

Heat-stress induced sesquiterpenes of *Chrysanthemum nankingense* attract herbivores but repel herbivore feeding

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Abstract

Plants are frequently exposed to heat stress as a result of global warming. Heat stress leads to a series of physiological responses including stress volatile elicitation, but how heat stress-induced volatile cues affect the behavior of herbivores is poorly understood. In this study, the polyphagous herbivore *Spodoptera litura* (tobacco cutworm, TCW) and perennial forb *Chrysanthemum nankingense* were selected as the model to elucidate the interactions between herbivore behavior and heat stress-induced plant physiological changes. Photosynthetic characteristics and volatile emissions were measured in *C. nankingense* control plants (25 °C) and in plants exposed to moderate (35 °C for 3 h), and severe (45 °C for 3 h) heat stresses. Net photosynthetic rate (A_n) decreased by more than two-fold after exposure to 45 °C due to non-stomatal inhibition of photosynthesis. 45 °C treatment induced emissions of the monoterpene camphor and sesquiterpene (*E*)- β -caryophyllene. Exposure to 35 °C had minor effects on photosynthetic characteristics and did not induce terpene emissions. Using dual-choice olfactometer bioassays, we found that 45 °C treatment enhanced the attractiveness of the plants to TCW. Moreover, the leaf concentrations of 9 sesquiterpenes were increased and the feeding of TCW was strongly inhibited after 45 °C treatment compared with control plants. Taken together, our study highlights the impact of heat stress on the behavior of the herbivore mediated by the accumulation and emission of sesquiterpenes and suggests altered pest-host interactions under future warmer climates. Modulation of terpenoid emissions and contents should be considered in developing future ecological pest control strategies in agricultural fields.

Introduction

Global environmental change is associated with unprecedentedly rapid increases in global mean temperature and enhanced frequency and duration of extreme heat stress episodes (Jagadish et al. 2021; Parmesan & Yohe 2003; Harvey et al. 2020). Thus, heat stress is becoming one of the most frequent and harmful stressors among different abiotic factors in nature (Perkins et al. 2012), and its impact on vegetation is expected to gradually increase in the future as global warming proceeds (IPCC 2013).

The response of plants to heat stress typically involves several stages from initial response to recovery and acclimation for moderately severe stress to necrosis and death for extremely severe stress (Morimoto 1998; Baniwal et al. 2004; Hamerlynck et al. 2000; Sung et al. 2003; Wang et al. 2018; Satpal Turan et al. 2019). Heat stress acclimation is associated with a series of physiological and biochemical reactions, including changes in plant morphology, photosynthetic characteristics, respiration, hormone production, volatile organic compound (VOC) production and antioxidant capacity (Larkindale & Knight 2002; Peñuelas & Llusà 2003; Niinemets 2010; Asseng et al. 2011; Hüve et al. 2012; Casal & Balasubramanian 2019; Ma et al. 2021). Photosynthesis is typically the first cellular function to be impaired by heat stress (AL-Khatib & Paulsen 1984; Kepova et al. 2005; Schrader et al. 2007; Hüve et al. 2011; Hüve et al. 2019; Hüve et al. 2011; Zou et al. 2017). Inhibition of foliage photosynthetic activity by mild heat stress is typically readily reversible, while severe heat stress can lead to sustained inhibition or even continuous reduction in photosynthesis after return to the lower temperature (Hüve et al. 2011; Turan et al. 2019).

Several studies have reported that the irreversible reduction of photosynthetic activity is related to the release of stress-induced VOC, including rapid release of short-chained oxygenated compounds such as methanol, acetone, acetaldehyde, and various lipoxygenase pathway-derived compounds (volatile LOX products, also called green leaf volatiles) (Loreto et al. 2006; Copolovici et al. 2012; Kask et al. 2016; Turan et al. 2019). Apart from stress-induced LOX-emissions, heat stress can also result in changes in the emissions of terpenoids that are stored or de novo synthesized (Monson & Fall 1989; Llusia & Peñuelas 1999; Copolovici et al. 2012; Turan et al. 2019). Stress-induced onset of the release of de novo synthesized terpenoids needs the expression of corresponding terpene synthases, hence, the stress-induced terpene emissions usually occur with a time delay (Pazouki et al. 2016).

Terpenoids represent the largest and most diverse group of plant specialized metabolites (Aharoni et al. 2005) and many terpenes play key roles in the interaction of plants with their environment. Thus, the stress-induced terpene release has many biological implications. In particular, volatile terpenoids emitted from plants can affect herbivore behavior and serve as an indirect defense mechanism by attracting the natural enemies of the herbivores to protect the plant from further damage (Abbas et al. 2017; Eberl et al. 2018; Dobson 1994; Köllner et al. 2008). In addition, synthesis of volatiles stored in plants can be enhanced by different abiotic and biotic stresses, and these stored compounds can play a major role as direct defenses deterring herbivore feeding (Mahajan et al. 2020; Agliassa and Maffei 2018; Wang et al. 2021). Drought, waterlogging, and high salinity are some of the abiotic stresses that have been studied most and can indirectly affect the performance, diversity and abundance of feeding insect through changes in plant physiology (Wearing 1967; Mattson and Haack 1987; Huberty & Denno 2004; Han et al. 2014; Hoang et al. 2016). However, there are few studies on the impact of volatile cues on orientation and feeding behavior of herbivores after heat stress (Harvey 2015).

