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RESEARCH ARTICLE



Effects of heat stress on growth, photosynthetic pigments, oxidative damage and competitive capacity of three submerged macrophytes

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ABSTRACT

There is an information gap regarding heat stress-induced oxidative damage and the species-specific behavior of plants under stress conditions. The present study was designed with the hypothesis that heat stress may induce species-specific oxidative damage that determines the competitive capacity of common submerged macrophytes. We conducted two laboratory experiments to simulate mono- and mixed cultures of three submerged macrophytes with the application of two heat shock treatments. The results showed that both heat shocks had significant effects on growth, photosynthetic pigments and the ability to induce strong oxidative damage for all three species. The comparative results of mono- and mixed cultures showed that *P. crispus* had an advantage in both the control and high-temperature treatments over the other two species as a strong competitor in the mixed culture. Further, the competitive capacity of *P. crispus* increased in the moderately high-temperature condition compared to the control.

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Heat stress; oxidative damage; competitive capacity; hydrogen peroxide

Introduction

The elevation of temperature over an optimal level, which can cause non-reversible damage to plant growth and development, is termed heat stress. Elevated water temperature can severely affect submerged macrophytes; the magnitude and intensity of temperature fluctuations and the tolerability of plant species determine the severity of these effects (Wahid et al. 2007).

The effects of the deviation of abiotic factors on the growth and development of aquatic macrophytes have been discussed in the literature (Santamaría & van Vierssen 1997; James et al. 2004; Declerck et al. 2005; Smolders et al. 2006; Ellawala et al. 2011b; Zaman & Asaeda 2013). Further, biotic and abiotic deviation-induced oxidative stress under elevated concentrations of reactive oxygen species (ROS) has been described in relation to water flow, turbulence, salinity, UV radiation, drought, heavy metals, nutrient deficiency, air pollutants, herbicides and pathogen attacks (Halliwell & Gutteridge 1985; Mittler 2002; Shin et al. 2005; Cruz de Carvalho 2008; Rucińska-Sobkowiak 2008; Ellawala et al. 2011a; Nawkar et al. 2013).

However, survival against oxidative stress depends on the equilibrium between the production and destruction of ROS by a series of enzymatic and non-enzymatic detoxification mechanisms. Enzymatic detoxification mechanisms that principally minimize cellular levels of superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2) include the production of antioxidant enzymes such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and superoxide dismutase (SOD) (Sairam & Tyagi 2004). The non-enzymatic defense mechanism includes the activity of ROS-scavenging proteins such as thioredoxin and metallothioneins (Steffens et al. 2013). The overproduction of ROS beyond equilibrium may cause severe damage to lipids, cellular proteins, nucleic

acids and enzymes, leading to the death of cells induced by programmed cell death (PCD) (Shah et al. 2001; Gill & Tuteja 2010).

Each plant has an optimum set of environmental temperatures for proper growth and development; the temperature optimum for one species may be highly stressful for another species. High-temperature-induced changes in morphology and biomass allocation may lead to increased growth and competitive capacity (Pilon & Santamaría 2002; Riis et al. 2012). For the structure and proper functioning of freshwater ecosystems, competition can be considered a paramount factor determining the species distribution in aquatic macrophyte communities (Moss et al. 2003, Doyle et al. 2007).

Furthermore, heat stress-induced oxidative stress has been widely discussed for terrestrial vegetation throughout the literature (Anderson 2002; Chaitanya et al. 2002; Mazorra et al. 2002; Mei & Song 2010, Kipp & Boyle 2013). Although dynamics in morphology, growth performance, photosynthesis and cellular respiration under the influence of heat stress are commonly cited, less attention has been paid to evaluating oxidative stress and the performance of antioxidant enzymes governed by heat stress. The full mechanism of the thermotolerance of aquatic macrophytes is largely unknown.

Further, there are few cases where the competitive interactions between different species of aquatic macrophytes have been measured (Herb & Stefan 2006), although competitive interactions between different macrophyte species are customary phenomena in natural environments. Most of these studies focused on the competition of various macrophyte species under different light regimes (Spencer & Rejmánek 2010). There are fewer experimental studies on the warming-stimulated competitive capacity of submerged macrophytes. Additionally, most studies on competition

between aquatic macrophytes are based on variations in the basic morphology and morphometry of plants. The oxidative damage induced by the interactive effects of abiotic stresses, such as temperature, and competition is largely unknown for submerged macrophytes.

Therefore, the present study was designed with the hypothesis that heat stress may induce species-specific oxidative damage, which determines the competitive capacity of common submerged macrophytes.

Materials and methods

The experimental plant species *Elodea nuttallii*, *Potamogeton crispus* and *Vallisneria asiatica*, collected from the Moto-Arakawa River in Japan, were rinsed with clean water to remove debris, and the attached algae were separated using forceps. The cleaned plants were cultured in glass aquaria under laboratory conditions for approximately one month.

To determine the competitive capacity of the three selected species, two separate experiments, a mono- and a mixed culture, were conducted with the same temperature treatments. For the monoculture experiment, plant apical tips (*E. nuttallii* and *P. crispus*) and small plants (*V. asiatica*) approximately 5 cm in height were separately planted in PVC pots with well-washed river sand as the substrate. For the mixed culture experiment, three apical tips or small plants from the three species were planted in the same PVC pot. Several PVC pots were prepared following the same design, and the prepared PVC pots were separately acclimatized under controlled conditions until the plants became rooted.

