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Extracts of the Brown Seaweed *Ascophyllum nodosum* Induce Gibberellic Acid (GA_3)-independent Amylase Activity in Barley

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Abstract Extracts of the brown seaweed *Ascophyllum nodosum* have been used as a biostimulant to promote growth and productivity in a number of agricultural production systems. Although the extracts have been shown to improve seedling emergence and vigor in a variety of plants, including barley, the mechanism(s) of this growth-promoting effect is(are) largely unknown. In our study, *A. nodosum* extract induced amylase activity in barley seed-halves; a significant difference in amylase activity was observed in seeds without an embryo. The addition of activated charcoal to the treatment media negated the bioactivity of the extracts suggesting the organic nature of bioactive compounds in *A. nodosum* extracts. The extracts induced amylase activity in a gibberellic acid (GA)-deficient barley mutant (*grd2*). LC-MS-MS analysis failed to detect the presence of GA_3 in the extracts. ABA supplementation of the medium caused a significant reduction of amylase activity in GA-treated seeds compared with those treated with the *A. nodosum* extract. Taken together, our results suggest that the organic components of *A. nodosum* extract induce amylase activity independent of GA_3 and might act in concert with GA-dependent amylase production leading to enhanced germination and seedling vigor in barley. Being derived from a renewable resource, the bioactive compounds from *A. nodosum* could be used to improve crop productivity in sustainable agricultural systems.

Keywords *Ascophyllum nodosum* · Seaweed extract · Amylase activity · Barley · Barley mutant · Abscissic acid · Sustainable agriculture

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Introduction

More than 15 million metric tons of seaweed products are used annually as nutrient supplements and biostimulants in agriculture and horticultural crop production (FAO 2006). Seaweeds and seaweed extracts have long been used in Canadian and European coastal agricultural zones as soil conditioners and foliar sprays to increase crop growth, yield, and productivity (Bokil and others 1974; Stephenson 1974; Senn 1987; Crouch and van Staden 1992; Verkleij 1992; Norrie and Keathley 2006). Further, a variety of seaweeds are used as soil conditioners to improve organic matter and preserve moisture and mineral content of soil (Gandhiyappan and Perumal 2001). *Ascophyllum nodosum* (L.) Le Jolis is a brown kelp, commonly known as rockweed, that dominates the rocky intertidal shores of Atlantic Canada and northern Europe (Taylor 1957; Ugarte and others 2006). The seaweed is well documented for its physiological tolerance to extremes of temperature, salt, and other environmental factors (Keser and others 1981). This alga is also an important source of organic matter in the coastal waters and is of significant ecological importance as the most used seaweed for agriculture in Canada and Europe (Filion-Myklebust and Norton 1981; Cousens 1984).

Exogenous applications of seaweed extracts have been observed to increase the yield and productivity of crop plants, but the mechanisms for such responses remain argely unknown (Blunden and others 1979; Abetz and Young 1983; Featonby-Smith and van Staden 1987). Earlier studies have reported positive effects of seaweed extracts on root growth when applied to the rhizosphere or as a foliar spray (Blunden and Wildgoose 1977; Finnie and van Staden 1985). An extract of a brown alga, *Sargassum wightii* Greville, improved yield and fruit quality of Indian jujube (*Zizyphus mauritiana* Lamk.) when applied as foliar spray (Rao 1991). Although seaweed extracts contain trace elements (Senn 1987), it has been argued that their levels in the commercial seaweed products are insufficient to produce a significant growth or biological response (Blunden 1977). Chemical analysis of seaweeds and their extracts have revealed the presence of a wide variety of plant growth regulators such as auxins and cytokinins in varying amounts (Senn and Kingman 1978; Jameson 1993; Zhang and Ervin 2004, 2008).

In monocotyledonous cereals such as barley successful growth and development following germination is accomplished through the mobilization of food reserves stored in the endosperm that provide nourishment to meristems (Brink and Cooper 1947) and support growth and differentiation of the growing embryo (Olsen and others 1998). The aleurone layer surrounding the endosperm secretes a range of hydrolytic enzymes such as α-amylase, β-amylase, α-glucosidase, (1-4)-β-xylan endohydrolase, glucanases, proteases, and nucleases that break down starch reserves, proteins, lipids, and cell wall components of the endosperm (Fincher 1989; Mrva and others 2006). During germination, the mobilization of starch reserves involves the breakdown of polysaccharides to simple sugars such as glucose and fructose through a multistage process (Fincher 1989; Lovegrove and Hooley 2000). This hydrolytic breakdown results in the generation of energy and a continuous supply of carbon skeletons for biosynthesis of the primary cellular components of the developing embryo (Nanjo and others 2004). α-Amylase is involved in the initial and critical step of starch hydrolysis (Lovegrove and Hooley 2000; Zentella and others 2002).

