

EFFECTS OF SHADING AND SODIUM HYDROSULFIDE, A HYDROGEN SULFIDE DONOR, APPLICATION ON PHYSIOLOGICAL RESPONSES IN CACAO UNDER CHILLING STRESS

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ABSTRACT – Due to global warming, cacao cultivation has been beginning at subtropical regions such as Okinawa Island, which is located at around 26-27° north latitude. During winter, the minimum temperature on Okinawa Island often falls below 10°C, and overcoming chilling stress remains the biggest challenge to cacao cultivation in Okinawa Island. So, cultivation methods for overcoming chilling stress are currently demanded in Okinawa Island. Chilling stress generally causes oxidative stress in the leaves, which leads to leaf damage. Thus, the effects of shading at approximately 80% and hydrogen sulfide (H₂S) application, which have the effects of reducing oxidative stress, on the physiological responses in one-year old cacao seedling grown in unheated greenhouse at Kyoto University were determined from October to December 2021 and October to November 2022. In the greenhouse, the minimum temperatures fell below 10°C from late October. Shading enhanced photosynthetic rate, relative chlorophyll content (SPAD value) and maximum quantum yield of photosystem II (Fv/Fm), which can be used as an indicator of leaf damage, in cacao under chilling stress, while transpiration rate and stomatal conductance were not enhanced. The application of sodium hydrogen (NaHS), H₂S donor, to the soil did not enhance photosynthetic rate, SPAD value and Fv/Fm, though transpiration rate and stomatal conductance were enhanced slightly. Therefore, under chilling stress where the minimum temperatures were lower than 10°C, shading has the effect of reducing chilling stress, while H₂S application has no effect. Shading is a relatively inexpensive method and easy to introduce for farmers on Okinawa Island.

Keywords: Light intensity, low temperature, maximum quantum yield of photosystem II, oxidative stress, photosynthetic rate

INTRODUCTION

Due to global warming, many fruit crops have been reported physiological damaged by high temperatures (Sugiura, 2018). Replacing currently cultivated fruit crops with more heat-tolerant crops is one potential solution. In Okinawa main Island, located at 26.1–26.9°N, subtropical area, cultivation of cacao, a tropical fruit crop, has begun in plastic greenhouses without heater, though the area is limited. Over 75% of the world's cacao is cultivated within 8° of the Equator, and the major production areas located between 20°N and 20°S (Wilson, 1999). Additionally, the lowest temperature that will not permanently damage cacao is 10°C (Wilson, 1999), while at Okinawa main Island minimum temperature often falls below 10°C during winter. Therefore, overcoming chilling stress is a significant challenge for cacao cultivation in Okinawa main Island, sub-tropical area.

Chilling stress causes oxidative stress in plants (Wise, 1995; Shen *et al.*, 1999). Photoinhibition in plants cause oxidative stress, and photoinhibition increases as the extent of the utilization of absorbed

light energy decreases at low temperature (Sonoike, 1998). Thus, decrease light intensity should lighten chilling stress via decreasing photoinhibition. In avocado, low temperature injury decreased with shading treatment, due to decrease of photoinhibition and oxidative stress (Kondo & Honsho, 2018). Then, the effects of shading on damage by chilling stress in cacao plant were testified in this study.

Reducing oxidative stress also might be effective for reducing chilling stress. Recently, hydrogen sulfide (H₂S) has been reported to reduce oxidative stress in many kinds of species (Sunda *et al.*, 2002; Mikami *et al.*, 2011; Shatalin *et al.*, 2011; Jinzenji *et al.*, 2022). In passion fruit, sodium hydrosulfide (NaHS), H₂S donor, application enhanced vegetative growth under chilling stress conditions (Kondo, 2021). Thus, the effects of NaHS application on damage by chilling stress in cacao plant were also testified.

MATERIALS AND METHODS

Experiment 1

Eight-month-old 12 cacao (*Theobroma cacao* L.) seedlings were used for experiment 1. The seeds were harvested from the tree grown at the cacao orchard located at Ogimi town, the north part of Okinawa main Island (128.2°E, 26.7°N). The seeds were sowed to 5 L plastic pots filled with mixed decomposed granite soil and bark compost in a volume ratio of 4:3, in March 2021. The seedlings were cultivated in a greenhouse at Kyoto University (135.8°E, 35.0°N). In the greenhouse, the temperature was not controlled. The application of fertilizer and irrigation were carried out appropriately. Six seedlings were applied 50 mL of 0.5 mM NaHS solution twice a week from October 23 to November 20, 2021 and the other six seedlings were applied 50 mL of tap water. The concentration of NaHS solution was determined according to Kondo (2021).

