweeds that are harvested from natural habitats with intrinsic climatic and environmental variability. Further, seaweeds harvested during different seasons of the year are at different stages of their life cycle. These differences could result in variable nutrient concentration, chemical composition and also varying amounts of bioactive molecules (or compounds) in the resulting products, ultimately affecting their performance in the field (Haug and Larsen 1958). Although the concentrations of major nutrients such as nitrogen, phosphorus and potassium in *A. nodosum* extracts can be estimated and corrected for uniformity, the detection of bioactive compounds in seaweed formulations is difficult. In principle, plants could be used as biosensors (bioassay model systems) for detecting the presence of bioactive molecules, testing and even assessing the effects of bioactivity, and could be used as a convenient system to ensure uniform bioactivity of seaweed products. However, different bioassays produce varying responses to the same product due to the complexity in interactions with different biologically active molecules present in the extracts. The present study was conducted to develop a standard bioassay that can produce rapid responses and therefore could be conveniently used to test the bioactivity of seaweed extract and also different fractions of seaweed extract.

The small mustard flowering plant *Arabidopsis thaliana* (Brassicaceae) was selected as a model plant for the rapid bioassay as it provides several advantages, such as small size of plant, easy growth under laboratory conditions, rapid life cycle, prolific seed production, small genome size and availability of large amounts of genetic information, making it an ideal laboratory model (Meinke et al. 1998 and references therein). In this study, our objective was to evaluate and develop a rapid bioassay for detecting the growth-promoting activity of *Ascophyllum nodosum* extracts using *Arabidopsis thaliana* and investigate the biochemical mechanisms of *A. nodosum* extract induced plant growth.

Materials and methods

Seeds of *Arabidopsis thaliana var.* Col-0 were purchased from Lehle Seeds (Round Rock, TX). The seeds were surface-sterilised in a 1.5 mL microcentrifuge tube with 2% sodium hypochlorite solution for 1 min and subsequently rinsed three times in sterile distilled water. The sterilised seeds were seeded in Petri dishes (9 cm diameter) containing half-strength Murashige and Skoog basal medium (catalogue no. M5524; Sigma, St. Louis, MO) supplemented with 1% sucrose and 0.8% agar. Seeds were evenly distributed in Petri dishes by placing individual seeds with a 100 μL micro pipette at the rate of 80–120 seeds per plate. Petri dishes were incubated in a low temperature incubator set at 4°C in dark for 48 h to facilitate uniform germination. After 2 days the Petri plates were taken out and staked vertically under a cool fluorescent light (100 μmol photons m^{-1} s^{-1}) with a 16:8 h (day:night cycle) at 22 ± 2°C for 7 days. At the end of 5–7 days the plants were about 2 cm long with well differentiated roots and shoots; uniform plants were then selected and used for all the experiments.

Preparation of *Ascophyllum nodosum* extracts

Different products of *A. nodosum* (soluble powder and liquid concentrate) were a gift from Acadian Seaplants Ltd (Dartmouth, NS, Canada). Test solution was prepared by dissolving 1g soluble powder of *Ascophyllum nodosum* extract type 1 or 2 (ANE1 or ANE2) in 20 mL sterile distilled water by constant stirring with a magnetic stirrer for 15 min. The solution was then filter sterilised with 0.22 μm SFCA syringe filters (Corning, NY) and stored in sterile centrifuge tubes (Corning) at 4°C until further use.

The organic extracts of seaweed products were prepared by extracting 10 g ANE1 or ANE2 powder in 40 mL methanol for 15 min. The solvent was then evaporated and the dried pellet re-suspended in 10 mL methanol was used as a stock solution. The solution was then filter sterilised with 0.22 μm SFCA syringe filters and stored in sterile centrifuge tubes (Corning) at 4°C until further use.

Arabidopsis plant bioassays

Plant growth medium, either liquid medium (half-strength MS basal medium containing 1% sucrose) or solid medium (with the addition of 0.8% agar in half-strength MS basal

medium) were used for these studies (Murashige and Skoog 1962). The plant growth medium supplemented with different concentrations of ANE (0.01, 0.1 g L^{-1}) or methanolic extracts (2 gL^{-1}) constituted the treatments. Plant growth medium without ANE served as the controls. The required concentration of extract was added to warm (50°C) agar medium prior to pouring in 9 cm diameter Petri dishes.

