International Journal of Agricultural Technology 2022Vol. 18(5):2123-2134
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Cellulase-enhanced fermented edible seaweed extracts as liquid organic fertilizers for lettuce (*Lactuca sativa* L. var. crispa)

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Opeña, J. M., Pacris Jr., F. A. and Pascual, B. N. P. (2022). Cellulase-enhanced fermented edible seaweed extracts as liquid organic fertilizers for lettuce (*Lactuca sativa* L. var. crispa). International Journal of Agricultural Technology 18(5):2123-2134.

Abstract The utilization of seaweeds as an agricultural bioresource such as liquid organic fertilizers posed opportunities in organic farming due to their potential plant growth-promoting contents. The Philippines has numerous species of edible seaweeds such as *Gracilaria bursa-pastoris, Acanthophora spicifera, and Sargassum ilicifolium* which served to be fertilizer potential in high value crops. The three edible seaweed species were formulated into cellulase-enhanced fermented extracts and applied to lettuce as liquid organic fertilizers. Results showed that lettuce applied with *G. bursa-pastoris, A. spicifera,* and *S. ilicifolium* extracts produced longer plants (52.60 mm, 28.56 mm, and 54.90 mm), heavier fresh weights (33.30 g, 20.28 g, and 19.74 g), and heavier dry weights (4.85 g, 2.47 g, and 3.42 g), respectively as compared to the unfertilized plants. Moreover, higher nitrogen, potassium, and iron contents were detected in the lettuce plants applied with *G. bursa-pastoris* (0.11% N, 0.60% K, and 1.38 mg kg^{-1} Fe), *A. spicifera* (0.59% N, 0.56% K, and 0.89 mg kg^{-1} Fe), and *S. ilicifolium* (0.94% N, 0.44% K, and 1.32 mg kg^{-1} Fe) extracts as compared to unfertilized plants. The promotive effects of cellulase-enhanced fermented *G. bursa-pastoris, A. spicifera,* and *S. ilicifolium* extracts were due to their nutrient and possible phytohormone contents. Results indicated that the fermented seaweed extracts can be applied as an alternative organic liquid fertilizers in growing lettuce.

Keywords: *Acanthophora spicifera, Gracilaria bursa-pastoris,* Growth, Nutritional content, *Sargassum ilicifolium*

Introduction

Coasts in the Philippines are very rich in edible seaweed species. *Porphyra sp.* (Rhodophyta) is a favorite edible seaweed along the Northern Luzon coasts and *Caulerpa racemosa* (Chlorophyta) is much preferred in the Southern part (Moreland, 1979). In the northernmost part of Luzon specifically in the Ilocos and Cagayan Valley regions, the seaweed farming industry promotes the growing of *Gracilaria sp.* (Rhodophyta) in seaweed nurseries (BFAR-2, 2018;

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Dilan, 2018), and the species were utilized in the manufacturing of carrageenan, alginates, and agars (McHugh, 2003). Some of the edible seaweed species growing on the coasts of northern Luzon, Philippines include *Gracilaria bursa-pastoris* (S.G. Gmelin) P.C. Silva (formerly *Gracilaria compressa* (C. Agardh) Greville), *Acanthophora spicifera* (M. Vahl) Borgesen (Rhodophyta), and *Sargassum ilicifolium* (Turner) C. Agardh (Phaeophyceae). They are utilized as food; however, farmers in Cagayan and nearby provinces have limited knowledge of their potential as a fertilizer.

Recently, the production of irradiated carrageenan from *Eucheuma sp.* (Rhodophyta) by the Philippine Nuclear Research Institute (PNRI) as a plant growth promoter was found to be effective in increasing the yield of mungbean (Abad *et al*., 2018) and rice (Carlos, 2015), hence now introduced and utilized by the farmers in the Philippines.