Photosynthetic rate of the youngest fully matured leaf located at sunny side was measured between 11:00AM-1:00PM on October 19, November 1, 15, and 29 using a photosynthetic rate measurement device (MIC-100, Masa International Co., Ltd., Japan). The measurement conditions of photosynthetic rate were as follows: PPFD 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, stabilization time 3 s, measurement start CO_2 concentration 400 ppm, and measurement CO_2 span 10 ppm. When the measurement was not finished within 60 s, we considered it to be below the lower limit of measurement and recorded as 0. At the same days and December 20, SPAD value of the same leaf was also measured using a chlorophyll-meter (SPAD-502, Konica Minolta Sensing, Inc., Japan). The maximum quantum efficiency of photosystem II (Fv/Fm) was measured between 8:00-9:00PM at the same days using a portable chlorophyll fluorometer (OS-30P; Optiscience, Inc., NH, USA). Fv/Fm can be used for an indicator of chilling damage, because Fv/Fm decreases when complexes such as photosystem II (PSII) is damaged by stress such as chilling. Air temperature was measured at 80 cm height, which was near the plant height, using thermometer with data logger (VP-4 and EM50; Meter Group Inc., WA, USA).

The data were analyzed by t-test. The significance level was $p < 0.05$.

Experiment 2

Eight-month-old 20 cacao seedlings were used for experiment 2. The seeds were harvested from the same tree as experiment 1 and sown in March 2022. The cultivation methods were the same as experiment 1. On October 9, 2022, shade clothes (Klark Corp., Aichi, Japan) were hung from a top of the greenhouse with height of 4 m to cover the east, south, and west sides. After that, 10 seedlings were moved under the shade

clothes, namely low light intensity treatment. The remaining 10 seedlings continued to be grown in a sunny area of the greenhouse, namely high light intensity treatment. Five seedlings in each of low and high light intensity treatments were applied 50 mL of 0.5 mM NaHS solution once a week from October 12 to November 8 and the other ten seedlings were applied 50 mL of tap water.

Photosynthetic rate, transpiration rate, and stomatal conductance of the youngest fully matured leaf located at sunny side were measured between 11:00AM-1:00PM on October 8, 20, 27, November 2, and 8 using a portable gas exchange system (LI-6400, LI-COR Inc., Lincoln, NE, USA). The measurement conditions were 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, 400 ppm CO_2 , and 300 $\mu\text{mol s}^{-1}$ flow rate. The chamber temperature was not controlled. At the same days, SPAD value and Fv/Fm were measured using the same methods as experiment 1. Air temperature and solar radiation of each light intensity treatment was measured at 80 cm height using thermometer and pyranometer with data logger (VP-4, PYR sensor, and EM50; Meter Group Inc., WA, USA).

The data were analyzed by two-way ANOVA. The significance level was $p < 0.05$.

RESULTS AND DISCUSSIONS

Experiment 1

When the treatment started, the minimum temperature often fell below 10°C (Figure 1). From around November 15, the third measurement day, the minimum temperature remained below 10°C. From around November 29, the fourth measurement day, the minimum temperature often fell below 5°C, though the daily fluctuation was large. The maximum temperature varied less than the minimum temperature and remained at around 25°C.

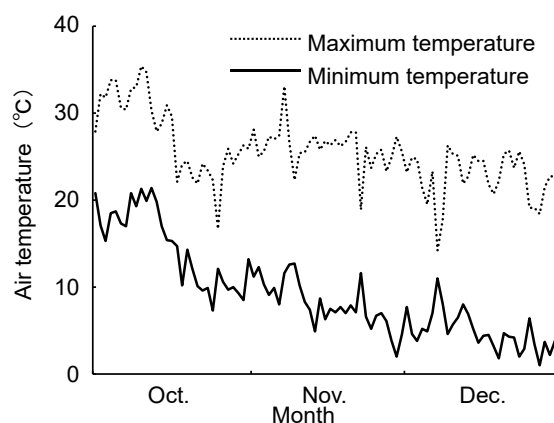


Figure 1 Daily maximum and minimum temperatures during the experiment 1.