Arabidopsis root-tip elongation bioassay

After 5 days of growth on half-strength MS basal solid medium, five uniform seedlings were transferred to treatment media containing 0.01, 0.1 g L^{-1} ANE1, ANE2 or methanolic extract at a concentration of 2 g L^{-1}. The plants were placed in such a way that they remained straight on the medium. The position of root tips was marked with a fine tip permanent marker on the base of the Petri plate at the start of the experimental period. Petri plates were staked vertically under a fluorescent light source (100 μmol photons m^{-1} s^{-1}) with a 16:8 h (day: night) photoperiod at a temperature 22 ± 2°C for 7 days. Observations on root elongation were made on 3rd, 5th and 7th day. Data reported here comes from five replicates, each comprising six plants (n=30). The experiment was repeated three times. Data were analysed using Fisher's least significant difference (LSD) at $P \leq 0.05$.

Arabidopsis liquid growth bioassay

Arabidopsis plants were grown on half-strength MS basal liquid medium for 7 days. Uniform seedlings were transferred to 12–well cell culture cluster plates (Corning, Cat.# 3513) containing 1.5 mL of liquid half strength MS basal medium supplemented with methanolic fractions of ANE to a final concentration 1 g L^{-1}. The plates were placed on an orbital shaker set at 90 rpm. Plants received light from cool fluorescent tubes with 16:8 h (day: night) photoperiod, the temperature was 22 ± 2°C. After 7 days, the plants were removed and observations on fresh weight were made. Observations were recorded from a total of 20 plants, wherein four seedlings from each treatment were randomized in a 12-well culture plate with five replications. The whole experiment was repeated three times. Data were analysed using Fisher's LSD at $P \leq 0.05$.

Greenhouse growth bioassay

Arabidopsis thaliana ecotype Col-0 (Lehle Seeds) were planted on presoaked Jiffy-7 pellets (Jiffy Products, Shippagan, NB, Canada) at the rate of two seeds per pellet. The peat pellets were arranged on plastic trays and grown in a greenhouse chamber. The greenhouse conditions were set as follows: temperature 22 ± 2°C, photoperiod of 16:8 h (day: night) with 100 μmol photons $m^{-1}s^{-1}$ incident light conditions, and humidity 65%. After germination, the plants were irrigated every other day. After 1 week, the plants were thinned to one plant per pellet.

Seaweed extract solutions were prepared in sterile distilled water to a final concentration of 1 g L^{-1}. The pellets with uniform plants were placed in individual plastic cups (5 cm diameter) to facilitate complete absorption of treatment solution by the pellets and to avoid cross contamination with other treatment solutions. Each plant was irrigated with 10 mL ANE solution starting with the first treatment after 14 days of germination and subsequently every week for 3 weeks. Additionally, the plants were irrigated on alternate days with distilled water to maintain uniform moisture for optimum growth of *Arabidopsis* plants. Observations on plant height and number of leaves were taken after 4 weeks. The experiment was conducted in a completely randomised block design with 15 plants per treatment (n=15). Data were analysed using Fisher's LSD at $P \leq 0.05$.

DR5: GUS plants and treatment

DR5: GUS transgenic plants was reported by Ulmasov et al. (1997). DR5, an artificial auxin response element with a β-glucuronidase (GUS) construct, i.e. DR5-GUS transgenic plants, were used to test the auxin response in *Arabidopsis* seedlings. Seedlings were grown for 7 days in half-strength MS basal liquid medium supplemented with 1.0% (w/v) sucrose, with shaking at 120 rpm at 22 ± 2°C under continuous fluorescent light of 100 μmol photons m^{-1} s^{-1}. The seedlings were then treated either distilled water (control) with indole acetic acid (IAA = auxin; 25 μM) or methanol extract in the medium for 24 h.

GUS staining

Histochemical GUS staining was performed by incubating whole seedlings in GUS staining buffer containing 50 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferrocyanide, 10 mM EDTA, 0.1% (v/v) Triton X-100, 2% (v/v) dimethyl sulfoxide, and 2 mm 5-bromo-4-chloro-3-indolyl-D-glucuronide at 37°C for 24 h (Jefferson 1987). The experiment was repeated twice with six replications per treatment.