The synthesis and secretion of α-amylase in the aleurone layer is induced by gibberellic acid (GA_3). In germinating seeds, the scutellar cells of the embryo produce gibberellins (GAs) which, through diffusion, reach the endosperm and induce hydrolytic enzymes in the aleurone layer. Gibberellins act as a signal in the germinating seed by activating the α-amylase genes in the aleurone cells which in turn secretes α-amylase (Sun and Gubler 2004). Abscisic acid (ABA) inhibits α-amylase synthesis and suppresses seed germination by blocking the activity of GA (Karssen and others 1983; Koornneef and Karssen 1994; Lovegrove and Hooley 2000). Thus, there is an antagonistic interaction between ABA and GA in the seed germination process (Sun and Gubler 2004).

Earlier studies have shown that application of seaweed extracts enhances seed germination and seedling vigor (Kambayashi and Watanabe 2005; Demir and others 2006; Economou and others 2007). Our recent studies on the enhancement of seedling growth and vigor showed that commercial extracts of *A. nodosum* improved root and shoot growth in the model plant *Arabidopsis thaliana* (Rayorath and others 2008). The focus of the present study was to investigate the effect of commercial *A. nodosum* extract and its organic subfractions have on seedling germination by studying α-amylase activity in germinating barley seeds.

Materials and Methods

Plant Material, Chemicals, and Seaweed Extract

Barley (cv. AC Sterling) seeds were obtained from the Department of Plant and Animal Sciences, Nova Scotia Agricultural College, Truro, NS, Canada. Soluble potato starch (Cat. No. S2630), activated charcoal (Cat. No. C7606), gibberellic acid (Cat. No. G7645), and abscisic acid (Cat, No. A1049) were purchased from Sigma Aldrich (Oakville, ON). Alkaline extract of *A. nodosum* (hereafter denoted ANE) was provided by Acadian Seaplants Limited, Dartmouth, NS, Canada. An aqueous solution of ANE was prepared by dissolving 1 g extract powder in 20 ml sterile distilled water by constant stirring with a magnetic stirrer for 15 min. The solution was then filter sterilized using a 0.22-μm SFCA syringe filter (Corning Inc., Lowell, MA) and stored in sterile centrifuge tubes (Corning) at 4°C until further use.

A methanol fraction of ANE was prepared by extracting 10 g ANE in 40 ml methanol for 15 min. The extract was then evaporated to dryness under a stream of nitrogen and resuspended in 10 ml methanol. The resultant solution was filter sterilized using a 0.22-μm SFCA syringe filter and stored in sterile centrifuge tubes (Corning) at 4°C until further use.

For subfractionation, a methanol fraction was prepared as per the above protocol and the dry solids after evaporation of the methanol were resuspended in 50 ml sterile distilled water to give an aqueous suspension. This was subfractionated by sequential extraction with three volumes (75 ml each) of chloroform and subsequently with three volumes (75 ml each) of ethyl acetate. The subfractions were dried under a stream of nitrogen and resuspended in a known volume of methanol.

LC-MS-MS Analysis of GA in ANE

The LC-MS-MS analysis was conducted on a Sciex API-4000 Triple Quadruple Mass Spectrometer fitted with an electrospray ion source and connected directly to an Agilent 1100 Binary HPLC. ANE was prepared for LC-MS-MS analysis using a mixed-mode solid-phase extraction procedure described previously (Dobrev and KamÍnek 2002). The resulting plant growth hormone-containing fractions were analyzed using an LC-MS-MS method in a multiple-reaction monitoring mode (Chiwocha and others 2003).

Nuclear Magnetic Resonance Spectroscopy of Organic Fractions of ANE

The methanol, chloroform, and ethyl acetate fractions of ANE were prepared as described above. Proton nuclear magnetic resonance (NMR) spectra of these fractions were obtained using a Bruker Advance DRX-500 spectrometer (Bruker, Canada Ltd.) at 500.13 MHz. The samples were dissolved in $CDCl_3$ (99.8% D, Sigma Aldrich, Oakville, ON) in a 5-mm NMR tube containing 0.03% of the internal reference trimethylsilyl propionic-2,2,3,3-d_4 acid (sodium salt), at 20°C. The spectrum was referenced to the signal at 7.26 ppm.

Barley Seed Emergence and Seedling Growth Studies

Barley (cv. AC Sterling) was used for greenhouse experiments to study the effects ANE and its organic subfractions on seedling emergence and establishment in vivo. Five barley seeds were planted in plastic pots (8-cm diameter) containing sterile vermiculite at a uniform depth of 2.5 cm from the surface. Each treatment had ten replicate pots ($n = 10$). Pots were irrigated with 50 ml aqueous ANE solution or an aqueous solution of the organic subfractions, each prepared to final concentrations equivalent to 0.1, 0.5, and 1 g l^{-1} of ANE with sterile distilled water. Subsequently, pots were watered daily with 10 ml distilled water. A 16:8 h (light:dark) photoperiod was maintained with a light intensity of 200–300 µmol m^{-2} s^{-1} at 25 ± 2°C and a relative humidity of 75%. Data on seedling emergence were recorded at 12-h intervals following first emergence. Fourteen days after treatment, seedlings were harvested and data on shoot and root length were collected. Harvested plant material was dried at 70°C for 2 days and shoot and root dry weight measurements were recorded.