The utilization of local marine bioresources in organic crop production is an opportunity. Farmers in the Philippines were encouraged to practice organic farming through the Organic Agriculture Act of 2010 (RA 10068) by finding alternative farm inputs with beneficial effects on the soil, crop yield, and crop quality. Lettuce needs to be grown organically, and the utilization of edible seaweeds as an alternative fertilizer to inorganic fertilizers needs to be further assessed and promoted. Our study wants to report the potential of cellulase-enhanced fermented *G. bursa-pastoris, A. spicifera,* and *S. ilicifolium* extracts as liquid fertilizers in lettuce production. Fermentation with the addition of cellulase as an enhancer in the seaweed extraction technique was least reported and was found different from the methods used in other studies (Trivedi *et al*., 2015; Rhein-Knudsen *et al*., 2015). Other methods of obtaining seaweed extracts/saps were mechanical (Pise and Sabale, 2010), heat (Kavipriya *et al.*, 2011; Rao and Chatterjee, 2014), and solvent/aqueous extractions (Kumari *et al.*, 2011; Torres *et al.*, 2018).

Interestingly, seaweeds contain various phytohormones (Tarakhovskaya *et al*., 2007), and the species of *Gracilaria* (Horrocks *et al*., 1995), *Acanthophora* (Fong *et al*., 2001), and *Sargassum* (Matanjun *et al*., 2009) contain a favorable amount of nutrients. Nutrient composition of *G. bursa-pastoris* (Valente *et al*., 2006), *A. spicifera* (Ganesan *et al*., 2020), and *S. ilicifolium* (Rohani-Ghadikolaei *et al*., 2012) were reported. The popularity of *Sargassum* sp. as fertilizer was reported in several studies (Erulan *et al.*, 2009; Williams and Feagin, 2010; Salmal *et al*., 2014) which demonstrated benefits in crop production. Moreover, the utilization of seaweed fertilizers in lettuce production was also reported (Crouch *et al*., 1990; Möller and Smith, 1998; Silva *et al*., 2019). The research finding aimed to investigate the effects of the formulated cellulase-enhanced fermented edible seaweed extracts on the growth and nutrient content of loose-leaf lettuce plants grown under greenhouse conditions.

Materials and methods

Collection and preparation of the seaweeds

Fresh/Ceylon Moss/Gulaman/Agar-Agar [*Gracilaria bursa-pastoris* (S.G. Gmelin) P.C. Silva], Erect sea moss/Kulot/Rag-ragatirit [*Acanthophora spicifera* (M. Vahl) Borgesen], and Aragan/Lusay-lusay [*Sargassum ilicifolium* (Turner) C. Agardh] were collected in the coasts of Gonzaga, Cagayan, Philippines. Identification of the edible seaweed species was verified through the descriptions and illustrations of Titlyanov *et al*. (2016).

Preparation of the cellulase-enhanced fermented edible seaweed extracts

Collected fresh seaweeds were washed with running tap water to remove the sand and then rinsed with distilled water thrice. The seaweeds were chopped finely and ground using a blender. Seaweed fertilizers were formulated by separately mixing 1.0 kg chopped fresh *G. bursa-pastoris*, *A. spicifera*, and *S. ilicifolium* with 33.33 mL kg^{-1} cellulase, 333.33 g kg^{-1} brown sugar, and 666.67 mL kg^{-1} distilled water. The mixtures were fermented at room temperature (22-25^{o}C) for 14 days. After fermentation, the seaweed extracts were filtered using double-layered fine cheesecloth, placed in sterile glass containers, and stored in the refrigerator for several days.

Experimental set-up

Our study was conducted from March to June 2021 at the greenhouse of Cagayan State University-Gonzaga Campus (CSU-G), Flourishing, Gonzaga, Cagayan, Philippines (Latitude: 18.25016^{o}N, Longitude: 122.00068^{o}E, Elevation: 86.87 m asl). The experiment was laid out employing the Completely Randomized Design with three replications of 10 sample plants each. The lettuce plants were applied with various kinds of fertilizer treatments (no fertilization, NPK fertilizer (14-14-14), irradiated carrageenan, cellulase-enhanced fermented *G. bursa-pastoris* extract (GSF), cellulase-enhanced fermented *A. spicifera* extract (ASF), cellulase-enhanced fermented *S. ilicifolium* extract (SSF), and mixture of GSF, ASF, and SSF). The lettuce plants were grown in the greenhouse for 35 days after transplanting (DAT). The growth (shoot length, root length, leaf number, shoot weight, root weight, and whole plant fresh and dry biomass) and nutritional content (nitrogen, phosphorus, potassium, copper, zinc, iron, and manganese) of the applied plants were evaluated at 35 DAT.

