Effect of UV-B Radiation Intensity on *Hsp70*, *mtHsc70-1*, and *cpHsc70-2* Gene Expression in *Synedrella nodiflora* (L.) Gaertn. Leaf Under Field Conditions

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ABSTRACT
Increased expression of genes encoding Heat Shock Protein 70 (Hsp70) is one of the plant defense responses against UV-B stress. *Synedrella nodiflora* may be tolerant to relatively high UV-B intensity. The research has been conducted to compare expression levels of genes encoding cytosolic, mitochondrial, and plastid Hsp70 (*Hsp70*, *mtHsc70-1*, and *cpHsc70-2*) in *S. nodiflora* leaves against environmental conditions with different UV-B radiation intensities in Faculty of Mathematics and Natural Sciences campus area, Universitas Indonesia, Depok. Alpha Tubulin (*TUB*) was used as a reference gene to normalize the cDNA quantity of each Hsp70 gene. The levels of relative gene expression were analyzed using the Pfaffl method. Average UV-B intensity in control, shaded, and open locations were 18.4 ± 0.1, 44.1 ± 0.6, and 260.1 ± 78.3 mW/m^2, respectively. Expression levels of *Hsp70* and *cpHsc70-2* were relatively higher in leaves collected from shaded locations, while *mtHsc70-1* was relatively lower. Inversely, expression levels of *Hsp70* and *cpHsc70-2* were relatively lower in leaves collected from open locations, while *mtHsc70-1* was relatively higher. *S. nodiflora* in an open location may have acclimatized to environmental conditions with relatively higher temperature, light intensity, and UV-B intensity. This acclimatization caused *S. nodiflora* in an open location to experience an increase in minimum temperature, light intensity, and UV-B intensity required to induce an increase in *Hsp70* and *cpHsc70-2* gene expression. The *Hsp70*, *mtHsc70-1*, and *cpHsc70-2* genes have different expression patterns in different environmental conditions, such as different UV-B radiation intensities.

Keywords: *cpHsc70-2, gene expression, Hsp70, mtHsc70-1, Synedrella nodiflora, UV-B radiation*

1. INTRODUCTION

Plants use sunlight as a source of energy and environmental cues that regulate the processes of growth, development, and adaptation [1, 2]. The ultraviolet-B (UV-B: 280–320 nm) radiation is a part of sunlight that can induce stress responses in plants [2]. UV-B-induced stress is a rare event under field conditions [3]. Decrement of the stratospheric ozone layer due to anthropogenic factors, such as the use of chlorofluorocarbons (CFC) [1, 2, 4], cause changes in the spectral UV-composition and increases in the flux of the UV-B reaching the surface of Earth [4, 5], which increase the amount of biologically active UV-B [1]. High dose UV-B radiation over the ambient level (1–3 μ mol m^{-2} s^{-1} or higher) causes cellular damage by high production of Reactive Oxygen Species (ROS) in a short time that can induce Programmed Cell Death (PCD) [1, 2].

Plants are sessile organisms that respond and adjust to various stresses, such as UV-B-induced stress [6, 7]. Increased expression levels of genes encoding Heat Shock Proteins 70 (Hsp70s) are one of the plant defense

reactions upon UV-B stress [8, 9, 10, 11]. These proteins generally act as molecular chaperones associated with protein folding, preventing the irreversible aggregation of misfolded proteins, facilitating renaturation of aggregated proteins, and helping protein translocation into the subcellular compartments. Hsp70s are found in some subcellular locations in a plant cell, such as cytosol, mitochondria, and plastid [6, 12].

