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# Efficacy of carbon dioxide treatments for the control of the two-spotted spider mite, *Tetranychus urticae*, and treatment impact on plant seedlings

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**Abstract** To develop a new control method for the two-spotted spider mite (TSSM), *Tetranychus urticae*, we investigated the effect of controlled atmospheres of carbon dioxide ($CO_2$) on TSSM mortality under different concentrations and treatment periods, and evaluated the impact of treatments on seedlings of five host plants of TSSM. Egg hatching rate of TSSM was reduced to 37.7, 5.4 or 0% after 24 h treatment involving concentrations of 16.7, 33.3 or 50%, respectively. Mobile stages (nymphs and adult) of TSSM were completely controlled after 24 h treatment at concentrations higher than 33.3%. After 4 h at concentrations of 33.3 or 50%, 1st-day survival rate for all mobile stages was 45.3 or 36.0%, respectively, whereas after 8 or 16 h treatments, all values were decreased to zero. Seedlings of four major host plants of TSSM (cucumber, eggplant, rape, green peppers) were damaged to varying degrees after 24 h at the three concentrations, but strawberry, another host plant, was not damaged. Cucumber suffered the most serious damage, resulting in wilting and death. In conclusion, controlled atmospheres of $CO_2$ can kill TSSM, particularly at high concentrations and with long treatment times. It can be used to control TSSM on strawberry, but should be used cautiously on other host plants.

**Keywords** Carbon dioxide · Controlled atmospheres · *Tetranychus urticae* · Pests control · Safety evaluation

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## Introduction

The two-spotted spider mite (TSSM), *Tetranychus urticae* Koch (Acari: Tetranychidae), is a highly polyphagous pest, causing serious damage to multiple vegetables, ornamentals and fruit trees (Castilho et al. 2015; Sun et al. 2012; Van Leeuwen et al. 2013). Management of TSSM is one of the major challenges for many crops, including strawberry production (Gerson and Weintraub 2012; Gonzalez-Dominguez et al. 2015; Nyoike and Liburd 2013). The control of TSSM largely relies on acaricides (Van Leeuwen et al. 2010), but their extensive use has led to resistance of TSSM to almost all types of acaricides and limited the efficacy of chemical control (Bi et al. 2016; Khajehali et al. 2011; Kwon et al. 2015; Van Leeuwen et al. 2008, 2010; Van Nieuwenhuyse et al. 2009). For instance, TSSM developed 10,000-fold field resistance to a newly developed acaricide, bifenazate, over a short time interval (Van Leeuwen et al. 2008). Development of new acaricides is required. Biological control using predatory mites and microorganisms has proven to be useful in the management of TSSM (Castilho et al. 2015; Gigon et al. 2016; Howell and Daugovish 2013; Wu et al. 2016). To reduce the cost and efficacy of biological control, combinations of natural enemies and compatible acaricides have been recommended (Abraham et al. 2013; Rhodes and Liburd 2006). Other control methods have also been developed, such as the use of resistant strain of crops (Costa et al. 2017; Gonzalez-Dominguez et al. 2015), intercropping garlic plant with strawberry (Hata et al. 2016), ultraviolet-B (Koveos et al. 2017; Tanaka et al. 2016) and regulation of humidity (Suzuki et al. 2012).

Outbreaks of TSSM have usually been caused by a failure to completely remove TSSM in host plant transport, and a delay in implementing control measurements after planting (Jeppson et al. 1975). In the case of strawberries, TSSMs have had a high likelihood of being transferred to seedlings due to transfer during propagation from stock plants. Although acaricides are used on seedlings before planting, TSSM eggs persist because they are not affected much by acaricides (Gong et al. 2013). Methods that can remove all stages of TSSM from seedlings should help reduce the frequency of outbreaks of TSSM after planting.

