The effect of postharvest carbon dioxide and cold quarantine treatment on *Tuta absoluta* mortality and tomato fruit quality

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ABSTRACT

Tuta absoluta is an invasive pest species that affects tomato and other solanaceous crops and is found in Europe and other Mediterranean areas. Hitherto, fumigation with methyl bromide is the only measure used to control this pest during the postharvest period. However, according to the Montreal Protocol on substances that deplete the ozone layer, methyl bromide should be replaced by alternatives if they are economically and technically feasible. Therefore, the objective of this study was to determine the ability of *T. absoluta* to complete its preimaginal development on tomato fruit during the postharvest period and evaluate the effectiveness of different high carbon dioxide (CO_2) atmospheres and cold storage treatments on *T. absoluta* control. *T. absoluta* was unable to complete its development from egg to adult in three tomato varieties. In contrast, *T. absoluta* completed its preimaginal development when more mature larvae were provided with tomato fruit only. The exposure of *T. absoluta* to a modified atmosphere of 95% CO_2 at 25 ºC for 48 h was effective for the control of all its developmental stages. An increase in exposure time to 72 h was necessary to obtain the same level of control at 40% CO_2. Cold storage at 1 ºC for a total of 10 days was also effective for the control of *T. absoluta* eggs. These treatments did not negatively affect fruit quality. Therefore, they are feasible alternatives to methyl bromide for postharvest tomato treatment.

Keywords

Tomato leaf miner, invasive pests, fumigation, methyl bromide alternatives, modified atmospheres, cold quarantine treatments

1 Introduction

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a pest species of solanaceus crops and is native to South America where it is widely distributed. It was first detected in Spain at the end of 2006 and from there it rapidly spread to Mediterranean areas, Europe, Africa and the Middle East (Desneux et al., 2010). Initially it was assumed that low temperatures associated with winters in Western Europe prevented this species from overwintering. However, recent studies indicated that *T. absoluta* was likely to overwinter successfully between two successive tomato crops in commercial greenhouses in this area (Van Damme et al., 2015). The main host for *T. absoluta* is tomato (*Solanum lycopersicum* L.). This moth species lays eggs in all aboveground plant organs (leaves, shoots and flowers) including the fruit (Desneux et al., 2010). On leaves, larvae feed between the epidermal layers causing irregular galleries that after some days may become necrotic. Larval feeding causes galleries on fruit that can be infected by secondary pathogens leading to decay. Pupation can occur in the soil, on leaf surfaces, or within mines (OEPP/EPPO, 2005). Depending on environmental conditions, the moth can complete its life cycle in 29–38 days and there may be 10–12 generations per year. An adult female lays about 260 eggs during its lifetime (Desneux et al., 2010).

This pest is still absent from countries such as the United States of America (USA), Canada, Japan, Australia, and New Zealand (CABI, 2015). For this reason, shipments of tomatoes from countries with *T. absoluta* must meet import requirements. For example, USA requirements include a phytosanitary certificate of inspection issued by the national plant protection organization of the origin country and a declaration that tomato fruits in the shipment originate from an area recognized as free of *T. absoluta* or that tomatoes have been produced in accordance with an APHIS approved systems

approach, which ensures that that tomatoes are free of *T. absoluta* (APHIS-USDA, 2011).

Among treatments accepted for quarantine measures, fumigation with methyl bromide is effective for the control of a number of pests present in fresh fruits and vegetables after harvest. It has been proposed for the fumigation of tomatoes that are produced in *T. absoluta*-infested areas (APHIS-PPQ, 2015). However, methyl bromide is considered a significant ozone depleting substance and has already been replaced by alternatives for a number of applications including soil, structures and commodities fumigations (UNEP, 2014). Cold treatments offer a commercially feasible alternative to chemical fumigation for quarantine disinfestation of perishable fruit products (Gould, 1994). Temperatures at or slightly above 0°C are effective for controlling a variety of insects, especially fruit flies, with minimal impact on commodity quality (Benschoter, 1984; Willink et al., 2006; de Lima et al., 2007). In addition, controlled atmospheres (CAs) and modified atmospheres (MAs) based on high carbon dioxide (CO_2) and/or reduced oxygen (O_2) offer a safe and environmentally friendly alternative to fumigation for arthropod pest control in perishable commodities (Ke and Kader, 1992). Data on the effects of different types of CA/MA treatments and dosages on key pests are available for many species and stages under specific conditions and for different commodities (Mitcham et al., 2003; Neven, 2008). Furthermore, CA methods are effective alternatives or complements to other disinfestation methods, including cold treatments (Neven and Rehfield-Ray, 2006; Follett and Neven, 2006; Palou et al., 2007). At the same time, storage under CAs/MAs is an ideal preservation technique for controlling product deterioration (Mangaraj and Goswami, 2009) and combined with low temperature can slow down ripening and senescence (Ahvenainen, 1996; Jacxsens et al., 1999).

The objectives of this study were to 1.) Determine the risk of spread of *T. absoluta* by assessing its ability to complete preimaginal development using only tomato fruit and 2) Evaluate the effectiveness of high CO_2 atmospheres, 3) cold storage and 4) CO_2 then cold treatments on *T. absoluta* control.

