Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 02 (2022): jtbb69178
DOI: 10.22146/jtbb.69178

Research Article

Biostimulant Activity of *Sargassum* sp. Extracts on Early Growth of *Zea mays* L. and the Phytohormones Content Analysis

Fauziatul Fitriyah[1], Muhammad Abdul Aziz[1], Sri Wahyuni[1], Hana Fadila[1], Insyiah Meida Luktyansyah[2], Sulastri[2], Priyono[1], Siswanto[1]*

1) Indonesian Research Institute for Biotechnology and Bioindustry, Jl. Taman kencana no 1, Bogor, Jawa Barat, Indonesia 16128
2) PT Pupuk Kalimantan Timur, Jl. James Simanjuntak No.1, Guntung, Bontang Utara, Kota Bontang, Kalimantan Timur, Indonesia 75314
* Corresponding author, email: siswanto99@yahoo.com

Keywords:
early growth
maize
liquid extract
germination
plant growth regulator
Sargassum sp.
Submitted:
22 September 2021
Accepted:
23 April 2022
Published:
20 June 2022
Editor:
Ardaning Nuriliani

ABSTRACT

Seaweed has been gaining global interest in agriculture for the development of marine-based plant biostimulants. This research aimed to study the effect of three different liquid extracts of *Sargassum* sp., acidic, alkaline, and water extract, on the germination and early growth of maize and to evaluate the phytohormones content responsible for the growth. Phytohormones content including Indole-3-acetic acid (IAA), gibberellins (GA), kinetin and zeatin were analyzed using high-performance liquid chromatography (HPLC) and bioassay was performed twice on maize. Parameters observed on the bioassay were germination percentage, number of roots, shoot length, shoot weight and root weight under 4 different concentrations with 0.5; 1.5; 3.5; and 5% in the first bioassay and 3.5% concentration in the second bioassay. Both bioassays following randomized complete design and the data were analyzed using one-way ANOVA using post hoc test of Duncan Multiple Range Test (DMRT) at error probability of 5% in Genestat software. Phytohormones content in the seaweed extract indicated that alkaline extract was rich in IAA, gibberellin, and zeatin content, while water extract showed the highest kinetin content. The first bioassay indicated that lower concentration of the seaweed extracts gave better growth in all extracts, therefore a 3.5% concentration was chosen for the second bioassay with higher replication for each treatment. The second bioassay confirmed alkaline extract resulted in the highest germination while the highest seedling height, number of roots, shoot and root weight were resulted from acidic extract treatment. In conclusion, *Sargassum* sp. extracts obtained from acidic, alkaline, and water-based extraction methods, were able to improve the shoot and root growth of maize plants. The acidic extract showed the highest growth promotion among other extracts with the lowest phytohormones content.

INTRODUCTION

Seaweed has been gaining global interest for over the last decades, commonly due to its potential for natural products (Stengel & Connan 2015). Numerous algae-based products have been developed for Agri-horticultural, food, pharmaceutical and nutraceutical products. A recent review reported approximately 3200 seaweed-based products, covering 13% of natural compounds reported in marine organisms (Stengel & Connan 2015).

Seaweed was usually categorized into three phyla based on the pigmentation, including green algae (Chlorophyta), red algae (Rhodophyta), and brown algae (Ochrophyta) (Sangha et al. 2014). Among marine algae, the extracts of brown algae were reported to possess higher bioactive compounds, such as betaines, brassinosterols, jasmonates, isoprenoid cytokinin, polyamines, salicylates, and signal peptides (Craigie 2011). Thus, they have been widely used continuously in agriculture and horticulture as natural plant growth stimulants or bio-fertilizers (Craigie 2011). The current commercial agricultural products mainly were produced from brown seaweeds of *A. nodosum*, *Laminaria* spp., *Ecklonia maxima*, *Sargassum* spp., and *Durvillaea* spp. (Craigie 2011). One of the brown algae, *Sargassum* sp. (Sargassaceae) has been attracted great interest for the higher phytohormones, macro- and micronutrients contents than other species in different phyla (Hernández-Herrera et al. 2014).