Photosynthesis rates decreased with decreasing temperature, and there were no differences among the treatments (Figure 2). On November 29, photosynthesis rate of all seedlings was below the measurement limit. SPAD (Figure 3) and Fv/Fm (Figure 4) decreased after November 29, and there were no differences among the treatments.

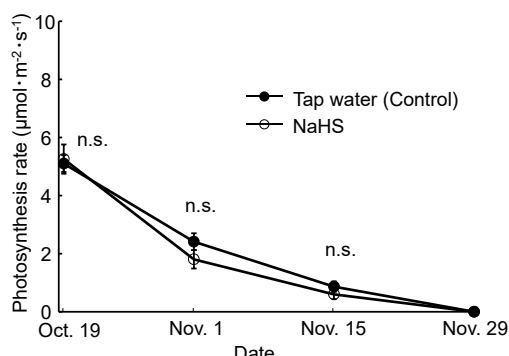


Figure 2 Effect of NaHS, a H_2S donor, application on photosynthesis rate in cacao seedling. At November 29, photosynthesis rate were not measured because of lower value than limit. n.s. indicate not significant by t-test ($p < 0.05$). Bars indicate standard error ($n=6$).

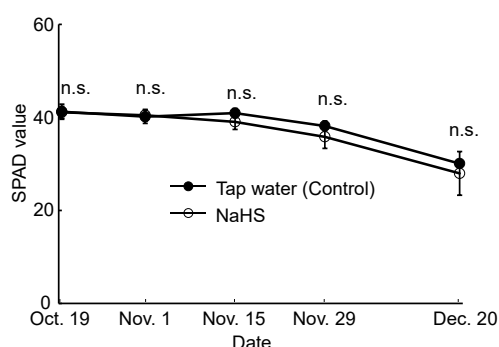


Figure 3 Effect of NaHS, a H_2S donor, application on SPAD value in cacao seedling. n.s. indicate not significant by t-test ($p < 0.05$). Bars indicate standard error ($n=6$).

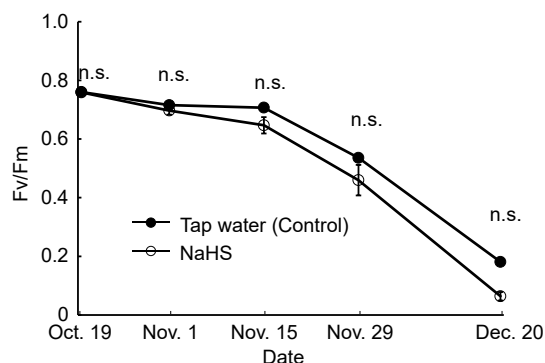


Figure 4 Effect of NaHS, a H_2S donor, application on Fv/Fm in cacao seedling. n.s. indicate not significant by t-test ($p < 0.05$). Bars indicate standard error ($n=6$).

Experiment 2

The minimum temperature fell below 10°C for the first time on October 18 and remained below 10°C on most days thereafter (Figure 5). The minimum temperature was approximately $0.3\text{--}0.5^\circ\text{C}$ higher in the low light intensity treatment. The lowest temperature during the experimental period was recorded on November 7, with 6.2°C and 6.6°C in the high and low light intensity treatment respectively. On sunny days, the solar radiation in the high light intensity treatment was approximately $250\text{--}400\text{ W}\cdot\text{m}^{-2}$, while in the low light intensity treatment it was about 20% of that (Figure 6).

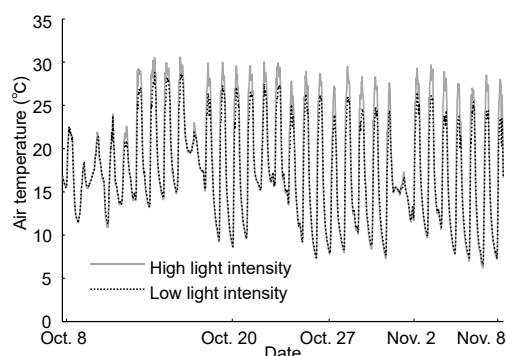


Figure 5 Air temperature during the experiment 2.