All treatments were conducted in glass microcosms (15.7 × 15.7 × 24.5 cm), and 5% Hoagland nutrient solution (HNS), as recommended by Atapaththu and Asaeda (2015) for better growth of submerged macrophytes, was used as the nutrient media in the microcosms. To stimulate heat shock, the PVC pots with planted apical tips were directly transferred from the acclimatization tank to the pre-prepared glass microcosms set to temperatures of 25°C (as a control), 30°C and 35°C. Laboratory-scale portable heaters (IC AUTO NEO Type 180, Japan) provided heat energy. The pH and dissolved oxygen concentration of all microcosms were maintained within the ranges of 7.08–7.44 and 8.2–8.6 mg/L, respectively, over the experimental period. All treatments were subjected to a photoperiod regime of 12 h dark and 12 h light with a light intensity of 100–120 μmol m⁻² s⁻¹ using white fluorescent lamps. The short-term effects of heat shock treatments were determined after 48 h.

Growth measurements

The initial and final lengths of apical tips were measured using a ruler. The relative growth rate (RGR) was calculated with the following formula:

$$\text{RGR} = \frac{(\text{FL} - \text{IL})}{\text{Time}},$$

where FL is the final length and IL is the initial length.

Determination of photosynthetic pigments and chlorophyll fluorescence

The pigments were extracted from fresh plant samples in 5 mL of N,N-dimethylformamide in the dark for 24 h and

measured spectrophotometrically (Shimadzu, UV Mini 1210). Quantification of the described pigments was performed using coefficients published by Wellburn (1994). Chlorophyll fluorescence was measured using a chlorophyll fluorescence imaging technique (FC 1000-H; Photon Systems Instruments, Czech Republic) with auto image segmentation. Initially, plant segments were dark-adapted for 20 min, and the maximum quantum efficiency of photosystem II photochemistry (F_v/F_m) was calculated using the following equation (DeEll & Toivonen 2003):

$$\frac{F_v}{F_m} = \frac{(F_m - F_o)}{F_m},$$

where F_v , F_m and F_o are the variable, maximum, and minimum fluorescence in the dark-adapted state, respectively.

Plant tissue preparation for stress assay

Stress assays, which included H₂O₂, CAT, APX and POD, were extracted by grinding the frozen (with liquid nitrogen) fresh plant samples (~500 mg) with ice cold pH 6.0, 50 mM phosphate buffer. Polyvinylpyrrolidone (PVP) was added to the extraction to mask the effects of phenolic compounds in the plant tissues. The extractions were centrifuged at 5000 × g and 4°C for 15 min, and the supernatant was separated and incubated at –80°C until further analysis. For each analysis, every extract was evaluated in triplicate, and the results were used to determine concentrations by fresh weight (FW).

H₂O₂ concentration

The concentration of H₂O₂ was determined using a pre-prepared standard curve for a known concentration series. The reaction mixture contained 750 μL of enzyme extract and 2.5 mL of 0.1% TiSO₄ in 20% H₂SO₄ (v/v); the mixture was centrifuged at 5000 × g for 15 min at room temperature. The intensity of the yellow color that developed in the reaction was measured spectrophotometrically at 410 nm (Jana & Choudhuri 1982).

The H₂O₂ concentrations produced at each temperature treatment were determined for each category as H₂O₂ produced by species-specific competition and H₂O₂ produced by heat stress. The concentrations of H₂O₂ produced by competition were derived from the differences in H₂O₂ concentrations between the mono- and mixed culture experiments at each temperature treatment. Further, the concentrations of H₂O₂ produced by heat stress were determined from the differences in H₂O₂ produced under the heat treatments (30°C and 35°C) and the control treatment (25°C).

CAT activity

The CAT activity was measured according to Aebi (1984): 100 μL of 10 mM H₂O₂ and 2.0 mL of 100 mM potassium phosphate buffer (pH 7.0) were added to a cuvette before adding 500 μL of the enzyme extract to initiate the reaction. The absorbance reduction at 240 nm was recorded every 10 s for 3 min. Finally, the CAT activity was calculated using an extinction coefficient of 40 mM⁻¹ cm⁻¹ and results were expressed as μmol/min/mg protein (μmol/min/mg pr.).

APX activity

The APX activity was determined according to Nakano and Asada (1981). The reaction mixture contained 100 μL of enzyme extract, 200 μL of 0.5 mM ascorbic acid in 50 mM potassium phosphate buffer (pH 7.0) and 2.0 mL of 50 mM potassium phosphate buffer (pH 7.0). The reaction was started by adding 60 μL of 1 mM H_2O_2 . The decrease in absorbance at 290 nm was recorded every 10 s. The APX activity was calculated using an extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ and results were expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein ($\mu\text{mol}/\text{min}/\text{mg}$ pr.).

POD activity

The POD activity was spectrophotometrically measured based on the oxidation of guaiacol in the presence of H_2O_2 (MacAdam et al. 1992). The reaction mixture contained 3.0 mL of pH 6, 50 mM potassium phosphate buffer, 40 μL of 30 mM H_2O_2 and 50 μL of 0.2 M guaiacol. The reaction was started by the addition of 100 μL of crude enzyme extract, and the increase in absorbance at 420 nm was recorded every 10 s for 3 min. The rate of change in absorbance was calculated, and the POD activity was determined using an extinction coefficient of $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ and results were expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein ($\mu\text{mol}/\text{min}/\text{mg}$ pr.).