Seaweed extract generally consists of useful bioactive compounds, including polyphenols, terpenoids, polysaccharides, polypeptides and free amino acids, pigments, vitamins, phytohormones, macro- and micro-nutrients (Mahmoud et al. 2019). Seaweed extracts were considered as plant biostimulants that act as metabolic enhancers to increase the effectiveness of conventional mineral fertilizers (Craigie 2011). Recent study by Sunarpi et al. (2021) evaluated bioactive and phytohormones roles of *Sargassum* extract as plant biostimulants in rice plant. The result suggested that *Sargassum* is a potential raw material for development of organic fertilizers which increased growth and yield of rice plants

Seaweed extracts have shown accelerated germination, vegetative growth, flowering and yield of many crops. Seaweed extracts of *Ascophyllum nodosum* at 0.5 g/L concentration improved total anthocyanin, yield and fruit size of strawberry (Roussos et al. 2009). Combination of *Sargassum wightii* and *Caulerpa chemnitzia* aqueous extract at low concentration (20% from 1 kg fresh seaweed in 1 L aquadest) reported to promote germination, vegetative growth and flowering of *Vigna sinensis* (Sivasankari et al. 2006). Enhancement of leaf chlorophyll content by various brown seaweed extracts application has also been reported in cowpea, red-radish and oil palm (Sivasankari et al 2006; Santoso et al 2011; Mahmoud et al. 2019). Improved vegetative and generative development and increase in crop growth treated by foliar spray of *Sargassum* extract of 10 ppm by 3 times application led to an increase in yield of harvested soybean (Sari et al. 2019). Foliar spray of *Sargassum* sp. extract at 2% concentration also reported to improve yield, vegetative and generative growth of rice and corn (Santoso et al. 2011). Vegetative growth of plantation crops, including oil palm and sugarcane were also improved after treatments of *Sargassum* sp. extract (Santoso et al. 2011; Putra et al. 2017). It was suggested that the stimulatory effect of seaweed extract may be controlled by phytohormones, such as auxins, cytokinins, or gibberelins, were probably in control for the improved growth and yield of the plants (Jannin et al. 2013).

This research was part of a grand research in the production of humic fertilizer supplemented with seaweed extract. Extraction of seaweed was developed to discover the suitable method with the highest biostimulant activity, which is suitable with humic acid extraction from lignite. Humic acid act as soil ameliorant which contains minerals to physically and chemically restore disturbed soil (Van Oosten et al. 2017). Application of humic acid to the crops directly improved abiotic stress tolerance in plants, for example application of humic acids to common bean under high salinity showed better adaptation to saline environments (Aydin et al. 2012). Supplementation of humic acid with seaweed extract was superior showed by increment of leaf hydration under dry soil conditions as well as improved vegetative growth and yield (Van Oosten et al. 2017). This study was conducted to evaluate the effect of *Sargassum* sp. liquid extracts under three extraction condition, which are acidic, alkaline, and water, on the germination and early growth of maize and evaluate the phytohormones content responsible for the growth.

MATERIALS AND METHODS

Materials

The seaweed *Sargassum* sp. was obtained from farmers in Gunungkidul coastal area, Yogyakarta, Indonesia, washed with fresh water to remove sand, stones and other debris and then dried under sunlight inside a plastic house for two days. The water contents were measured by weighing seaweed samples before and after drying. Commercial hybrid maize seeds of Bonanza F1 (Cap Panah Merah) was used for bioassay in this study. The production of seaweed extracts and bioassay was performed at the Laboratory of Biochemistry, Indonesian Research Institute for Biotechnology and Bioindustry, Bogor, Indonesia. The phytohormones analysis was performed at Agrochemical Material Residues Laboratory-The Agricultural Environment Research Institute (Balingtan), Bogor Indonesia. The research was performed from November 2020 until March 2021.