Starch Zone Clearing Assay for Quantification of Amylase Activity

An in vitro starch zone clearing bioassay was used to quantify the effect ANE had on inducing amylase activity in barley. Barley seeds (cv. AC Sterling) were surface sterilized for 30 min with 5% sodium hypochlorite (NaOCl) solution containing 0.01% sodium dodecyl sulfate (SDS). Seeds were then rinsed with several changes of sterile distilled water, soaked in sterile distilled water for 1 h, and then resterilized with 2.5% sodium hypochlorite solution for 10 min. After rinsing, seeds were cut transversely into two halves such that only one half contained the embryo. Seed-halves were then placed on a pad of sterile wet filter paper to prevent desiccation.

Amylase activity of the barley seed-halves was tested by measuring the area of clear zone produced by degradation of starch in solidified starch medium after treatment with iodine. Briefly, filter-sterilized (0.22-µm SFCA syringe filter) aqueous ANE or organic subfractions were added to autoclaved molten starch medium (0.5% soluble potato starch and 0.5% agar) to final concentrations equivalent to 0.1, 0.5, and 1 g l^{-1} of ANE or 1 g l^{-1} equivalent of methanol, chlorophyll, and ethyl acetate fraction, respectively. Fifteen-milliliter aliquots of the warm media were then transferred into 9-cm petri plates and allowed to cool on a level surface. A uniform thickness of media was essential to obtain reproducible data. The seed-halves without embryos were then placed on the surface of the solidified media so that the cut ends were in contact with the medium. All procedures were carried out under sterile conditions.

Three seed-halves were placed in each petri plate and incubated at room temperature (25 ± 2°C) in the dark for 48 h. Following incubation, seed-halves were removed and the plates were flooded with iodine solution (6 g KI + 0.6 g I_2 dissolved in 1 L of 0.05 N HCl). The iodine reacted with the starch in the medium to give a blue color. A clear zone, indicating the hydrolysis of starch, appeared around the seed-halves that had secreted amylase. The area of the cleared zone correlated directly with the amount of amylase secreted by the seed-halves (Ho and others 1980).

Charcoal Treatment of Extract to Adsorb Organic Components

The ability of activated charcoal to adsorb organic compounds has been used to test the role of organic compounds in eliciting biological responses (Callaway and Aschehoug 2000; Bais and others 2005). To determine if the components of the organic fractions of ANE are responsible for the observed biological activity, the chloroform and ethyl acetate fractions were treated with activated charcoal. A total of 10 ml of the relevant organic fraction was treated with 5 g finely powdered activated charcoal for 15 min with occasional shaking. The solution was then centrifuged at 3000 *g* for 5 min and the supernatant was filter sterilized and stored in sterile centrifuge tubes at 4°C until further

use. The bioactivity was tested using the starch zone clearing assay as described above.

The Effect of ANE and Organic Fraction on Amylase Production in GA-deficient Mutants

The barley parent line cv. Himayala and *grd2* mutant M463 were gifts from Dr. M. P. Chandler, CISRO, Canberra, Australia. Barley M463 is a *grd2* (GA-responsive dwarf) mutant that carries a mutation in the last step of the GA biosynthetic pathway. The *grd2* mutant plants are highly responsive to exogenous application of GA. The starch zone clearing bioassay was carried out as described earlier, using ANE (0.5 g l^{-1}) and the methanol fraction of ANE (1.0 g l^{-1}). Distilled water and 0.1 mM GA were used as controls in the experiment. Petri plates were incubated at room temperature (25 ± 2°C) for 48 h in the dark, stained with iodine solution, and the area of the clear zone was measured.

Confirmatory Test for GA-independent Amylase Activity of ANE

Gibberellins (GAs) and abscisic acid (ABA) play an important and antagonistic role in modulating growth and development of plants (Razem and others 2006). ABA negatively affects expression of α-amylase genes and neutralizes the activity of GA in inducing amylase synthesis and secretion (Jacobsen and others 1995). To test if amylase induction activity of ANE and its organic fraction was due to GAs present in them, the starch zone clearing bioassay was conducted with starch media supplemented with 0.1 mM ABA and 0.1 mM GA_3.