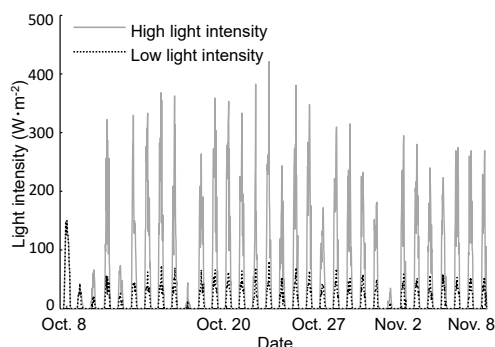


Figure 6 Light intensities during the experiment 2.

There was no interaction between light intensity and NaHS application on all measuring. Therefore, the effects of light intensity and NaHS application will be discussed separately below. Photosynthesis rate was higher in the low light intensity treatment on October 20 and November 2 (Figure 7). There was no effect of NaHS application on photosynthesis rate. Light intensity had no effect on transpiration rate and stomatal conductance (Figure 8, for transpiration rate data not shown). On October 27 and November 2, SPAD values were higher in the low light intensity treatment (Figure 9). There was no effect of NaHS application on SPAD values. Fv/Fm decreased in the high light intensity treatment after October 20, it did not decrease in the low light intensity treatment (Figure 10). On November 8, Fv/Fm decreased due to NaHS application.

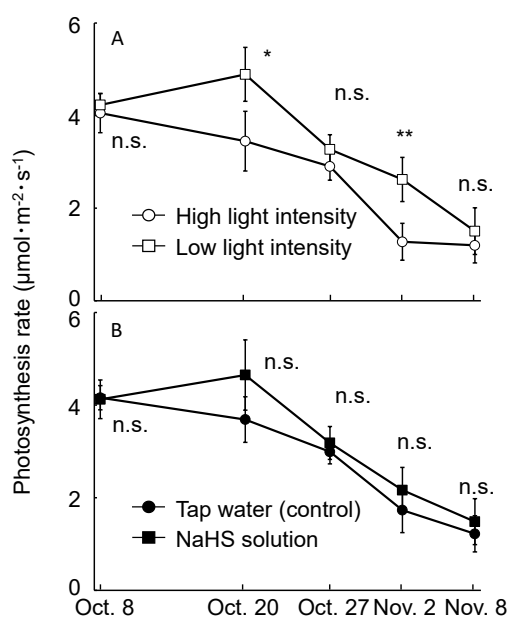


Figure 7 Effects of light intensity (A) and NaHS, a H_2S donor, application (B) on photosynthesis rate in cacao seedling. The pot experiment was conducted with a two-factor factorial design. By two-way ANOVA, interaction was not observed, **, *, and n.s. indicate significant differences at $p < 0.01$, $p < 0.05$, and not significant, respectively. Bars indicate standard error ($n=10$).

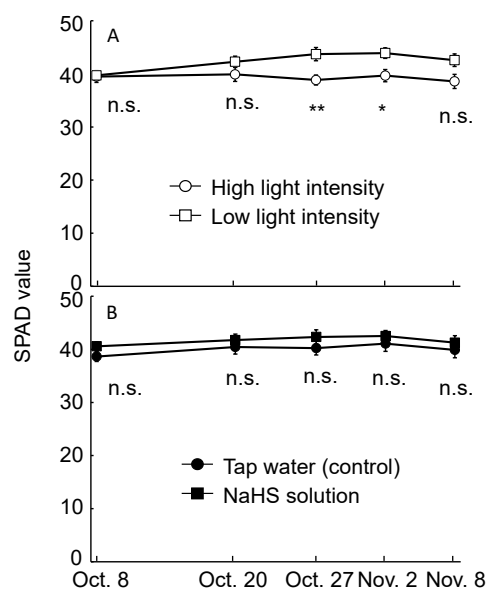


Figure 9 Effects of light intensity (A) and NaHS, a H_2S donor, application (B) on SPAD value in cacao seedling. The pot experiment was conducted with a two-factor factorial design. By two-way ANOVA, interaction was not observed, **, *, and n.s. indicate significant differences at $p < 0.01$, $p < 0.05$, and not significant, respectively. Bars indicate standard error ($n=10$).