Seaweed extraction

The *Sargassum* sp. extraction protocol was modified from Sharma et al. (2013). The dried material was mechanically milled with 40 mesh filter. *Sargassum* sp. meal (2 g) was mixed with 200 mL of aquadest and vortexed for water extract (WE). Alkaline and acidic extract were produced by mixing 2 g of *Sargassum* sp. meal with 200 mL aquadest pH 9.0 and pH 3.0, respectively. pH was adjusted by adding 2 M sulfuric acid or 2 N potassium hydroxide into aquadest to reach the designated pH. pH was adjusted before mixing with *Sargassum* sp. meal and measured by pH meter (Sartorius; PB-10 basic pH meter). The mixtures were incubated at 85°C in water bath for 2 hours and let them cool down at room temperature. The extracts were filtered with cheese cloth and then centrifuged at 6000 rpm for 5 minutes. The crude extracts were stored in a refrigerator for further analysis.

Phytohormones analysis

Sargassum sp. extracts were adjusted to pH 5.8 as the final product of seaweed extract and then sent to Balingtan Bogor, Indonesia for phytohormones analysis. Phytohormones analysis was performed using in-house protocol from Barendse (1987) in HPLC and the separation of compounds, hypersil gold C18 column was used. Standard solution (0.1%) of kinetin, GAs, zeatin and kinetin, were prepared. The mobile phase used was methanol/water (65:35, v/v). The flow was 0.5 mL.min^{-1} and 5-10 μL of sample was loaded onto the column. The temperature of the column was maintained at 40°C. Detection was carried out at the wavelength of λ= 254 nm. Samples prior to analysis were mixed with methanol 65% and centrifuged at 4000 rpm for 30 min. The supernatants were filtered with a syringe filter (0.45 µm) and the filtrate was ready to inject onto the column. Phytohormones content was calculated by comparing between chromatogram peak of sample and standard.

Bioassay in maize

Three different *Sargassum* sp. extracts were tested for germination and early growth activity of maize in two bioassays. The first bioassay, maize seeds were germinated in water, acidic, and alkaline *Sargassum* extracts following the experiment design of randomized complete design with 4 concentrations (0.5; 1.5; 3.5; and 5%) in 3 replicates and aquadest was used as negative control. Maize seeds were soaked according to the treatment for 1 hour, followed by germination in Petri dish (10 seeds per Petri dish) covered with wet tissue paper. Germination was observed when negative control was germinated at 30-50%. Observation of germination was performed twice (during negative control germinated 30-50%) and the significant result between treatment was chosen. The germination rate was calculated by the percentage of number of germinated seeds (when negative control is 30-50% germinated) divided by total number of germinated seed at the end of germination period. All germinated seeds were grown for the next 7 days to observe the shoot length, number of roots, shoot and root weight. The optimum concentration was chosen for the next bioassay with higher replications number.

The second bioassay was performed under the same procedure but only one concentration of *Sargassum* sp. extract was used. Maize seeds were germinated in Petri dish soaked with treatment solutions, aquadest as negative control, and treatment solution including water, acidic, and alkaline extract in six replicates (15 seeds per Petri dish). Germination was observed following the first bioassay condition. The growth parameter was observed 4 days after germination including shoot length, number of roots, shoot and root weight.

Data analysis

Quantitative data including germination and early growth parameters from first and second bioassay were statistically analyzed using Genstat software in

one-way ANOVA and Duncan's multiple range test was performed at α = 0.05.

RESULTS AND DISCUSSION

Seaweed extract production

Seaweed extraction was performed using aquadest as the solvent with three different pH, namely acidic, neutral and alkaline. The seaweed used in this study is *Sargassum* sp. obtained from farmers in Gunungkidul coastal area (including Sepanjang beach, Krakal beach, Drini beach, Nglolang beach, Pok tunggal beach, Sanglen beach, Porok beach etc.; Personal communication with the supplier), Yogyakarta (Indonesia) which was harvested from the nature (Figure 1A). The seaweed was then dried until water content was around 5-10% (Figure 1B). The dried seaweed was milled mechanically with 40 mesh filter chosen (Figure 1C). Under this milling condition, it was able to obtain seaweed meal with particle size of < 2 mm. Sharma et al. (2013) reported that for agricultural purposes, seaweed was preferred in a coarse size grade (1–4 mm) for extraction, while the feedstock industry utilizes the finer particle for supplement.