Statistical analysis

The collected data were tested for normality using the Shapiro–Wilk test before statistical analysis. The percentage variations (increment or reduction) of the growth rate, total chlorophyll and H_2O_2 concentrations were calculated from the differences between the values of the prescribed parameters in the mono- and mixed cultures, with reference to the monoculture. The statistical analyses were conducted using SPSS 16v, and all results were presented as the mean \pm SD of three replicates. The measured parameters in the mono- and mixed cultures were tested separately per species with one-way analysis of variance (ANOVA) using temperature as an independent variable. Two-way ANOVA tests were performed with temperature and culture condition combinations as independent variables per species.

Results

The variations in the RGR of the three species under the two different heat shocks compared to the control condition are shown separately in Figure 1 for the mono- and mixed cultures.

Among the three considered species, the lowest height increment rate, which ranged from 0.042 ± 0.007 to $0.098 \pm 0.008 \text{ cm}/\text{day}$, was recorded for *V. asiatica* in both the mono- and mixed cultures. In both the mono- and mixed culture experiments, the height increments decreased with applied temperature for *P. crispus* and *V. asiatica*; these variations were statistically significant ($F = 15.850$, $p < .05$ and $F = 27.043$, $p < .05$ for *P. crispus* and *V. asiatica*, respectively) with applied temperature. A different kind of variation in the RGR was obtained for *E. nuttallii*, increasing in the moderate temperature treatment (30°C) and decreasing in the highest temperature treatment (35°C). A similar trend was observed in both the mono- and mixed cultures, but this variation was not significant ($F = 2.585$, $p > .05$) in the mixed culture. The height increment rates of *E. nuttallii* and *V. asiatica* were lower in the mixed cultures compared to the monocultures at each temperature treatment, and the variables were statistically significant for both species ($F = 7.89$, $p < .05$ and $F = 34.89$, $p < .05$ for *E. nuttallii* and *V. asiatica*, respectively). For *P. crispus*, the significant, highest RGR values ($F = 32.66$, $p < .05$) were recorded in the mixed culture compared to the monoculture in each temperature treatment (Table 1).

The variations in chlorophyll a (chl *a*), chlorophyll b (chl *b*) and total carotenoids in the three species under the two different heat shocks compared to the control condition are shown in Figure 2 separately for the mono- and mixed cultures.

The lowest values of chl *a*, chl *b* and total carotenoids were obtained for *V. asiatica* among the considered plant species. A similar trend of decrease was obtained for chl *a* and chl *b* between the control treatment and high-temperature treatments for *P. crispus* and *V. asiatica*. This same trend could be observed in both the mono- and mixed cultures. Different trends were observed in the variations of chl *a* and chl *b* for

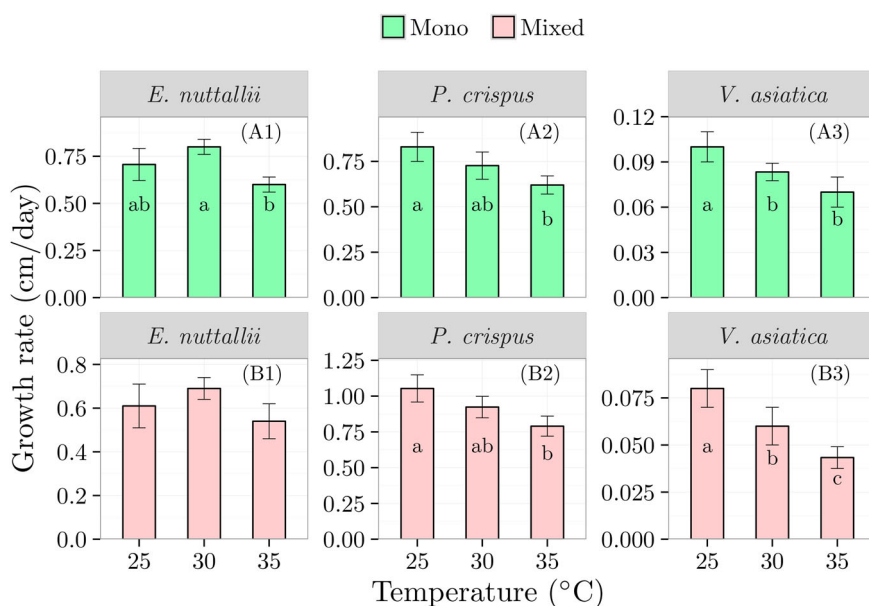


Figure 1. Variations in the RGR of *E. nuttallii*, *P. crispus* and *V. asiatica* with heat shock treatments ($n = 3$). The results are shown separately for the monoculture (A) and the mixed culture (B).

Table 1. Results of two-way ANOVA of the effects of culture method (mono- and mixed culture) and applied heat shock temperature treatment (25°C, control; 30°C; and 35°C) on the growth rate of *E. nuttallii*, *P. crispus* and *V. asiatica* ($n = 3$).