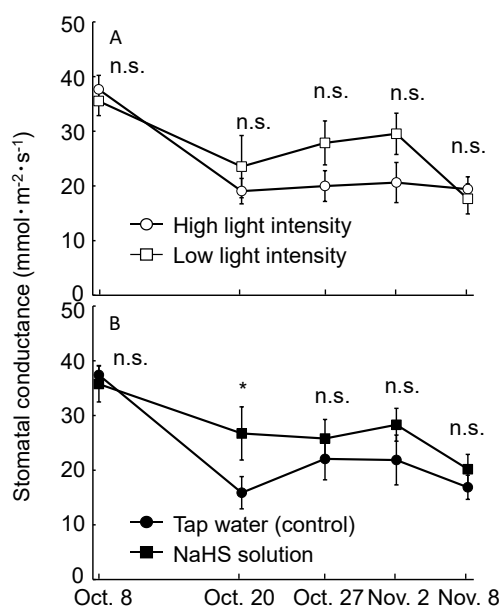


Figure 8 Effects of light intensity (A) and NaHS, a H_2S donor, application (B) on stomatal conductance in cacao seedling. The pot experiment was conducted with a two-factor factorial design. By two-way ANOVA, interaction was not observed, *, and n.s. indicate significant differences at $p < 0.05$, and not significant, respectively. Bars indicate standard error ($n=10$).

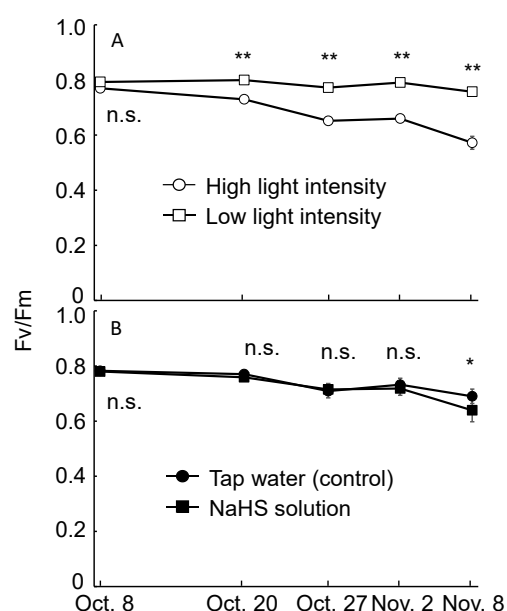


Figure 10 Effects of light intensity (A) and NaHS, a H_2S donor, application (B) on Fv/Fm in cacao seedling. The pot experiment was conducted with a two-factor factorial design. By two-way ANOVA, interaction was not observed, **, *, and n.s. indicate significant differences at $p < 0.01$, $p < 0.05$, and not significant, respectively. Bars indicate standard error ($n=10$).

Thus, even under low temperatures where the minimum temperature fell below 10°C and reached 6.6°C, shading treatment suppressed PSII damage and maintained relatively high photosynthesis rates in cacao seedlings. Generally, chilling stress is caused by oxidative stress resulting from excessive photophosphorylation reactions (Sonoike, 1998). In this study, photophosphorylation reactions should be suppressed due to low light intensity, leading to reduced oxidative stress. Similarly, under chilling stress conditions of approximately -5°C, leaf damage was lightened by shading in avocado (Kondo & Honsho, 2018). On the other hand, in passion fruit under chilling conditions shading did not enhance growth and lighten leaf damage (Kondo, 2021).

Additionally, in this study, the minimum temperature in the low light intensity treatment was slightly higher. Similar situations were also observed in Kondo & Honsho (2018). Therefore, it remains unclear whether the suppression of leaf damage in the low light intensity treatment was due to the influence of light or temperature. However, even under shading treatment the minimum temperature reached low enough to damage cacao plant, so the light intensity likely had a significant effect. Thus, the finding that shading can suppress leaf damage should be valuable information for cacao cultivation in sub-tropical areas. Since 2000, at the northern region of Okinawa main Island, the lower temperature than 6.6°C, which was observed in this experiment, has been recorded only once at 2016 (Japan Meteorological Agency, 2025). Therefore, in Okinawa Island as well, it may be possible to cultivate cacao in open fields with shading trees.

On the other hand, NaHS application had no effect on leaf damage by chilling stress. In passion fruit, H₂S reduced oxidative stress, thereby promoting photosynthetic rate and growth under chilling conditions (Kondo, 2021). Therefore, cacao may be less responsive to the effects of H₂S.

CONCLUSIONS

Under chilling stress where the minimum temperatures were lower than 10°C and reached 6.6°C, shading has the effect of reducing chilling stress, while H₂S application has no effect in cacao seedling. Shading is relatively inexpensive and easy to introduce for farmers, especially at unheated greenhouse. At open field, further studies, on the density and positioning of shade trees, are needed.

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