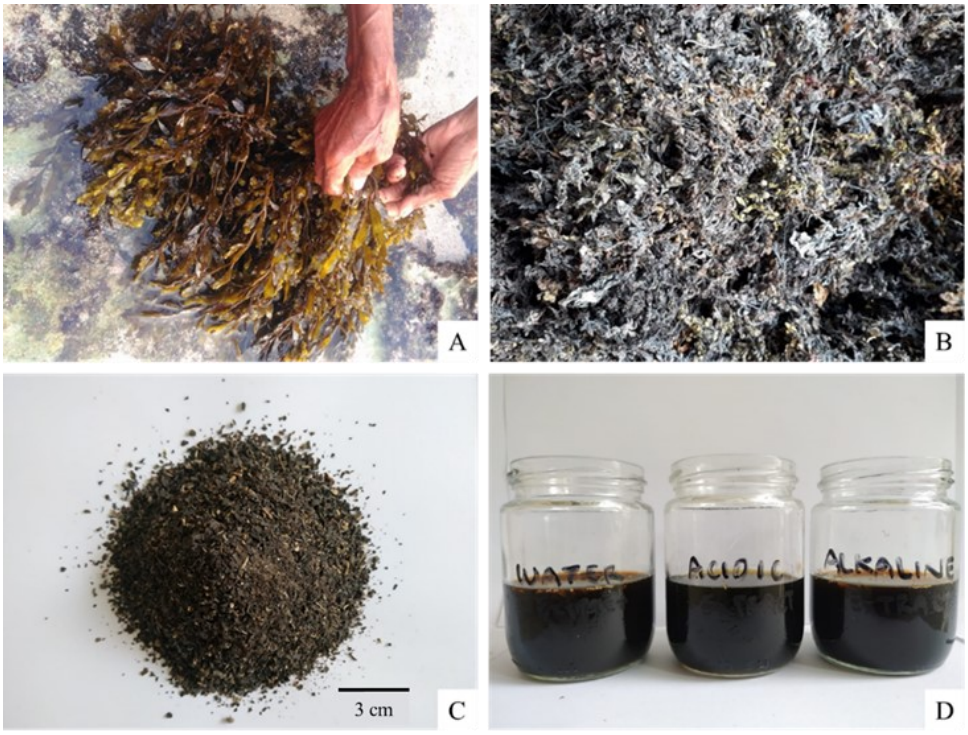

Figure 1. Seaweed used in this study: (A) Intertidal seaweed *Sargassum* sp. harvested from the nature, (B) Dried *Sargassum* sp., (C) *Sargassum* sp. meal, and (D) *Sargassum* sp. Extract.

Sargassum sp. which grows in intertidal zone may be exposed to unfavorable environmental conditions, including extreme variations in temperature, salinity, and light, which lead to the production of a wide range of secondary metabolites to survive in these environments (Shukla et al. 2016). Goñi et al. (2016) reported that different extraction protocols produce bi-

ostimulants with different bioactive compounds and activities. Various commercial plant biostimulant products have been utilizing the hydrolysis process of seaweed (Shukla et al. 2019).

Phytohormones content in *Sargassum* sp. extract

Seaweed extraction protocol in this study resulted in three different solutions, they are acidic, alkali, and water extract, (Figure 1D) with dark brown color and the pH was adjusted to 5.8 before dilution with 2 N KOH or 1 M H_2SO_4. The phytohormones content in liquid extract of *Sargassum* sp. respectively as shown in Table 1.

Table 1. Phytohormones content of *Sargassum* sp. Extract.

Seaweed extract	Concentration (mg.L^{-1})			
	IAA	Gibberellins	Zeatin	Kinetin
Acidic extract	10.64	18.89	34.92	4.09
Water extract	11.04	29.63	35.71	4.73
Alkaline extract	11.49	30.88	38.25	4.33