2 Materials and methods

2.1 *Insect rearing*

Original field populations of *T. absoluta* used in the experiments were collected in 2010 in the coastal area of Barcelona (Catalonia, Spain) and Castelló de la Plana (Valencia, Spain), and reared in climatic chambers inside ventilated cages (120 × 70 × 125 cm) with tomato plants at 25 ± 2 ºC, 70 ± 10% relative humidity (RH) and 16:8 (Light (h):Dark (h), L:D) photoperiod. Weekly, groups of six tomato plants, measuring about 30 cm in height, were placed inside the cages for feeding and oviposition. Plants were left undisturbed for five weeks; a period long enough to enable moths to emerge from the plants so artificial re-infestation was unnecessary (González-Cabrera et al., 2011). When needed, eggs were taken directly for experimentsor gently transferred using a fine paintbrush to young tomato plants located in similar cages at the same environmental conditions where, after the appropriate time period, larvae, pupae, or adults were collected and used in experiments.

2.2 Development of *Tuta absoluta* in tomato fruit

T. absoluta eggs and larvae were used to determine their capacity to develop to adulthood in different commercial varieties of tomato fruits. For the egg bioassay, five 24-h-old eggs were gently placed on tomato fruit (cvs. Pitenza, Daniela and Syrem) near

the peduncle using a fine paintbrush. Each tomato fruit (or a bunch of five tomato fruits in the case of cv. Syrem) was isolated in a ventilated plastic cage and kept in a climatic chamber at 25 ± 2 ºC, 70 ± 10% RH and 16:8 L:D. The control treatment consisted of small tomato plants (cv. Bodar) of three to five leaves isolated in ventilated cylindrical cages with eggs of *T. absoluta* placed on the upper side of the largest leaf. The number of eggs hatched on each tomato fruit or plant was assessed periodically using a magnifying glass over the course of 7 days and the number of adults emerged per replicate was recorded at the end of the experiment after 25 days. Twenty replicates were conducted for each treatment. Likewise, for the larvae bioassay, three second or third instar were gently placed on the surface of tomato fruit (cv. Pitenza) using a fine paintbrush. Larvae were placed on the upper side of small tomato plants (cv. Bodar) in control treatments. The number of adults that emerged per replicate was recorded at the end of the experiment after 17 days. 20 replicates were conducted for each treatment.

2.3 Effect of CO_2 treatments on *Tuta absoluta* mortality

Two different methods were used to assess the effect of CO_2 on *T. absoluta* mortality. The first method consisted of treating *T. absoluta*-infested tomatoes to a high CO_2 atmosphere (98%) at 25 ºC for short exposure times (10 and 20 h). The second method consisted of two CO_2 concentrations (95 and 40%) at two different temperatures (25 and 19 ºC) at longer exposure times (24, 48 and 72 h).

For the first approach, *T. absoluta* eggs, larvae (third to fourth instar), pupae, or adults were exposed to CO_2 in chambers that consisted of hermetic Perspex cabinets (82 × 62 × 87 cm), fitted with inlet and outlet ports through which CO_2 passed at a rate adjusted to yield a concentration of 98% (v/v) inside the cabinet. Gas was allowed to

escape from the outlet port through a bubble tube to maintain the proper gas mixture in the chamber. The desired gas concentrations were regularly reached 25–30 min after closing the door of the cabinets. CO_2 and O_2 levels, temperature, and RH were continuously monitored using a Control-Tec® system (Tecnidex S.A., Paterna, Spain). Cabinets were installed inside a 40-m^3 standard storage room that was also set to the experimental temperature of 25 ºC. The following CO_2 treatments were applied: i) air atmosphere at 25 ± 1 ºC (control, samples located in the standard cold room), ii) atmosphere containing 98% CO_2 in air at 25 ± 1 ºC for 10 h, and iii) atmosphere containing 98% CO_2 in air at 25 ± 1 ºC for 20 h. In all cases, the RH during the exposure period was 85 ± 5%. Treatments were applied to eggs from stock colonies that were individually transferred to tomato leaflets (five eggs per leaflet). Each replicate consisted of two leaflets (10 eggs) deposited on a layer of agar (2% wt, adaxially) in 90-mm diameter Petri dishes. Five Petri dishes (50 eggs) were used per treatment. In the case of larvae, the treatments were applied to artificially infested tomato fruit. Third to fourth instars were individually obtained from young tomato plants. A plug of tomato pericarp (peel and flesh) measuring 10 mm in diameter and 20 mm deep was punctured and removed with a cork borer from the equator of each early-red tomato (cv. Daniela) and five larvae were gently introduced into the hole using a fine paintbrush. The pericarp plug that was previously removed was then returned to the fruit and sealed with warm paraffin applied with a soft paintbrush. Five tomatoes (replicates) were used per treatment (25 larvae). Pupae and adults were obtained from eggs that were transferred to individual young tomato plants. For CO_2 exposure, five pupae were deposited in a 90-mm diameter Petri dish. Similarly, 15 adults were transferred into a 140-mm diameter Petri dish where a smear of honey had been previously placed on the inner surface as a food source. Each treatment was applied to six Petri dishes (30 pupae or 90 adults).