Atmospheric control can serve as an alternative and environmentally friendly method to control pests of agricultural commodities (Miyata et al. 2016; Seki and Murai 2011, 2012a, b; Wong-Corral et al. 2013). Control is achieved by maintaining high carbon dioxide ($CO_2$) and low oxygen levels, placing the pest under the stresses of hypercarbia and hypoxia (Donahaye 2000; Seki and Murai 2011). Treatments using high levels of $CO_2$ are widely applied for control of grain and stored product pests (Banks and Annis 1977; Carli et al. 2010; Fields and White 2002; Hashem et al. 2012; Liu 2003; Seki and Murai 2012b; Wong-Corral et al. 2013). However, little is known about whether they might also be applied to control pests on seedlings (Miyata et al. 2016; Oyamada and Murai 2013; Seki and Murai 2012a, b). In this study, we therefore evaluated the effect of controlled atmospheres (CAs) involving $CO_2$ on TSSM, and we evaluated potential damage to seedlings of five host plants of TSSM.

## Materials and methods

### Source population

The TSSMs used in this study were previously collected from strawberry in a greenhouse in Daxing District, Beijing, China. The stock colonies were reared in a phytotron for 2–3 generations at 25 ± 2 °C, with 55 ± 5% relative humidity and a 16:8 h light-dark photoperiod.

### Host plants of TSSM

Seedlings of five host plants (strawberry, eggplant, green pepper, cucumber and rape) were used for evaluating the impact of CAs on plants under different concentrations and treatment times (Table 1). All plants were grown in a greenhouse under normal atmospheric conditions prior to laboratory tests.

### General settings of the experiments

All tests were conducted in insect rearing cages (l × w × h = 45 × 45 × 0 cm) at 25 ± 2 °C, 55 ± 5% RH and 16L:8D photoperiod. The cages were sealed using plastic sheets to form airtight spaces.

We used $CO_2$ ice as the source of carbon dioxide. The mass of $CO_2$ ice needed in each treatment was calculated based on levels of $CO_2$, the volume of the cage (101.25 L), molar mass (44 g/mol) and molar volume of $CO_2$ (22.4 L/mol). Thus, 9.77, 99.44, 198.88 or 298.33 g of $CO_2$ ice were used to generate concentrations of 16.7, 33.3, 50 or 66.7% of $CO_2$, respectively. Four replicates were included in each treatment. Controls involved the normal atmospheric concentration of $CO_2$. Temperature in the cage (25.1 ± 0.7, 25.7 ± 1.3, 25.6 ± 1.1 and 25.1 ± 0.8 °C in each treatment concentrations of 16.7, 33.3, 50 or 66.7% of $CO_2$, respectively) was monitored using a thermo recorder (RS-14; Espec MIC, Omido, Japan). The $CO_2$ concentration was confirmed using a combustible gas detector (XP-3140; New Cosmos Electric, Osaka, Japan). The monitored concentration at initial time was 17.0, 34.0 and 50.3% in each of the concentration, respectively. After 24 h, the concentration of $CO_2$ was dropped by 10, 11.2 and 13.9% in treatment of 16.7, 33.3, 50 and 66.7%, respectively.

**Table 1** Plants used for safety evaluation of carbon dioxide

| Species | Cultivar | Development stage (number of leaves) |
|---|---|---|
| Strawberry (*Fragaria x ananassa*) | Hongyan | 5–6 |
| Eggplant (*Solanum melongena*) | Jingqie no. 6 | 4–5 |
| Green pepper (*Capsicum annuum*) | Jingtian no. 3 | 7–8 |
| Cucumber (*Cucumis sativus*) | Lvxiu | 4–5 |
| Rape (*Brassica campestris*) | Lvxia | 6–7 |

### Hatching rate of eggs of TSSM

Double-side adhesive tape was cut into 2×2 cm squares which was pasted on a glass slide. One hundred and twenty eggs (obtained 48 h after laying) were pasted on the tapes, and then moved into insect rearing cages along the inner edges of the cages. $CO_2$ ice was placed at the center of a cage, which was then sealed. Eggs were exposed to the different levels of $CO_2$ for 24 h at 25±2 °C. After treatment, eggs were placed into Petri dishes covered with cling film to maintain humidity and held at 25±2 °C under a 16L:8D photoperiod. The hatching rate of eggs was scored after 10 days.