Establishment of the experimental plants

'Grand Rapid' loose leaf lettuce seeds (Known You Seeds, Philippines) were germinated in planting beds filled with garden soil and carbonized rice hull (1:1) for 21 days inside the greenhouse. The lettuce seedlings (84.626±6.07 g shoots; 38.669±4.22 mm roots; 3.0±0.15 true leaves; 0.412±0.06 g whole plant fresh weight; 0.021±0.001 g whole plant dry weight) were transplanted in planting beds with garden soil (pH 6.81, 1.69% OM, 17.48 ppm P, 439.0 ppm K, 0.96 ppm Cu, 0.10 ppm Zn, 12.40 ppm Fe, and 3.60 ppm Mn) following the 15.0 cm x 15.0 cm distance of planting. The plants were acclimatized for 14 days in the greenhouse of CSU-G before treatment application.

Seaweed extracts application to the lettuce plants

The 35-day old (days after sowing) lettuce plants were applied with diluted cellulase-enhanced fermented edible seaweed extracts namely: *G. bursa-pastoris* extract (GSF), *A. spicifera* extract (ASF), *S. ilicifolium* extract (SSF), a mixture of GSF, ASF, and SSF, and Irradiated Carrageenan at 10 mL L^{-1} solution. Application of seaweed extracts as liquid fertilizers was done early in the morning at 14, 21, and 28 DAT, applied by wetting the leaves and the whole plant and drenching in the plant base (25 mL^{-1} plant). Lettuce plants without fertilization were irrigated with tap water while inorganic fertilizer-treated plants were applied with 5.0 g^{-1} plant NPK fertilizer (14-14-14) at 14 DAT. Lettuce plants applied with seaweed extracts as liquid fertilizers were not fertilized with NPK fertilizer. The plants were misted thrice a week.

Growth of the lettuce plants

The growth of the lettuce plants at 35 DAT was measured following the measurement of Opeña and Sotto (2020). Leaves per plant that are green, fully developed, expanded, and not dried were counted. Shoot and root were measured from the base of the plant to the tip of the longest leaf and root, respectively using a vernier caliper. Fresh weights (shoot, root, and whole plant) were determined by washing the plant with tap water and blotting with dry tissue paper to remove the foreign materials attached to the plants. The lettuce plants were oven-dried at 70 °C for 48 h and dry weights (shoot, root, and whole plant) were gathered using a digital weighing scale.

Lettuce and seaweed extract nutrient analyses

The nutrient content of the lettuce plants (leaves and stem) at 35 DAT and various cellulase-enhanced fermented edible seaweed extracts were determined following the procedures of the Bureau of Soils and Water Management (2021). The total nitrogen, total phosphorus, total potassium, and total trace elements (zinc, copper, manganese, and iron) of the lettuce plants were analyzed using Steam Distillation, Vanadomolybdophosphoric acid colorimetric, Flame Photometric, and Microwave Plasma – Atomic Emission Spectroscopic tests, respectively. Meanwhile, the nutrients of the cellulase-enhanced fermented edible seaweed extracts were measured through the Kjeldahl Jaudber-Gunning method (total nitrogen), Vanadomolybdate test (total phosphorus), Flame Atomic Emission test (total potassium), and Atomic Absorption Spectrophotometric test (copper, zinc, iron, and manganese contents). The nutrient analysis was performed under 25°C ± 2.0 temperature and 40-60% relative humidity.

Data analysis

Data were subjected to Analysis of Variance (ANOVA) using the Statistical Tool for Agricultural Research 2.0.1, 2013 (International Rice Research Institute, Los Baños, Laguna, Philippines). Statistical differences among fertilizer treatments were determined using Tukey's Honest Significant Difference Test ($P \leq 0.05$). Standard errors were also determined for the error bars.