After CO_2 exposure, Petri dishes containing eggs, pupae or adults, or tomatoes containing larvae, were removed from gas cabinets and transferred to a ventilated climatic chamber set at the same environmental conditions used for rearing (25 ± 2 ºC, 70 ± 10% RH and 16:8 L:D) and were maintained until egg hatching, larvae pupation or adult emergence from pupae. Adult survival was individually checked two days later. In each case, five control replicates were performed.

For the second approach, *T. absoluta* eggs, pupae and adults were isolated separately with a tomato leaf into a glass vial (petiole down) filled with water inside 300 × 210 mm and 59 µm-thick plastic bags (Cryovac® BB4L, Sealed Air, Elmwood Park, NJ, USA). The plastic bags were permeable to O_2 and CO_2 of 30 and 150 cm^3 m^{-2} day^{-1} bar^{-1} measured under conditions standardized at 23 ºC and 0% RH, respectively. Three different high CO_2 atmospheres were tested with exposures of 24, 48 and 72 h: (i) 40% CO_2 with a residual 3% O_2 and 57% balance N_2; (ii) 70% CO_2 with 3% O_2 and 27% balance N_2; and (iii) 95% CO_2 with 3% O_2 and 7% balance N_2. Plastic bags were filled with the desired atmosphere, which was previously prepared with a gas mixer (Witt KM 100-3M/MEM, Witt-Gasetechnik GmbH & Co. KG, Witten, Germany) using food grade gases (Freshline®S.E., Carburos Metálicos, Air Products Group, Barcelona, Spain), using a vacuum-packaging machine (Multivac A 300/16; Multivac Verpackungsmaschinen, Wolfertschwenden, Germany) (Riudavets et al., 2009). A gas analyzer (Abiss model TOM 12, Viry-Chatillon, France) was used to determine CO_2 and O_2 levels inside plastic bags at the start and at the end of the exposures. Treatments were performed in climatic chambers at 19 ± 2 ºC and 25 ± 2 ºC. After exposure to the atmospheres, plastic bags were opened to release the atmosphere and insects and leaves were kept in ventilated plastic cages in a climatic chamber at 25 ± 2 ºC, 70 ± 10% RH and 16:8 L:D up to 10 days to allow eggs and pupae to reach adulthood. When

evaluating the effect of treatments on adults, the cages were kept for 24 h after the treatment only. In all cases, the number of living adults in each cage was counted and mortality was calculated based on the initial number of specimens placed in each cage. Five replicates were prepared for each developmental stage, CO_2 level and exposure time with 10 specimens per replicate.

2.4 Effect of cold treatments on the mortality of *Tuta absoluta* eggs

For cold treatments, *T. absoluta* eggs were exposed to a temperature of 1 ºC for different time periods. Approximately 24-h-old eggs were transferred from stock colonies to tomato leaflets (five eggs per leaflet) placed in 90-mm diameter agar Petri dishes. Each dish contained two leaflets. Four replicates (40 eggs) were used per treatment. Dishes were placed in climatic cabinets located into a 40-m^3 cold storage room and exposed in the dark to 1.0 ± 0.5 ºC and 80% RH for 0 (Control), 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h. After exposure, dishes with eggs were removed from the cold room and transferred to a ventilated climatic chamber set at the same environmental conditions than those described for insect rearing where they were maintained until egg hatching. For each exposure time, control eggs (four dishes, 40 eggs) were maintained in these chambers during the exposure period.

2.5 Effect of consecutive CO_2 and cold treatments on *Tuta absoluta* egg mortality

Tests to evaluate the consecutive effect of CO_2 and cold treatments were conducted by following the general method previously described for each of these treatments. Eggs on tomato leaflets in agar Petri dishes were exposed to 98% CO_2 at 20 ºC for 24 h in

hermetic Perspex cabinets followed by exposure in cold storage chambers at a temperature of 1.0 ± 0.5 ºC and 80% RH for 0 (CO_2 stand-alone treatment), 3, 5, 7 and 10 days. After treatment application, eggs were maintained in climatic chambers under rearing conditions until egg hatching was determined. Control eggs (five dishes, 50 eggs) were maintained in these chambers during the exposure period. Therefore, six different treatments were considered and each was applied to five replicates (dishes) of 10 eggs each.

2.6 Fruit quality assessment

Intact tomato fruit (cv. Daniela) grown under the Spanish integrated production directive in commercial orchards in the Valencia area was used for the assessment of external and internal quality at harvest (initial quality; Tables 4, 5) and after each treatment. These fruit are not organic, but only field pesticides listed in the directive are used under a strict control. Fruit were harvested at two different commercial maturity stages, green and early-red, and did not receive any postharvest treatment. In the case of high CO_2 treatments, fruit quality was determined after exposure of early-red tomatoes to 0, 40, 70 and 90% CO_2 at 19 or 25 ºC for either 24 or 48 h plus a storage period of eight days at 5 ºC and an additional shelf life period of seven days at 20 ºC. In the case of cold treatments, fruit quality was determined after exposure of early-red tomatoes to 1 ºC for seven or 14 days plus a shelf life period of seven days at 20 ºC. In the case of CO_2 and cold complementary treatments, fruit quality was determined on both green and early-red tomatoes after exposure to 98% CO_2 at 20 ºC for 24 h plus either 3, 5, 7, or 10 days at 1 ºC followed by a 7-day shelf life period at 20 ºC. The following quality

attributes were determined: peel color, fruit firmness, weight loss, soluble solids concentration (SSC), acidity, and decay and physiological disorders.

