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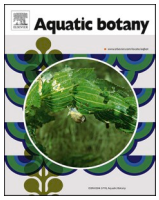

Enhancements provided by the use of an *Ascophyllum nodosum* extract can be transferred through archeospores in the red alga *Neopyropia yezoensis* (Ueda) L.-E. Yang & J. Brodie

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ABSTRACT

The use of seaweed extracts as biostimulants to promote enhancements in other seaweed crops is gaining momentum. Here we examined if the seaweed-derived biostimulant *Ascophyllum* marine plant extract powder – AMPEP, enhanced growth and thermal tolerance of cultured thalli of *Neopyropia yezoensis* when grown under optimal and sub-optimal temperature conditions. We also examined if enhancements could be transferred to new blades through archeospore germination. Area, specific growth rate, reactive oxygen species (ROS) and protein content of thalli were measured as indicators of potential enhancement. The application of AMPEP significantly increased growth rates in thalli of *N. yezoensis* grown under optimal temperature conditions, whilst the thalli showed no indications of improved thermal tolerance. The collated data suggested that growth enhancement could be transferred from treated thalli to newly formed blades, which developed from archeospores. This study provides new evidence of the far-reaching potential of using extracts of selected seaweeds as biostimulants to support the cultivation of economically important *Neopyropia* species.

1. Introduction

Neopyropia (Bangiales, Rhodophyta; gim in Korean and nori in Japanese) mariculture is one of the most economically important marine aquaculture industries in Asia, with China and Korea leading the market (Kim et al., 2017; Wu et al., 2017). Continued increasing demand for this biomass supports the development of extensive, selective breeding programs that have resulted in improved strains with higher growth rates, better flavor and appearance, and higher tolerances to diseases than the wild types of this genus (Hwang et al., 2019, 2020). The continued interest in *Neopyropia* as a food or feed product, and as a source of value-added by-products, with animal and human health benefits prompted research on other *Neopyropia/Pyropia/Porphyra* species with farming potential in America and Europe (Kim et al., 2017; Park et al., 2021). Despite progress made in Asia, *Neopyropia/Pyropia/Porphyra* farming elsewhere is still in its early stages, and selective breeding programs to improve local strains are far on the horizon (Kim et al., 2019).

Multiple studies have shown that applications of plant growth regulators and biostimulants derived from selected seaweeds could be used

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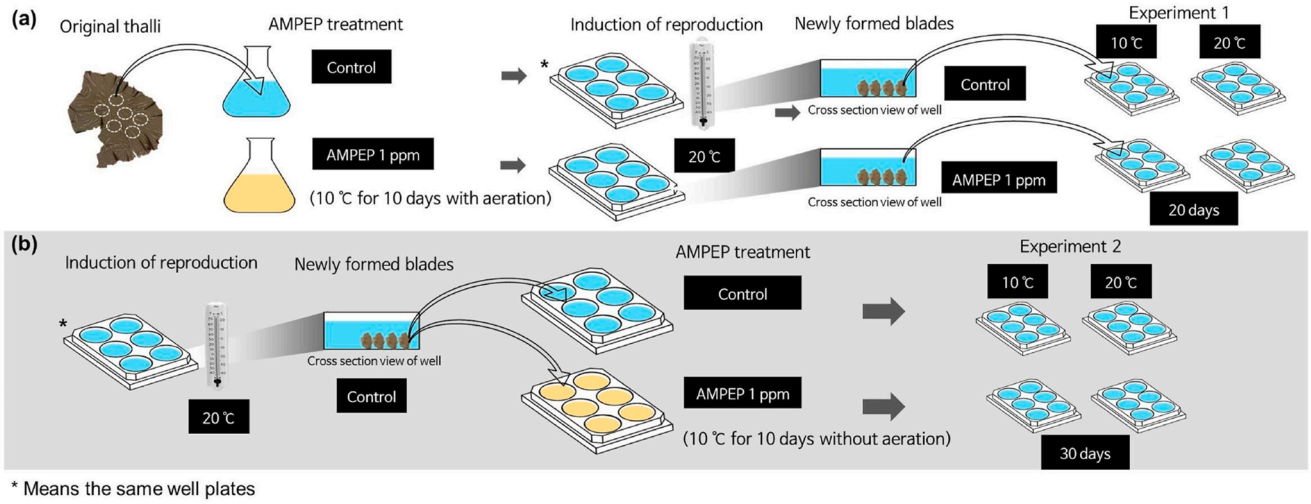