Results

Barley Seed Emergence and Seedling Growth Studies

Barley seeds treated with an aqueous solution of ANE and its organic fractions showed significantly higher percent emergence, shoot lengths, and dry weights (Table 1). Ninety-six hours after planting, seeds treated with ANE showed 87% emergence compared to only 69% in water control. Seeds treated with methanol, chloroform, and ethyl acetate subfractions also showed significantly higher emergence over the control seeds (methanol, 73%; chloroform, 84%; and ethyl acetate, 85%). After 2 weeks of germination, seeds treated with organic fractions of ANE showed increases of 17, 20, and 13% in shoot length for methanol, chloroform, and ethyl acetate fractions, respectively. Our results also indicated that the greater shoot length contributed to a greater dry mass accumulation. The

Table 1 Seedling emergence (after 96 h) and shoot length and dry weight (after 2 weeks) of Barley cv. AC Sterling treated with *A. nodosum* extract (ANE) and organic subfractions of ANE

Treatments	Emergence (%)	Shoot length (cm)	Shoot dry weight (mg)
Control	69 ± 4.9	13.32 ± 1.21	95.9 ± 6.7
ANE	87 ± 4.1	14.49 ± 0.53	108.4 ± 3.2
Methanol fraction	73 ± 6.2	15.59 ± 0.28	134.0 ± 5.2
Chloroform fraction	84 ± 4.5	15.92 ± 0.79	164.9 ± 2.3
Ethyl acetate fraction	85 ± 4.7	15.01 ± 0.59	109.8 ± 4.6

ANE treatment resulted in a 13% greater dry weight, whereas the methanol, chloroform, and ethyl acetate fractions exhibited 40, 72, and 15% increases in shoot dry weight compared to the control seedlings. No significant difference was observed in root growth (root length or dry mass) between treatments. Furthermore, lower concentrations of ANE (0.1 and 0.5 g l^{-1}), showed no significant difference in any of the growth parameters (data not shown). This suggested that the positive responses of ANE and its organic fractions were dose-dependent and that the maximum effect on seed emergence and seedling vigor was at a concentration of 1 g l^{-1} in greenhouse conditions.

Effect of ANE and Its Organic Fractions on Induction of α-Amylase Activity in Barley Seeds in vitro

ANE induced α-amylase activity in barley seeds as observed by an increase in the area of the starch clearing zone. This α-amylase activity was significantly higher ($p < 0.001$) than that of the untreated control (Fig. 1). At a concentration of 0.5 g l^{-1}, ANE showed a 13-fold increase in α-amylase activity; the methanol fraction induced a 7-fold increase in α-amylase activity when tested at the equivalent of 1 g $l^{-1}$of ANE. When compared to the water control, the chloroform and ethyl acetate fractions showed a 4-fold increase in α-amylase activity (Fig. 1). These results suggest that the bioactive compound(s) that induce the α-amylase activity in barley are organic compounds that can be readily extracted into organic solvents.

Confirmation of the Organic Nature of the Bioactive Compounds in ANE Using Activated Charcoal

To test if the ANE-induced α-amylase activity is elicited by organic components, the starch zone clearing assay was carried out with the starch medium supplemented with chloroform and ethyl acetate fractions of ANE that had been treated with activated charcoal. When the charcoal-treated fractions were tested for α-amylase induction activity, they were found to be inactive (Fig. 2). This result suggested that

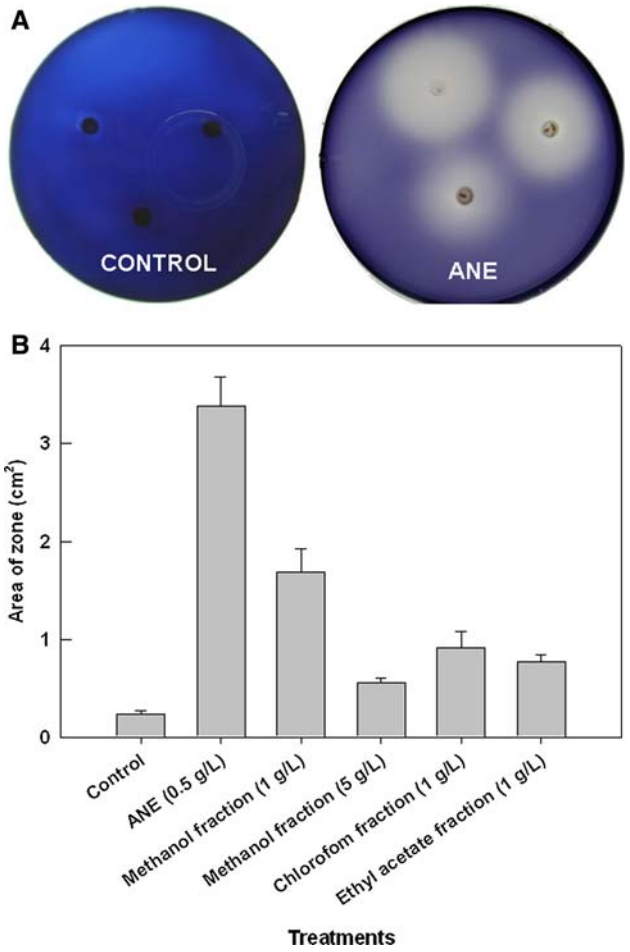

Fig. 1 (**a**) Starch zone clearing assay with *Ascophyllum nodosum* extract (ANE) (0.5 g l^{-1}) and water control. (**b**) Effect of ANE and its organic subfractions in inducing α-amylase activity in barley seed-halves without embryos in the starch zone clearing bioassay. Each value represents an average of 15 replicates. Bars represent standard error