2.6.1 Peel color

Peel color was measured as CIELab parameters (Commission Internationale de l'Eclairage) with a colorimeter (Minolta CR-300, Konica Minolta Business Technologies, Inc., Tokyo, Japan). Values of lightness (L*) ranged from 0 (black) to 100 (diffuse white). Negative and positive values of a* (from -60 to +60) indicated greenish and reddish tonalities, respectively. For each treatment, three measurements along the equatorial area of 30 fruit were obtained.

2.6.2 Fruit firmness

For high CO_2 experiments, the firmness of 20 fruit per treatment was determined using a penetrometer (Model PCE-PTR 200, PCE Instruments UK Ltd, Southampton, Hampshire, UK) equipped with a standard 1.0-cm^2 probe operated at a constant penetration speed. For each fruit, the penetration force in two points along the equatorial area was measured after removing approximately 2 cm^2 of peel for each measurement. For cold and combined CO_2 and cold experiments, the firmness of 20 fruit per treatment was determined using an Instron Universal Testing Machine (Model 4301, Instron Corp., Norwood, MA, USA). Each fruit was compressed between two flat surfaces that closed together at a rate of 5 mm min^{-1}. The machine gave the deformation (mm) after application of 10 N to the equatorial region of the fruit. Results were expressed as percentage deformation related to initial diameter.

2.6.3 Weight loss

For each treatment, 20 fruit were used to determine percent weight loss. Weight loss was expressed as a percentage.

2.6.4 Soluble solids concentration

For each treatment, six fruits that were previously weighed were squeezed to obtain juice that was filtered through cheesecloth. The SSC from three samples per fruit was measured using a digital refractometer (Model DR-101, Optic Ivymen System, Barcelona, Spain) and expressed as a percentage.

2.6.5 Acidity

For the high CO_2 experiments, acidity was determined as a measure of CO_2 dissolution in the fruit after treatments. Four measures of pH were determined in six different tomatoes per treatment using a pH meter (Crison Basic 20+, Crison Instruments S.A., Barcelona, Spain) and a penetration electrode (Crison 5232, Crison Instruments S.A.). For cold and combined CO_2 and cold experiments, titratable acidity (TA) was determined from a 5-ml aliquot by titration with 0.1 N NaOH with phenolphthalein indicator and results were recorded as g of citric acid per 100 ml (%). For each treatment, the juice was obtained from three samples of six fruit each.

2.6.6 Decay and physiological disorders

Fruit decay and external rind injuries potentially produced by CO_2 or cold exposure were assessed by naked eye at the end of the shelf life period on 30 randomized fruit for each treatment. Each fruit was classified into one of the three following categories according to external appearance: 1 = healthy, 2 = moderately decayed/injured, and 3 = severely decayed/injured. Results on incidence and severity of disorders were converted to a ponderate average value per treatment (1–3 scale).

2.7 Statistical analysis

Insect mortality was calculated for all experiments based on the initial number of specimens. The number of hatched eggs and the number of adults emerged per replicate was analyzed using a Kruskal-Wallis test and a post-hoc Wilcoxon test with Bonferroni correction to compare the mortality of *T. absoluta* when developing from egg or larvae to adult in fruits from the different tomato cultivars considered. For the cold quarantine treatments, reduction of egg hatching (%) for each exposure time (h) was corrected, transformed into probits and the corresponding probit line fitted (LeOra Software Inc., Petaluma, CA, USA). A chi-square test was used to test the goodness of fit. The results of mortality of *T. absoluta* and changes in fruit quality parameters due to the application of different CO_2 or complementary treatments were analyzed by analysis of variance (ANOVA) after checking the data for normality and homoscedasticity. When needed, treatment mortalities were corrected for control mortality using Abbott's formula and were arcsine transformed prior to ANOVA. Where appropriate, means were separated by Tukey test ($P<0.05$). Statistical analyses were performed with JMP 8.0.1 software (SAS Institute Inc., Cary, NC, USA).

3 Results

3.1 Development of *Tuta absoluta* in tomato fruit

T. absoluta was not able to complete its development from egg to adult in fruit from three different tomato cultivars (cvs. Syrem, Pitenza and Daniela) (Fig. 1). In comparison, over half of the specimens tested were able to develop from egg to adult when eggs were placed on tomato plants. Reduction of egg hatching was less than 18% and not different between treatments (i.e. tomato fruit from the three cultivars and tomato plants) ($\chi^2 = 1.89$; d.f. = 3; $P = 0.59$). Although there was a high mortality, some *T. absoluta* were able to develop from larva to adult when larvae were placed on the top of tomato fruit (cv. Pitenza) (Fig. 2). Mortality was also high in the tomato plants (control treatment), probably due to the high sensitivity of the larvae to handling, but lower than in tomato fruit ($\chi^2 = 4.48$; d.f. = 1; $P<0.05$).