Results

Nutrient composition of cellulase-enhanced fermented edible seaweed extracts

The nutrient contents of the cellulase-enhanced fermented *G. bursa-pastoris* (GSF), *A. spicifera* (ASF), and *S. ilicifolium* (SSF) extracts were presented in Table 1. Among the three fermented edible seaweed extracts, ASF contained the highest amount of macronutrients (0.20% N, 0.18% P, and 2.25% K) while both GSF and SSF contained the lowest amount of nitrogen (0.04%). Moreover, GSF and SSF contained the lowest phosphorus (0.05%) and potassium (0.95%) contents, respectively. Meanwhile, GSF contained the highest amount of zinc (7.50 ppm) and iron (55 ppm) while the lowest amounts (2.50 ppm Zn and 17.50 ppm Fe) were tested in ASF. Both GSF and ASF contained the highest amount of manganese (35 ppm) while SSF contained the lowest (10 ppm). All the fermented edible seaweed extracts contained the same amounts of copper (2.50 ppm).

Table 1. Formulations and nutrient content of cellulase-enhanced fermented edible seaweed extracts used in the study

Code	Formulation	Nutrient Content						
		N (%)	P (%)	K (%)	Zn (ppm)	Cu (ppm)	Mn (ppm)	Fe (ppm)
GSF	1.0 kg *Gracilaria bursa-pastoris* (S.G. Gmelin) P.C. Silva + 333.33 g kg^{-1} brown sugar + 33.33 mL kg^{-1} cellulase + 666.67 mL kg^{-1} distilled water	0.04	0.05	1.54	7.50	2.50	35.00	55.00
ASF	1.0 kg *Acanthophora spicifera* (M. Vahl) Borgesen + 333.33 g kg^{-1} brown sugar + 33.33 mL kg^{-1} cellulase + 666.67 mL kg^{-1} distilled water	0.20	0.18	2.25	2.50	2.50	35.00	17.50
SSF	1.0 kg *Sargassum ilicifolium* (Turner) C. Agardh + 333.33 g kg^{-1} brown sugar + 33.33 mL kg^{-1} cellulase + 666.67 mL kg^{-1} distilled water	0.04	0.10	0.95	5.00	2.50	10.00	30.00

n=3

Growth response of loose-leaf lettuce applied with cellulase-enhanced fermented edible seaweed extracts

Lettuce plants applied with various cellulase-enhanced fermented edible seaweed extracts as fertilizers produced 0.84 to 3.17 more leaves than untreated plants while NPK fertilizer and irradiated carrageenan-applied lettuce plants produced 1.84 and 2.34 more leaves than untreated plants, respectively (Figure 1a). Lettuce plants produced longer shoots (33.43 mm to 64.0 mm longer) and shorter roots (1.13 mm to 23.0 mm shorter) when applied with various fermented edible seaweed extracts as compared to untreated plants.

Lettuce plants applied with SSF (64.0 mm longer), NPK fertilizer (71.9 mm longer), and GSF (53.73 mm longer) significantly produced the longest shoots, followed by lettuce plants applied with ASF, and seaweed extract mixture (GSF, ASF, and SSF) which produced 36.33 mm, and 33.43 mm longer shoots, respectively. The shortest shoots were measured in the controlled plants. No significant differences were noted in the root length of the lettuce plants. The longest plants were observed in lettuce plants applied with NPK fertilizer, SSF, and GSF. NPK-fertilized lettuce plants were 77.46 mm longer as compared to untreated plants, while SSF and GSF-fertilized lettuce plants were 54.9 mm, and 52.6 mm longer than the controlled plants (Figure 1b).