Polyploid species in the genus *Chrysanthemum* are commercially important ornamental plants that have been cultivated in China for more than 3000 years (Cheng et al. 2010). Temperature is one of most critical factors in the field production of *Chrysanthemum* spp. Generally, the growth of *Chrysanthemum* spp. will be disturbed above 32 °C and be terminated at 40 °C (Kong et al. 2019). The expected increase of the occurrence of extremely high temperatures above 40°C due to global warming is predicted to have a major negative influence on field production of *Chrysanthemum* spp., and this could be further exacerbated by potential enhancement of pest attacks (Sagheer 2019; Lemoine et al. 2014; Hamann et al. 2020). The diploid species *C. nankingense* is a wild relative of *C. morifolium* (Cheng et al. 2010). Because most of the ornamental chrysanthemums are tetraploid or hexaploid and have a complicated genetic background, *C. nankingense* can be used as a simple model to study the response of plants from Compositae to abiotic stress. Besides, chrysanthemum plants are susceptible to a broad array of herbivores, such as leaf miners, beet armyworms and aphids, which affect the vegetative organs and also decrease the quality of flowers during their growth and development (Visser et al. 2007; Xia et al. 2014). It has been observed that the larvae of the polyphagous moth *Spodoptera litura* Fabr. (tobacco cutworm, TCW) occurred on about 29 species of Compositae (Lim et al. 2012). TCW is a major pest that feeds on plant leaves in tropical and subtropical areas of Asia (CABI 2021). Given the severe damage of TCW to

Chrysanthemum spp., TCW can be regarded as an ideal model herbivore for the volatile-mediated biological assay.

In this study, we first address the impact of heat stress of different severity (mild vs. severe) on photosynthetic traits and volatiles emissions of *C. nankingense* leaves. We further explored the influence of heat stress-mediated changes in volatile emissions and volatile contents on the orientation and feeding behavior of TCW in *C. nankingense*. We hypothesized that foliage photosynthetic activity decreases and volatile emissions increase in heat stress severity-dependent manner. We also hypothesized that heat stress-induced volatiles reduce the attractiveness of *C. nankingense* leaves to TCW, and heat stress-dependent changes in secondary metabolite content reduce the feeding of TCW. We observed that paradoxically heat stress enhanced the attractiveness of *C. nankingense* leaves to TCW, but the heat-stressed leaves were less palatable to the herbivore. The results could be used to develop new strategies for ecological control of pests in the agricultural fields.

Methods And Materials

Plant materials

Chrysanthemum nankingense plants were obtained from the greenhouse at the Chrysanthemum Germplasm Resource Preserving Centre, Nanjing Agricultural University, China (118° 98' N, 32° 07' E). Healthy shoot cuttings of similar size were grown under controlled conditions in a growth chamber (Ningbo Southeast Instrument Co., Ltd.). Light intensity at plant level was 12.6-19.8 $\mu\text{mol m}^{-2} \text{s}^{-1}$, photoperiod length 16 h, air temperature 25 °C (day)/18 °C (night), relative humidity 68-75%. Morphologically uniform seedlings with 10-15 leaves were used for all treatments.

Heat stress treatment

Three treatments were applied: 25 °C (control), 35 °C (moderate heat stress), and 45 °C (severe heat stress) applied for 3 h in a growth chamber (light intensity 19.8 $\mu\text{mol m}^{-2} \text{s}^{-1}$, relative humidity 68-75%). Three different plants were used for each treatment.

Photosynthetic trait measurements

After heat stress treatment, from each plant, a fully-expanded fifth leaf from the top was selected to measure leaf photosynthetic traits with a portable photosynthesis system (LI-6800, LI-COR Biosciences, USA). The leaf was clipped in the leaf chamber and the following environmental conditions were established and maintained during the measurements: chamber CO_2 concentration, 400 $\mu\text{mol mol}^{-1}$; air flow rate, 600 $\mu\text{mol s}^{-1}$; leaf temperature, 25 °C; relative humidity, 50%-75%; light intensity, 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The leaf was kept as the standard conditions for 30 min., and net assimilation rate (A_n), stomatal conductance to water vapor (G_s), and intercellular CO_2 concentration (C_i) were recorded in 5 min intervals for 30 min.

Organic extraction of leaf terpenoids

The leaves collected from 45 °C treated and control plants were powdered in liquid nitrogen. Ethyl acetate (Macklin Technology, Shanghai, China) was added to the powder in a 5:1 (volume to mass) ratio, with 0.002% nonyl acetate (CAS:143-13-5, ≥ 98%, Sigma Aldrich, St Louis, MO, USA) included as an internal standard. After shaking at 200 rpm at room temperature for two hours and centrifugation at 5000 rpm for five minutes, the organic phase was collected for subsequent GC-MS analysis.

Volatile collection and identification by GC-MS

Volatiles emitted from the heat stress-treated and control plants were collected with an open headspace sampling system (Analytical Research Systems, Gainesville, FL, USA) as previously reported (Yuan et al. 2008). The volatiles were collected for 4 h by sucking air through a volatile collection trap filled with Super Q adsorbent (Alltech Assoc., USA) and were eluted with 100 µL of CH₂Cl₂ containing 0.001% nonyl acetate as the internal standard (Jiang et al. 2018). 1 µL of the eluent was injected into a gas chromatography-mass spectrometry (GC-MS) system (Agilent Intuvo 9000 GC system coupled with an Agilent 7000D Triple Quadrupole mass detector) for separation and identification of terpenes. The separation was performed on an Agilent HP 5 MS capillary column (30 m length × 0.25 mm inner diameter). Helium with a flow rate of 5 mL min⁻¹ was used as the carrier gas. Splitless injection (injection temperature 250 °C) with a temperature gradient of 6 °C min to 300 °C was applied. The temperature of the injection port was 260 °C, with a split mode (5:1), and a linear temperature gradient was used for compound separation. The column initial temperature was 40 °C and the temperature was increased to 250 °C at a rate of 5 °C min⁻¹. The MS was operated in the electron impact mode with ion source temperature set at 230 °C, ionization energy was 70 eV, and mass scan range 40-500 amu. Terpenoid products were identified using the National Institute of Standards and Technology mass spectral database (NIST 17.0) and by comparison of retention times and mass spectra with available authentic standard compounds. Quantification was performed based on peak areas of mass chromatograms (Zhang et al. 2020).