3.2 Effect of CO_2 treatments on *Tuta absoluta* mortality

The first treatment approach consisting of short exposures (10 or 20 h) to a high CO_2 atmosphere (98%) was only effective for the control of adults and larvae (Fig. 3). In both stages mortality reached 100% after 20 h exposure and was significantly higher than that observed in the control treatments (adults: $F = 250.74$; d.f. = 2, 15; $P<0.001$; larvae: $F = 5.66$; d.f. = 2, 14; $P<0.05$). Furthermore, a significant decrease in egg hatching was observed after treatment with high CO_2 conditions. However, reduction of egg hatching only reached about 40% after 10 and 20 h of exposure ($F = 40.62$; d.f. = 2, 17; $P<0.001$). However, CO_2 treatments did not increase pupae mortality, even after an exposure period of 20 h ($F = 0.91$; d.f. = 2, 16; $P = 0.43$) (Fig. 3).

Longer exposures to CO_2 at 25 ºC (second treatment approach) also led to significant increases in mortality (Table 1). Reduced egg hatch and increased pupal mortality (i.e. the most resistant stages when treated with the first approach at 98% CO_2) were found after 48 h and 72 h when treated with 95% and with either 70 or 40% CO_2, respectively. A 24 h exposure was enough to control adults of *T. absoluta* at the lowest CO_2 content (40%) at 25 ºC. *T. absoluta* larvae were not tested due to the high sensitivity showed by this developmental stage in the first treatment approach and the high mortality attributed to handling.

Overall, in the second set of treatments a decrease in the treatment temperature to 19 ºC required an increased exposure time to obtain the same mortality for all *T. absoluta* development stages (Table 2). In adults, a 24-h exposure time was needed for 100% mortality at 95 and 70% CO_2. However, a longer exposure of 48 h was needed to control the adults at the lowest CO_2 level tested (40% at 19 ºC). For eggs, a 72-h exposure time was needed to achieve complete control irrespective of the CO_2 level, while only a lower mortality was recorded after a 48-h exposure with all three CO_2 atmospheres tested. In contrast with the treatment at 25 ºC, complete control of pupae was not possible even after 72 h of exposure in all three CO_2 atmospheres tested at 19 ºC.

3.3 Effect of cold treatments on egg mortality

The exposure of *T. absoluta* eggs to a 1 ºC cold quarantine treatment led to a general reduction in hatching as the exposure time increased (Fig. 4). The following probit line fit the data: $y = 2.58x - 5.414$ ($\chi^2 = 29.36$; d.f. = 7). The estimated time required for half the eggs to die (LT_{50}) was 125.41 h ($\chi^2 = 29.362$; 95% confidence lower and upper

limits of 74.49 h and 195.50 h, respectively), whereas the period of time at 1 ºC required to cause 100% mortality was 10 days. In the control treatment the number of eggs that fail to hatch (0 days of cold exposure) was approximately 5%.

3.4 Effect of complementary CO_2 and cold treatments on egg mortality

The combined treatment consisting of an initial exposure to a modified atmosphere of 98% CO_2 at 20 ºC for 24 h followed by cold treatment at 1 ºC significantly decreased egg hatching as the duration of cold exposure increased from 0 to 10 days (240 h) (F = 114.5; d.f. = 5, 29; P<0.0001; Fig. 5). A clear synergistic effect between exposure to CO_2 and low temperature was observed. While the number of eggs that fail to hatch in the control treatment was approximately 5% and after treatment with CO_2 alone was about 30%, it was about 70 and 90% after treatment with CO_2 followed by three and five days at 1 ºC, respectively. No significant differences were found among cold exposure periods of 5, 7 and 10 days. 100% egg mortality was only achieved with the combination of CO_2 plus 10 days of exposure at 1 ºC (Fig. 5).

By comparing these results with those obtained with cold treatments alone, the synergistic activity between complementary CO_2 and cold treatments results were even more evident. While reductions of egg hatching after 72, 120, 168, and 240 h exposure to 1 ºC were about 40, 50, 50, and 100%, respectively (Fig. 4), they were about 70, 90, 95, and 100%, respectively, when exposure was preceded by 24 h of exposure to 98% CO_2 (Fig. 5).

3.5 Effect of postharvest treatments on fruit quality

3.5.1 Quality of tomatoes exposed to high CO_2 treatments

In general, quality attributes of early-red tomatoes (cv. Daniela) were little affected when exposed to modified atmospheres with different levels of CO_2 at 19 or 25 ºC for either 24 or 48 h and subsequently stored at 5 ºC for eight days followed by a shelf life period of seven days at 20 ºC. Decreases in quality attributes were only observed in tomatoes exposed to 70 and 95% CO_2 at 25 ºC compared with tomatoes stored under the same conditions but not exposed to CO_2. For these tomatoes there was a significant decrease of L* (F = 18.2; d.f. = 7, 652; P<0.0001) and a* values (F = 29.4; d.f. = 7, 652; P<0.0001) and a decrease in firmness (F = 7.2; d.f. = 7, 291; P<0.0001) (Table 3).