Fig. 1. Experimental design for two sequential experiments assessing the effects of AMPEP and temperature: (a) juvenile thalli via asexual reproduction (archeospores) derived from parental thalli of *Neopyropia yezoensis* (AMPEP treated and untreated) were cultivated at different temperature conditions; (b) juvenile thalli via asexual reproduction (archeospores) derived from the untreated parental thalli were exposed to AMPEP or no exposure to AMPEP and then cultivated at different temperature conditions.

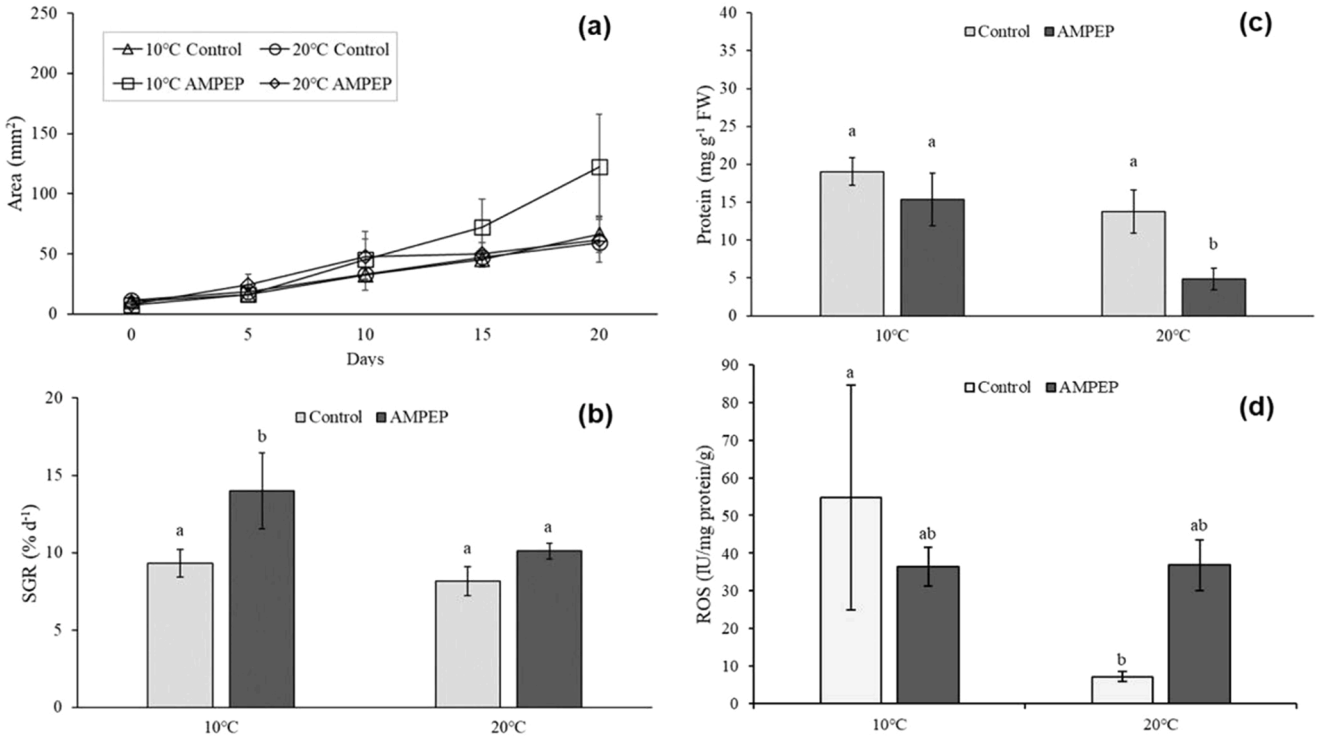

Fig. 2. Total area (a), specific growth rate (SGR) (b), protein (c), and reactive oxygen species (ROS) content (d) from experiment one in which parental thalli received AMPEP or control treatments, and measurements were made on juvenile thalli derived asexually from archeospores. Error bars show standard error of n = 3 replicates.

as an alternative approach to enhance multiple traits and the overall performance of various seaweed crops especially when exposed to suboptimal temperatures. For example, the application of different biostimulants derived from the temperate fucoid *Ascophyllum nodosum* was shown to enhance shoot formation by shortening the time to their appearance and increasing percentage emergence in the carrageenophyte *Kappaphycus alvarezii* (Ali et al., 2018). Longer erect shoots were detected when the biostimulant was supplemented with IAA and kinetin (Tibubos et al., 2017). Further studies also reported an increase in the thermal tolerance of *K. alvarezii*, possibly driven by enhanced antioxidant enzymatic activity (Loureiro et al., 2014). *Ascophyllum* extracts similarly stimulated shoot formation of *Eucheumatopsis isiformis* (Umanzor et al., 2020), and higher pigment concentrations in *Laurencia catarinensis* (Souza et al., 2019).