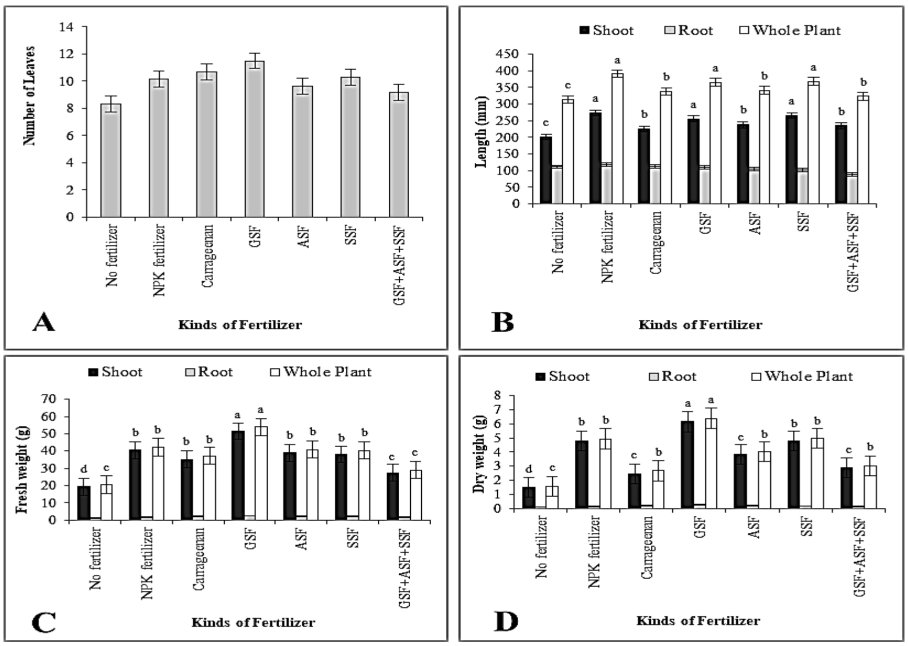

Figure 1. Growth [number of leaves (A), lengths (B), fresh weights (C), and dry weights (D)] of loose-leaf lettuce applied with various cellulase-enhanced fermented edible seaweed extracts as liquid fertilizers at 35 DAT: Cellulase-enhanced seaweed extracts are GSF – fermented *G. bursa-pastoris* extract; ASF – fermented *A. spicifera* extract; SSF – fermented *S. ilicifolium* extract. Means of the same letter are not significant (HSD test, ($P \leq 0.05$). n=30, error bars ± StdError

Heaviest fresh biomass was recorded in lettuce plants applied with GSF (32.14 g heavier shoot and 33.30 g heavier whole plant fresh weight). Improved fresh biomass was also observed in lettuce plants applied with NPK fertilizer (21.11 g heavier shoot and 21.61 g heavier whole plant fresh weight), ASF (19.51 g heavier shoot and 20.28 g heavier whole plant fresh weights), SSF (18.75 g heavier shoot and 19.74 g heavier whole plant fresh weights), and irradiated carrageenan (15.87 g heavier shoot and 16.60 g heavier whole plant fresh weights) as compared to the unfertilized plants (Figure 1c). Furthermore, heaviest dry biomass was observed in lettuce plants applied with GSF (4.68 g heavier shoot and 4.85 g heavier whole plant dry weights). Heavier dry biomass as compared to unfertilized plants was also recorded in lettuce plants applied with SSF (3.33 g heavier shoot and 3.42 g heavier whole plant dry weights), NPK fertilizer (3.31 g heavier shoot and 3.39 g heavier whole plant dry weights), ASF (2.36 g heavier shoot and 2.47 g heavier whole plant dry weights), seaweed extract mixture (1.39 g heavier shoot and 1.46 g heavier whole plant dry weights), and irradiated carrageenan (0.99 g heavier shoot and 1.12 g heavier whole plant dry weights) (Figure 1d).

Plant nutrients of loose-leaf lettuce applied with cellulase-enhanced fermented edible seaweed extracts

Lettuce plants applied with cellulase-enhanced fermented edible seaweed extracts such as seaweed extract mixture, SSF, and ASF obtained 1.38, 0.94, and 0.59% more total nitrogen content than the unfertilized plants, respectively. An increase in the potassium content was also noted in lettuce plants applied with seaweed extract mixture (0.74% more total K), GSF (0.60% more total K), ASF (0.56% more total K), and SSF (0.44% more total K) as compared to unfertilized plants. Lettuce plants fertilized with NPK fertilizer and irradiated carrageenan contained higher potassium content than the unfertilized plants. Meanwhile, the phosphorus content of the lettuce plants was not significantly improved by fertilizer treatments (Figure 2a).

