Enhancement of phenolic and flavonoid compounds in cabbage (*Brassica oleraceae*) following application of commercial seaweed extracts of the brown seaweed (*Ascophyllum nodosum*)

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Brassica crops are rich is phytochemical compounds and frequent consumption of these vegetables has been associated with a lower risk in cancer, heart disease, hypertension and stroke. The effect of three commercial extracts of the brown seaweed, *Ascophyllum nodosum*, on phytochemical content and yield in cabbage plants was tested under field conditions in two consecutive crops. Total phenolic content was higher in all seaweed treated plants, with the highest increase recorded with AlgaeGreen™ (3.5 l ha^{-1}) with a 2 fold increase relative to the control. The other commercial seaweed extract, XT achieved a lower increases of 1.3 fold (3.5 l ha^{-1}). Similar increases were recorded in total flavonoid content. No statistically significant increases in yield were recorded with any of the seaweed extracts tested. The results suggest that seaweed extracts stimulated an increased accumulation of phytochemicals in cabbage but had no significant effect in yield under these experimental conditions.

Key words: Seaweed, total phenolics, flavonoids, cabbage, phytochemicals, *Ascophyllum nodosum*

Introduction

There is an increasing demand by consumers for the production of high value and healthy vegetables whose production has minimum impact on the environment (Spinelli et al. 2010). These attributes are particularly important with horticultural crops that receive relatively large amounts of external inputs, in particular the application of inorganic fertilisers and plant protection products. In addition, excess of nutrient input in the soil can result in environmental problems such as eutrofication in sea and lakes (Spinelli et al. 2010). Biostimulants include diverse formulations of compounds, substances and other products that are applied to plants or soils to regulate and enhance the crop's physiological processes, thus making them more efficient. These biostimulants act on plant physiology through different pathways than nutrients to improve crop vigour, yield, quality and post-harvest shelf life (Vernieri et al. 2006, European Biostimulants Industry Council 2012).

Seaweed extracts are biostimulants that have been traditionally used as soil conditioners in improving plant growth in agricultural crops (Hurtado et al. 2009). Numerous studies have revealed the benefits of seaweed extracts on plants including early seed germination, improved crop performance and yield, enhanced shelf life and better resistance to biotic and abiotic stress (Norrie and Keathley 2006, Eyras et al. 2008, Jayaraman et al. 2011). Seaweeds have received increased interest as promising products for providing both novel biologically active substances and essential compounds for human nutrition, with high potentially economical impact in food and pharmaceutical industry (Norrie and Keathley 2006). Recent studies have shown that cotton seeds soaked in seaweed solution (1:500 *Sargassum wightii* for 12 h) provided seedlings with considerable resistance against *Xanthomonas campestris (*Raghavendra et al. 2007). An even greater resistance was provided when seed treatment was combined with spraying (up to 74%). Seaweed extracts have also been found to have an impact on nematode populations in soil as well as insect pests such as aphids and spider mites (Khan et al. 2009). Many of these commercial extracts are derived from the brown alga *Ascophyllum nodosum*, which grows abundantly in France, Ireland, Norway, UK and Canada. In recent years there has been an increased interest in *A. nodosum* due to its unique nutraceutical properties (Apostolidis and Lee 2011). *A. nodosum* contains several bioactive compounds such as polysaccharides, vitamins, antioxidants, peptides, and secondary metabolites (e.g., phlorotannins) (Kandasamy et al. 2012). Current uses of *A .nodosum* include use as a source of alginates, fertilisers, but also in animal and human consumption (Apostolidis and Lee 2011, FAO 2011). *A. nodosum* extracts are a source of plant growth regulator-like compounds (Jameson 1993), organic osmolites, minerals, vitamins and amino acids (Jameson 1993, Spinelli et al. 2010). There are a number of reports that indicate enhanced yield and health of crops sprayed with seaweed