The data in Table 1 shows that even with slight difference from each extract, it was feasible that alkaline extract showed the highest IAA, gibberellins, and zeatin content. While kinetin content was almost the same in all extract and was the lowest phytohormone content among others. Gibberellins and zeatin content were considered high compared to IAA and kinetin in all extracts. Sunarpi et al. (2021) reported that different Sargassum extracts produced different phytohormones contents. Extract from *S. polycystum* produced the highest gibberellins and kinetin content of 8 mg. L^{-1} and 10 mg. L^{-1} respectively, compared to *S. cristaefolium* and *S. crassifolium* (Sunarpi et al. 2021). It was also reported that IAA is the most common growth hormone found in all *Sargassum* species (Sunarpi et al. 2021). Indole-3-Acetic Acid (IAA) has chemically poor solubility in water or aqueous buffers unless the pH of the solvent is alkaline (Yamakawa et al. 1979). This explained, IAA content of alkaline extract was slightly higher compared to other extracts. Previous research from Sumera & Cajipe (1981) reported that alkaline hydrolysis for *S. polycystum* extraction increases the auxin activity of algal extracts due to liberation of bound auxins or inactivation of co-existing inhibitory substances during hydrolysis.

Bioassay in maize

The result of bioassay in maize was shown in Table 2, indicates that the application of seaweed extracts improved germination and early growth of maize significantly compared to negative control. The highest germination percentage was achieved by soaking maize seeds in 3.5% *Sargassum* alkaline extract. The highest number of roots and shoot height showed by treatment of 1.5% *Sargassum* alkaline extract, which was not significant with 1.5% acidic extract. Shoot weight and root weight showed the highest value under treatment of 0.5% acidic extract.

Table 2. The effect of liquid extracts of *Sargassum* sp. on germination and early growth of maize (first bioassay).

Treatment		Germination (%)		Number of roots		Shoot height (cm)		Shoot weight (g)		Root weight (g)	
Extract	Concen-tration	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Acidic extract	0.50%	62.50^{abcd}	14.6	6.97^{b}	1.0	14.47^{abc}	0.1	3.32^{c}	0.2	1.46^{b}	0.1
	1.50%	62.96^{abcd}	8.6	7.23^{b}	0.8	15.61^{c}	1.0	3.06^{bc}	0.8	1.03^{ab}	0.2
	3.50%	52.38 ^{abcd}	8.0	6.70^{b}	0.0	12.91^{abc}	0.9	2.64^{abc}	0.4	0.93^{ab}	0.3
	5%	61.64^{abcd}	16.7	4.97^{a}	1.4	10.77^{a}	2.4	1.83^{ab}	0.7	0.89^{ab}	0.4
Alkali extract	0.50%	39.01^{ab}	15.1	6.40^{ab}	0.5	14.38 ^{abc}	1.3	2.46^{abc}	0.7	1.06^{ab}	0.3
	1.50%	74.60^{cd}	4.1	7.27^{b}	0.4	16.42^{c}	0.6	2.18^{abc}	0.2	0.88^{ab}	0.2
	3.50%	81.06^{d}	3.5	7.03^{b}	0.8	15.39^{bc}	2.4	2.41^{abc}	0.9	1.10^{ab}	0.4
	5%	66.67^{bcd}	14.7	6.87^{b}	0.7	14.53 ^{abc}	0.8	2.81^{abc}	0.1	1.22^{ab}	0.1
Water extract	0.50%	59.72^{abcd}	10.9	5.93^{ab}	0.1	13.86 ^{abc}	1.4	2.33^{abc}	0.3	1.15^{ab}	0.3
	1.50%	48.68^{abc}	4.3	6.07^{ab}	0.8	13.43 ^{abc}	2.1	2.79^{abc}	0.6	1.00^{ab}	0.2
	3.50%	55.73^{abcd}	9.1	6.43^{ab}	0.3	15.89^{c}	2.5	2.95^{abc}	0.4	0.99^{ab}	0.3
	5%	76.67^{cd}	17.0	6.67^{b}	0.6	16.18^{c}	1.3	2.76^{abc}	0.4	1.02^{ab}	0.3
Negative control		34.52^{a}	17.6	6.00^{ab}	0.3	11.50^{ab}	2.9	1.67^{a}	0.5	0.76^{a}	0.3

Based on Table 2, it was visible that different concentrations on the same extract were not significantly different towards germination and early growth of maize. However, based on the germination result which was the first activity observed during maize growth, it is showed that the highest concentration did not ally with high growth or germination. Lower concentration of the extracts gave higher germination and growth instead. Thus, to narrow down the effect of each extract towards maize growth in the second bioassay, lower concentration of 3.5% was chosen.