Y-tube olfactometer bioassay

Third instar larvae of *S. litura* (tobacco cutworm, TCW) were used as model herbivores to infest plants. Larvae of TCW were purchased from Henan Jiyuan Baiyun Industry Co., Ltd. We used a Y-tube olfactometer equipped with a Y-shaped glass tube (one 20 cm arm and two 15 cm branched arms, 1 cm diameter) to explore the olfactory orientation behavior of the TCW larvae. The branched arms were connected to two glass bottles as odor sources. One arm was connected to a bottle containing a control plant, another to a bottle with a plant exposed to 45 °C for 3 h or to an empty bottle. Each glass bottle was supplied by air with a low-pressure air pump through a charcoal filter at a rate of 100 mL min⁻¹. Three instar TCW larvae were collected from rearing cages in a separate insectary room and starved for 20 h before each trial, then a group with 10 larvae was released into the base of the tube and each larva entered the Y-tube one at a time. If a larva crossed the bifurcation point of 3 cm within one arm, a positive

or a negative response was recorded. If the larva did not make a clear choice after 5 min, it was considered as unresponsive. Besides, the time spent by larvae in the different arms of the olfactometer was recorded. After passage of five larvae, the olfactometer was rotated 180° to exclude the position interference. Each experiment was conducted with three replicate plants from control and 45 °C heat treatment (Sun et al. 2015).

Evaluation of damage severity by herbivore feeding

Three instar TCW larvae were collected from rearing cages in an insectary room and starved for 20 h before each trial. Seven larvae were put carefully on the leaves of each plants after the 45 °C treatment at 5:00 pm. After 16 h of larval infestation, the leaves fed by the larvae in each plant were collected, and the infested area was calculated using image J (1.52v, National Institutes of Health, USA).

Statistical analysis

One-way ANOVA was used to separate differences in photosynthetic traits, and t-test was used to separate differences in terpenoid concentration, Y-tube olfactometer bioassay and feeding area (SPSS version 19.0).

Results

Effects of heat stress on photosynthetic traits of *C. nankingense* leaves

Compared with the control treatment (25 °C), 3 h exposure to 35 °C did not affect net assimilation rate (A_n), but A_n was reduced by about 70% in the plants exposed to 45 °C (Figure 1a). Stomatal conductance to water vapor (G_s) was initially reduced in plants exposed to 35 °C, but it reached similar levels as G_s in control plants during 30 min measurement period (Figure 1b). G_s was not affected by exposure to 45 °C (Figure 1b). Similarly to G_s , the intercellular CO₂ concentration (C_i) was initially lower for plants exposed to 35 °C than for plants in the control treatment, and reached a similar level as the measurements continued (Figure 1c). At the end of the measurements, plants exposed to 45 °C had a higher C_i than controls plants and plants exposed to 35 °C (Figure 1c).

Heat stress treatment induced the terpene emission in *C. nankingense*

There is no modification of the terpene profile with the exposure to 25 °C and 35 °C. However, heat treatment at 45 °C enhanced both emissions of mono- and sesquiterpenes (Fig. 2a). The monoterpene camphor and sesquiterpene (*E*)- β -caryophyllene were the key induced terpenes emitted after the 45 °C treatment with the emission rates of $5.45 \pm 0.59 \text{ ng h}^{-1} \text{ g}^{-1}$ and $9.56 \pm 2.16 \text{ ng h}^{-1} \text{ g}^{-1}$, respectively (Fig. 2b).

Heat stress enhanced TCW visitation of *C. nankingense* leaves

Y-tube olfactometer assays showed that the control plants were not attractive to the larvae of TCW (no choice difference between control plants and clean; Fig. 3). The plants exposed to 45 °C were more attractive to TCW ($65.00 \pm 3.33\%$; $P < 0.01$) than control plants (Fig. 3). These results clearly revealed the determinant role of volatile emissions from heat stress treated plants in TCW attraction.

Heat stress increased the content of terpenoids in *C. nankingense* leaves

No monoterpenes were detected in leaf extracts of either the control or 45 °C-treated plants. 11 sesquiterpenes were detected in *C. nankingense* leaves (Fig. 4). Total sesquiterpene concentration, and the concentrations of 9 sesquiterpenes, α -farnesene, β -copaene, γ -elemene, γ -muurolene, (*E*)- β -caryophyllene, (*E*)- β -farnesene, isogermacrene D, germacrene D and germacrene D-4-ol were higher in heat-treated than in the control plants (Fig. 4).

Heat stress-treated leaves repel the TCW-infestation

TCW preferred the control plants (Fig. 5a) and after 16 h feeding, TCW consumed a higher total leaf area in the control plants than in the plants under the 45 °C treatment (Fig. 5b). The percentage of total leaf area consumed ($4.57\% \pm 1.36\%$ in heat-stressed vs. $58.91 \pm 3.35\%$ for control plants) was also greater in control plants ($P < 0.01$).

Correlations between the contents of different terpenoids and the leaf area consumed by TCW

Pearson correlation analysis showed that the total area consumed by TCW scaled negatively with total sesquiterpene concentration ($r = -0.988$ for total area, $r = -0.991$ for percentage of total area, $P < 0.01$). In addition, the concentrations of 8 sesquiterpenes were negatively correlated with the area consumed by TCW larvae. Concentrations of β -copaene, γ -elemene, γ -muurolene, isogermacrene D, and germacrene D were most strongly correlated with feeding area (r ranging between -0.967 to -0.987 , $P < 0.01$). Concentrations of three other sesquiterpenes ((*E*)- β -caryophyllene, (*E*)- β -farnesene, germacrene D-4-ol) were also negatively correlated with the feeding area (r ranging from 0.877 to -0.895 , $P < 0.05$), whereas the correlation of α -patchoulene and consumed area was positive ($r = 0.852$, $P < 0.05$).