Species	Variable	Source	F	p
<i>E. nuttallii</i>	Growth rate (cm/day)	Culture	7.899	.016
		Temperature	9.308	.004
		Culture × Temperature	0.254	.078
<i>P. crispus</i>	Growth rate (cm/day)	Culture	32.661	.000
		Temperature	15.850	.000
		Culture × Temperature	0.188	.831
<i>V. asiatica</i>	Growth rate (cm/day)	Culture	34.897	.000
		Temperature	27.043	.000
		Culture × Temperature	0.782	.479

E. nuttallii, for which chl *a* and chl *b* significantly increased in the 30°C heat shock treatment and decreased in the 35°C heat shock treatment. For *E. nuttallii* and *V. asiatica*, the chl *a* and chl *b* concentrations decreased in the mixed cultures compared to the monocultures, while they increased in the mixed cultures for *P. crispus*.

Total carotenoids increased from the control treatment (25°C) to the high-temperature treatment for all three species. For *E. nuttallii* and *V. asiatica*, the carotenoid concentrations increased from the monocultures to the mixed cultures, and this difference was statistically significant ($F = 171.589$, $p < .05$) for *V. asiatica* but not statistically significant ($F = 0.921$, $p > .05$) for *E. nuttallii*. Further, the carotenoid concentrations of *P. crispus* at each temperature treatment significantly decreased ($F = 50.676$, $p < .05$) in the mixed cultures compared to the monocultures (Table 2).

The variations in H₂O₂ concentrations generated by the inter-species competition and by the heat shock treatments of *E. nuttallii* and *V. asiatica* are illustrated in Figure 3.

The H₂O₂ concentrations of *E. nuttallii*, *P. crispus* and *V. asiatica* increased with applied heat shock treatments in both the mono- and mixed cultures. Further, for *E. nuttallii* and *V. asiatica*, the highest values for H₂O₂ were obtained in the mixed cultures compared to the monocultures at every temperature treatment. The H₂O₂ concentration decreased in the mixed culture compared to the monoculture for *P. crispus*. When considering the % H₂O₂ formation, the H₂O₂ formed by heat shock decreased while that formed by competition among plants increased with increased temperature for *E. nuttallii*. In contrast, the H₂O₂ formed by heat shock increased and that formed by competition among plants decreased with increased temperature for *V. asiatica*.

The variations in H₂O₂ concentrations with applied temperature in each treatment of each plant species are shown in Figure 4.

Figure 4 illustrates that there were significant positive correlations between the H₂O₂ concentrations and applied temperatures for all species under both the mono- and mixed cultures.

Varying patterns of growth, photosynthetic pigments and primary ROS were observed; H₂O₂ provided certain clues for the stress induced by inter-specific competition among the considered species in the mixed culture experimental setup at each temperature treatment. Figure 5 illustrates the variations in percentage increments or reductions in the growth rate, total chlorophyll and H₂O₂ concentration in the mixed cultures compared to the monocultures of the three plant species with applied temperature treatments.

According to Figure 5, there were reductions in the RGR and total chlorophyll in the mixed cultures compared

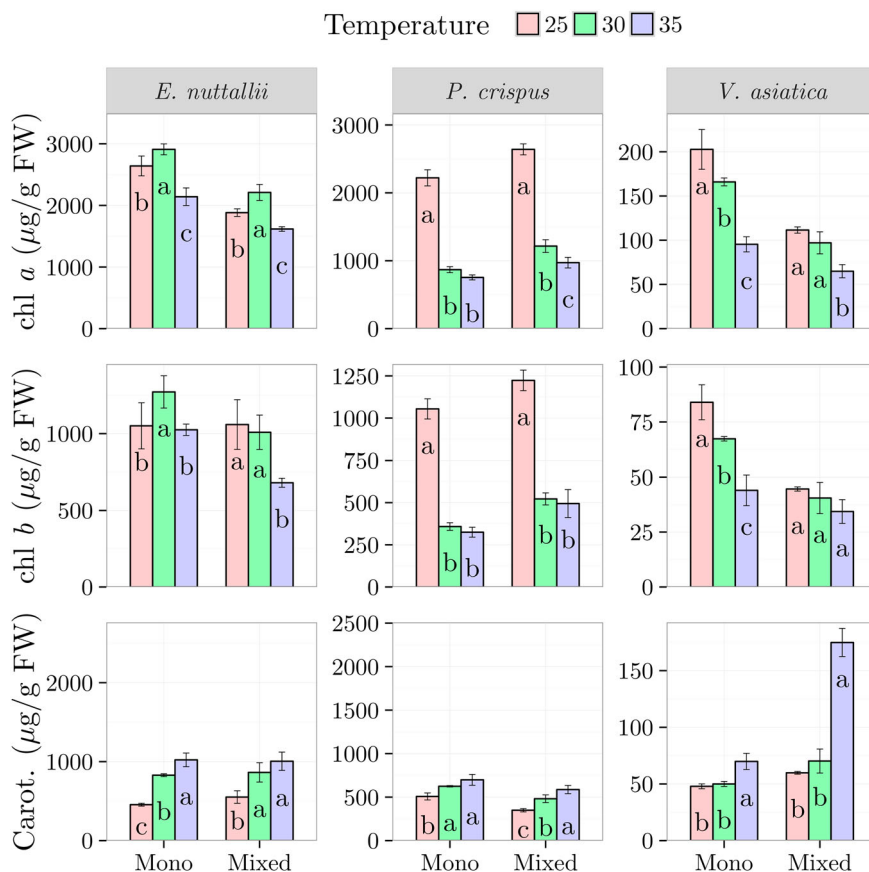


Figure 2. Variations in the chl *a*, chl *b* and total carotenoids of *E. nuttallii*, *P. crispus* and *V. asiatica* with heat shock treatments. The results are shown separately for the mono- and mixed cultures.