Differences in weight loss were small and did not correlate with exposure times, temperatures or CO_2 concentrations. Furthermore, treatments had no impact on SSC (F = 1.38; d.f. = 7, 76; P = 0.23) and minimal or no impact on acidity (pH) (F = 5.29; d.f. = 7, 307; P<0.0001). The observed values for both parameters were within the reference values for tomatoes, thus confirming that the different CO_2 treatments did not hasten the ripening of the tomatoes.

3.5.2 Quality of tomatoes exposed to cold treatments

External and internal physico-chemical quality attributes of early-red tomatoes (cv. Daniela) were minimally affected by cold exposure. After exposure to 1 ºC for 7 or 14 days followed by a shelf life period of 7 days at 20 ºC, significant differences compared to fruit quality attributes at harvest (initial) were only observed for peel color parameters [L (F = 21.41; d.f. = 59 P= 0.0002), a (F = 40.15; d.f. = 59; P<0.0001)], SSC (F = 201.5; d.f. = 8 P<0.0001), and TA (F = 27.43; d.f. = 8 P = 0.0010) (Table 4). Peel color after 7 and 14 days at 1 ºC was slightly more intense and reddish as indicated by a significant decrease in L* value (from 45 to 41) and a significant increase in a*

value (from 9 to up to 22). Although significant, the SSC decrease after 14 days (from 5.0 to 4.6%) was not important and lacked practical impact. None of the cold treatments caused peel damage, as there were no significant differences in fruit firmness, weight loss, and incidence of decay and external physiological disorders

3.5.3 Quality of tomatoes exposed to complementary CO_2 and cold treatments

Combined CO_2 and cold treatments caused clear adverse effects on the external quality of both green and early-red tomatoes (cv. Daniela) (Table 5). Irrespective of the initial maturity stage, fruit first exposed to 98% CO_2 at 20 ºC for 24 h and then to 3, 5, 7 or 10 days at 1 ºC had a more reddish and intense peel color than at harvest after a shelf life period of 7 days at 20 ºC, especially in fruit that were exposed to low temperature for 3 or 5 days [L (F = 33.2; d.f. = 99 P<0.0001), a (F = 117.4; d.f. = 99; P<0.0001)]. Peel deformation (indicating lower fruit firmness, F = 8.98; d.f. = 99; P = 0.0002) and weight loss (F = 8.7; d.f. = 75; P<0.0001) significantly increased on treated fruit. The most important negative effect was an increase in severe injuries on the peel of fruit subjected to combined CO_2 and cold treatments. In the case of green tomatoes, the disorder ponderate index varied significantly between cold exposure periods of 3 or 5 days (values up to 1.9, moderate damage) and 7 or 10 days (values up to 2.6, close to severe damage) (F = 8.48; d.f. = 79; P<0.0001). In the case of early-red tomatoes, the injuries were severe (index of 2.6 to 2.9), irrespective of cold exposure duration (F = 43.54; d.f. = 99 P<0.0001). In general, all the fruit rated as injured (index higher than 2) showed a very high incidence of postharvest decay caused by fungi at the end of the shelf life period at 20 ºC. The diseases that were most frequently observed on treated tomatoes were blue mold, black spot and grey mold, caused by *Penicillium* spp.,

Alternaria spp. and *Botrytis cinerea,* respectively. In contrast to external quality, internal quality was not adversely affected by the combined treatments. The differences in SSC, although significant, were minimal and had no practical relevance (F = 21.7; d.f. = 14; P<0.0001).

4 Discussion

Based on our results, there is no notable risk of *T. absoluta* spread in shipments of three cultivars of tomato fruit if they are infested with eggs or very young larvae. Although eggs can hatch on the surface of tomato fruit, first instar would not be able to survive and complete their preimaginal development. In comparison, the presence of more mature larvae on/in tomato fruit presents a threat for *T. absoluta* spread during shipment. As shown in our experiment, larvae can develop to adulthood by feeding on tomato fruit, and then colonize new growing areas. Moreover, tomato shipments containing sepals, leaves, shoots, stems or other plant parts also present a chance for the pest to spread. Shipments of tomato fruit from infested areas to non-infested areas should be inspected to ensure they do not contain plant parts other than fruit. The USA has established restrictions to prevent the introduction and establishment of *T. absoluta.* For example, all tomato fruit from areas where the pest is present must be imported without vines, stems, or calyces (APHIS, 2011).

If a phytosanitary treatment is required to control this insect pest, both high CO_2 modified atmosphere and cold storage treatments can be considered alternatives to the use of methyl bromide during postharvest processing.