Synedrella nodiflora (L.) Gaertn. is a plant species belonging to the Asteraceae family and native to tropical America. However, this plant is now widely distributed in every tropical and subtropical region as an invasive weed [13, 14]. *S. nodiflora* can grow in various environmental conditions. For example, this species grows in shaded and open areas [13]. Most *S. nodiflora* populations in the Universitas Indonesia campus area, Depok, tend to grow in open areas with full sunlight exposure [15]. UV-B radiation intensities in open areas are relatively higher than in shaded areas [16]. The expression levels of *Hsp70* genes are known correlated with stress resistance [10, 17]. Therefore, it can be assumed that *S. nodiflora* in open areas may be tolerant

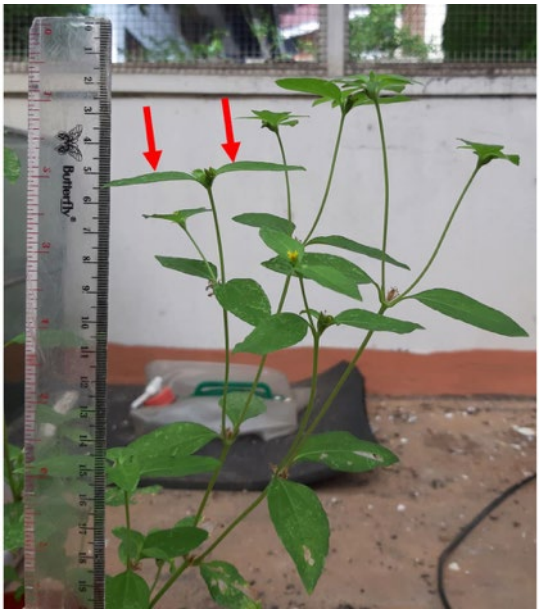

Figure 1. Position of *S. nodiflora* young leaves to relatively high UV-B intensity.

Studies investigating the effect of UV-B stress on *Hsp70* genes expression were mostly carried out indoors with low intensities of Photosynthetically Active Radiation (PAR) [16]. This experimental condition did not represent growth conditions that occur naturally in the field. However, how different UV-B intensities affect the expression levels of genes that encode Hsp70 in naturally occurring *S. nodiflora* has not been characterized yet. Here, we compare expression levels of genes encoding cytosolic, mitochondrial, and plastid Hsp70 (*Hsp70*, *mtHsc70-1*, and *cpHsc70-2*) in *S. nodiflora* leaves against environmental conditions with different UV-B radiation intensities. mRNA amounts of *Hsp70*, *mtHsc70-1*, and *cpHsc70-2* were quantified by Reverse Transcription–quantitative real-time Polymerase Chain Reaction (RT-qPCR).

2. MATERIALS AND METHODS

2.1. Plant Materials and Environmental Factors Measurement

The research was conducted from February to June 2021. The first to third of young, healthy *S. nodiflora* leaves below the bud used as samples for RNA isolation (Figure 1). The young leaves were collected from five *S. nodiflora* individuals chosen randomly in each location to represent five replicates of total RNA isolation (one plant per replicate). Young leaves from *S. nodiflora* were collected from three locations in the Faculty of Mathematics and Natural Sciences campus area, Universitas Indonesia, Depok (Figure 2). *S. nodiflora* population that grew under a *Syzygium myrtifolium* plant near the Department of Geography Building was chosen to represent shaded conditions. The *S. nodiflora* population that grew on an open area in the park between the Department of Biology Building and the Faculty of Pharmacy Building was chosen to represent open conditions. Five *S. nodiflora* individuals were also collected randomly from the Faculty of Mathematics and Natural Sciences campus area, Universitas Indonesia, Depok, as control plants. These individuals were grown in pots filled with a mixture of manure and fuel husk (1:1 in volume) media. Control plants were acclimatized for a week and were maintained in Green House, Department of Biology, Universitas Indonesia, Depok. Therefore, there were 5 leaf samples from 5 plants per location and 15 leaf samples from 15 plants in total. The sample collecting time was from 12.00 to 13.00. Leaf samples were immediately put in a 1.5 mL microfuge tube that contains 1 mL of Nucleic Acid Preservation (NAP) buffer [18]. The leaf samples were then stored at -20°C or directly used for total RNA isolation.