Discussion

Impact of heat stress on photosynthetic characteristics of *C. nankingense*

Photosynthesis is a critical plant physiological process that is responsible for plant growth and survival, and accordingly it is of vital importance of plant photosynthesis responds to stress. Photosynthesis is one of the most sensitive physiological processes to heat stress (Allakhverdiev et al. 2008; Ashraf & Harris 2013; Hüve et al. 2019; Okereke et al. 2022). Several studies have demonstrated that heat stress reduces leaf photosynthetic activity due to inhibition of multiple rate-limiting processes and damage to key components of photosynthetic machinery (Ashraf & Harris 2013; Salvucci & Crafts-Brandner 2004; Sharkey 2005; Schrader et al. 2007; Hüve et al. 2011; Zhu et al. 2018). Typically, moderate heat stress results in readily reversible changes in photosynthetic activity, primarily as the result of reductions in

stomatal conductance to water vapor (G_s) (Hüve et al., 2019; Okereke et al., 2022), and severe heat stress leads to non-stomatal slowly reversible or non-reversible inhibition of photosynthesis due to damage of the photosynthetic machinery (Salvucci and Crafts-Brandner 2004; Sharkey 2005; Schrader et al. 2007; Hüve et al. 2011; Zhu et al. 2018). Photosynthetic electron transport, in particular, PSII has been considered to be a highly heat-sensitive component of photosynthetic apparatus (Gombos et al. 1994; Schrader et al. 2004; Hüve et al. 2011).

In our study, net assimilation rate, A_n , was not affected by plant exposure to 35°C, although there was evidence of reduced rate of increase of G_s after leaf enclosure to leaf chamber and increase in light level (Fig. 1a, b). Such delayed responses of photosynthetic characteristics have been observed after heat exposure (Hüve et al. 2019), and are expected to reduce leaf carbon gain in fluctuating environments. Leaf exposure to 45°C treatment resulted in a major reduction of A_n without concomitant reductions in G_s , indicating non-stomatal reduction of foliage photosynthetic activity (Fig. 1a, b) as was also confirmed by increased intercellular CO₂ concentration (Fig. 1c). This result is in agreement with the reports showing that severe heat stress can reduce the cyclic transport of electrons and thylakoid permeability (Schrader et al. 2004; Sharkey 2005). Our results are in accordance with previous reports suggesting that severe heat stress leads to non-stomatal inhibition of photosynthesis, and milder heat stress to stomatal limitations (Zou et al. 2017; Turan et al. 2019).

Heat stress-dependent stimulation of leaf volatile organic compound (VOC) emissions and concentrations in *C. nankingense*

Under optimal conditions for photosynthesis, volatile organic compounds (VOC) emitted by plants account for a few percentage of terrestrial fixation, but this percentage can greatly increase during stress conditions that inhibit net primary productivity and stimulate emissions of some class of volatiles (Niinemets 2010; Jardine et al. 2020). In several species, the increase in temperature has been demonstrated to strongly increase the emission of various terpenes (Staudt and Bertin 1998; Grote et al. 2013; Loreto & Schnitzler 2010; Maja et al. 2016). To understand the impact of temperature on terpene emissions, it is important to consider that only some species are strong constitutive terpene emitters, and terpenes might be emitted right after their synthesis (de novo emissions) or from specialized storage (storage emissions) (Grote et al. 2013; Copolovici & Niinemets 2016). Moderately high temperatures can enhance constitutively synthesized de novo terpene emissions primarily by reversibly enhancing the activity of terpene synthases and substrate availability (Niinemets et al. 2002; Grote et al. 2013). In addition, heat stress can lead to induction of expression of terpene synthases and start of terpene release in non-constitutive emitters (Copolovici & Niinemets 2016). In addition, in storage emitters, severe stress can enhance terpene emissions by breaking the trichomes or increasing the permeability of glandular trichome outer surfaces (Guenther et al. 1993; Jansen et al. 2009; Grote et al. 2013). In addition, larger molecular size volatiles and semi-volatiles might be released from leaf surface waxes, especially when temperature increases (Himanen et al. 2015; Joensuu et al. 2016). Both induction of emissions as well as

enhancement of storage emissions have been observed in heat-shocked *Solanum lycopersicum* and *Nicotiana tabacum* (Copolovici et al. 2012; Pazouki et al. 2016; Turan et al. 2019).

Chrysanthemum nankingense leaves have small amount of glandular and non-glandular trichomes, and thus volatile emission might come from both de novo synthesis and specialized storage (Guo et al. 2020). In our study, no terpene emissions were observed in control plants at 25 °C, and the release of mono- and sesquiterpenes was initiated only after 3 h 45 °C treatment (Fig. 2). Given that the dominant monoterpene induced by heat stress, camphor, was not found in leaf extracts (Fig. 4), we conclude that the release of monoterpenes reflects de novo induction of monoterpene synthesis in heat-stressed leaves. Given that sesquiterpenes were also observed in leaf extracts (Fig. 4), the heat stress-induced emissions of the sesquiterpene (*E*)- β -caryophyllene could be related to the breakage of glandular trichomes or increases permeability of storage cells. However, leaf extracts demonstrated presence of 10 other sesquiterpenes, but only (*E*)- β -caryophyllene was observed in leaf emissions (Fig. 2). This suggests that the emitted (*E*)- β -caryophyllene might also come from de novo synthesis as has been observed in response to different stresses (Hansen & Seufert 2003; Jiang et al. 2017; Kanagendran et al. 2018).