In general, the enhancements derived from the application of extracts of selected seaweeds have been attributed to their phytohormonal content (i.e., gibberellins, IAA, kinetin), micro- and macro-nutrients, or the presence of algal-specific compounds (i.e., betaines, polysaccharides, phenolic compounds) that would stimulate naturally occurring processes in treated land plant crops (Khan et al., 2009). A

Table 1
Comparative analysis of the effects of AMPEP and temperature in experiments one and two using two-way ANOVA.

Experiment 1	df	MS	F	P
Area				
AMPEP	1	2014.369	1.245	0.109
Temperature	1	772.594	3.245	0.297
AMPEP*Temp	1	767.453	1.236	0.298
Error	8	620.766		
SGR				
AMPEP	1	32.955	16.46	0.004
Temperature	1	19.034	9.507	0.015
AMPEP*Temp	1	5.693	2.843	0.13
Error	8	2.002		
Protein				
AMPEP	1	118.77	18.526	0.003
Temperature	1	186.945	29.16	0.001
AMPEP*Temp	1	20.635	3.219	0.111
Error	8	6.411		
ROS				
AMPEP	1	96.942	0.403	0.543
Temperature	1	1684.328	7.008	0.029
AMPEP*Temp	1	1724.522	7.176	0.028
Error	8	240.331		
Experiment 2	**df**	**MS**	**F**	**P**
Area				
AMPEP	1	3984.022	6.273	0.037
Temperature	1	6881.358	10.835	0.011
AMPEP*Temp	1	2037.996	3.209	0.111
Error	8	635.075		
SGR				
AMPEP	1	8.93	29.955	0.001
Temperature	1	3.269	10.965	0.011
AMPEP*Temp	1	0.006	0.019	0.895
Error	8	0.298		
Protein				
AMPEP	1	61.17	4.14	0.076
Temperature	1	759.468	51.407	0
AMPEP*Temp	1	60.123	4.07	0.078
Error	8	14.774		
ROS				
AMPEP	1	2,398,751.064	3.051	0.119
Temperature	1	19,537,458.13	24.854	0.001
AMPEP*Temp	1	8,924,294.994	11.353	0.01
Error	8	786,090.049		

recent study conducted to understand possible mechanisms driving enhancements of land plants showed that extracts, albeit mainly derived from *Ascophyllum*, affected the endogenous balance of plant hormones by adjusting hormonal homeostasis. These extracts also regulated transcription transporters, thus modulating nutrient uptake and assimilation, stimulating and protecting photosynthetic apparatus, and reducing stress-induced responses in (land) plants (Wally et al., 2013).