### Survival rate of mobile stages of TSSM

Strawberries were manually infested with TSSM. After 7 days, each strawberry seedling was placed along the edge of a cage where 177–784 TSSM were then distributed evenly. $CO_2$ ice was placed at the center of a cage, and the cage sealed. After 24 h of exposure, the mortality of mites was investigated on days 1, 3, 7 and 14. Based on results from the above experiments, two levels of $CO_2$ (33.3 and 50%) were used to investigate the effect of exposure times (4, 8 and 16 h) on mortality of TSSM.

### Safety evaluation of carbon dioxide to seedlings of plants

Five seedlings of strawberry, eggplant, green pepper, cucumber and rape were placed into a cage at about 40 cm spacing, and exposed to four levels of $CO_2$ for 24 h. Leaf damage was evaluated on day 1, 7 and 10 with five damage levels recognized:

Level 0 (−): no damage observed.
Level 1 (+): less than 10% of leaf showing a slight discoloration or recoverable in 3–4 days, suggesting little impact.
Level 2 (++): 11–20% of leaf showing a clear discoloration or recoverable in 7–10 days, indicating slight damage to plants.
Level 3 (+++): 21–50% of leaf showing a clear discoloration or unrecoverable in 10 days, indicating moderate damage to plants.
Level 4 (++++): more than 50% of leaf showing a clear discoloration or unrecoverable, indicating severe damage to plants.

### Statistical analysis

The survival rate of mites under different treatments was compared using Kruskal–Wallis tests implemented in the R package stats, followed by Dunn's test in the R package dunn.test (R Core Team 2017). In the two-way design, rank-based tests were conducted using the raov function from the package Rfit (Kloke and McKean 2012) to test the effect of concentrations, exposure duration and their interaction.

## Results

### Hatching rate of TSSM eggs

The hatching rate of eggs was significantly different among treatments (Kruskal Wallis test: $\chi^2 = 17.78$, $df = 4$, $p < 0.001$). The hatching rates were 37.7, 5.4 or 76.2% under concentrations of 16.7, 33.3 or 0% (control) of $CO_2$. Zero eggs hatched after fumigation with 50 or 66.7% $CO_2$, treatments which differed from the control and 16.7% $CO_2$ treatments ($p < 0.05$, Table 2).

### Mortality of mobile stages of TSSM

After exposure to various levels of $CO_2$ for 24 h, the survival rate of mobile stages of TSSM decreased as the concentration of $CO_2$ increased, with a significant difference among treatments (Kruskal Wallis test: $\chi^2 = 18.64$, $df = 4$, $p < 0.001$). The survival rate of mobile stages of TSSM under 16.7% $CO_2$ was 46.4, 27.4, 22.7 and 31.6% on day 1, 3, 7 and 14, respectively. All mobile stages of TSSM died under the three high concentrations of $CO_2$ on day 1, 3, 7 and 14 (Table 2).

We tested the effect of exposure duration under two levels of $CO_2$ (33.3 and 50%) that can lead to 100% control efficacy after 24 h exposure. In most cases, the survival rate decreased as exposure duration increased (Table 3), with a difference among exposure duration treatments after 14 days (Kruskal Wallis test: $\chi^2 = 12.71–14.50$, $df = 3$, $p < 0.001$). After exposure for 4 h, the survival rate of mobile stages of TSSM under 33.3% $CO_2$ was 45.3, 46.7, 45.4 and 83.0% on day 1, 3, 7 and 14, respectively, whereas the survival rate under 50% $CO_2$ was 36.0, 37.3, 46.7 and 86.0%, respectively (Table 3). The effect of exposure duration on survival was significant across 14 days ($F = 27.824$, $df = 2$, $p < 0.001$), whereas the effect of concentration was not significant after day 7 ($F = 0.407–0.747$, $df = 1$, $p = 0.40–0.51$).

### Safety to plants

Damage was observed on cucumber, eggplant, rape and green pepper after 24 h of exposure, and was aggravated with a rising concentration of $CO_2$. A concentration of 50% or higher caused a damage level up to 4 (++++) in all plants except for strawberry (Table 4, Fig. 1). Cucumber was the most sensitive plant to $CO_2$, given that the lowest concentration (16.7%) caused level 4 damage at day 7. The $CO_2$ treatment caused less damage on eggplant, followed by rape and green pepper. Almost all of concentrations did not cause obvious damage to strawberry seedlings. The highest concentration of $CO_2$ caused minor wilting on strawberry that recovered over a short time.