Samples collection, measurement of environmental factors (UV-B radiation intensity, temperature, and light intensity), and weather observation were carried out simultaneously in each location for 10 minutes. UV-B radiation intensity, temperature, and light intensity data were collected using temperature probe (Vernier), light sensor (Vernier), and UV-B sensor (Vernier), respectively. All sensors were connected to a LabQuest Stream (Vernier). The data collection settings were set using the Graphical Analysis Application v5.7.1-309 (Vernier), which was installed in a smartphone and was connected to the LabQuest Stream via Bluetooth. Time-Based and minutes (min) were chosen for Mode and Time Units options, respectively. The data collection

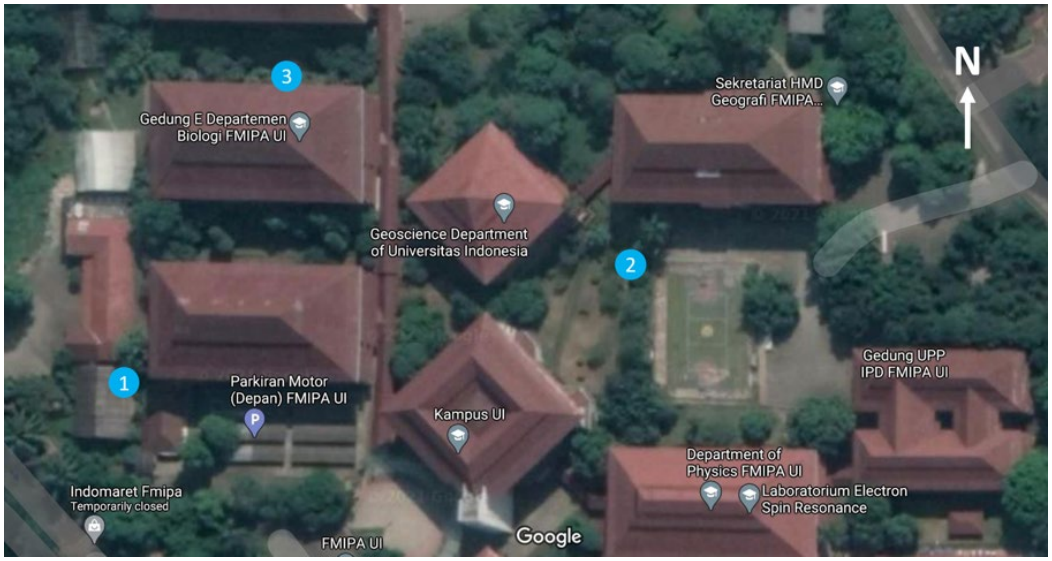

Figure 2. Locations of *S. nodiflora* sampling; 1 = Green House (control condition); 2 = *S. myrtifolium* near Department of Geography Building (shaded condition); 3 = park between Department of Biology Building and Faculty of Pharmacy Building (open condition).

was started manually and was ended after 10 minutes duration. The environmental factors data collection rate was 1200 samples/min (interval 0.00083333 s/sample).

2.2. Total RNA Isolation and cDNA Synthesis

Total RNA isolation has been done in Molecular Biology Preparation Laboratory, Department of Biology, Universitas Indonesia, Depok. Total RNA was isolated from 30-90 mg *S. nodiflora* leaves using a Plant Total RNA Extraction Miniprep System Kit (Viogene) following the manufacturer's protocol. Measurement of total RNA purity and concentration, RNA integrity assessment, DNase treatment, and complementary DNA (cDNA) synthesis were done in Integrated Instrumentation Laboratory, Department of Biology, Universitas Indonesia, Depok. The purity of total RNA and its concentration were measured with NanoPhotometer® spectrophotometer (Implen). The integrity of total RNA was checked with 1% agarose gel electrophoresis stained with GelRed (1 µL/20 mL agarose). The agarose gel was then visualized under trans-UV light using a Gel Documentation System (BioRad). Genome DNA (gDNA) elimination from total RNA samples was performed using RNase-*free* DNase I (ThermoFisher Scientific), following the kit instructions. The total RNA samples were stored at -80°C or used immediately in the following step. The total RNA (400–700 ng) that was already isolated was reverse-transcribed with oligo $(dT)_{18}$ primer (100 µM) using a RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Rapid cooling on ice for 1-2 minutes after incubation of total RNA sample, oligo $(dT)_{18}$ primer, and Nuclease Free Water (NFW) mixture at 65°C for 5 minutes was performed before other cDNA synthesis components were added. Incubation of M-MuLV Reverse Transcriptase (200 U/µL) was performed for 60 minutes at 45°C, and the cDNA was stored at -80°C or used directly for real-time PCR.