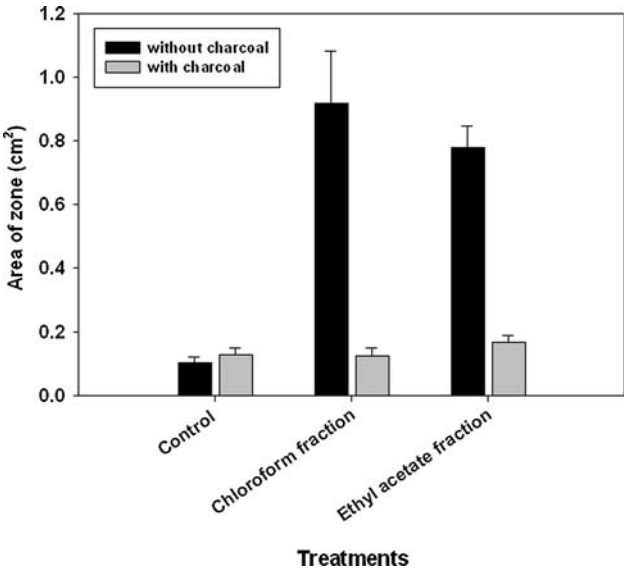

Fig. 2 Comparison of the bioactivity of the subfractions of *Ascophyllum nodosum* extract (ANE) before and after charcoal treatment. Each value represents an average of 15 replicates. Bars represent standard error

the activated charcoal adsorbed the organic compounds present in the chloroform and ethyl acetate fractions that were responsible for the previously observed α-amylase bioactivity. Inspection of the proton NMR spectra of the chloroform and ethyl acetate fractions before and after charcoal treatment supported this hypothesis as the chloroform and ethyl acetate supernatants after the charcoal treatments showed only the peaks responsible for water and the internal standard (Fig. 3). These results indicate that the bioactive compounds of ANE which induce α-amylase activity in barley seeds are organic compounds.

Thermal Stability of Components of ANE that Induce α-Amylase Activity

The thermal stability of the component(s) in ANE that induced α-amylase activity was tested by adding the required concentrations of ANE to the starch media which were subsequently autoclaved at 121°C for 20 min. Using the starch zone clearing bioassay it was determined that the autoclaved ANE showed a reduced level of α-amylase activity compared to ANE that had not been autoclaved. These results indicated that the bioactive compound(s) present in ANE are thermally labile, but the proton NMR spectra of the autoclaved and unautoclaved fractions did not show any difference in peaks (data not shown).

Validation of Function of Bioactive Compounds in ANE Using GA-deficient Mutants

The functional characteristics of the bioactive compounds of ANE that induced α-amylase activity were confirmed using *grd2* (GA-responsive dwarf) mutants, which are naturally deficient in GA production. The starch zone clearing assay with ANE (0.5 g l^{-1}) and its methanol fraction (1 g l^{-1}) showed significantly higher α-amylase activity than the untreated water controls (Fig. 4b). The bioactivity was standardized with the *grd2* parental line cv. Himalaya. ANE (0.5 g l^{-1}) and its methanol fraction (1 g l^{-1}) were found to induce α-amylase activity in both *grd2* mutant and wild-type barley seed-halves. Chromatogram analysis showed that ANE does not have a significant amount of GA_3 and any amount present may be below the detectable limit of a standard LC-MS-MS. These results support the hypothesis that the bioactive compound(s) present in ANE are capable of reverting the abnormalities caused by GA deficiency in *grd2* mutants. When *grd2*