Analysis of leaf extracts further demonstrated that total sesquiterpene concentration, and the concentrations of most individual sesquiterpenes detected increased after plant exposure to 45 °C (Fig. 4). There is limited information of heat stress impact on terpene contents, and the results are controversial. In conifers *Pinus sylvestris* and *Picea abies*, foliage terpene concentrations were enhanced by growth at higher temperature (Sallas et al. 2003), but in the annual herb *Artemisia annua* terpenoid content was not affected by growth temperature (Daussy & Staudt 2020). In the conifer *Pseudotsuga menziesii*, growth under high temperature reduced foliage terpene concentrations in one provenance and did not affect the concentrations in another provenance (Duan et al. 2019). In the perennial herb *Valeriana jatamansi* grown in a Free Air Temperature Increase Experiment, the concentration of the sesquiterpene globulol was increased by high temperature, but not the concentration of other compounds (Kuandal et al. 2018). Clearly more work is needed to gain an insight into the impact of heat stress of foliage terpenoid contents.

Heat stress increases the attractiveness of *C. nankingense* leaves to *Spodoptera litura* (tobacco cutworm, TCW)

Our study showed that the volatile terpenoids emitted from *C. nankingense* after 3 h exposure 45 °C increased plant attractiveness for TCW. Enhanced attractiveness of the plants to TCW could be mediated by the induced emissions of (*E*)- β -caryophyllene. However, (*E*)- β -caryophyllene is typically associated with attraction of pest and herbivore enemies rather than attraction of herbivores themselves. For example, (*E*)- β -caryophyllene emitted from maize (*Zea mays*) roots strongly attracted an entomopathogenic nematode of the pest *Diabrotica virgifera virgifera* (Rasmann 2005; Köllner et al. 2008). In addition, *Z. mays* leaves infested by TCW emitted (*E*)- β -caryophyllene that strongly attracted a larval parasitoid *Cotesia marginiventris* (Köllner et al. 2008). On the other hand, it has been also observed that (*E*)- β -caryophyllene could attract the pest *D. virgifera virgifera* larvae to locate the maize plant (Robert et al.

2012). Moreover, (*E*)- β -caryophyllene could function as a host location signal for the rice (*Oryza sativa*) pest *Sogatella furcifera* (Wang et al. 2015), and it also attracted the whitefly (*Bemisia tabaci*) to the leaves of rosemary (*Rosmarinus officinalis*) (Sadeh et al. 2017). Our results are consistent with the observations that (*E*)- β -caryophyllene could serve as host location signal for TCW.

Inhibition of TCW feeding by heat-dependent increases of *C. nankingense* leaf sesquiterpene concentrations

The comparison of feeding area between control plants and 45 °C-treated plants suggested that elevated concentrations of sesquiterpenes could inhibit the infestation of TCW (Fig. 5). In particular, total sesquiterpene concentration and concentrations of γ -elemene, β -copaene, isogermacrene D, γ -muurolene, germacrene D, (*E*)- β -caryophyllene, (*E*)- β -farnesene, and germacrene D-4-ol were negatively correlated with the total leaf area consumed and the percentage of leaf area removed by TCW. This implies that acclimation to heat stress resulted in higher resistance to TCW larval infestation. Anti-feedant activity and toxicity of sesquiterpenes to related herbivore *Spodoptera littoralis* larvae has been observed in multiple studies, including 38 sesquiterpenes extracted from leaves of different *Celastraceae* species (Gonzalez et al. 1997), two eremophilane sesquiterpenes isolated from the forb *Senecio adenotrichus* (Ruiz-Vasquez et al. 2017), and terpenoids from the forb *Origanum vulgare* (Agliassa & Maffei 2018). It has been further observed that among eight tropical woody species, the essential oil extracted from leaves of *Piper pseudolanceifolium* and *Ocimum campechianum* most strongly repelled the food grain pest *Tribolium castaneum*; in these species, germacrene D and (*E*)- β -caryophyllene were the dominant components of the essential oil (Caballero-Gallardo et al. 2014). In our study, the concentrations of these two sesquiterpenes increased after heat stress in *C. nankingense* leaves, and this was associated with reduced TCW feeding area, underscoring the high repellence of germacrene D and (*E*)- β -caryophyllene.

Conclusions

In summary, our study indicates that (*E*)- β -caryophyllene can serve as host location cue for TCW. However, together with other sesquiterpenes, it also serves as feeding deterrent for TCW. Thus, such attraction to (*E*)- β -caryophyllene might seem maladaptive. However, sesquiterpene synthesis is elicited by multiple stresses, some of which can lead to a systemic defense response in all leaves, and some to localized response in only impacted leaves. In the case of a localized stress, such as herbivory on single leaves, attraction to (*E*)- β -caryophyllene could be advantageous as the herbivores might feed on as yet undamaged leaves. In the case of a systemic stress such as whole plant heat stress, however, all leaves are impacted and the (*E*)- β -caryophyllene-emitting plant will be a lower quality food source than a non-emitting plant.

Our findings could provide new opportunities for pest management. Heat stress exposed *C. nankingense* will have elevated emissions of (*E*)- β -caryophyllene, and this could effectively attract TCW. This could indirectly protect the adjacent crops that cannot produce (*E*)- β -caryophyllene by reducing their herbivore infestation. Further studies should focus on the mechanisms underlying the terpene metabolism in *C.*

nankingense by functional identification of the terpene synthase genes responding to high temperature. It is also necessary to explore the ecological function of individual terpenoids on the behavior of herbivores. In particular, electroantennogram (EAG) analyses would be very useful to understand the relationships between olfactory stimuli of individual terpenes released after heat stress and after herbivory and resultant behavioral responses of TCW.