The complexity of the mode(s) of action(s) involved suggests that some of the effects prompted by using seaweed-derived biostimulants could be transferred from one generation to another or, in seaweeds, from parental thalli to newly formed thalli developed through archeospores (asexual-related spores). However, to our knowledge, this aspect has not yet been explored. Inheritance or transference of enhanced traits resulting from the application of biostimulants could be a quick and low-cost method to improve strains and universal practice, at least temporarily, to enhance particular traits in economically important seaweed crops.

2. Materials and methods

2.1. Experimental setup

We conducted two sequential experiments in which *Neopyropia yezoensis* growth and traits were compared between standard laboratory culture conditions and those same conditions with a 10-day exposure to AMPEP. *Ascophyllum* Marine Plant Extract Powder (Acadian Seaplants Ltd, Canada), also known as AMPEP, is a so-called biostimulant derived from the intertidal fucoid, *Ascophyllum nodosum*. This extract is commonly used commercially to enhance the development, growth, resistance, and vigor in agricultural, horticultural, and, more recently, seaweed crops (Pereira et al., 2020).

The two experiments differed by when exposure to AMPEP occurred. In the first experiment, thalli were exposed to AMPEP prior to archeospore formation and release. In the second experiment, we exposed newly formed blades derived from archeospores obtained after the first experiment. For both experiments, we tested the performance of thalli at two different temperatures (i.e., an optimal of 10 °C and sub-optimal 20 °C). AMPEP exposure ended prior to temperature treatments. For both experiments, we measured specific growth rates as a function of area, production of reactive oxygen species (ROS), and protein content as indicators of possible enhancements provided by the application of 1 ppm AMPEP. The extract solution was prepared by dissolving 1 g of AMPEP into 1000 ml of sterilized seawater. Once homogenized, the solution was filtered through a glass microfiber GF/F (Whatman) 0.7-μm filter to remove undissolved particles and residues. The experimental concentration of AMPEP tested was chosen based on preliminary trials comparing thalli growth at different concentrations (Table S1) where lower concentrations of this product showed no significant effects on *N. yezoensis*. VSE (von Stosch Enriched solution, as cited by Ott, 1966) was used in both control and treatment conditions.

2.2. Experiment one

Approximately 1 kg of *Neopyropia yezoensis* strain PY-SeC-ST1 was originally collected from a *Neopyropia* aquaculture farm, at Seocheon, Korea (36° 08'N, 126° 30'E) in January 2019. Culturing was set up in 2 L Erlenmeyer flasks filled with sterilized and enriched seawater (30 psu, VSE), 80–100 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), and a 12:12, L:D, provided by cool-white, fluorescent bulbs, with continuous aeration from zp-40 Zephyros pumps. *N. yezoensis* grows well at temperatures ranging from 8 to 10 °C, with signs of stress, including low photosynthetic performance and slow growth, detectable at 20 °C and above (Le et al., 2019). Although mutant strains have been developed to grow well at temperatures above 20 °C (Zhang et al., 2011), this is not the case for the strain used in these experiments.

In January 2020, 8 g (fresh weight) of *Neopyropia* were randomly sampled and sectioned using a cork borer of approximately 1.5 cm in diameter. Disks of thalli were then placed in a 500 ml flask containing either the control, or AMPEP solutions, so that each flask had 40 disks (design with potential pseudo-replication). Thalli were then exposed to a temperature of 10 °C in a growth chamber for ten days (Fig. 1a). Studies performed on other red seaweeds, particularly Eucheumatoids, showed positive results both if thalli were exposed to seaweed extracts for short periods (i.e., 30–45 min, Hurtado et al., 2012) or constantly (Umanzor et al., 2020). The application of AMPEP necessitates an extra step in the production of *Neopyropia* and given that the addition of AMPEP increases media turbidity, we considered a 10-day exposure to balance both issues, while still expecting to obtain measurable benefits. All flasks were provided with continuous aeration. Media were changed on day five after the initial exposure to avoid nutrient limitation. At day ten, the disks were individually transferred to 6-well plates (6 plates per solution). Each well contained one disk in 5 ml of the control medium only.