Several studies have reported that seaweed-based plant biostimulants were effective in low concentration. Khan et al. (2011) reported higher growth in roots and shoots of *Arabidopsis* following application of *Ascophyllum nodosum*-based biostimulants at 0.3% concentration. A similar trend was found on the evaluation of different concentrations (0.2; 0.4; 0.6; 0.8; and 1.0%) of seaweed extract (*S. tenerrimum*) on seed germination and growth of tomato plant (Sasikala et al. 2016). Full seed germination was only observed at 0.8% treatment together with a maximum number of leaves, root length, and total dry weight. While the maximum shoot length and total plant height were found at 0.6% concentration.

Figure 2 showed different growth of maize seedlings under different concentrations of acidic, alkali, and water extract compared to the negative control. The seedlings were grown for 7 days after germination. The non-significant difference in many treatments probably attributed to the over-grown seedling. Therefore, in the second bioassay, maize seedlings were grown for 4 days after germination in order to see more diverse effect of each extract towards early growth of maize seedling. Determination of concentration for the second bioassay was quite difficult due to no significant

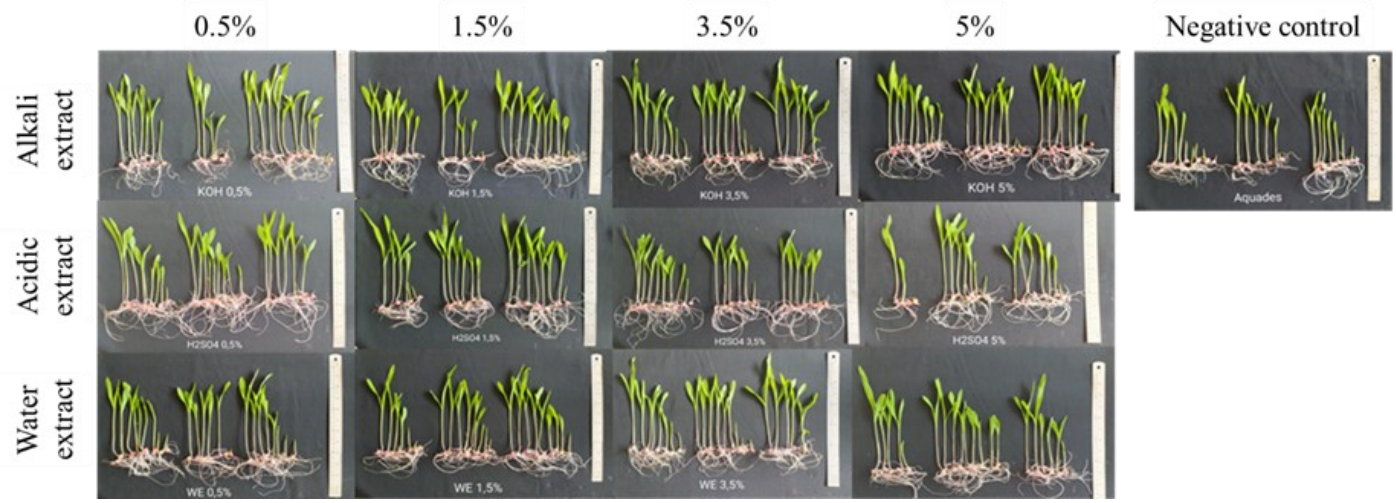

Figure 2. The growth of maize seedling under different treatments of *Sargassum* sp. Extracts.

different in all treatment, therefore, middle concentration given the highest germination was chosen, assumed that germination was the first stage influenced by the treatment.

The second bioassay was mainly performed to confirm the different actions of each extract in the germination and early growth of maize. A non-significant result from different treatments in the first bioassay making it difficult to determine the seaweed extraction method, therefore, the second bioassay was performed in higher replicates and minimum treatment. The result of the second bioassay is shown in Table 3 and Figure 3.