Declarations

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Availability of Data and Material

All data are included in the manuscript.

Conflicts of Interest/Competing Interests All authors declare that they have no conflict of interest.

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Figures

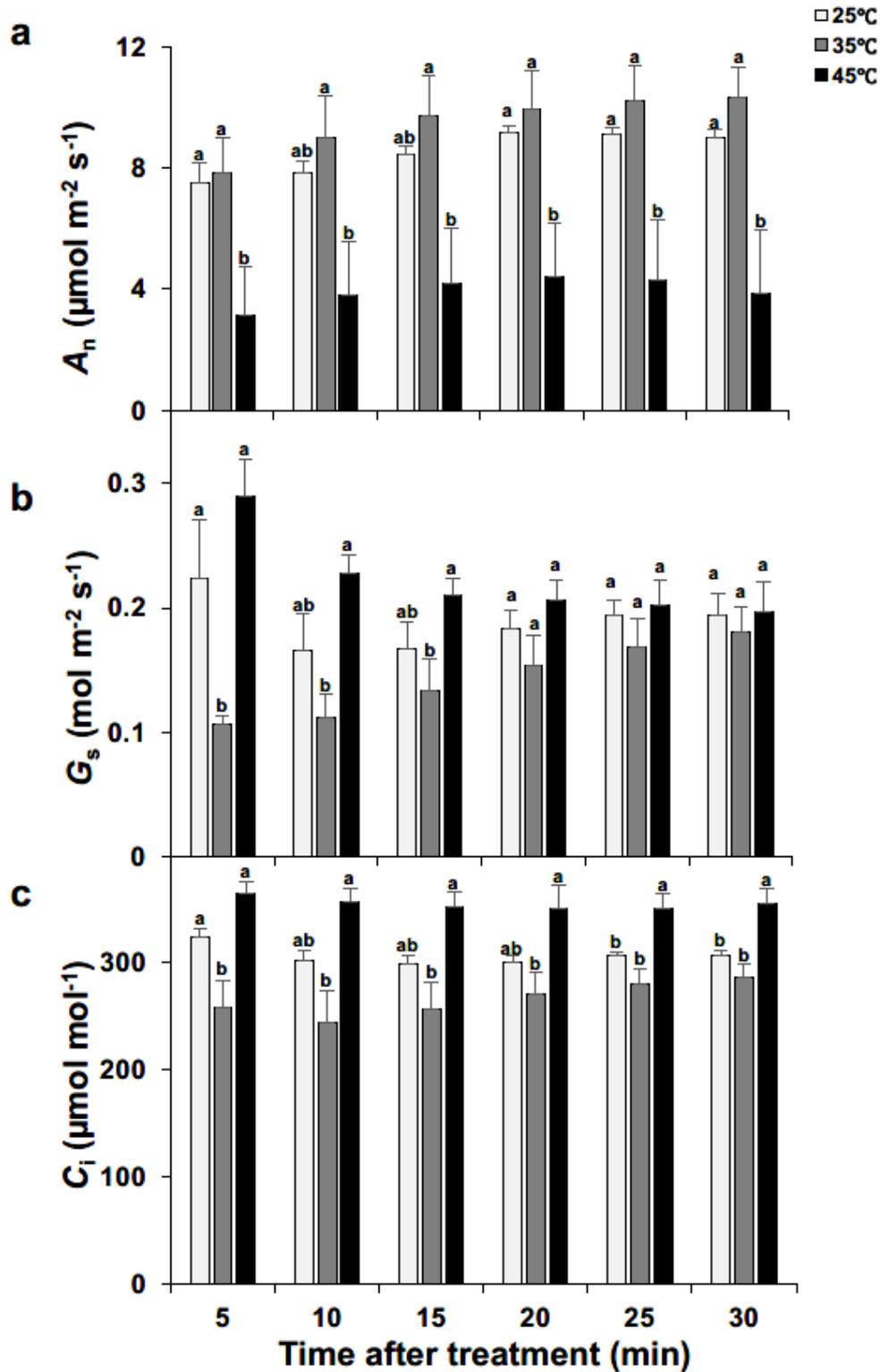


Figure 1

Effect of moderate (exposure to 35 °C for 3 h) and severe (45 °C for 3 h) heat stress on average \pm SE net assimilation rate (A_n)(a), stomatal conductance to water vapor (G_s)(b), and the intercellular CO_2 concentration (C_i)(c) of the perennial herb *Chrysanthemum nankingense*. The control plants were kept at 25 °C for 3 h under otherwise identical conditions. Different letters indicate statistically significant differences among the means according to ANOVA analysis ($n = 3$, $P < 0.05$).