Table 2. Results of two-way ANOVA of the effects of culture (mono- and mixed culture) and applied heat shock temperature treatment (25°C, control; 30°C; and 35°C) on the chl *a*, chl *b* and total carotenoid concentrations of *E. nuttallii*, *P. crispus* and *V. asiatica* ($n = 3$).

Species	Variable	Source	F	p
<i>E. nuttallii</i>	chl <i>a</i> (µg/g FW)	Culture	154.758	.000
		Temperature	55.148	.000
		Culture × Temperature	1.748	.000
	chl <i>b</i> (µg/g FW)	Culture	14.367	.003
		Temperature	10.467	.002
		Culture × Temperature	4.094	.044
	Carotenoids (µg/g FW)	Culture	0.921	.356
		Temperature	57.063	.000
		Culture × Temperature	0.681	.524
<i>P. crispus</i>	chl <i>a</i> (µg/g FW)	Culture	75.072	.000
		Temperature	684.514	.000
		Culture × Temperature	2.416	.131
	chl <i>b</i> (µg/g FW)	Culture	45.174	.000
		Temperature	364.456	.000
		Culture × Temperature	0.006	.994
	Carotenoids (µg/g FW)	Culture	50.676	.000
		Temperature	41.308	.000
		Culture × Temperature	0.493	.623
<i>V. asiatica</i>	chl <i>a</i> (µg/g FW)	Culture	132.854	.000
		Temperature	67.468	.000
		Culture × Temperature	10.369	.002
	chl <i>b</i> (µg/g FW)	Culture	89.129	.000
		Temperature	29.645	.000
		Culture × Temperature	10.341	.002
	Carotenoids (µg/g FW)	Culture	171.589	.000
		Temperature	157.013	.000
		Culture × Temperature	72.565	.000

to the monocultures for *E. nuttallii* and *V. asiatica*, while there was an increment of both parameters for *P. crispus*. The percentage reduction of the RGR for *E. nuttallii* further decreased, but not significantly ($F = 0.816$, $p > .05$), with applied temperature, and it significantly increased ($F = 26.610$, $p < .05$) for *V. asiatica*. The increment of the RGR for *P. crispus* was unchanged with applied temperature.

In the case of total chlorophyll, the percentage reduction for *E. nuttallii* significantly increased ($F = 12.086$, $p < .05$) with applied temperature treatment, while for *V. asiatica*, it significantly decreased ($F = 9.493$, $p < .05$). In contrast, there were increments of total chlorophyll (18–35%) for *P. crispus*,

which significantly increased ($F = 24.137$, $p < .05$) with applied temperature.

A completely different trend was obtained for the H_2O_2 concentrations. There were increments of H_2O_2 in the mixed cultures relative to the monocultures of *E. nuttallii* and *V. asiatica*, demonstrating that oxidative stress was induced by inter-specific competition. However, the percentage increment of *E. nuttallii*, which ranged from 39% to 43%, increased but was not significantly different ($F = 0.135$, $p > .05$) with applied temperature. In the case of *V. asiatica*, the percentage increment (22–58%) significantly decreased ($F = 19.189$, $p < .05$) with applied temperature. In contrast, there was a reduction of H_2O_2 in *P. crispus* in the mixed culture with applied temperature, but these values were not statistically significant ($F = 7.763$, $p > .05$).

Variations in the activity of the primary antioxidant enzymes, CAT and APX, with applied temperature treatments are separately presented for the mono- and mixed cultures in Figure 6 for the three considered species.

According to Figure 6, the activities of CAT and APX increased with the applied temperature treatments, both in the monocultures and mixed cultures, for all considered species. The CAT and APX activities in the mixed cultures increased compared to the monocultures for *E. nuttallii* and *V. asiatica*. The mean CAT activity in the monoculture was significantly different ($F = 6.50$, $p < .05$) from that in the mixed culture, while the mean APX activity in the monoculture was not significantly different ($F = 1.269$, $p > .05$) from that in the mixed culture for *E. nuttallii*. In the case of *V. asiatica*, the mean values of both the CAT and APX activities in the monocultures were not significantly different from those in the mixed cultures ($F = 1.64$, $p > .05$ and $F = 1.24$, $p > .05$ for the CAT and APX activities, respectively). In contrast, for *P. crispus*, the CAT and APX activities decreased in mixed cultures compared to the monocultures, but the mean values were not significantly different ($F = 2.32$, $p > .05$ and $F = 1.27$, $p > .05$ for the CAT and APX activities, respectively). Additionally, significant positive correlations were obtained for the H_2O_2 concentrations and CAT or APX activities for all three species.