The twelve plates remained at a temperature of 20 °C for approximately 90 days, to induce archeospore release, germination, and blade formation. Temperature was reduced to 15 °C as soon as blades were detected and further reduced to 10 °C 24 h later to avoid thermal stress (Shin et al., 2018). Nine juvenile thalli (derived from archeospore pseudo-replicates of the single disk per well) of approximately 5 mm were then transferred once more to individual 5 ml wells within 6-well plates and cultivated at either 10 or 20 °C for 20 more days. In total, each treatment consisted of three replicates with nine pseudo-replicates (Fig. 1a). Media were changed every five days to avoid nutrient

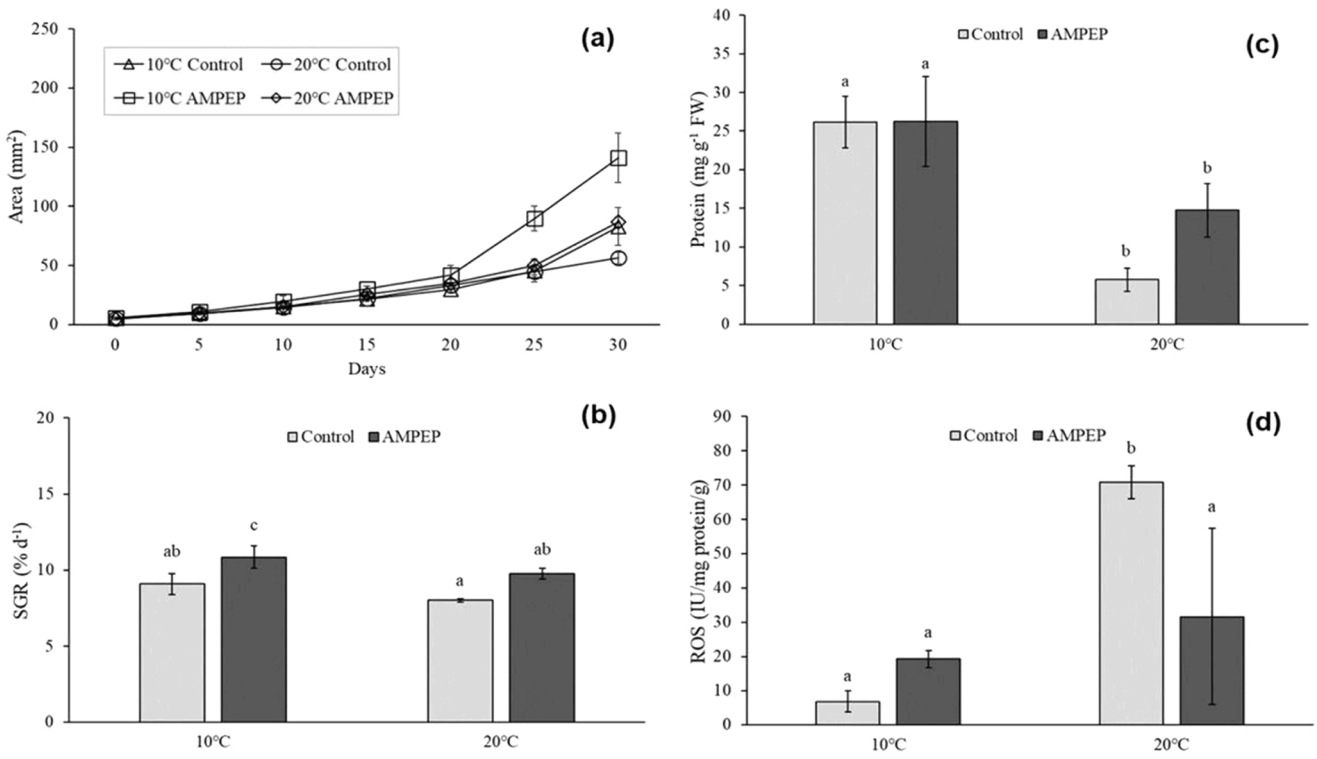

Fig. 3. Total area (a), specific growth rate (SGR) (b), protein (c), and reactive oxygen species (ROS) content (d) from experiment two in which thalli received AMPEP or control treatments, and measurements were made on newly formed blades derived asexually from archeospores from which parental thalli previously exposed to control treatments. Error bars show standard error of n = 3 replicates.

limitation. On the same day, thalli were photographed with an HK6.3E3S digital camera (KOPTIC, Korea) installed onto a Nikon Ts2R microscope (Nikon, Japan). The area of each thallus was measured using the binary imaging function in Image-J software (version 1.52a, Java 1.8.0_112, Rasband, 1997–2018) and used to calculate specific growth rates (SGR) as a function of changes in area over time. SGR was calculated using the following equation,

$$SGR = \frac{ln \quad A_{20} - ln \quad A_0}{T_{20} - T_0} \times 100$$

where A_{20} and A_0 correspond to the total area of the disks at days T_{20} and T_0, respectively.

By the end of the 20-day period, thalli were collected and processed for protein content and ROS determination.

2.3. Experiment two