Our results confirm that a modified atmosphere of 98% CO_2 for 20 h at 25 ºC is an effective treatment for the control of *T. absoluta* adults and larvae. However, a minimum of 48 h would be necessary to achieve 100% egg and pupae mortality at the

same temperature with 95% CO_2. The exposure time should be increased to 72 h to obtain the same level of mortality if the content of CO_2 is reduced to 70 and 40%. The highest CO_2 levels (95 and 70%) at 25 ºC had a negative effect on tomato quality. Reducing the temperature to 19 ºC did not change the time needed to control eggs but did increase the exposure time to more than 72 h to reach 100% pupae mortality at all three CO_2 concentrations tested. At this temperature we did not find any negative effects on the quality of treated cv. Daniela tomatoes. According to a study by Ke and Kader (1992) on the use of low O_2 and/or high CO_2 atmospheres for postharvest disinfestation of fruits and vegetables, the exposure time required to achieve 100% mortality varies between insect species, developmental stages, temperature and CO_2/O_2 levels but for many pest species this time is shorter than the time it takes for detrimental effects occur in the host commodity. Tolerance of fresh horticultural produce to CO_2 atmosphere levels higher than 50% is limited to potential injuries that usually occur after 3 to 8 days of exposure. On the other hand, since the effects of CA exposure and insecticidal treatments on produce quality can vary considerably depending on species and cultivar, testing on many varieties of fruits and vegetables is important to establish tolerance to these postharvest quarantine treatments (Follett and Neven, 2006). In our case, further research should focus on the effects of CA on the quality of commercially important tomato cultivars exports other than cv. Daniela. Moreover, the cultivar is also important from the point of view of the survival and development of the pests. Results from recent research showed that life table parameters of *T. absoluta* varied significantly on different cultivars. Therefore, in severely affected areas, the commercial use of resistant tomato cultivars can be used to limit the spread of *T. absoluta* (Gharekhani and Salek-Ebrahimi, 2014).

A temperature of 1 ºC for a total of 10 days was also effective for the control of *T. absoluta* eggs. This result was consistent with recommendations for the control of other species such as *Ceratitis capitata* Wiedemann and *Anastrepha suspensa* (Loew), which require 14 days in quarantine (Hallman, 1995; De Lima, 2012). In our experiment we did not find any relevant negative effects on external and internal quality indicators at low temperature, except for a slight decrease in SSC. According to several authors, the SSC/TA ratio is a good indicator of tomato flavor. A low SSC is correlated with sweetness and TA is correlated with both sourness and sweetness (Stevens et al., 1977; Kader et al., 1978). According to Majidi (2014), the storage of green-mature harvested tomatoes at 13 ºC combined with the use of a CA (gas levels are strictly maintained at all times) and modified atmosphere packaging (gas mixture is flushed into the package once and changes with time) significantly retarded the ripening process in comparison with conventional cold storage.

Our results suggest that there is a synergistic effect on the mortality of eggs with the combined treatment of CO_2 (98% at 20 ºC for 24 h) followed by subsequent cold treatment at 1 ºC. However, a total of 10 days of cold storage was necessary to reach 100% mortality, the same exposure time as without the initial exposure to CO_2. Moreover, combined treatment led to a decrease in tomato quality. Since in the previous set of experiments, tomatoes exposed to 1 ºC for up to 14 days did not have significant changes in external and internal quality measures and organoleptic characteristics and a maximum of 10 days at 1 ºC was used for the combination of treatments, the observed severe peel damage can be mostly attributed to CO_2 exposure. It was also because of these results from cold treatment experiments that we did not consider in the design of the combination experiments the use of additional control treatments consisting of no CO_2-treated fruit exposed to different cold periods. Therefore, although combined

treatments have been proven as a proper approach for insect control, often superior to stand-alone control methods (Yahia, 2006), in this particular case, the combination of 98% CO_2 with cold storage cannot be considered a feasible alternative for the control of *T. absoluta* in tomatoes.

In conclusion, postharvest CO_2 and cold quarantine treatments can be considered alternatives to methyl bromide for the control of *T. absoluta*. However, before these treatments can be accepted for quarantine procedures, efficacy and confirmatory tests are needed. According to the International Plant Protection Convention (IPPC), probit-9 mortality of a pest is often seen as the benchmark for the efficacy of a phytosanitary treatment, this means the treatment of several thousand insects in the selected commodity with no survivors (Follett and Neven, 2006).

5 Acknowledgements

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Table 1. Reduction of egg hatching and mortality of adults and pupae of *Tuta absoluta* (%) (mean ± SEM) in sealed plastic bags exposed to different modified atmospheres (40, 70 or 95% CO_2 (balanced with N_2 and 3% O_2) for 24, 48 and 72 h at 25 ºC. Five replicates were prepared for each developmental stage, CO_2 level and exposure time with 10 specimens per replicate.

Developmental stage	% CO_2	Exposure time (h) 24	48	72
Eggs	40	28 ± 8.0bc	94 ± 4.0ab	100
Eggs	70	48 ± 6.6b	76 ± 6.8b	100
Eggs	95	90 ± 3.2a	100a	
Pupae	40	40 ± 9.5bc	98 ± 2.0a	100
Pupae	70	20 ± 3.2c	88 ± 5.8ab	100
Pupae	95	38 ± 6.6bc	100a	
Adults	40	100a		
Adults	70	100a		
Adults	95	100a		
P		<0.001	<0.01	
F		57.43	4.63	
d.f.		8; 44	5; 29	

Significant differences between developmental stages and CO_2 concentration in a given column are indicated by different letters ($P<0.05$, Tukey's test after ANOVA).

Table 2. Reduction of egg hatching and mortality of adults and pupae of *Tuta absoluta* (%) (mean ± SEM) in sealed plastic bags exposed to different modified atmospheres (40, 70 or 95% CO_2 (balanced with N_2 and 3% O_2) for 24, 48 and 72 h at 19 ºC. Five replicates were prepared for each developmental stage, CO_2 level and exposure time with 10 specimens per replicate.