The second bioassay result showed improvement in germination and all growth parameters observed in this study for acidic, alkali, and water extract compared to negative control except for number of roots at water extract treatment. The highest germination occurred on alkali extract treatment but with no significant difference in all treatments. Acidic extract treatment occurred to give the maximum number of roots, shoot height, shoot weight, and root weight. The acidic extract was the only treatment with a significant difference in the number of roots compared to negative control than other treatments. Statistical analysis in the second bioassay helped in the determination of the optimum method to achieve high biostimulant activity from *Sargassum* sp. extract.

Table 3. The effect of liquid extracts of *Sargassum* sp. on germination and early growth of maize (second bioassay).

Treatment	Germination (%)		Number of roots		Shoot height (cm)		Shoot weight (g)		Root weight (g)	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Acidic extract	52.22[a]	9.4	4.87[b]	0.5	4.94[c]	1.9	4.94[c]	0.7	4.94[c]	0.3
Alkali extract	56.67[a]	6.3	3.89[a]	0.5	2.40[ab]	0.3	2.40[ab]	0.3	2.40[ab]	0.1
Water extract	56.67[a]	3.1	3.21[a]	0.7	3.26[b]	1.0	3.26[b]	0.3	3.26[b]	0.3
Negative control	44.44[a]	11.3	3.62[a]	0.3	1.78[a]	0.4	1.78[a]	0.5	1.78[a]	0.3

* Concentration used was 3.5% for all extracts, negative control is aquadest

** Means in the same column followed by different letters are significantly different according to Duncan's multiple range test at $\alpha = 0.05$

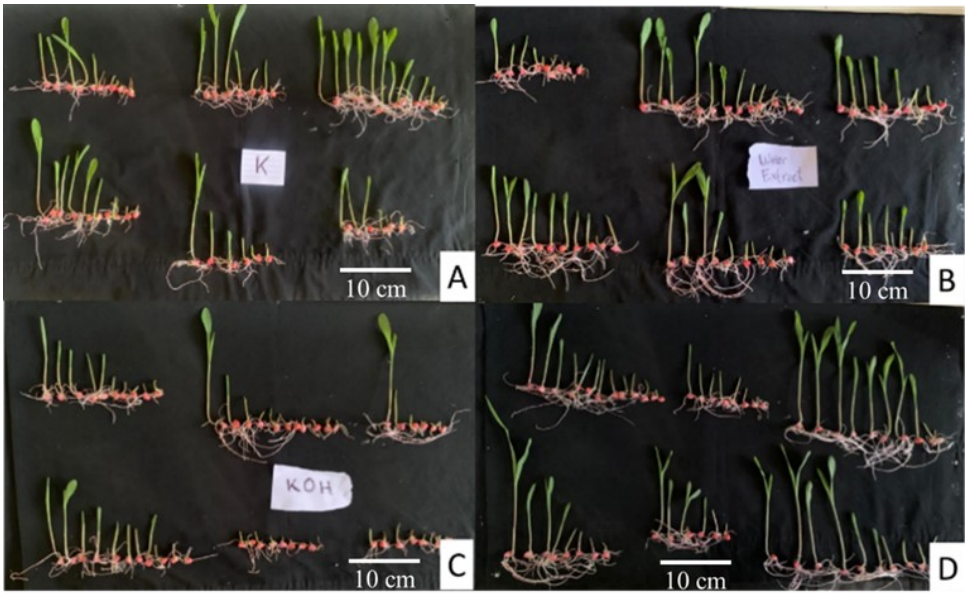

Figure 3. The growth of maize seedling under different treatments of *Sargassum* sp. extracts: (A) negative control (aquadest), (B) water extract (3.5%), (C) alkali extract (3.5%), and (D) acidic extract (3.5%).