Newly formed blades (approximately 5 mm) obtained from archeospores, without AMPEP treatment in experiment one, were transferred to individual 5 ml wells within fresh 6-well plates, containing either a control or AMPEP solution (as in the first set of experiments). Each plate (n = 3) held six-pseudo-replicates per treatment. All thalli remained exposed to their assigned solution for ten days, at a constant temperature of 10 °C, without aeration. Following the exposure period, thalli were placed in fresh, individual 6-well plates with 5 ml of control solution only and then exposed to either 10 or 20 °C for 30 days (Fig. 1b). Media changes, measurements, and data acquisition followed the same periodicity and methodology as described above.

2.4. Analysis of protein

Protein was measured to determine if thalli exposed to AMPEP showed similar content when growing at 10 vs 20 °C. Samples were processed according to Bradford (1976) using 0.150 g (fresh weight) of tissue per treatment. First, we created a protein standard curve by preparing solutions of 20, 40, 60, 80, and 100 μg protein/ml of Bovine Serum Albumin protein (BSA, Sigma-Aldrich) mixed with 1.5 ml Bradford's reagent. This reagent was prepared to a volume of 250 ml with distilled water, 0.025 g of Coomassie Blue G250 dye, 12.5 ml of 95% ethanol, and 25 ml H_3PO_4. Then, 30 μl of each standard mixture were added to individual wells within a 48-well plate so that each concentration had three replicates. In addition, one well was filled with 30 μl of distilled water used as a blank. The plate was incubated for 30 min at room temperature. After incubation, the absorbance per well was measured using a Synergy™ HTX Multi-Mode Microplate Reader (Bio-Tek, USA) at 595 nm. Once the standard curve was obtained, 0.150 g of tissue per treatment per plate (n = 3 plates) were placed in individual conical tubes. Each tube was supplied with 1 ml of 50 mM potassium phosphate buffer, containing 0.25% Triton X-100% and 1% polyvinylpyrrolidone, and homogenized to extract proteins. Homogenized samples were centrifuged at 3134 rcf (relative centrifugal force) for 40 min.