2.3. Real-time PCR

Real-time PCR was done in Integrated

Table 1. Primer sequences for real-time PCR of each target gene and reference gene

Gene	GenBank Accession Number	Primer Sequence 5' to 3' (Forward/Reverse)	Amplicon Length (bp)	Subcellular Location	Reference
Hsp70	AP002055/ AJ002551	TCAAGCGGATAAGAGTCAC/ CTCGTCCGGGTTAATGCT	862*	Cytosol	[19]
mtHsc70-1	AL035538	GCTGCTGCACTATCATATG G/CACGGAGGATACCACCTT	613*	Mitochondrion matrix	[19]
cpHsc70-2	AB024032/ AF217459	GGTGATCCTTGTTGGTGG/ ATCTCAACGCTTGTCTGTC	306*	Plastid stroma	[19]
TUB	GT051159	ATGCTTTCGTCTTATGCCC/ CTCTTGGTTTTGATGGTTGC	215	-	[20]

*The amplicon length of *Hsp70*, *mtHsc70-1*, and *cpHsc70-2* were determined using Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) based on Refseq mRNA database.

Instrumentation Laboratory, Department of Biology, Universitas Indonesia, Depok. Real-time PCR of the *Hsp70, mtHsc70-1,* and *cpHsc70-2* gene was performed using the Rotor-Gene Q (QIAGEN) in a 36-well rotor. All real-time PCR reactions were set up manually. Each real-time PCR reaction had a final volume of 20 µL that contained 10 µL qPCR Master Mix 2X KAPA SYBR FAST (KAPA BIOSYSTEMS), 200 nM of each gene-specific primer (Macrogen), cDNA (final concentration < 20 ng), and NFW. Primer sequences for target genes (*Hsp70, mtHsc70-1,* and *cpHsc70-2*) and reference genes (Alpha Tubulin, *TUB*) were determined based on [19] and [20], respectively. Primer sequences used for real-time PCR are shown in Table 1. Real-time PCR amplification was performed by applying enzyme activation of 3 minutes at 95°C, and 40–45 cycles of denaturation, annealing, and extension (data acquisition) for 3 seconds at 95°C, 20 seconds at 55°C, and 20 seconds at 72°C. The thermal profile was set using the Q-Rex Software 1.1.0 (QIAGEN). The program file and manual of Q-Rex Software 1.1.0 can be downloaded from www.qiagen.com/Q-Rex-software. Total RNA from five individuals in each location was used for real-time PCR analysis. Real-time PCR reaction was run in triplicate for each cDNA sample.

2.4. Data Analysis

The average value of amplification efficiencies (*E*) of all real-time PCR reactions was used to determine the *E* of each target and reference genes. The amplification efficiency of each reaction was calculated by Q-Rex Software 1.1.0 (QIAGEN) based on the 4 data points following the take-off point. The take-off point is the cycle where the run transitioned into the exponential phase [21]. The Analysis of Variance (ANOVA) single-factor at $\alpha = 0.05$ was conducted to ensure that the mean of *E* from each gene was not significantly different from another. The Least Significant Difference (LSD) test at $\alpha = 0.05$ would be conducted if the mean of *E* from each gene were significantly different from another. ANOVA was performed using Microsoft Excel 2019, while the LSD test was performed manually. Calculation of quantification cycle (C_q) values was started from the first cycle. C_q threshold fluorescence at 0.1 was determined to calculate C_q values. cDNA quantity of each gene was normalized to *TUB* (reference gene). Levels of relative gene expression were analyzed using the Pfaffl method [22].

3. RESULTS AND DISCUSSION

3.1. Environmental Conditions of Sampling Locations

The data of UV-B radiation intensity, temperature, light intensity, and weather conditions during leaf samples collected in each location are shown in Table 2. Average UV-B intensity, temperature, and light intensity were highest in an open location. Average UV-B intensity was lowest in the control location, while average temperature and light intensity were lowest in the shaded location. The open location had the highest Standard Deviation (SD) for all environmental factors.