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extracts (Colapietra and Alexander 2006, Rayorath et al. 2008, Jayaraman et al. 2011). Seaweed extracts can also improve nutrient availability and productivity and can be used as foliar or soil applications (Aziz et al. 2011). Seaweed products can be found in their pure form (such as two of the extracts used in this study), or in formulations with or without the addition of other nutrients (Aziz et al. 2011). These extracts have also been shown to improve stress tolerance in many plant species through an increase in the concentration of bioactive molecules including antioxidants in treated plants (Rayirath et al. 2009, Fan et al. 2011). However the mode of action of seaweed extracts on plant growth and stress tolerance is not well understood (Mercier et al. 2001, Aziz et al. 2011).

Phytochemicals are compounds that possess antioxidant, antimicrobial and anticancer activities (Gupta and Abu-Ghannam 2011, Picchi et al. 2012). Plants in the genus *Brassica* are associated with prevention of carcinomas, especially of stomach, colon and recto, and with the prevention of cardiovascular diseases (Sousa et al. 2009). Epidemiological studies have shown that ingestion of three or more half cup serving, with a cup being the equivalent of 80 g of vegetables (Agundo 2005) such as broccoli or cabbage per week can significantly reduce the risk for prostate cancer by 40% when compared to one or less servings per week (Jeffery et al. 2003). These properties are attributed to the presence of phytochemicals and especially to the glucosinolates and their derived break down products, isothiocyanates, which have been shown to be anti carcinogenic (Jeffery et al. 2003, Sousa et al. 2009). An increase of the phenylopropanoid metabolism and the amount of phenolic compounds can be observed under different environmental factors and stress conditions (Michalak 2006). The synthesis of some flavonoids is induced when plants are infected, injured (Takahama and Oniki 2000) or under low nutrient conditions (Michalak 2006). In general, phytochemicals are involved in defence against an array of biotic and abiotic stresses such as drought, ultraviolet radiation, pathogen and insect attack (Dai and Mumper 2010).

The aim of this study was to investigate and compare the effect of three commercially available seaweed products on the phytochemical content and yield of cabbage on two consecutive crops, a winter and spring cabbage planting. We are reporting initial results on two differently processed seaweed extracts. AlgaeGreen™ (AG) is produced with the ``cell burst'' technology, where the algal cells are ruptured using high pressure and low temperatures, while XT (Brandon Products Ltd) is produced by heating the seaweed at high temperatures with alkaline sodium or potassium solutions.

Materials and methods

Experimental site

The experiment was conducted at a site in Kinsealy (53º 25' N Lat 6 º10' W), located in north county Dublin, Ireland. Soil type was characterised as loam to clay loam belonging to the grey brown podzolic soil group. (Altitude: 28 metres O.D., Slope: 1º, Drainage: Moderately well drained).

Experimental setup

Cabbage seeds (cv. 'Caraflex') were placed in cell trays (23x23x35mm) containing the potting mix Shamrock compost (peat 0–14 mm, N,P,K and pH 5.3–5.7) and sown at 1.5 cm deep. Cell trays were placed in an unheated greenhouse and were grown for 7–10 weeks prior to planting in the field. The experiment was repeated in two consecutive pointed cabbage crops, a winter and spring crop and was a complete randomised block design with 5 replications. Each experimental unit (plot) consisted of 30 plants (13 treatment plants row^{-1} and 4 buffer plants). Cabbage heads from all experimental plants (26 per plot) were collected for assessment. Each experimental plot was 6.8 m long, distance between the plants was 45 cm. All cabbage harvested in each treatment was used to assess total yield. For phenolics and flavonoid content analysis cabbage heads were subsampled from each replication, immediately after weighing, by randomly choosing three healthy cabbage heads from each plot. Cabbage heads were harvested 15 days after the last seaweed application. The selected cabbage samples were placed in Ziplock bags (Sparks) and stored at −20 °C until further use. Spray applications were conducted with a spray hood when necessary. Seaweed treatments were applied once a month with a calibrated knapsack sprayer (Solo 485). Each seaweed treated plot received 2.6 ml of seaweed extract for the 3.5 l ha^{-1}, 3.7 ml for the 5 l ha^{-1} application rate. Control plots received only water. Each of the seaweed application rates were diluted in equivalent water based on the knapsack calibration. The seven treatments were: control (water), XT 3.5 l ha^{-1}, XT 5 l ha^{-1}, AG1 3.5 l ha^{-1}, AG1 5 l ha^{-1}, AG2 3.5 l ha^{-1}, AG2 5 l ha^{-1}. The first crop was planted on the 28 September and cabbage was harvested in mid June 2011. The second crop was planted on the 14 July 2011 and harvested in October 2011.