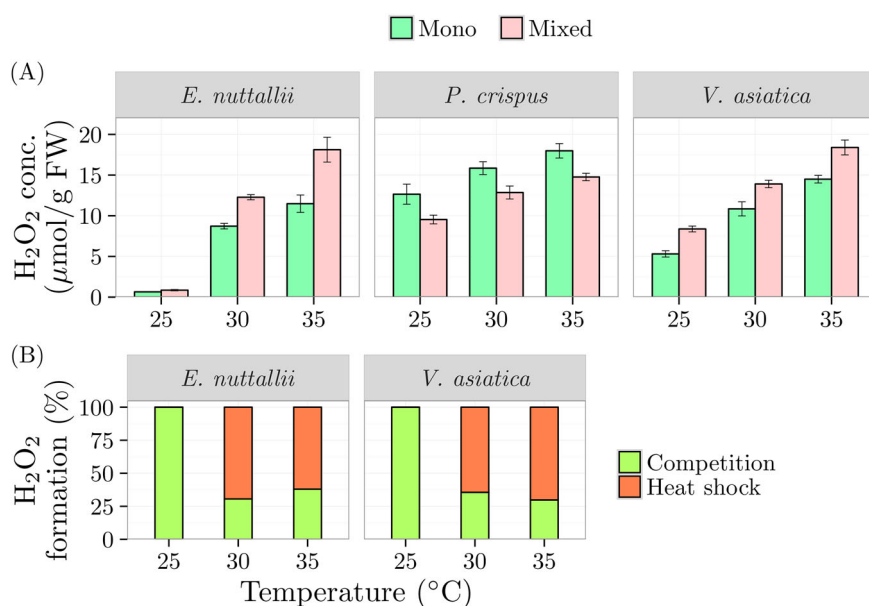


Figure 3. Variations in the H_2O_2 concentration of *E. nuttallii*, *P. crispus* and *V. asiatica* with heat shock treatments (A), and % H_2O_2 formation by competition and heat shock treatments for *E. nuttallii* and *V. asiatica* (B). The results of the different heat shock treatments are shown separately.

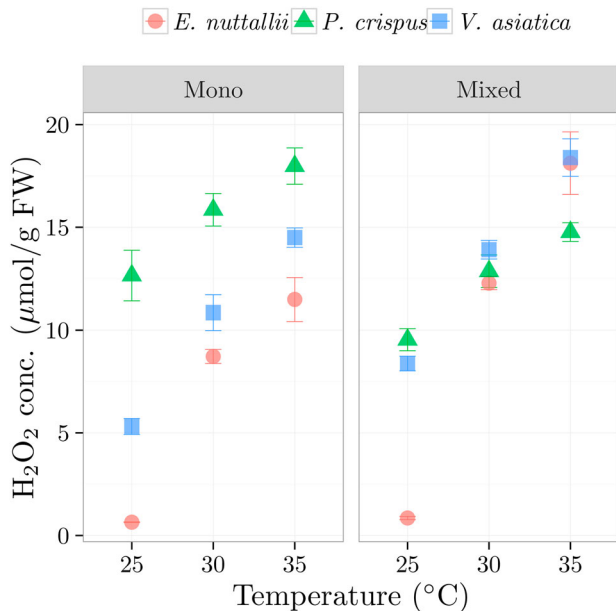


Figure 4. Variations in H₂O₂ concentrations with applied temperature for each treatment for *E. nuttallii*, *P. crispus* and *V. asiatica* ($n = 3$).

Discussion

In the present study, two laboratory experiments were designed to investigate the effects of elevated water temperature or heat stress on three plant species with three different morphologies by means of their individual effects and competitive ability. In this experiment, the accumulation of ROS was significantly higher in plants grown under elevated temperature conditions compared to the control conditions (room temperature, 25°C) in both the mono- and mixed culture experiments. Similarly, the activities of the antioxidant enzymes (CAT and APX) were also significantly higher in plants grown under high-temperature treatments. Further, the accumulation of ROS and the prescribed antioxidant activities were significantly higher in the mixed cultures in comparison to the monocultures of certain plant species. However, the applied temperature values and H₂O₂ (a strong ROS) had a positive relationship in each treatment for each species.

It was clear that heat stress can lead to overproduction of ROS, as supported by earlier findings by Wahid et al. (2007). The ROS are generated in various sub-cellular loci by the partial reduction of oxygen to radical and non-radical oxygen species. Cellular levels of ROS can determine the plant's survival ability under unfavorable conditions, as high concentrations of ROS can damage important biomolecules (including proteins and nucleic acids). Meanwhile, low or moderate concentrations can serve as secondary messengers in intracellular signaling cascades and regulate various responses (Takeda et al. 1995). Because it is the only ROS that can diffuse through aquaporins in membranes and over longer distances within cells, H₂O₂ has received prime attention as a signal ROS molecule that can regulate biological processes under environmental deviations in plants (Bienert et al. 2007). Additionally, H₂O₂ is a reasonably stable ROS compared to other ROS.

Excess accumulation of H₂O₂ in plant cells is mediated by the activities of various types of enzymatic and non-enzymatic compounds. The two major antioxidant enzymes that can efficiently scavenge stress-induced H₂O₂ generated in

plants are CAT and APX. CAT was the first characterized enzyme that can catalyze the dismutation of two molecules of H₂O₂ into water and oxygen. APX is considered the most efficient scavenger of H₂O₂ due to its higher affinity for H₂O₂ compared to CAT. Carotenoids, which are non-enzymatic lipophilic antioxidants, have the ability to detoxify H₂O₂. In addition to their direct deactivation of H₂O₂, carotenoids also quench triplet sensitizers and excited chlorophyll (Sharma et al. 2012). When plants are exposed to a continuous stress, the equilibrium between H₂O₂ production and the activity of antioxidant mechanisms becomes unbalanced, leading to excessive accumulation of H₂O₂ in the plant body. This excess accumulation of H₂O₂ further increases the levels of CAT, APX and carotenoids, as they are continually involved in rebalancing the equilibrium. Therefore, the activity of CAT and APX and the concentration of carotenoids exhibited positive relationships with the H₂O₂ concentration in the present study. A positive relationship between H₂O₂ concentration and temperature was also observed, indicating higher stress in plants due to excess accumulation of H₂O₂.