According to Paul & Gwynn-Jones [16], an open area has a relatively higher UV-B intensity than a shaded area. The relatively higher temperature in an open area is frequently accompanied by higher light intensity [23]. The highest SD values for all environmental factors indicated more frequent UV-B intensity, temperature, and light intensity fluctuations in the open location than in control and shaded location. These fluctuations were also caused by cloudy conditions that happened during sample collection in an open location. Cloud cover generally contributes to reducing UV-B that reaches the ecosystems [16].

Table 2. UV-B radiation intensity, temperature, light intensity, and weather conditions recorded in control, shaded, and open location

Trait	Location (Date, Time of Samples Collection)		
	Shaded (April 13th 2021, 12.01-12.11)	Control (April 19th 2021, 12.19-12.29)	Open (April 27th 2021, 12.00-12.10)
UV-B intensity (mW/m²)			
Minimum – Maximum	42.1–44.9	18.1–18.6	176.8–378.4
Mean ± SD	44.1 ± 0.6	18.4 ± 0.1	260.1 ± 78.3
Temperature (°C)			
Minimum – Maximum	32.3–32.9	32.3–34.0	33.4–35.6
Mean ± SD	32.5 ± 0.2	33.2 ± 0.5	34.3 ± 0.7
Light intensity (lux)			
Minimum – Maximum	4110.7–5882.5	7828.4–8459.9	19850–78990
Mean ± SD	4546.1 ± 242.1	8439.6 ± 80.7	43640 ± 23400
Weather condition(s)	Sunny	Sunny	Sunny – cloudy – sunny – cloudy

SD: Standard Deviation

3.2. Amplification Efficiency

Average amplification efficiencies (*E*) of target and reference genes are shown in Figure 3. *mtHsc70-1* had the highest average *E* value (1.87 ± 0.12), while *Hsp70* had the lowest average *E* value (1.62 ± 0.15). The average *E* value of *TUB* and *cpHsc70-2* was 1.86 ± 0.12 and 1.7 ± 0.15, respectively. Single-factor ANOVA result at α = 0.05 indicated significant differences among four average *E* value, i.e., *Hsp70*, *mtHsc70-1*, *cpHsc70-2*, and *TUB*. LSD test result at α = 0.05 revealed the average *E* of *TUB* and *Hsp70*, *TUB* and *cpHsc70-2*, *Hsp70* and *mtHsc70-1*, *Hsp70* and *cpHsc70-2*, *mtHsc70-1* and *cpHsc70-2* were significantly different. Meanwhile, the average *E* of *TUB* and *mtHsc70-1* were not significantly different.

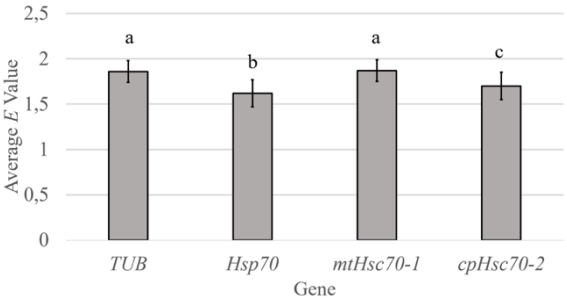

Figure 3. Average amplification efficiencies (*E*) of target and reference genes. Error bars indicate the standard deviation of the mean. The difference in letters in each column indicates a significant difference based on the LSD test (α < 0.05)

Average amplification efficiencies (*E*) for all four genes ranged from 1.62 ± 0.15 to 1.87 ± 0.12, representing poor efficiency. The acceptable range of *E* value is 90–110% (*E* = 1.9–2.1) [24]. *Hsp70* showed the lowest average *E* value with the longest amplicon among four genes (see Table 1). In general, the longer the amplicon, the lower the *E* value [25]. *TUB* had the shortest amplicon among the four genes (see Table 1), but the average *E* value was ranked second. Evaporation of some reactions can cause this to happen during real-time PCR. Evaporation of the sample can decrease the number of reaction components resulting in incomplete amplification of specific genes during each cycle of the exponential phase [26]. Amplicon length of *mtHsc70-1* was longer than *cpHsc70-2* (see Table 1). However, the average *E* value of *mtHsc70-1* was relatively higher than *cpHsc70-2*. These results indicate that amplification efficiency is determined by amplicon length and sample evaporation [26], formation of primer-dimers, thermocycling conditions, and/or real-time PCR reagent concentrations [24].