Results

Arabidopsis root-tip elongation bioassay

Addition of *Ascophyllum nodosum* extracts (ANE) to growth medium enhanced *Arabidopsis* root tip growth. Growth was influenced dramatically by the extracts. At

3 days after treatment (DAT), with 0.01 g L^{-1}, ANE1 (*Ascophyllum nodosum* extract type 1) showed a 23% increase in root length, while ANE2 (*Ascophyllum nodosum* extract type 2) showed a 32% increase in root length over untreated controls. At 5 DAT, ANE1 showed a 20% increase in root length, while ANE2-treated plants at this stage showed a 37% increase in root length. After 1 week of *A. nodosum* extract treatment, ANE1 and ANE2 showed 15% and 38% increases in root length, respectively (Fig. 1a). At ten times higher concentration (0.1 g L^{-1}), ANE2 again performed better than ANE1. An increase of 45% in the root length was observed with ANE2, while ANE1 caused a 42% increase in root length at 3 DAT. At 5 DAT, ANE1 and ANE2 extract treatments resulted in increases of 39% and 54%, respectively. Finally, after 1 week of treatment with *A. nodosum* extracts, ANE1 and ANE2 showed root length increases of 16.9% and 34%, respectively (Fig. 1b). Statistical analysis using Fisher's LSD test ($P \leq 0.05$) confirmed that treatments varied significantly over controls at both 0.01 and 0.1 g L^{-1} concentrations. We also tested the effects of exogenous auxin (IAA) on root growth. We found that addition of exogenous IAA to the growth medium at concentrations of 10^{-4} and 10^{-5} M resulted in increased lateral root numbers while suppressing growth of primary root of *Arabidopsis* plants (data not shown).

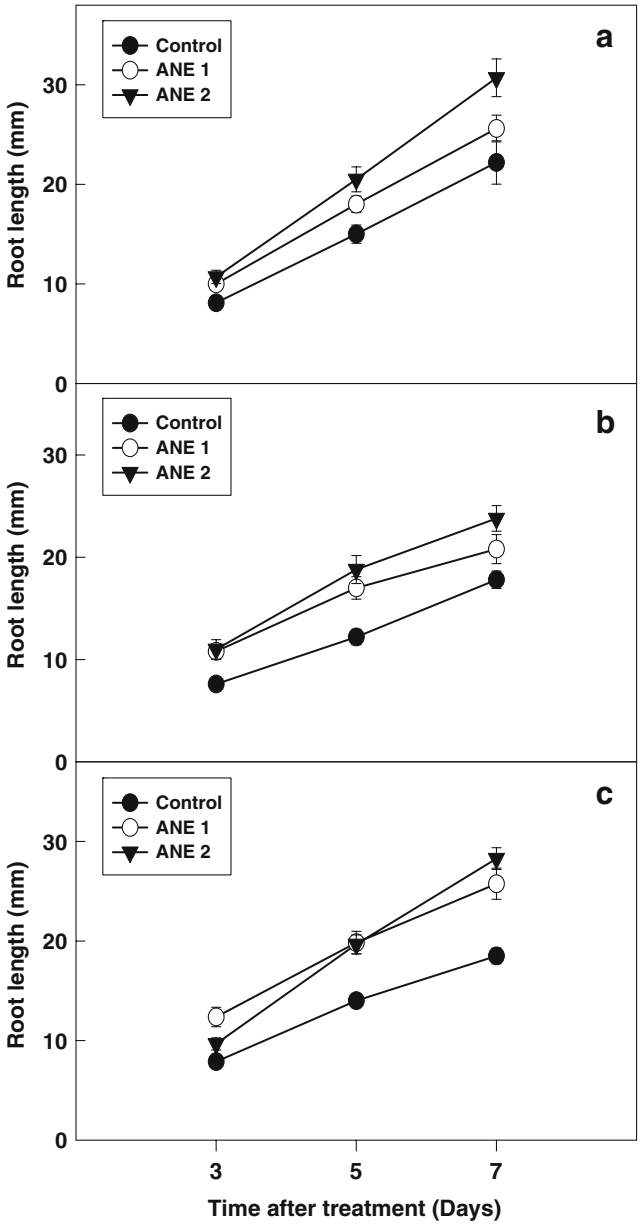

Fig. 1a–c The effect of different concentrations of *Ascophyllum nodosum* extract (ANE) on *Arabidopsis thaliana* root tip growth. **a** ANE (0.01 g L^{-1}). **b** ANE 0.1 g L^{-1}). **c** Methanolic fraction of ANE (g L^{-1} equivalent). The values represent mean of 30 plants (*bars* indicate standard error). The treatments were significantly different [$P \leq 0.05$; Fisher's least significant difference (LSD)] test from the control