In the present study, the H₂O₂ concentration gradually increased with increased temperature for all species in both the mono- and mixed cultures, suggesting that heat stress-induced oxidative damage was significant at 30°C and 35°C. Further, the H₂O₂ concentration of *P. crispus* decreased in the mixed culture in comparison to the monoculture, while it increased for *E. nuttallii* and *V. asiatica* in the present study. The increased H₂O₂ concentration in the mixed cultures compared to the monocultures of *E. nuttallii* and *P. crispus* indicated that growing under mixed culture conditions placed extra stress on the plants. The H₂O₂ formation data (Figure 3) showed that the H₂O₂ formed due to competition among species and increased with increased temperature for *E. nuttallii*, suggesting that warming further suppressed the competitive ability of the plant, which triggered high oxidative damage. Conversely, in the case of *V. asiatica*, the portion of H₂O₂ produced by competition among species decreased with increased temperature. Thus, although temperature itself could damage *V. asiatica* under mixed culture conditions, ROS production was low at high temperatures. In contrast, the lower production of H₂O₂ in the mixed culture in comparison to the monoculture of *P. crispus* indicated that growing under mixed culture supported *P. crispus* in both the control and high-temperature conditions. This finding led to *P. crispus*'s status as a strong competitor over *E. nuttallii* and *V. asiatica*, thereby creating a type of allelopathic effect on them. The previous literature has reported that allelopathic stress can induce oxidative damage mediated by the overproduction of ROS (Ding et al. 2007). Therefore, the excess accumulation of H₂O₂ in the mixed cultures of *E. nuttallii* and *V. asiatica* can be considered an indicator of allelopathic stress. Further, the percentage reduction of H₂O₂ for *P. crispus* under the mixed culture was significantly reduced for the 30°C heat shock compared to the control condition, suggesting the improvement of the competitive capability of *P. crispus* through a reduced stress level under moderately high temperature.

Reductions in chl *a* and chl *b* were observed in *P. crispus* and *V. asiatica* exposed to moderate and high temperatures, while *E. nuttallii* exposed to moderate temperatures had higher concentrations of chl *a* and chl *b* compared to the control under both the mono- and mixed cultures. Reductions in

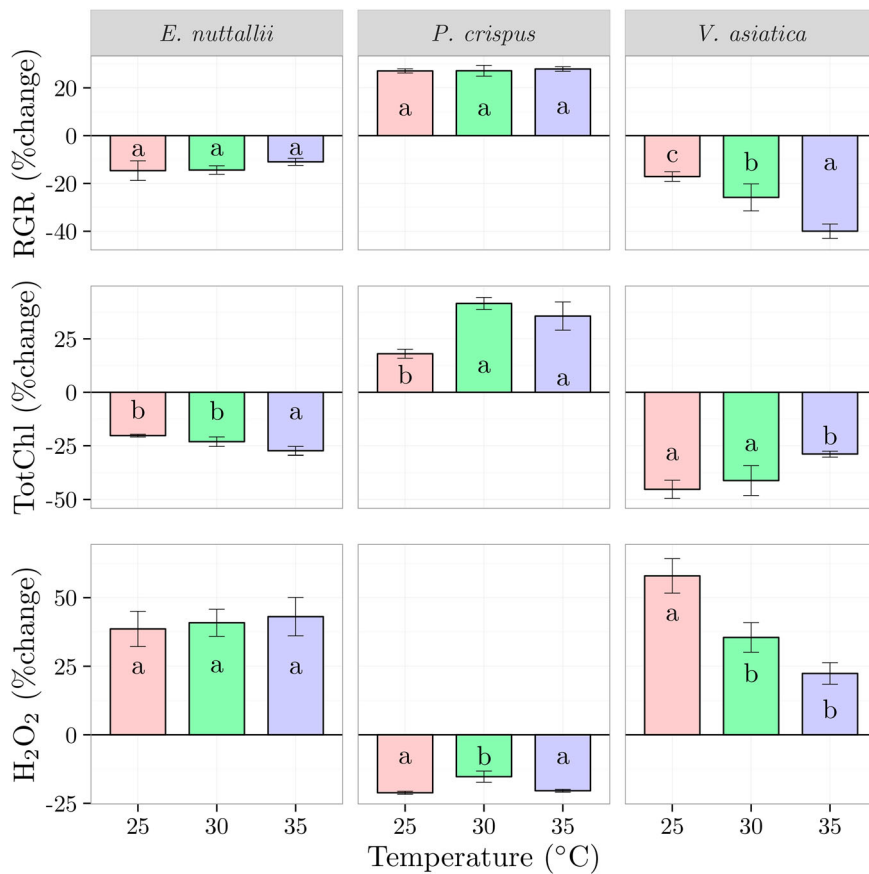


Figure 5. Variations in the percentage increments or reductions of the RGR, total chlorophyll and H₂O₂ concentration in the mixed cultures relative to the mono-cultures of the three plant species with applied temperature treatments.