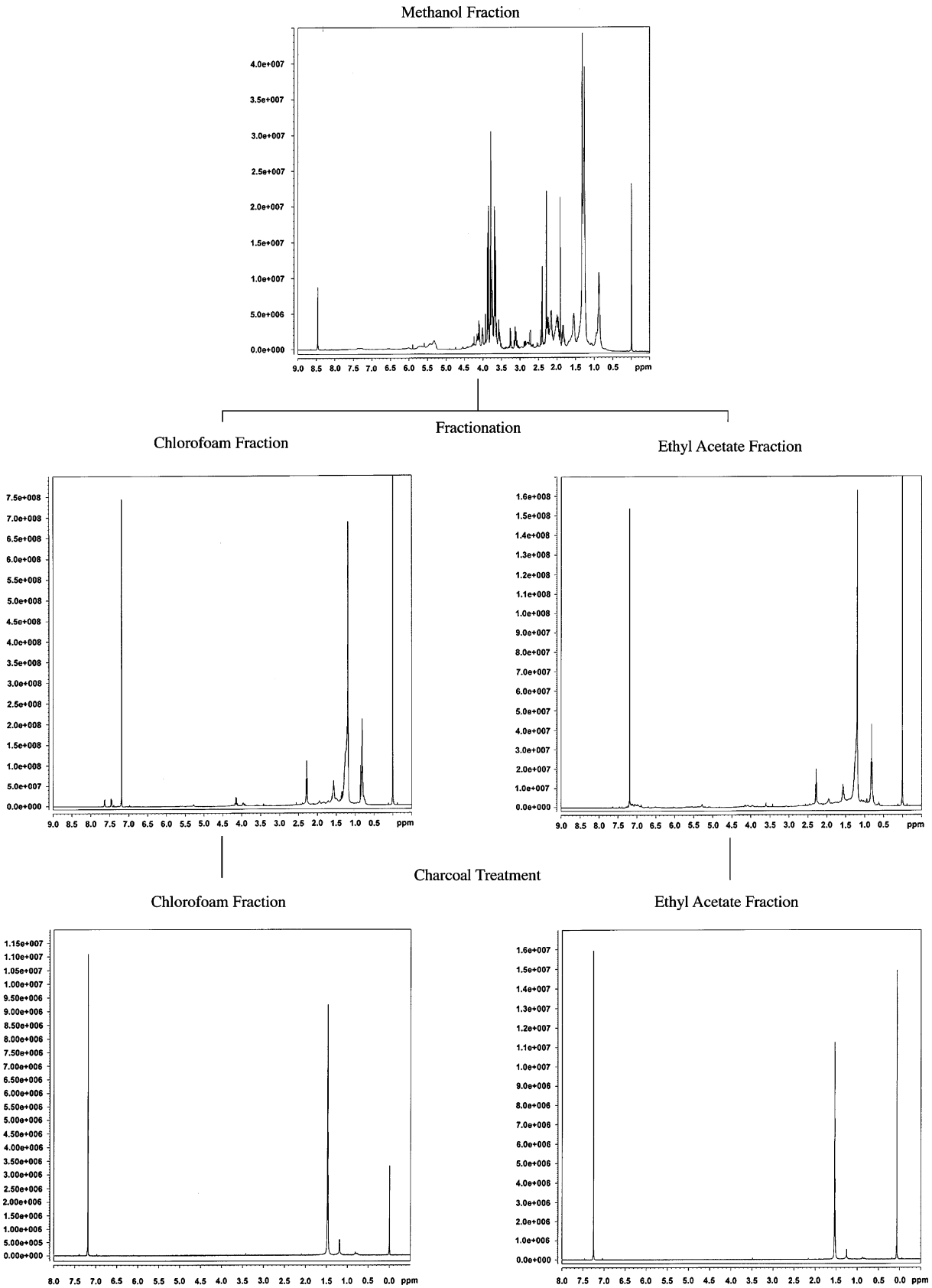

Fig. 3 Proton NMR spectra of the methanol organic extract of *Ascophyllum nodosum* extract (ANE) and the chloroform and ethyl acetate extracts before and after charcoal treatment

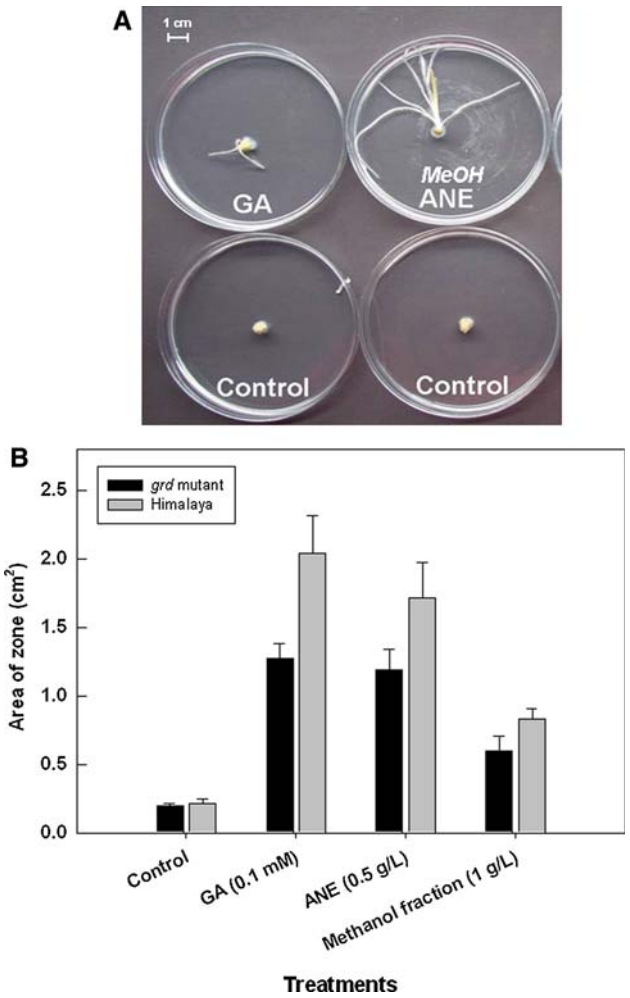

Fig. 4 (**a**) Growth response of *grd2* barley mutant seed-halves with embryos to methanol extract of *Ascophyllum nodosum* extract (ANE) (1 g l^{-1}) compared to GA (0.1 mM) and water controls. (**b**) Effect of ANE (0.5 g l^{-1}) and its methanol fraction (1 g l^{-1}) in inducing α-amylase activity in *grd2* (GA-responsive dwarf) mutant as compared to GA_3 (0.1 mM) and water controls. Each value represents an average of 10 replicates. Bars represent one standard error

mutant seed-halves containing embryos were treated with methanol fractions of ANE (1.0 g l^{-1}), embryo germination was restored and root growth was significantly increased compared to GA_3-treated seeds. The control seeds showed no embryo germination or root growth (Fig. 4a).