Fertiliser and pesticide application

Fertilisers were applied at the following rates: 50 kg ha^{-1} N, 60 kg ha^{-1} P and 250 kg ha^{-1} K. The herbicide pendimethalin (Stomp Aqua) was applied on the 27 September at the recommended rate of 3.3 l ha^{-1}. Prior to planting, trays were drenched with chlorpyrifos (Dursban) for cabbage root fly control, at the manufacturers recommended rates. In the second crop, fertiliser rates, chlorpyrifos (Dursban) and pendimethalin (Stomp Aqua) were the same as above. Plants were sprayed with deltamethrin (Decis) (29/8/1) for caterpillars, glufosinate ammonium (Basta) (7/9/11) between the beds for weeds and pirimicarb (Aphox) (23/9/11) for aphids, at the manufacturers recommended rates. Two different commercial seaweed extracts formulations of AlgaeGreen™ were used (Oilean Glas Teo Ltd, Donegal, Ireland) and XT, an alkaline extract (Brandon Products, Ltd).

Total phenolics

The total phenolic content (TPC) was analysed using the Folin-Ciocalteu method (Singleton and Rossi 1965). Briefly methanolic extracts of cabbage tissue were prepared as follows: cabbage tissue was ground into fine powder with liquid nitrogen, 0.5 g of ground tissue was placed in a 50 ml falcon tube where 5 ml of 80% Methanol (Lennox) was added. Tubes were vortexed and left to stand for 20 minutes. The tubes were then mixed by inversion and 2 ml of the suspension was transferred into a clean 2 ml micro-centrifuge tube (Anachem). The tubes were centrifuged at 12,000 rpm at 4 °C for 5 minutes. The supernatant was placed in a clean eppendorf tube and stored at −20 ºC until further use. For measurement of total phenolics samples were prepared as follows: in a 2 ml micro-centrifuge tube 150 µl of extract, 150 µl of 80% MeOH, 150 µl of Folin-Ciocalteu reagent (Sigma-Aldrich) and 1050 µl of 20% Na_2CO_3 (Lennox) were placed. Tubes were vortexed immediately and placed in the dark for 20 minutes. They were then centrifuged at 13000 rpm for 3 minutes. Absorbance readings were taken with a spectrophotometer at 735 nm (Jenway 6300). Gallic acid was used as a standard and the results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g FW. A calibration curve was prepared.

Total flavonoids

Total flavonoid content (TFC) of fresh cabbage methanolic extracts was spectrophotometrically determined by the aluminium chloride method using catechin as standard, modified from (Zhuang 1992). Briefly 150 µl of methanolic extract, prepared as above, were mixed with 600 µl of H_2O and 45 µl of 5% $NaNO_2$ (Sigma-Aldrich). The solution was incubated for 5 minutes at room temperature and then 45 µl of 10% $AlCl_3$ were added (Sigma-Aldrich) and incubated for one more minute. Finally 300 µl of 1M of NaOH (Sigma-Aldrich) and 300 µl of H_20 were added. Samples were measured with a spectrophotometer at 512 nm (Jenway 6300) and expressed as mg catechin equivalents l^{-1}. A calibration curve was prepared.