3.3. Expression Levels of Hsp70 Genes

Relative gene expression ratio (*R*) of *Hsp70*, *mtHsc70-1*, and *cpHsc70-2* that were calculated using the Pfaffl method [22] are shown in Figure 4. Pfaffl method was chosen to measure relative gene expression because the average *E* values of all genes were different (see Figure 3). *S. nodiflora* leaves from the shaded location had *Hsp70, mtHsc70-1,* and *cpHsc70-2* expression levels that increased 85.54; 2.41; and 30.58-fold relative to *TUB*. Leaves from open locations had *Hsp70, mtHsc70-1,* and *cpHsc70-2* expression levels that increased 2.01, 9.46, and 2.50-fold relative to *TUB*. These results show that *Hsp70* genes could be expressed in the natural environment [10, 17].

Interestingly, we found that *Hsp70* and *cpHsc70-2* expression levels were relatively higher in leaves collected from shaded locations, while *mtHsc70-1* was relatively lower. Inversely, *Hsp70* and *cpHsc70-2* expression levels were relatively lower in leaves collected from open locations, while *mtHsc70-1* was relatively higher. These results were surprising since most studies reveal that UV-B radiation, temperature, and/or light stress induce the expression of *Hsp70* genes. Swindell et al. [8] showed that UV-B and high-temperature stress increased expression levels of some Hsp70 genes in Arabidopsis leaves. Different *Hsp70* genes had different induction responses to UV-B and high-temperature stress. Barua & Heckathorn [23] demonstrated that Hsp content in *Solidago altissima* in the open area was significantly greater than shaded area. Recently, Kim et al. [11] revealed that *Hsp70* genes expression patterns varied in *Lactuca sativa*. For example, *LsHsp70-23* and *LsHsp70-39* were unresponsive to high light exposure but were induced by UV exposure. Inversely, *LsHsp70-32* did not change to UV stress but was induced by high intensity light.

Gene expression patterns of *S. nodiflora Hsp70* and *cpHsc70-2* under field conditions may be similar to expression patterns of *HSFA2* and *CP-sHSP* genes in *Potamogeton perfoliatus* [27], which were determined by the heat acclimation process. It could be assumed that responses of *S. nodiflora Hsp70*, *mtHsc70-1*, and *cpHsc70-2* to environmental stress may be determined by the acclimatization process. Acclimation is a physiological phenotype alteration of an individual after exposure to one or two distinct environmental parameters. This phenomenon usually happens under controlled laboratory conditions. Acclimatization is a physiological phenotype alteration of an individual after being subjected to various natural conditions [28, 29].

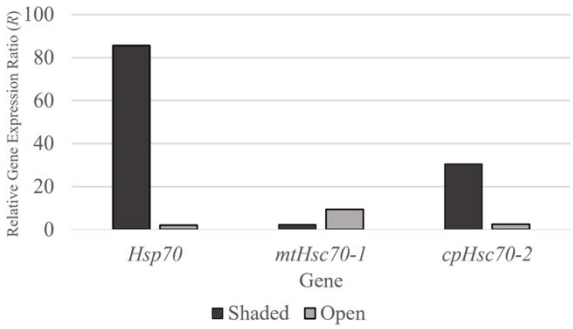

Figure 4. Relative gene expression ratio (*R*) of *Hsp70*, *mtHsc70-1*, and *cpHsc70-2* in *S. nodiflora* leaves collected from shaded and open locations. Calculation of *R* values were performed using the Pfaffl method.

S. nodiflora may be sensitive to a relatively higher temperature, light intensity, and UV-B intensity. This assumption is reinforced by the statement of CABI, which states that *S. nodiflora* tends to grow in slightly shaded areas [13]. *S. nodiflora* growing in shaded locations is thought to have not acclimatized to relatively high light intensity, temperature, and UV-B intensity. This unacclimatized situation makes them more susceptible to cell damage if there is an increase in UV-B, temperature, and light intensity. Cellular damage due to abiotic stress is generally caused by increased ROS production [30]. Therefore, the expression of genes that help increase ROS scavenging activity should be induced at a lower temperature, light intensity, and UV-B intensity.