Second 48-well plates were prepared with triplicates of the five standard solutions described above. After centrifugation of the conical tubes, the supernatant of each sample was recovered and added to the wells of the second plate such that each standard concentration was supplemented with aliquots of 25 μl of the samples, in addition to 75 μl of distilled water and 2.5 ml of Bradford's Reagent. The content was thoroughly mixed and incubated for at least 5 min at room temperature. Wells used as blanks consisted of 100 μl of distilled water and 2.5 ml of Bradford's Reagent. Measurements were conducted using a Synergy™ HTX Multi-Mode Microplate Reader at the absorbance of 595 nm. Protein content per sample were calculated as:

$$Protein \quad content \quad (mg/g) = OD/(m * 500 * g)$$

where OD is the optical density (i.e., absorbance of the sample at 595 nm), m is the slope (Y = mX), 500 is a factor, and g is weight of the sample.

2.5. Analysis of ROS

ROS was measured as a marker to detect oxidative stress driven by temperature stress. ROS analysis was performed following Cathcart et al. (1983). If AMPEP provided any thermal enhancement, we would expect elevated ROS levels only in thalli grown at 20 °C without AMPEP. First a dye solution was prepared by adding 0.5 ml of 1 mM DCFDA (2′, 7′-dichlorodihydrofluorescein diacetate) to 2 ml of 0.01 N NaOH and incubated for 30 min at room temperature. Then, the hydrolase was neutralized with 10 ml of 25 mM sodium phosphate buffer (pH 7.2) and stored on ice until used. Twenty-five mM sodium phosphate buffer (pH 7.2) was prepared by diluting 4.275 ml of 1 M Na_2HPO_4 and 1.976 ml of 1 M $NaH_2PO_4{\cdot}H_2O$ in 250 ml of distilled water and setting the pH to 7.2. Each tissue sample was weighed to obtain 0.150 g (fresh weight) and homogenized with 1 ml of potassium phosphate buffer containing 0.25% Triton X-100% and 1% polyvinylpyrrolidone. Homogenized samples were centrifuged at 3134 rcf for 40 min. After centrifugation, 20 μl of the supernatant per sample was recovered and mixed with 180 μl of the potassium phosphate buffer solution and 200 μl of dye solution in individual wells within a 48-well plate. The plate was incubated in the dark at 20 ℃ for 1 h. Fluorescence (λ_{ex} = 485 and λ_{em} = 535 nm) was measured immediately after using a Synergy™ HTX Multi-Mode Microplate Reader.

The concentration of ROS was calculated as:

$$ROS \quad (IU \Big/ mg \quad protein \Big/ g) \quad = \left[\frac{\{Fluorescence \quad change \quad (|ROS - blank|) * Total \quad reaction \quad volume\}}{EC * Supernatant} \right] * g$$

where, EC is a constant equal to 1 and g is the fresh weight of tissue used.

2.6. Data analysis

Differences in the responses of *N. yezoensis* were analyzed independently using two-way ANOVAs for experiments one and two. The concentration of AMPEP (0 and 1 ppm) and temperature (10 and 20 °C) were set as categorical and independent factors. Pseudo-replicates were averaged to obtain one value per true replicate. Normality (Shapiro–Wilk test), independence of variables (Durbin–Watson test), and homogeneity of variances (Cochran's test) were confirmed per factor and level. Data required no transformation. Tukey's honestly significant difference tests ($p < 0.05$) were conducted to further explore differences for all analyses even if factors showed no significant interaction. Data were analyzed with the statistical software SPSS 25.0.

3. Results and discussion

Overall, the application of AMPEP showed significant positive results for the performance of *Neopyropia yezoensis* when cultured at 10 °C. Specifically, although no significant differences were found in terms of thallus area in experiment one (Fig. 2a; p-value > 0.05; Table 1), SGR was enhanced in thalli treated with AMPEP and cultured at 10 °C. This was compared with control thalli at 10 °C, control and thalli treated with AMPEP at 20 °C. These results showed a significant improvement, influenced by the application of AMPEP (p-value < 0.05, F-value 16.46; Table 1) and temperature (p-value < 0.05, F-value 9.507; Table 1). Complementary to the Analysis of Variance, the Tukey's test showed an interactive effect of AMPEP and temperature (Fig. 2b; Tukey p-value < 0.05; Table S2).