Yield

After maturation cabbage was harvested and total weights were recorded. Immediately after weighing, subsamples were set aside for phytochemical analysis. Three healthy cabbages were randomly selected, which were cut in four quarters, from which only one (the same from each sample) was kept for analysis. They were then placed in ziplock bags (Sparks) and stored at −20 ºC until further use.

Statistical analysis

All data are expressed as mean ± standard deviation unless otherwise stated. Data were analysed using ANOVA and Friedman's test (Minitab 15). A significant difference was considered at the level of $p < 0.05$ or $p < 0.01$.

Results

Total phenolics

Results presented in Figure 1 show that in the first crop there were significant differences in the total phenolic content between the control and the seaweed extract treated plants (Friedman, df=6, $p<0.0001$). Statistically significant differences between the seaweed extracts were recorded only for the 5 l ha^{-1} application rate of the extract AG1 (Friedman, df=5, $F<0.03$). Similarly in the second crop (Fig. 1), there were statistically significant differences between the control and the seaweed treated plants (Friedman, df=6, $p<0.008$). There were significant differences at the lower application rate between the AG and the XT treatments (ANOVA, df=5, $p< 0.02$), with both AG products resulting in a higher concentration in total phenolics. There were no statistically significant differences at the higher application rate between the different seaweed products tested (ANOVA, df=5, $p<0.54$), the only differences recorded were between the untreated control and the seaweed treatments.

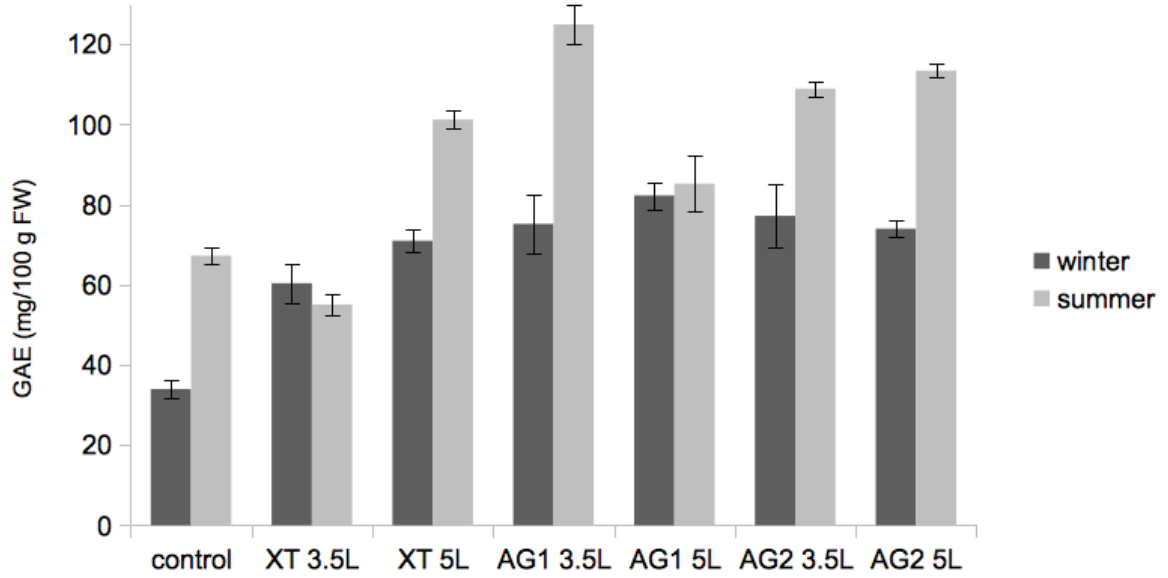

Fig. 1. Effect of three commercial liquid seaweed extracts (XT, AG1 and AG2) on total phenolic content on two consecutive cabbage crops. Treatment plants received two different seaweed application rates of 3.5 and 5 l ha^{-1}. Bars show the average of 5 replications ±SD. Results are expressed in Gallic Acid Equivalents (GAE mg/100FW).