The increased Hsp synthesis helps reduce ROS accumulation in cells [31]. The relative expression levels of *Hsp70* and *cpHsc70-2* at the shaded location were higher than at open ones. Scarpeci et al. [9] showed that Hsp70, localized in the cytoplasm of *A. thaliana* cells, contains relatively more cysteine residues than other Hsp. The cysteine residue in a protein plays an important role in regulating the redox conditions of the cell. Therefore, increased expression of *Hsp70* regulates redox status by regulating the levels of ROS produced due to the presence of abiotic stresses [11].

Increased expression of the gene encoding Hsp70 localized in chloroplasts is often associated with light and UV-B stress. High-intensity UV-B and light can damage the proteins that makeup photosystem II and trigger ROS production increase in the chloroplast [5]. The relatively lower average UV-B intensity, temperature, and light intensity are thought to cause the relative expression level of *mtHsc70-1* to be lower than the other two genes. Abiotic stresses are also known to trigger an increase in ROS production in the mitochondria [32].

S. nodiflora in an open location may have acclimatized to environmental conditions with relatively higher temperature, light intensity, and UV-B intensity, making it more resistant to cellular damage. The relative gene expression levels of *Hsp70* and *cpHsc70-2* at the open location were lower than at the shaded one. This indicates that *S. nodiflora* in open location increase in minimum UV-B intensity, temperature, and light intensity required to increase the expression of these two genes. A relatively higher average temperature, light intensity, and UV-B intensity are thought to cause a higher relative expression level of *mtHsc70-1* than the other two genes to protect mitochondria from increased ROS production [32].

Based on the above discussion, the *Hsp70* and *cpHsc70-2* gene expression patterns of *S. nodiflora* might be similar to the *HSFA2* and *CP-sHSP* gene expression patterns of *P. perfoliatus* [27]. Meanwhile, the expression pattern of *mtHsc70-1* gene is expected to have a similar expression pattern with members of the *Hsp70* gene family in most previous studies' results [8, 11, 23]. Our results support the evidence that all plant species have genes encoding Hsp, but they vary in their expression pattern. Plant species also vary in the minimum and maximum dose of abiotic stress for Hsp induction. The minimum dose of abiotic stress for Hsp induction is correlated with the environmental conditions in which species live [17]. In this study, we only observed the expression of *Hsp70*, *mtHsc70-1*, and *cpHsc70-2*, so we cannot explain its association with other Hsp gene families. Hsp70 have been reported to interact with other classes of Hsp, such as Hsp90 [33, 34, 35], Hsp100 [36, 37, 38], and small Hsp (sHsp) [35, 39]. Reference [6], [10], and [40] also provide a review about Hsp70 and their interaction with other chaperones.

Environmental factor(s) that induced *Hsp70*, *mtHsc70-1*, and *cpHsc70-2* and the minimum quantity needed to increase every gene's expression level is still unknown. According to Barua & Heckathorn [23], the accumulation of Hsp is influenced by the interaction between light and temperature. The intensity of UV-B radiation was not considered the sole factor determining the expression levels of *Hsp70*, *mtHsc70-1*, and *cpHsc70-2*. Those genes' expression levels may also be influenced by other factors, including temperature and light intensity. Therefore, further research is needed to determine which environmental factor has the most significant influence on the expression of *Hsp70*, *mtHsc70-1*, and *cpHsc70-2* genes in *S. nodiflora*.

The *Hsp70*, *mtHsc70-1*, and *cpHsc70-2* genes have different expression patterns under growth conditions of *Synedrella nodiflora*, both shaded and open conditions (different UV-B radiation intensities). The expression levels of *Hsp70*, *mtHsc70-1*, and *cpHsc70-2,* may also influence by other factors, including temperature and light intensity. More sampling locations and replications per location should be involved in future research to interpret more accurate and representative data. Experimental research is needed to determine the minimum UV-B intensity, temperature, and light intensity at which those genes are expressed. The relationship between these three genes with other Hsp genes still needs to be explored.

AUTHORS' CONTRIBUTIONS

M.C. collected, running the samples, analysed data, wrote the manuscript. A.E.M and A.S. designed the research, analysed the data and supervised all the process.

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