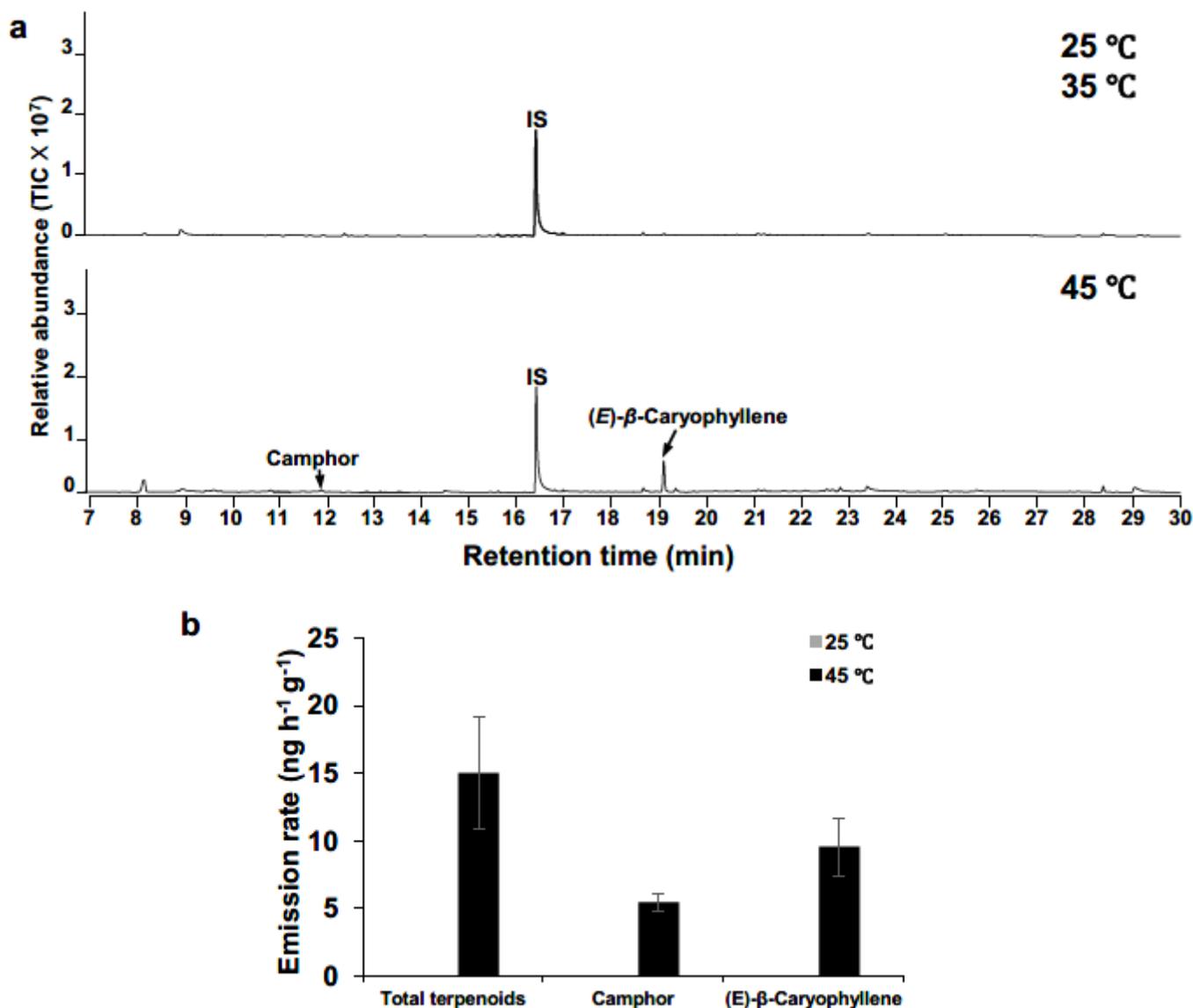


Figure 2

Chromatogram profiles (total ion chromatograms, TIC) of terpenoid compounds emitted from leaves of *C. nankingense* control plants (25 °C) and plants exposed to moderate (35 °C for 3 h) and severe (45 °C for 3 h) heat stress (a). The volatiles were collected from the headspace and analyzed with GC-MS. IS denotes the internal standard (nonyl acetate). Unlabeled peaks are not terpenoids. 1, camphor; 2, (*E*)-β-caryophyllene. Average ± SE emission rates of total terpenoids, and the monoterpene camphor, and the sesquiterpene (*E*)-β-caryophyllene at 4 h after the treatments (b). nd: no detection.

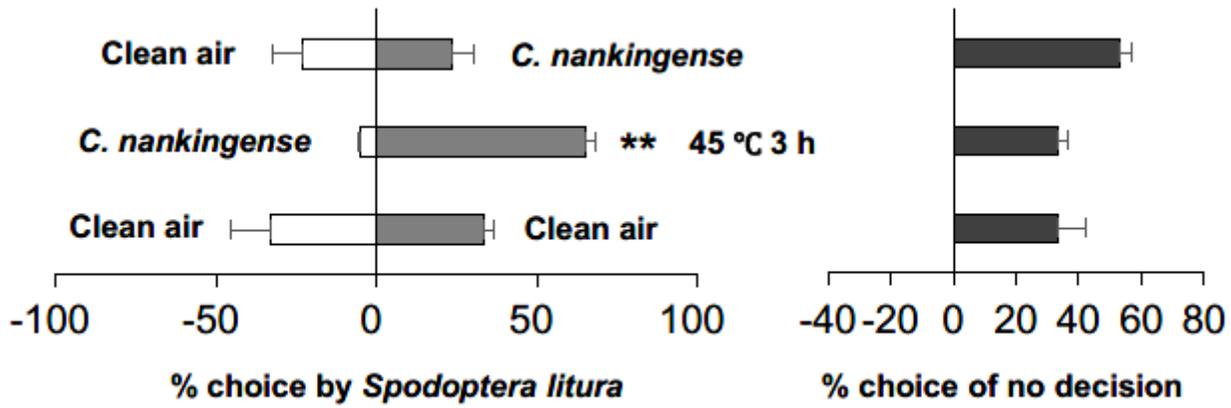


Figure 3

Spodoptera litura (tobacco cutworm, TCW) olfactory orientation in Y-tube olfactometer bioassays. Three groups of comparisons were conducted: clean air vs control (25 °C) *C. nankingense*, control *C. nankingense* vs 45 °C-treated *C. nankingense*, and clean air vs clean air. Clean air was used to eliminate the effect of the collection device itself on the tests. The bars represent the percentage of tested insects that made a particular choice. ** - significantly different at $P < 0.01$. Left bars show the percentage of larva that made no choice within 5 min after start of the test.