Total flavonoids

In the first crop (Fig. 2) there were no statistically significant differences between the different treatments (Friedman, df=6, p<0.06), although the average content in flavonoids in the control plants was much lower when compared to the seaweed treated plants. There were also no statistically significant differences between the different seaweed extracts. In the second crop (Fig. 2) we recorded statistically significant differences between the different treatments (Friedman, df=6, p<0.0001). At the lower application rate there were significant differences only between the two different OGT products (Friedman, df=5, p<0.02) but no statistically significant differences were detected with XT. Similarly at the higher application rate no statistically significant differences were detected either (Friedman, df=5, p<0.5).

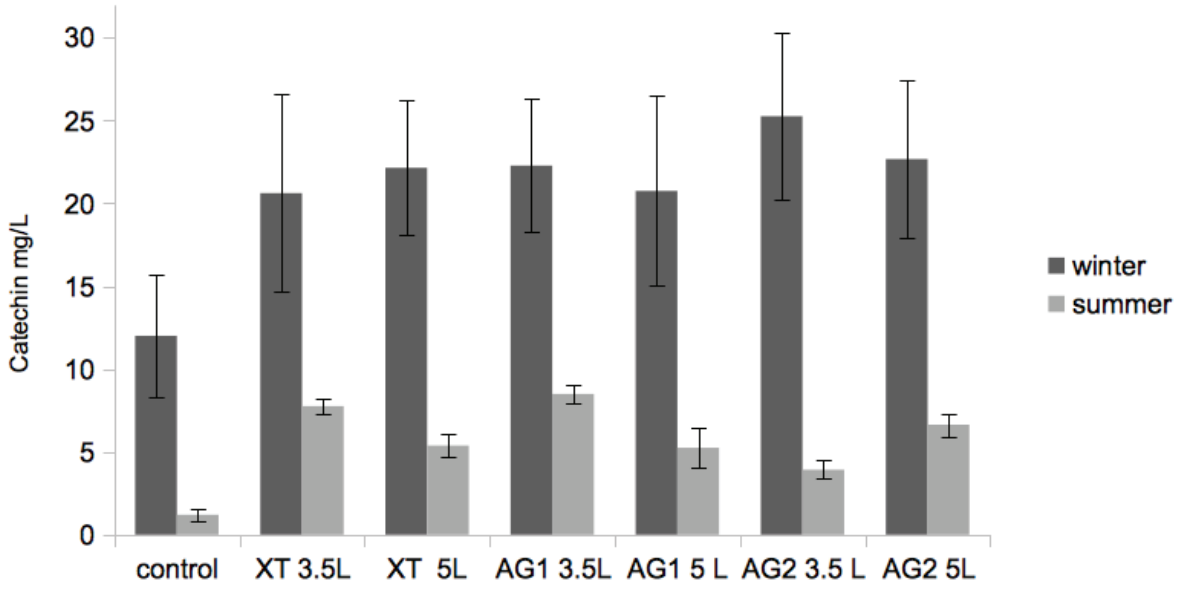

Fig. 2. Effect of three commercial liquid seaweed extracts (XT, AG1 and AG2) on total flavonoid content on two cabbage crops. Treatment plants received two different seaweed application rates of 3.5 and 5 l ha^{-1}. Bars show the average of 5 replications ±SD. Results are expressed as mg catechin equivalents l^{-1}.

Yield

Data presented in Figure 3 shows that in both years there was no significant difference in yield recorded between the different seaweed treatments and the control (Friedman, df=6, p<0.7 (Fig. 3) and Friedman, df=6, p< 0.6, (Fig.3).