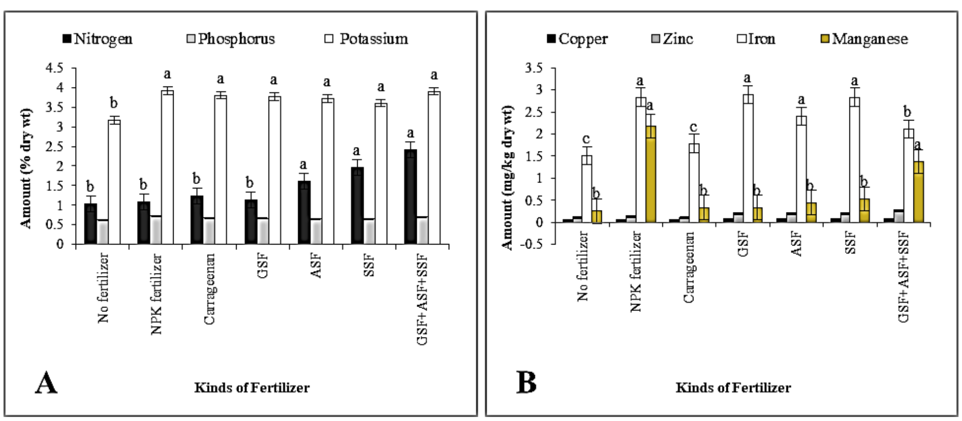

Figure 2. Nutrient content [Macronutients (A), Micronutrients (B)] of loose-leaf lettuce applied with various cellulase-enhanced fermented edible seaweed extracts as liquid fertilizers at 35 DAT: Cellulase-enhanced seaweed extracts are GSF – fermented *G. bursa-pastoris* extract; ASF – fermented *A. spicifera* extract; SSF – fermented *S. ilicifolium* extract. Means of the same letter are not significant (HSD test, ($P \leq 0.05$). n=3, error bars ± StdError

Moreover, the application of cellulase-enhanced fermented edible seaweed extracts (GSF, ASF, SSF) increased the iron content of the loose-leaf lettuce grown under greenhouse conditions. The highest iron contents were tested in lettuce plants applied with NPK fertilizer, GSF, SSF, and ASF while the lowest amounts were analyzed from plants applied with irradiated carrageenan and no fertilization. Lettuce plants applied with GSF and SSF contain 1.38 mg kg^{-1} and 1.32 mg kg^{-1} more iron as compared to unfertilized plants, respectively. Also, NPK-fertilized plants contain 1.32 mg kg^{-1} more iron than the unfertilized lettuce plants. On the other hand, more manganese content was measured in lettuce plants applied with NPK fertilizer (1.92 mg kg^{-1} more manganese) and seaweed extract mixture (1.12 mg kg^{-1} more manganese). The copper and zinc contents of the lettuce plants were not significantly improved by the fertilizer treatments (Figure 2b).