Confirmatory Test for GA-independent α-Amylase Activity of ANE

Previous reports have shown that ABA neutralizes the activity of GA in inducing α-amylase synthesis and secretion in cereals (Karssen and others 1983; Koornneef and Karssen 1994; Lovegrove and Hooley 2000). To confirm the mode of action of the bioactive compounds of

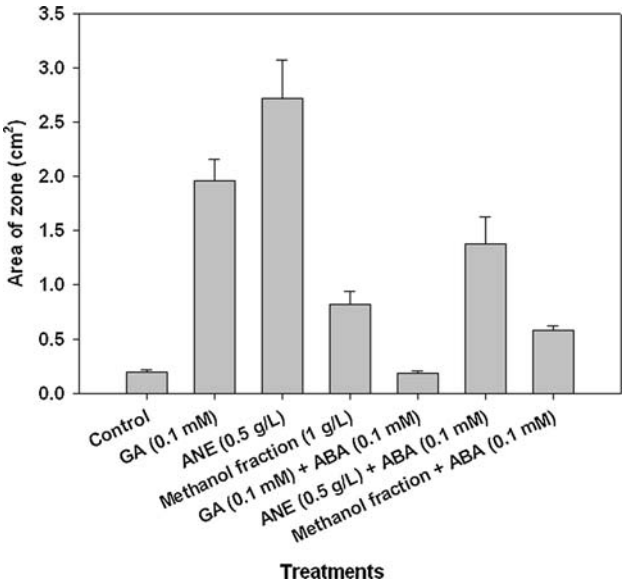

Fig. 5 Differential response of barley seed-halves to *Ascophyllum nodosum* extract (ANE) (0.5 g l^{-1}) or its organic subfractions (1 g l^{-1}) and GA_3 (0.1 mM) in inducing α-amylase in the presence of 0.1 mM ABA. Each value represents an average of 15 replicates. Bars represent standard error

ANE, ABA was used to nullify the effect of GA. When ANE (0.5 g l^{-1}), its methanol fraction (1.0 g l^{-1}), and GA (0.1 mM) were tested individually for activity in the starch zone clearing assay, a significant increase in α-amylase activity was observed (Fig. 5). When the same experiment was conducted with media supplemented with 0.1 mM ABA, no significant α-amylase activity was observed for GA, whereas the ANE and its methanol fraction retained bioactivity. Interestingly, ANE showed a higher level of α-amylase induction than the methanol fraction of ANE in both the cases. These findings suggest that the bioactive components present in ANE are not GAs and that these GA-like compounds exhibit a GA-independent activity as the presence of ABA had no effect on its bioactivity.

Discussion

The positive effect seaweed extracts have on crop growth, yield and quality, pest and disease resistance, and environmental stress tolerance have been previously reported (Featonby-Smith and van Staden 1987; Crouch 1990). Application of a commercial formulation of seaweed concentrate prepared from *A. nodosum* (Maxicrop Triple®, Maxicrop International, Lysaker, Norway) to the roots of hydroponically grown barley or applied as a spray were shown to increase growth by 56–63% over the control (Steveni and others 1992). This enhanced-growth effect is thought to be due to various organic compounds present in

the seaweed extract. More specifically it is thought to be due to the presence of phytohormones, mainly cytokinins, in the seaweed extracts. Wrightman and Thimann (1980) had observed root growth-promoting effects of cytokinins at concentrations ranging between 10^{-6} and 10^{-8} M. The findings of Steveni and others (1992) confirmed that Maxicrop Triple® had cytokinins within that range. They also proved that the effect of the seaweed extract, irrespective of whether it was applied as a foliar spray or supplemented in hydroponic solution, was independent of the addition of macro- and microelements (Steveni and others 1992). The bioactive compound(s) of *A. nodosum* that were responsible for improving the emergence and growth of barley seedlings have not been determined as yet. Recently, using an *Arabidopsis thaliana* model, we reported that plants treated with *Ascophyllum nodosum* extracts showed improved root and shoot growth over control plants (Rayorath and others 2008).

Our initial greenhouse studies indicated that ANE and its organic fractions and subfractions improved seed emergence, seedling vigor (exhibited by higher shoot length), and biomass accumulation (indicated by higher dry weight) in barley. Ninety-six hours after planting, seeds treated with ANE or its organic subfraction showed higher levels of emergence over control seedlings. There was a similar increase in shoot length after 2 weeks, which in turn was directly reflected in total biomass accumulation of plants treated with ANE and its organic fractions. Early seed emergence and improved seedling vigor have a large impact on seedling establishment, growth, and development. Early emergence assists the plant in better establishment in the field through swift transformation from a heterotroph, which relies upon stored food reserves, to an autotroph with functional photosynthetic machinery.