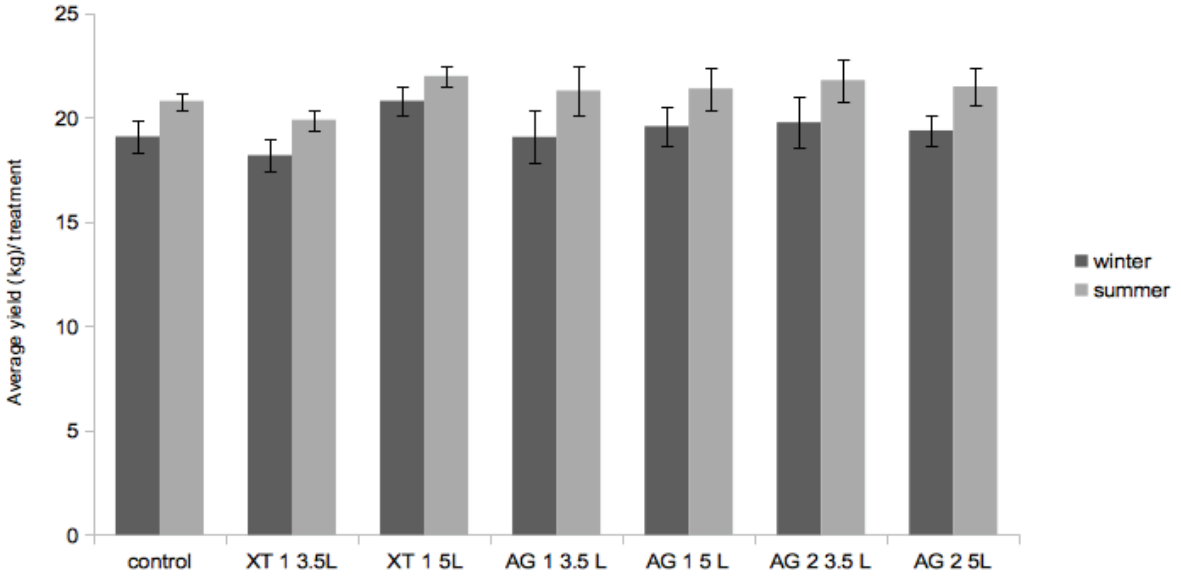

Fig. 3. Effect of three commercial liquid seaweed extracts (XT, AG1 and AG2) on the average yield of two cabbage crops. Treatment plants received two different seaweed application rates of 3.5 and 5 l ha^{-1}. Bars show the average of 5 replications ±SD. Results are expressed in kg

Discussion

Total phenolics

The quantity of health promoting compounds in cabbage plants treated with three different commercial seaweed liquid products, with different extraction processes (cold and heat extraction) was investigated in the present study. Cabbage plants sprayed with seaweed extracts showed an increased phytochemical content in both crops compared to the non treated water control. Phytochemicals, particularly polyphenols, have high free radical scavenging activity, which helps to reduce the risk of chronic diseases, cancer and age related neuronal degeneration (Lako et al. 2007, Teow et al. 2007, Picchi et al. 2012). Post harvest assessment of cabbage plants receiving 3.5 and 5 l ha^{-1} liquid seaweed, showed a significant increase in total phenolic content (Fig. 1). All three seaweed products increased total phenolic content with AlgaeGreen™ (AG1) achieving an average 2 fold increase in total compared to the water only control at the lower application rate of 3.5 l ha^{-1}. XT achieved a 1.3 and 1.8 fold increase for the 3.5 l ha^{-1} and 5 l ha^{-1} application rates respectively. Similar results are reported by Fan et al. (2011) who applied seaweed extracts (*A. nodosum*) on spinach plants at the rate of 1 and 5 g l^{-1}. Del Rio et al. (2003) and Botía et al. (2001) report a similar increase in phenolics, where they found that the use of 0.3 % Brotomax ® (plants extracts plus 5% urea, 1.7% Mg and 0.5% Zn) also increased phenolic composition in olive fruits and leaves. The seaweed extracts used in this study, although they are all manufactured from *A. nodosum* the processing method is very different and this should be taken into account when assessing their efficacy. AG is produced using the cell burst technology (cold extraction) while the XT extract is produced in the more traditional way of heating the seaweed at very high temperatures with alkaline sodium or potassium solutions. Our results are in agreement with (Guo et al. 2011) who also report an increased phenolic content in broccoli sprouts as a direct result from mannitol application (a component of seaweed extracts). Cold process seaweed extracts are naturally higher in antioxidants and mannitol, a sugar alcohol that evaporates when exposed to high temperatures. This can partly explain the increase in phenolic compounds observed with AG as well as the fact that secondary metabolites remain intact which would otherwise be lost as they are heat sensitive.