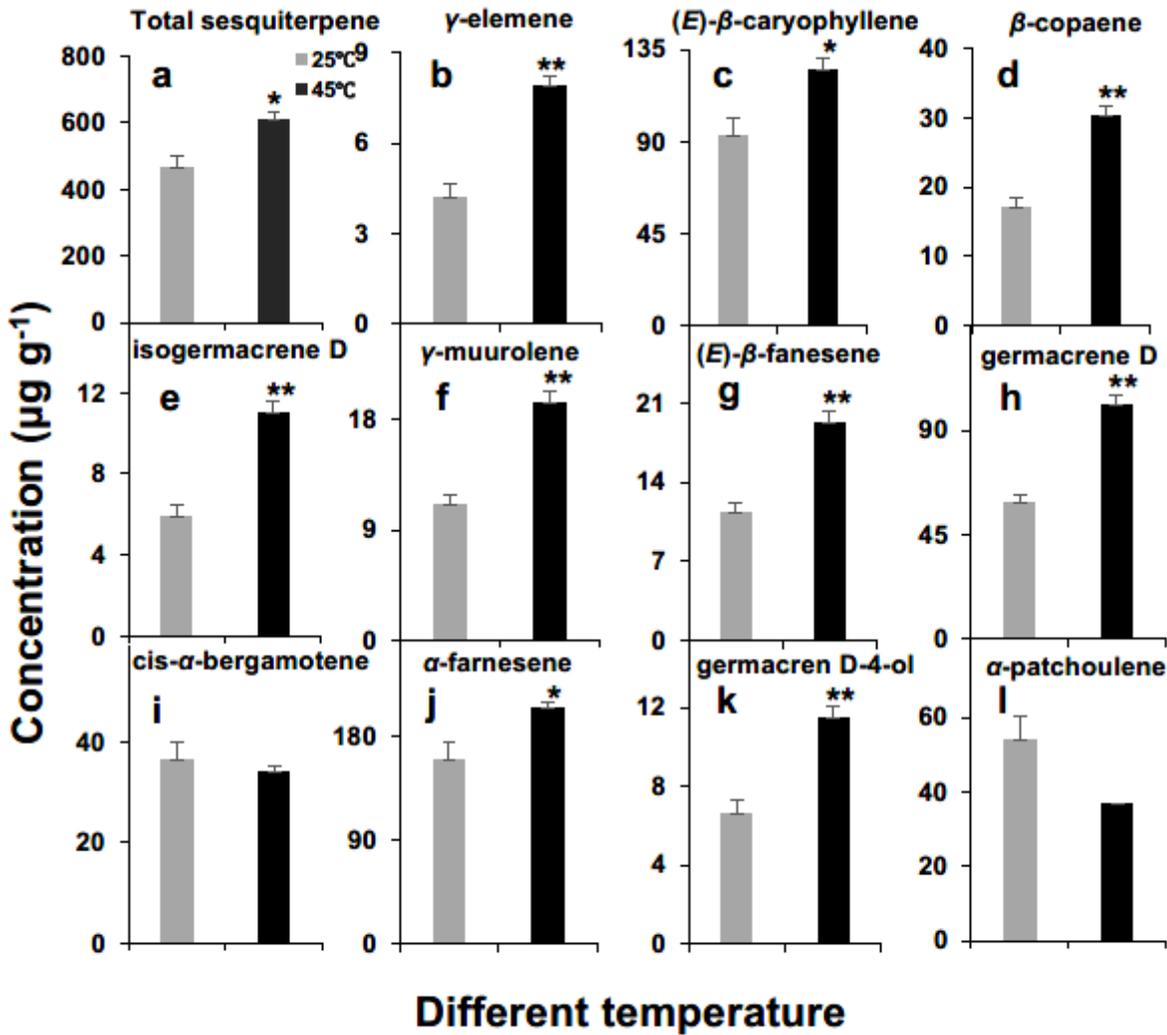


Figure 4

Effect of heat stress on the concentrations of total sesquiterpenes and 11 sesquiterpenes in leaf extracts of *C. nankingense*. Concentration of sum of all sesquiterpenes (a), and γ -elemene (b), (*E*)- β -caryophyllene (c), β -copaene (d) isogermacrene D (e), γ -muurolene (f), (*E*)- β -farnesene (g), germacrene D (h), *cis*- α -bergamotene (i), α -farnesene (j), germacrene D-4-ol (k), and α -patchoulene (l) from control and 45 °C exposed *C. nankingense* leaves. The asterisks indicate significant differences: * - $P < 0.05$, ** - $P < 0.01$.

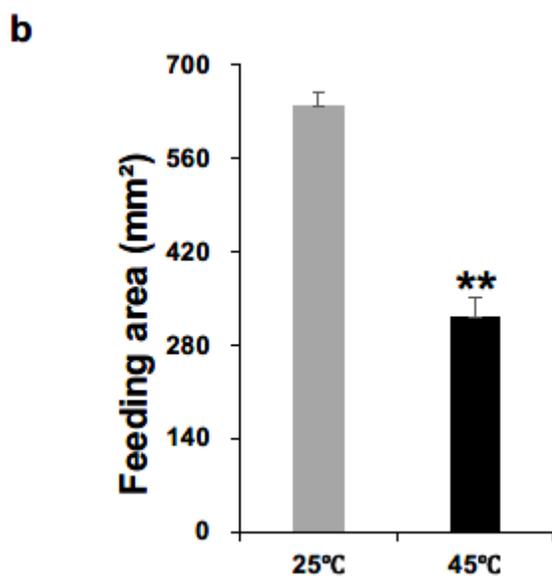


Figure 5

Effect of heat stress on the feeding area on *C. nankingense* after 16 h of infestation by *Spodoptera litura*. (a) Representative images of infested leaves from a control and 45 °C-treated plant. (b) Average \pm SE leaf area consumed in control and 45 °C-treated plants. ** - $P < 0.01$.