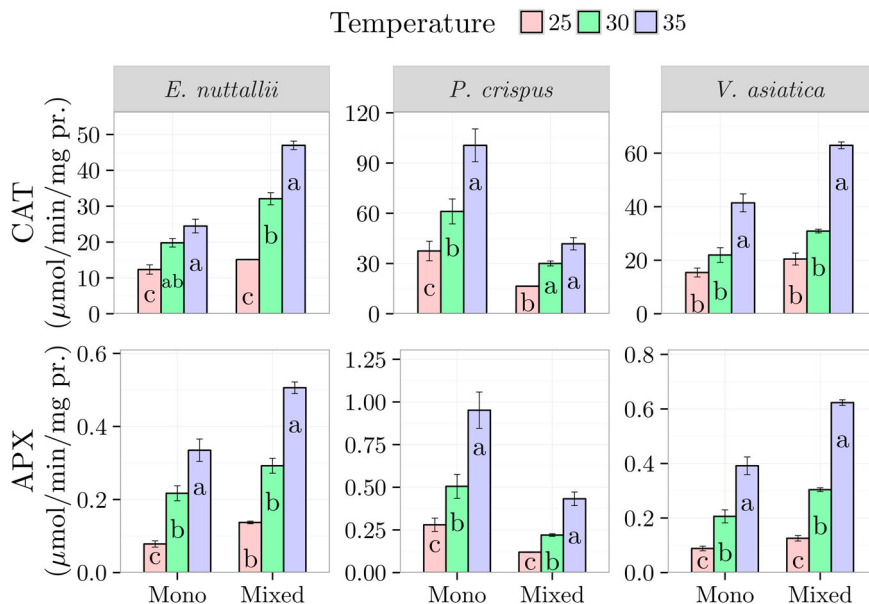


Figure 6. Variations in the CAT and APX activities with heat shock treatments for *E. nuttallii*, *P. crispus* and *V. asiatica*. The results are shown separately for the mono- and mixed cultures ($n = 3$).

chlorophyll pigments under heat stress were suggested to be associated with the production of ROS and thereby indirectly represent the stress level of the plants. Additionally, the activity of PSII, which is highly thermolabile, is significantly reduced under high temperatures (Camejo et al. 2005). The high concentrations of chl *a* chl *b* for *E. nuttallii* exposed to moderate (30°C) temperature indicate an improvement in the chlorophyll pigments. Any alterations in plant

photosynthetic attributes under heat stress can be considered reliable indicators of thermotolerance in plants (Wahid et al. 2007). Therefore, it can be suggested that *E. nuttallii* showed thermotolerance under moderate temperature (30°C), while *P. crispus* and *V. Asiatica* became highly stressed under moderate and high temperatures.

Moreover, the total chlorophyll of *E. nuttallii* and *V. asiatica* showed significant reductions in the mixed cultures in

comparison to the monocultures but increased in the mixed culture for *P. crispus*. The percentage increment of total chlorophyll in *P. crispus* significantly increased under the moderately higher temperature treatment (30°C). Therefore, the competitive ability of *P. crispus* increased in the warming condition, with *P. crispus* having an advantage over *E. nuttallii* and *V. asiatica* by being a strong competitor under moderate warming conditions. It was clear that both *E. nuttallii* and *V. asiatica* were suppressed by *P. crispus* under both the control and high-temperature conditions. The results showed that this suppression was further increased with increased temperature for *E. nuttallii* in the mixed culture because the reduction of total chlorophyll further increased with increasing applied temperature. However, in the case of *V. asiatica*, the reduction of total chlorophyll was lower than that for *E. nuttallii* with increased applied temperature, suggesting that warming confers certain advantages to overcome suppression by competition under moderate and high temperatures.

As they showed good correlation with plant growth, these adverse effects on photosynthetic attributes could limit plant growth at high temperatures. The shoot elongation of *P. crispus* and *V. asiatica* in both the mono- and mixed cultures decreased with temperature. The growth of *V. asiatica* was severely affected at 35°C, showing more than a 50% reduction in the growth rate compared to the control plants. In contrast, variations in the growth rate of *E. nuttallii* followed a similar trend as chlorophyll pigments, showing thermotolerance under moderately high temperatures. These findings were highly compatible with previous findings (JianMin et al. 2009) in which 25–30°C was considered the optimum temperature range for the growth of *E. nuttallii*. Further, it was found that when the water temperature was greater than 30°C, plant growth was inhibited.

Conclusions

In the present study, we observed that both *P. crispus* and *V. asiatica* exhibited reduced growth and photosynthetic pigment levels with increasing applied temperature due to increased physiological stress. High H₂O₂ production coupled with high antioxidant production was observed in plants exposed to high and moderate temperatures. *E. nuttallii* exhibited tolerance under moderately high temperatures and showed acquired thermotolerance, while it was adversely affected by the highest temperature treatment. Further, heat shock treatment-induced oxidative stress, which significantly impaired the plants' growth and photosynthesis, was pronounced in all the species subjected to these treatments. The comparative results of the mono- and mixed cultures suggested that *P. crispus* was the strongest competitor in both the control and warmer conditions; its competitive capacity increased in moderately high-temperature treatments. Further, elevated levels of H₂O₂ can be seen as an indicator of allelopathic and temperature stress.


Disclosure statement

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