Methanolic fractions of ANE1 and ANE2 were further tested in root elongation bioassays. At 3 DAT, ANE2 treatment resulted in a 23% increase in root elongation. On the other hand, at the same time point, ANE1-treated roots showed a 57% increase in elongation in comparison with untreated controls. By 5 DAT, treatment with ANE1 and ANE2 increased root elongation similarly by 41%. However, by 7 DAT, the effects of ANE2 treatment were clearly greater, with 53% increase in root length, while ANE1 increased root length by 40%. Analysis using Fisher's LSD test ($P \leq 0.05$) confirmed that treatments varied significantly over controls (Fig. 1c).

Arabidopsis liquid growth bioassay

Plants were grown in liquid media containing extracts in 12-well culture plates for 7 days. Methanolic fractions of ANE1 and ANE2 were used in this assay. Here, we observed increased fresh weight with methanol fractions of ANE1 in comparison to ANE2 and controls. Methanol fractions of ANE1 showed a 30% increase in average fresh weight while ANE2 showed an increase of 19% in fresh weight over untreated plants. Statistical analysis showed that ANE 1 showed significant differences from ANE2 and controls at $P \leq 0.05$ (Fig. 2a).

Greenhouse growth bioassay

In the greenhouse growth bioassay method, experiments were performed under greenhouse condition and treatments given in pots. Since roots are well established by the end of the experimental period, root length measurements are difficult to analyse without causing some injury to the plant. Plant height and number of leaves were chosen as growth parameters to test the effects of *A. nodosum* extract treatments. We observed that plants treated with ANE1 showed an increase in plant height of 57% over untreated

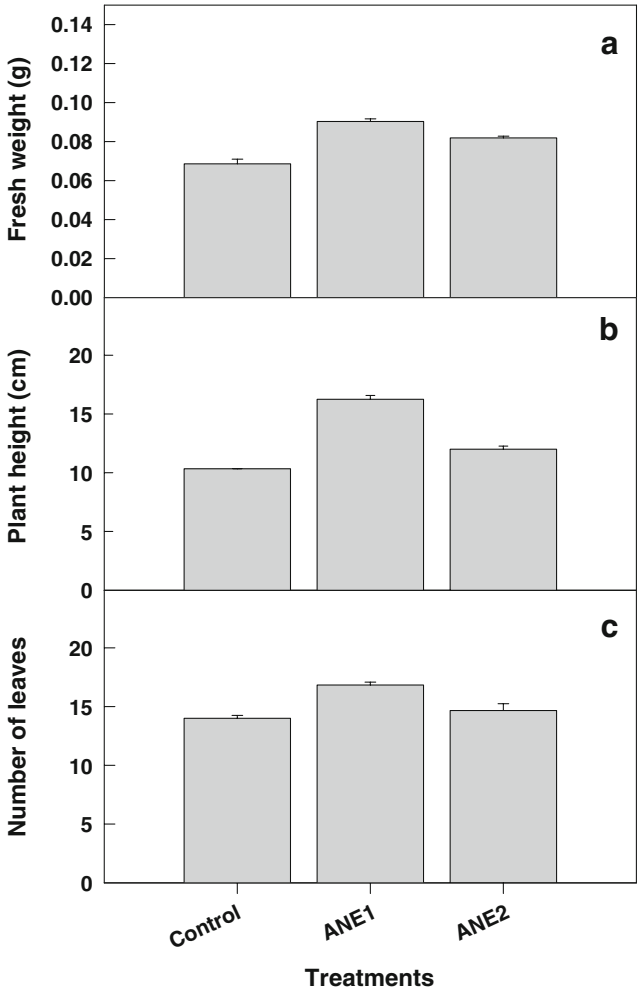

Fig. 2a–c The effect of *Ascophyllum nodosum* extract (ANE) on the biomass of *Arabidopsis thaliana* in in vitro hydroponic culture and in the greenhouse. **a** Effect of methanolic extract of ANE (1 g L^{-1}) on fresh weight in in vitro hydroponic cultures. **b** Effect of ANE (1 g L^{-1}) on plant height under greenhouse conditions. **c** Effect of ANE (1 g L^{-1}) on number of leaves under greenhouse conditions. The values represent the mean of 20 plants; *bars* standard error

controls. On the other hand, ANE2-treated plants showed a 16% increase over untreated controls (Fig. 2b). Treatment with ANE1 also resulted in an increase in the average number of leaves over control plants by 20%. However, treatment with ANE2 did not increase the average number of leaves over control plants significantly (Fig. 2c).