## Discussion

Controlled atmosphere (CA) treatments are efficient and environmentally friendly methods to control post-harvest pests. In this study, we investigated the effect of this

**Table 2** Mean (range in parentheses; n = 4) survival rate (%) of eggs and mobile stages of *Tetranychus urticae* after 24 h exposure to different carbon dioxide concentrations

| $CO_2$ (%) | Number | Eggs | Mobile stages of *T. urticae* | | | |
|---|---|---|---|---|---|---|
| | | | Day 1 | Day 3 | Day 7 | Day 14 |
| Control | 784 | 76.15 (63.33–83.33)a | 94.92 (92.57–97.61)a | 85.51 (70.21–95.83)a | 61.42 (49.66–69.5)a | 88.37 (71.62–102.84)a |
| 16.7 | 205 | 37.69 (26.67–50.0)ab | 46.38 (27.08–61.9)a | 27.39 (14.58–44.19)a | 22.7 (16.28–31.94)a | 31.58 (11.63–45.24)a |
| 33.3 | 290 | 5.38 (0.0–10.0)bc | 0.0b | 0.0b | 0.0b | 0.0b |
| 50.0 | 177 | 0.0c | 0.0b | 0.0b | 0.0b | 0.0b |
| 66.7 | 740 | 0.0c | 0.0b | 0.0b | 0.0b | 0.0b |

Means within a column followed by different letters are significantly different (Dunn's test, $p < 0.05$)

**Table 3** Effect of exposure duration of carbon dioxide on mean (range in parentheses; n = 4) survival (%) of mobile stages of *Tetranychus urticae*

| $CO_2$ (%) | Duration (h) | Day 1 | Day 3 | Day 7 | Day 14 |
|---|---|---|---|---|---|
| – | Control | 101.94 (94.29–106.92)a | 99.32 (88.48–111.54)a | 112.57 (98.18–137.69)a | 120.96 (96.97–156.15)a |
| 33.3 | 4 | 45.26 (23.71–55.81)a | 46.65 (23.71–57.27)a | 45.35 (21.65–58.18)ab | 82.97 (69.19–91.87)ab |
| | 8 | 0b | 0.13 (0–0.52)b | 14.51 (7.72–28.68)bc | 46.38 (40.44–52.41)bc |
| | 16 | 0b | 0.22 (0–0.51)b | 3.36 (0–6.06)c | 2.53 (0.85–4.53)c |
| – | Control | 101.94 (94.29–106.92)a | 99.32 (88.48–111.54)a | 112.57 (98.18–137.69)a | 120.96 (96.97–156.15)a |
| 50.0 | 4 | 36.01 (25.66–49.24)a | 37.29 (26.55–46.21)a | 46.71 (35.4–61.36)a | 86 (59.09–105.52)ab |
| | 8 | 0b | 0b | 5.59 (1.24–13.29)b | 26.79 (15.41–36.1)bc |
| | 16 | 0b | 0.18 (0–0.7)b | 7.75 (0.36–15.05)b | 8.6 (1.43–14.08)c |

Pairwise comparisons were conducted among treatments under the same $CO_2$ concentration. Means within a column followed by different letters are significantly different (Dunn's test, $p < 0.05$)

**Table 4** Damage degree of plants after exposure to four levels of carbon dioxide

| Plant | Concentration of $CO_2$ (%) | Day 1 | Day 3 | Day 7 |
|---|---|---|---|---|
| Cucumber | 16.7 | +++ | ++++ | ++++ |
| | 33.3 | ++++ | ++++ | ++++ |
| | 50.0 | ++++ | ++++ | ++++ |
| | 66.7 | ++++ | ++++ | ++++ |
| Eggplant | 16.7 | + | ++ | ++ |
| | 33.3 | +++ | +++ | ++ |
| | 50.0 | ++++ | ++++ | ++++ |
| | 66.7 | ++++ | ++++ | ++++ |
| Rape | 16.7 | + | + | + |
| | 33.3 | ++ | ++ | ++ |
| | 50.0 | ++++ | ++++ | ++++ |
| | 66.7 | ++++ | ++++ | ++++ |
| Green pepper | 16.7 | + | + | + |
| | 33.3 | ++ | + | + |
| | 50.0 | ++++ | ++++ | ++++ |
| | 66.7 | ++++ | ++++ | ++++ |
| Strawberry | 16.7 | – | – | – |
| | 33.3 | – | – | – |
| | 50.0 | – | – | – |
| | 66.7 | + | – | – |