In experiment two, the area and growth rates of AMPEP-treated thalli cultured at 10 °C were significantly greater than thalli in either control treatments or cultured at 20 °C (Fig. 3a and b; Tukey p-value < 0.05; Table S2). These findings provided insights as to enhancements on growth to *Neopyropia yezoensis* after the application of AMPEP. On the other hand, findings from experiment one, it also provided preliminary results showing the potential of having growth enhancement transferred to new blades via archeospores through experiment one. Results could directly contribute to the cultivation of *N. yezoensis* and possibly other commercially valuable species within this and related genera. Data collected aligns with the increasing body of evidence related to the benefits of using extracts of seaweeds in algal cultivation.

Seaweed extracts contain phyco-elicitors, such as IAA-like and cytokinin-like substances (Khan et al., 2009; Stirk et al., 2020) that coupled with other constituents (e.g., micronutrients), are recognized to influence numerous aspects of macroalgal growth, development, and physiology (Hurtado et al., 2012; Tibubos et al., 2017). Specifically, cytokinin (in the form of kinetin) induces cell division and differentiation (Letham, 1967), the analogous activity of which could explain the enhancement observed in the final total area and growth rates of the thalli treated with AMPEP. Still, this is only one of the possibilities as the mechanisms of action are unknown.

Despite the enhancements observed in growth, outcomes related to protein content and production of ROS did not support any significant enhancement in thermal tolerance that could have been attributed to the application of AMPEP (Figs. 2c and d; 3c and d). Heat stress is a frequent abiotic stress in the marine environment (Wernberg et al., 2016). It can cause protein denaturation, enhance the production of reactive oxygen species, and negatively influence the photosynthetic capacity of crops, resulting in metabolic imbalances affecting the overall performance, including farmed seaweeds (Cortleven et al., 2019; Le et al., 2019). Although we expected expression of ROS and low protein, only at elevated temperature without AMPEP, we found no differences in these response variables among any treatments, in either experiment. Outcomes show that the application of AMPEP may even have negative effects (Figs. 2c and d; 3d; Tukey p-value < 0.05) or no detectable effect (Fig. 3c; Tukey p-value < 0.05) on the performance of thalli at 20 °C. Further studies may benefit from a greater number of replicates and experimental temperatures.

Patterns of transference related to enhancements provided by seaweed extracts are not yet documented for macroalgal crops. Nonetheless, these patterns have already been reported for terrestrial crops. For instance, experiments conducted on wheat and chickpeas to which seaweed extracts were applied to prevent pathogenic fungi, documented that both crops showed changes in gene expression that were transferred from treated individuals to their progeny (Tsygankova, 2012). It is plausible that the application of AMPEP on thalli of *N. yezoensis* could have caused epigenetic changes that were transferred to their newly formed thalli via the archeospores. Such assessments were out of the scope of our work. The complex nature of the composition of seaweed-derived extracts and the wide range of compounds they contain (including natural and created in the reaction process) complicates understanding and defining the pathways in which AMPEP is responsible for the enhancements observed. However, this work provides a foundation to investigate further the modes of action and benefits of using seaweed-derived extracts to enhance seaweed cultivation.

Cultivation of *Neopyropia yezoensis* has been enhanced through biotechnological and technological innovations, including the development of long-term selective breeding programs (Hwang et al., 2020) and the use of bioreactors to sustain growth of free-living conchocelis (He and Yarish, 2006). The application of selected extracts of seaweeds is a

promising, rapid, and low-cost approach that could complement current optimization approaches in *Neopyropia* production. Seaweed extracts such as AMPEP could be applied to batches of *N. yezoensis* during the nursery and transferred to larger batches through archeospore production and germination.

Author statement

This manuscript or a very similar manuscript has not been published, nor is under consideration by any other journal.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquabot.2021.103481.

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