Auxin response of DR5:GUS transgenic lines

We also wanted to test whether the bioactives extracted from *A. nodosum* elicit enhanced growth through modulation of IAA responses through auxin-responsive promoter elements (AuxRE). Auxin is one the primary phytohormones known to play a fundamental role in plant growth and development (Weijers and Jürgens 2004; Kramer and Bennett 2006). Therefore, in this study, we chose a transgenic line that is responsive to auxin and studied the involvement of bioactives of *A. nodosum* extract in inducing DR5 AuxRE expression. The DR5:GUS reporter *Arabidopsis* line is a useful reporter because it harbours a highly active synthetic AuxRE and is ideal for studying auxin-responsive inducible process using *A. nodosum* bioactives. Such an analysis would provide a rapid dissection of molecular processes using transgenic lines. Seven-day old transgenic *Arabidopsis* seedlings containing the DR5:GUS reporter gene were treated with 25 μM IAA, distilled water (control), or methanolic extract of the ANE for 24 h and were then stained for GUS activity. In the untreated control plants, GUS activity was detectable mainly in roots. Some staining was detectable in the shoots. This observation indicates that DR5:GUS expression is more sensitive to auxin in roots than in shoots as reported by Ulmasov et al. (1997). When seedlings were treated with IAA, both shoots and roots stained for GUS activity. More interestingly, methanolic extract of *A. nodosum* induced DR5:GUS expression to a higher degree as compared to untreated controls, mainly in roots. Taken together, these observations show that DR5:GUS expression positively responds to compounds in *A. nodosum* extracts and indicate the presence of chemical component(s) in the methanolic extract that promote auxin distribution in roots (Fig. 3).

Discussion

Several biological effects of *Ascophyllum nodosum* have been reported in different plant systems, including enhancement of plant growth (Temple and Bomke 1989; Saravanan et al. 2003); increase in root growth and lateral root development (Metting et al. 1990); stress alleviation (Featonby-Smith and van Staden 1983; Nabati 1991; Nabati et al. 1994); and reduced nematode infestation (Wu et al. 1998). However, a rapid high-throughput system is essential to screen the growth-promoting activity of seaweeds as well as to test the effect of processing methods on its growth-promoting effects. The test plant should be small, easy to grow, fast growing and should ideally respond to external growth factors. *A. thaliana* is a model plant widely used in plant biology research that fits all the above criteria. and has been used in many previous studies, albeit for other purposes. For instance, studies on the interaction of ANE and rhizosphere organisms have been reported because of its thin, small and transparent root system (Wu et al. 1998). More importantly, to understand the molecular modes of action of *A. nodosum*, this model is best suited because its