To minimize the number of variables and closely monitor the mode of action of the ANE, an in vitro starch zone clearing bioassay was developed. Using this assay, it was clearly demonstrated that ANE and its organic fractions induce α-amylase synthesis and secretion over untreated controls. The results indicated that barley seed-halves without embryos were more responsive to lower concentrations of ANE than higher concentrations. Overall, a greater in vitro response was observed at lower concentrations of ANE (0.5 g l^{-1}) or its methanol fractions (1 g l^{-1}) than the higher concentrations of ANE (1.0 g l^{-1}) or its methanol fraction (5 g l^{-1}) (Fig. 1). These findings were in agreement with those of a previous study (Economou and others 2007), wherein a higher concentration of *A. nodosum* extracts showed an inhibitory effect on seed germination of *Orobanche ramosa*. The difference in response may be because these bioactive compounds perform better at optimum concentrations and possess an inhibitory effect at high concentrations. This is typical of the mode of action of currently known phytohormones (Kende and Zeevaart 1997).

Significant α-amylase activity was retained in the methanol fraction of ANE and the subsequent chloroform and ethyl acetate subfractions of the methanol fraction. The organic nature of the bioactive compounds in ANE responsible for α-amylase activity was confirmed by charcoal adsorption. When the chloroform and ethyl acetate subfractions were treated with activated charcoal, the resulting supernatants failed to induce significant α-amylase activity, indicating that the bioactive compounds of ANE are readily adsorbed by activated charcoal. These results and proton NMR spectra of the relevant ANE fractions confirm the organic nature of the bioactive compounds that induced α-amylase activity in barley.

Gibberellins are involved in a range of developmental processes in plants such as dormancy, germination, stem elongation, flowering, sex expression, and senescence (Davies 1995). During seed germination, gibberellic acid (GA_3) induces the synthesis and secretion of α-amylase that breaks down starch reserves in the endosperm into simple sugars, providing energy to the growing embryo. In monocots, GA_3 transported from the embryo to the aleurone layer of the endosperm activates the gene encoding α-amylase in the aleurone cells. The enzyme is largely responsible for starch hydrolysis in the endosperm and represents as much as 70% of the newly synthesized protein in germinating barley aleurone cells (Jones and Jacobsen 1991).

Experiments with the GA-deficient barley mutant *grd2* (GA-responsive dwarf) showed that ANE and/or its organic fractions contain bioactive compounds that are functionally analogous to GA_3. The mutant plants were highly responsive to ANE application and produced a similar effect to that of the positive GA controls (Fig. 4). LC-MS-MS analysis failed to detect the presence of GA_3 in ANE. This suggests that some compound(s) present in ANE other than GA is(are) responsible for the elicited α-amylase activity observed in barley seed-halves.

It is well known that α-amylase activity in cereals is controlled by GAs (Fincher 1989) and any variation in α-amylase activity between varieties may occur due to differences in GA_3 sensitivity. ABA, however, blocks the production of α-amylase and suppresses seed germination (Karssen and others 1983; Koornneef and Karssen 1994; Lovegrove and Hooley 2000). It has been reported that ABA can have an antagonistic effect on the activity of α-amylase genes (Jacobsen and others 1995). Although GAs promote the germination and postgermination processes, such events could be inhibited by ABA (Bethke and others 1999).

The confirmatory experiment for GA-independent activity of ANE showed that the ANE-treated barley seed-

halves exhibited α-amylase activity even in the presence of 0.1 mM ABA. The GA-treated positive controls completely lost their ability to induce α-amylase activity in the presence of ABA (Fig. 5). It was evident that 0.1 mM ABA neutralized the effect of GA but had no effect on the activity of ANE or its organic fractions (Fig. 5). These findings suggest that the bioactive compound(s) present in ANE is(are) not a GA because the presence of ABA had no effect. This suggests that the bioactive components in ANE act via a GA-independent pathway.

The existence of GA-like compounds has been reported previously (Chin and Beevers 1970; Rood and others 1983; Abad Farooqi and others 1994). Gibberellins are biosynthesized via diterpene hydrocarbon intermediates after a series of oxidations and rearrangements (Mander 1992). The presence of "GA-like substances" has been shown to be related to diterpene hydrocarbon levels in the foliage of the gymnosperm *Cryptomeria japonica* D. Don (Ogiyama and Pharis 1980). Because a variety of diterpene hydrocarbons are found in many marine algae (Schmitt and others 1998; Barbosa and others 2004), it is likely that novel compounds with GA-like activity might be present in ANE. Further studies should be conducted to delineate the mode of action of these compounds, and additional information about the chemical nature of these compounds will provide a better understanding of the role they play in plant growth and development.

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