Based on Table 1, it showed that acidic extract contained the lower phytohormones compared to alkali and water extract. However, it showed the highest biostimulant activity in the early growth of maize. Seaweed-based plant biostimulants were known to contain diverse compounds, ranged from single compounds such as amino acid and phytohormones, to complex matrices such as polysaccharides or polypeptides (Ugena et al. 2018). The activity of different groups of bioactive components in seaweed-based biostimulants has only been partly characterized. Understanding the mechanism of action requires multidisciplinary approach; not only based on phytohormones activity, but also from multiple interaction among different groups of bioactive compounds (El Boukhari et al. 2020). Therefore, further studies on the analysis of other bioactive compounds, such as bioactive peptides, amino acids, or polysaccharides, in *Sargassum* extract were highly recommended.

Previous study reported that acid hydrolysis of brown seaweed removed complex phenolic compounds and increased de-polymerization of polysaccharides (Flórez-Fernández et al. 2018). Generally, the acidic hydrolysis method was used for the extraction of fucose-containing sulfated polysaccharides, a class of bioactive compounds in seaweed extracts that promote plant growth (Shukla et al. 2016). Marais & Joseleau (2001) purified fucoidans from *Ascophyllum nodosum* by acid hydrolysis. AZAL5 is a commercially available biostimulant manufactured from *A. nodosum*, which is extracted through acid hydrolysis (Jannin et al. 2013).

The high germination rate under alkali extract treatment was linear to high gibberellins and zeatin content in the extract (30.88 and 38.25 mg. L^{-1} respectively) compared to other extracts. Several hormones induce germination and break the dormancy including Gibberellins (GAs), brassinosteroids, ethylene, and cytokinin (Kucera et al. 2005). Rayorath et al. (2008) suggested

that the organic components of the *A. nodosum* extracts also play important role in promoting seed germination, by inducing α-amylase activity independently from gibberellic acid-3 (GA3), that might act, in concert with GA-dependent α-amylase production, resulting in enhanced germination and seedling vigour in barley plant.

Alkali hydrolysis extraction methods have been used in biostimulant production (Craigie 2011; Sharma et al. 2013; Flórez- Fernández et al. 2018). Alkali extraction method underwent degradation, re-arrangement, condensation, and catalysis reactions which also produces novel compounds that are not initially present in the brown seaweed (Craigie 2011). Several commercial biostimulants are manufactured from brown algae including Maxicrop (United States), Seasol (Australia), and Acadian (Canada) (Shukla et al. 2019). Alkali hydrolysis under relatively low temperatures 70 and 100 °C breaks down complex polysaccharides into oligomers with smaller sizes and lower molecular weight (Craigie 2011). Alkali treatments of brown seaweed biomass also act on polyphenols in the tissue to produce a complex array of reaction products, which are dependent on the hydroxylation pattern of the original polyphenol (Craigie 2011).

Several reports demonstrated that treatment of seaweed extracts improved root vigour, plant growth and development, shoot/root biomass and leaf numbers, which resulted in the higher yields of various crops under normal or stress conditions (Sangha et al. 2014). Mahmoud et al. (2019) utilized *S. vulgare* extract by soaking the seeds before planting and indicated that the treatment gave significantly higher values of plant length, number of leaves, root diameter, and leaves and root weights as well as leaves and root dry weights than water-soaked seeds.

Considering the high activity of acidic *Sargassum* sp. extract on early growth of maize and humic acid extraction process with alkaline condition, it was suggested that extraction of *Sargassum* and humic acid should be conducted separately and mixed at the end of the process to neutralized pH. However, *Sargassum* sp. extract concentration is supposed to be adjusted to the humic acid formula to achieve the highest biostimulant activity in the further study.

CONCLUSION

In conclusion, *Sargassum* sp. extracts obtained from acidic, alkaline, and water -based extraction methods, were able to improve shoot and root growth of maize plants during the early stage of growth. The acidic extract showed the highest growth promotion among other extracts with the lowest phytohormones content.

AUTHORS CONTRIBUTION

FF, P, and Si designed the study; MAA, SW, IML, and Su collected the research materials; FF performed data collection; FF and Si analysed the data;

FF wrote the draft paper; MAA, SW, HF, and P preformed review and paper editing.

ACKNOWLEDGMENTS

The research was part of the grand research number PRJ-28/LPDP/2020 granted by Indonesia Endowment Fund for Education (LPDP), Indonesian Ministry of Finance.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest in this study.

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