Total flavonoids

In order to further investigate the quality of phenolics we also measured the concentration of flavonoids. A similar trend of increased total flavonoids on plants that have received application with seaweed extracts was observed with an increase of 2 fold recorded for the XT extract and between 2.2−2.5 fold increase for the AG products in both crops. Flavonoids are most widely found in fruits and vegetables and they tend to be potent antioxidants with quercetin inhibiting the proliferation of leukemia and tumour production (Simon 2002). Naguib et al. (2012) also report on an increase on total flavonoid content of broccoli plants receiving bio-organic fertilisers (50% *Azotobacter chrococcum* and 50% *Bacillus megaterium*).

Yield

Foliar application of *A. nodosum* on cabbage plants did not significantly increase total yield when compared to the control. We only recorded a minor increase in the second year, with the XT extract (Fig. 3). There are a number of studies indicating that application of seaweed extracts increases yield of vegetable and fruit crops. Bozorgi 2012 recorded an increase in aubergine plants both in terms of fruit yield and number of fruits per plant when plants were sprayed with an *A. nodosum* extract at the rates of 1 and 2 g l^{-1}. Similar results are reported by Yazied et al. (2012), who also recorded an increase in yield when *A. nodosum* was applied on bean plants at the highest rate of 750 ppm. Shehata et al. (2011) reports that application of seaweed extract, a mixture of *A. nodosum*, *Laminaria* and *Sargassum* significantly increased yield in celeriac plants, although the product they used was a mixture of three different seaweeds and was also enriched with plant hormones, nitrogen, calcium, manganese, iron and alganic acids, at a rate of 1000 and 2000 ppm. Similar results are reported by Zodape et al. (2011) who recorded a 61% increase on tomato yield when plants were sprayed with *Kappaphycus alvarezii* (5−15% concentration). On another study where an extract from *Eklonia maxima* was used in tomato, a 10% increase in total fruit number was recorded although it was not statistically significant from the control (Crouch and Staden 1992). These results do not agree with our observations on cabbage where we found no differences in yield between the control and seaweed treated plants. This could be due to the fact that pure cold seaweed extracts, as two of the extracts tested in this study are not complemented with NPK or plant hormones. In addition pure extracts are mainly used to help plants to better respond to either biotic or abiotic stress.

Conclusions

In the present study we used three different commercial seaweed extracts two different AlgaeGreen™ products (OGT) which is a pure *A. nodosum* extract and XT, another commercial seaweed extract also based on *A. nodosum.* Results from this study indicate that application of pure seaweed extracts can potentially increase the phytochemical content in cabbage. All seaweed products tested increased both the total phenolic and flavonoid content in cabbage but with some differences in doses/effects. Our results indicate that regular application of seaweed extracts can significantly increase the total phenolic and flavonoid content in cabbage plants, without the need of costly plant breeding programmes. Although there is a plethora of studies indicating the positive effects of seaweed extracts on yield increase and quality of vegetables, the exact compound within seaweed extracts that elicit the phenylpropanoid and flavonoid pathways in plants is not yet known (Fan et al. 2011). The elucidation of the mechanisms by which seaweed extracts exert such increases in phytochemical content in plants is of great interest and requires further research.

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