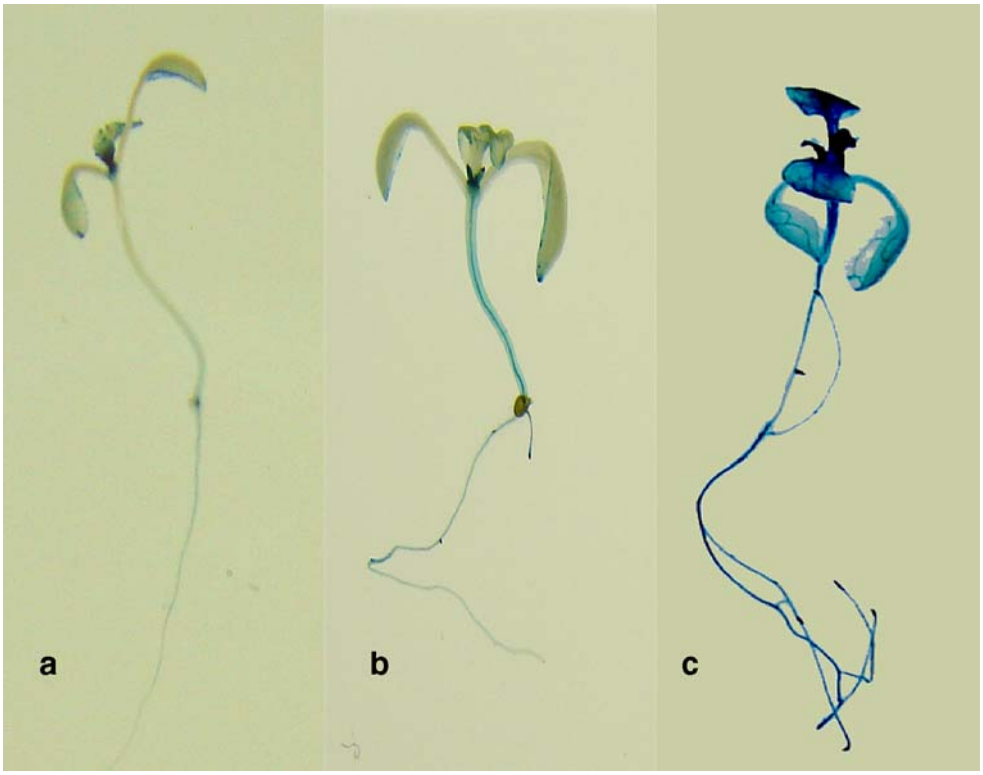

Fig. 3 Histochemical staining for β-glucuronidase (GUS) activity in DR5-GUS transgenic *Arabidopsis* control seedlings (**a**) or seedlings treated with methanolic fraction of ANE (**b**) or IAA (**c**)

genetics and genome information are well-established. Therefore, it would be possible to study the interaction of *A. nodosum* extracts on the biochemical pathways by analysing the whole genome of *A. thaliana*.

Previous assay systems, including the cucumber cotyledon, have been used for specific purposes, i.e. to study increased chlorophyll content. In this study, we used three rapid bioassay protocols: *Arabidopsis* root-tip elongation bioassay, *Arabidopsis* liquid growth bioassay and greenhouse growth bioassays, to test the plant growth-promoting effect of *A. nodosum* extracts. Two different *A. nodosum* seaweed extracts were tested for their bioactivity using all three methods. Under the experimental conditions reported here, ANE2 performed better than ANE1 in the root-tip elongation bioassay. On the other hand, ANE1 performed better in biomass growth assays including the liquid growth assay and greenhouse growth assay. Overall, our results indicate that both extracts promoted root and shoot growth over control plants. This is encouraging, because different extract combinations could be analysed and differentiated based on the reported methods for rapid screening. There are advantages and disadvantages of the different methods reported in this study for rapid screening purposes. The root growth elongation assay has the advantage that the effect could be tested more rapidly than with the other methods. The root growth elongation assay could be used as an indicator for abiotic stress tolerance studies like salt stress (Halfter et al. 2000). However, it is not an ideal system for studies under water-deficit and drought conditions. For such studies, shoot growth can be used because shoot growth is more sensitive than root growth to soil drying (Sharp and Davies 1989). This is possible with liquid growth and greenhouse assays. Greenhouse assays could be used to test several parameters of plant shoot growth such as fresh weight (FW) and dry weight (DW), which could be tested simultaneously. While greenhouse assays are very time consuming and need a larger space, the liquid growth assay should be given preference as a rapid assay for screening plant growth and development following extract treatments. Therefore, specific assays could be used depending on the need and facilities availability for studying the effects of *A. nodosum* extracts on plant growth and development.

Furthermore, detailed studies at the biochemical and molecular level could be performed using the *Arabidopsis* model system. In this study, we were also able to determine, using the transgenic DR5:GUS line, auxin inducible expression by GUS staining using bioactive compounds from *A. nodosum* extract, confirming that transgenic lines could be used to test expression patterns induced by bioactive compounds. This is significant considering the large collection of transgenic lines, T-DNA lines and other knockout *Arabidopsis* lines available, hence providing a unique means to study phenotype–genotype interactions. Therefore, the present study clearly demonstrated that *Arabidopsis* could be an ideal experimental choice for studying the potential beneficial effects of *Ascophyllum nodosum*.

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