Discussion

Improving crop production with less chemical application and ensuring better crop quality were the main objectives of organic agriculture. Organic farming promotes the utilization of local bioresources in promoting better agricultural production and value addition. The utilization of seaweed liquid fertilizers must be promoted to reduce soil pollution due to the excessive application of chemical fertilizers. Our study showcases technology for utilizing edible seaweeds in organic vegetable production where local vegetable farmers can improve their yield by application of seaweed-based liquid fertilizers. Moreover, our study describes the nutrient contents of cellulase-enhanced fermented *G. bursa-pastoris*, *A. spicifera*, and *S. ilicifolium* extracts and their beneficial effects as liquid organic fertilizers that can increase the leaf number, size, and biomass of the loose-leaf lettuce grown under greenhouse conditions. Our results revealed that the cellulase-enhanced fermented *A. spicifera* extract contained the highest amounts of nitrogen, phosphorus, and potassium while cellulase-enhanced fermented *G. bursa-pastoris* extract contained the highest amounts of zinc and iron. Moreover, the highest manganese content was tested in cellulase-enhanced fermented *G. bursa-pastoris* and *A. spicifera* extracts. The nutrient contents of the seaweed extracts aided better growth which promoted longer plants and heavier biomass in fertilized loose-leaf lettuce plants. The longest lettuce plants were observed with GSF and SSF application while the heaviest plants were noted with GSF application. Improved growth and yield were also found in *Gracilaria* (Shah *et al.*, 2013; Rao and Chatterjee, 2014) and *Sargassum* (Kavipriya *et al.*, 2011; Kumari *et al.*, 2011) extract/liquid fertilizer-applied crops. Also, NPK fertilizer improved shoot and whole plant lengths however better growth (improved length and biomass) was measured in plants treated with cellulase-enhanced fermented *G. bursa-pastoris* extract. With the fact that NPK fertilizer contains a high amount of macronutrients (14% N-P-K), fermented edible seaweed fertilizers do contain a good amount of micronutrients that can enhance growth and development. Hänsch and Mendel (2009) listed the beneficial effects of Fe, Zn, Cu, and Mn in plant growth, especially in plant hormone synthesis. Moreover, the species were also believed to contain phytohormones. Verkleij (1992) reported the plant hormone properties of seaweed extracts from various seaweed species.

The nutrient/mineral contents of the loose-leaf lettuce plants applied with cellulase-enhanced fermented edible seaweed extracts as fertilizers were also investigated. Our study is one of the scientific reports that disclosed the nutrient enhancement of leafy vegetables through the application of seaweed extracts/saps as fertilizer. Cellulase-enhanced fermented *G. bursa-pastoris*, *A. spicifera*, and *S. ilicifolium* and seaweed extract mixture increased the nitrogen, and potassium contents of the lettuce plants. Furthermore, cellulase-enhanced fermented *Gracilaria, Acanthophora,* and *Sargassum* extracts enriched the iron content while the application of seaweed extract mixture increased the manganese content of the lettuce plants grown under greenhouse conditions. The beneficial effect of cellulase-enhanced fermented edible seaweed extracts application on the improvement of lettuce nutritional contents posed a comparable result with NPK fertilizer application. Increased nutrient levels were also observed in tomato applied with *Kappaphycus alvarezii* (Rhodophyta) seaweed sap (Zodape *et al.*, 2011) and wheat applied with *K. alvarezii* and *Gracilaria edulis* saps (Shah *et al*., 2013). Enhancement of the nutritional composition in lettuce plants applied with cellulase-enhanced fermented *G. bursa-pastoris*, *A. spicifera*, and *S. ilicifolium* extracts was evident. Improved nutrient contents of the lettuce plants were possibly due to the absorption of fermented edible seaweed extract nutrients which promoted higher nutrient accumulation in the plants since the seaweed extracts were directly applied to the leaves.

The findings on improved growth and nutrient content assured the beneficial effects of applying fermented edible seaweed extracts as liquid fertilizers in loose-leaf lettuce, thus an effective fertilizer for organic lettuce production. We also reported the nutrient contents of the various fermented edible seaweed extracts as fertilizers which have potential in other crops that are grown organically. The cellulase-enhanced fermented *G. bursa-pastoris*, *A. spicifera,* and *S. ilicifolium* extracts can be substitutes for inorganic NPK fertilizers in lettuce production where these liquid fertilizers can promote organic crop production, and improve crop growth and quality. Their utilization in agriculture can lead to reduced soil degradation in vegetable farms, thus promoting an environmentally-sound farming technology.

Acknowledgements

The authors would like to offer particular thanks to the Cagayan State University (CSU) through the leadership of the University President, Dr. Urdujah G. Alvarado; Regional Soils Laboratory of the Cagayan Valley Integrated Agricultural Laboratory, Department of Agriculture Region 2.; Atty. Benefroi P. Pascual, and several agriculture students of the Cagayan State University-Gonzaga, Flourishing, Gonzaga, Cagayan, Philippines.

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(Received: 22 March 2022, accepted: 30 July 2022)