Damage levels are defined in text


**Fig. 1** Damage of cucumber (**a**), eggplant (**b**), rape (**c**) and green pepper (**d**) caused by 50% concentration of carbon dioxide after 24 h exposure

technology on TSSM and seedlings of its host plants. We found that the mortality of TSSM and damage level of plants increased with concentrations and exposure times of $CO_2$.

Treatment using high concentration $CO_2$ was tested in another spider mite species, pacific spider mites, *Tetranychus pacificus* (Zhou and Mitcham 1998). Sequential treatment with 65–90% $CO_2$ followed by 8 or 20% $CO_2$ at 0 °C caused complete mortality of all stages of *T. pacificus*; these treatment combinations show potential for use as quarantine treatments, which need Probit 9 (99.9968%) mortality of a pest (Zhou and Mitcham 1998). Studies using controlled atmosphere for quarantine control were conducted in other species, such as the leafroller *Platynota stultana*, western flower thrips

*Frankliniella occidentalis*, and green peach aphids *Myzus persicae* (Mitcham et al. 1997; Shelton et al. 1996). Some studies tested the control efficacy of controlled atmosphere on mortality of pests at high temperature (Miyata et al. 2016; Whiting and van Den Heuvel 1995). We aimed at the development of a novel method in control of TSSM on seedlings of crops. Therefore, relatively low concentrations were tested under room temperature of 25 °C in our study.

During the CA treatments, both the TSSM and agricultural commodities were exposed to high $CO_2$ and low oxygen, resulting in hypercarbia and hypoxia. The tolerance of hypoxic condition varies among stages of TSSM and among plants. Generally, metabolically active stages or organs are more sensitive to hypoxia than inactive stages or organs (Wong-Corral et al. 2013). In this study, CA treatments of 33.3% $CO_2$ with 24 h of exposure was sufficient to kill 100% of TSSM adults, whereas the eggs still maintained a 5.4% hatch rate under the same condition. This indicates that TSSM adults are more sensitive than eggs to high concentration of $CO_2$, as found in thrips (Seki and Murai 2012a, b). This can explain the resurgence of TSSM after a CA treatment of 33.3 or 50% $CO_2$ for 8 and 16 h exposure (Table 3): a number of eggs survived and hatched leading to the recovery of the population. However, a higher concentration (> 50%) caused complete mortality of both eggs and adults, which was consistent with Held et al. (2001).

Factors that may influence the response of plants to CA treatments include atmosphere composition, exposure duration, temperature and relative humidity. Held et al. (2001) found that a high level of $CO_2$ (> 99%) caused severe damage to several bedding plants. In this study, slight or moderate damage was observed on most vegetable seedlings after CA treatments with low levels of $CO_2$ (16.7%), whereas control efficacy was also low under these conditions. However, iceberg lettuce showed only minor damage after 4 days of exposure to 6% $CO_2$ at 10 °C (Liu 2003) even though mites were controlled. Thus, further studies on other interacting factors, including temperature and relative humidity, may help identify conditions that decrease damage of living plants but increase control efficacy.

The strawberry seedlings exhibited little damage even under the highest concentration of $CO_2$ used in our study (66.7%). A 24-h exposure period to 60% $CO_2$ has previously been shown to have no adverse effects on the primary flower clusters of strawberry (Oyamada and Murai 2013). Strawberry plants may therefore be relatively resistant to $CO_2$ treatments. However, the safety of high concentrations of $CO_2$ to other cultivars of strawberry under field conditions needs to be evaluated before the approach can be adopted widely. Large-scale trials are also needed to explore the feasibility and cost of CA treatments for producing TSSM-free seedlings of strawberry when compared to currently-used approaches for TSSM management.

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