Developmental stage	% CO_2	Exposure time (h) 24	48	72
Eggs	40	14 ± 5.1d	35 ± 5.1bc	100a
Eggs	70	70 ± 7.1b	74 ± 7.5b	100a
Eggs	95	50 ± 7.1bc	25 ± 3.1c	100a
Pupae	40	42 ± 4.9bcd	46 ± 2.5bc	72 ± 8.0b
Pupae	70	24 ± 11.3cd	26 ± 16.9c	70 ± 3.2b
Pupae	95	44 ± 10.3bcd	32 ± 12.0c	76 ± 5.1b
Adults	40	69 ± 4.6b	100a	
Adults	70	100a		
Adults	95	100a		
P		<0.001	<0.001	<0.001
F		27.67	16.33	22.54
d.f.		8; 44	6; 34	5; 29

Significant differences between developmental stages and CO_2 concentrations in a given column are indicated by different letters ($P<0.05$, Tukey's test after ANOVA).
Mortality in the control treatments are not shown (<20%).

Table 3. Quality attributes of early-red tomatoes cv. Daniela exposed to 0, 40, 70 or 95% CO_2 at 19 or 25 ºC for either 24 or 48 h and stored at 5 ºC for 8 days followed by a shelf life period of 7 days at 20 ºC.

Exposure temperature (ºC)	Treatment (% CO_2)	Exposure time (h)	Peel color[a]		Firmness[b]	Weight loss	SSC[c]	Acidity
			a*	L*	(kg cm^{-2})	(%)	(%)	pH
-	Initial	0	19.6	41.3	0.9	-	4.6	4.2
19	0	24	17.1abc	42,6a	0.9a	1.4c	4.4a	4.5a
		48	18.1abc	43.0a	1.0a	2.8a	4.3a	4.4b
	40	24	18.2ab	42.1ab	0.7a	1.8bc	4.3a	4.4ab
		48	16.7bc	40.8bc	0.6a	2.5ab	4.3a	4.4ab
	70	24	17.1abc	43.4a	1.9a	2.3abc	4.1a	4.5ab
		48	16.1c	40.1c	0.7a	2.8a	4.2a	4.5ab
	95	24	18.0abc	43.5a	0.9a	2.5ab	4.4a	4.4ab
		48	19.0a	39.2c	0.9a	2.9a	4.2a	4.5a
25	0	24	20.9a	40.9bc	1,2a	2,2ab	4,2a	4,4b
		48	20.1ab	41.6b	0,9bc	1,9b	4.2a	4.4b
	40	24	20.3ab	40.6cd	0.9b	2.1b	4.4a	4.4b
		48	19.3bc	41.6b	0.7cd	2.1b	4.2a	4.4b
	70	24	17.1abc	44.0a	1.0ab	2.4ab	4.1a	4.5b
		48	15.0d	39.8d	0.6d	3.1a	4.1a	4.7a
	95	24	18.0c	43.8a	0.9b	2.8ab	4.0a	4.5ab
		48	14.4d	40.9bc	0.6d	1.9b	4.3a	4.5ab

Significant differences between treatments in a given column for 19 and 25 ºC separately are indicated by different letters (P<0.05, Tukey test after ANOVA).

[a] CIELab parameters.

[b] Penetration force (PCE penetrometer).

[c] SSC = soluble solids concentration.

Table 4. Quality attributes of early-red tomatoes cv. Daniela at harvest (initial) and after cold exposure at 1 ºC for 7 or 14 days followed by a shelf life period of 7 days at 20 ºC.

	Peel color[a]		Firmness[b]	Weight loss	SSC[c]	Acidity[d]	Decay and physiological disorders
Days at 1 ºC	a*	L*	(%)	(%)	(%)	TA (%)	(1-3 scale)[e]
Initial	9.1b	45.1a	5.3a	-	5.0a	0.4a	1.0a
7	18.7a	42.2b	4.6a	1.5a	5.0a	0.4a	1.0a
14	22.3a	41.8b	5.3a	2.3a	4.6b	0.4a	1.4a

Significant differences between treatments in a given column are indicated by different letters (P<0.05, Tukey's test after ANOVA).

[a] CIELab parameters.

[b] Deformation under compression load of 10 N (Instron testing machine).

[c] SSC = soluble solids concentration.

[d] TA = titratable acidity in % citric acid.

[e] Refer to the text for scale description.

Table 5. Quality attributes of green and early-red tomatoes cv. Daniela at harvest (initial) and after exposure to complementary treatments of 98% CO_2 at 20 ºC for 24 h plus 3, 5, 7 or 10 days at 1 ºC followed by a shelf life period of 7 days at 20 ºC.

Maturity stage	Days at 1 ºC	Peel color[a]		Firmness[b]	Weight loss	SSC[c]	Acidity[d]	Decay and physiological disorders
		a*	L*	(%)	(%)	(%)	TA (%)	(1–3 scale)[e]
Green	Initial	-6.9c	50.3a	3.4b	-	4.3a	0.4a	1.0c
	3	19.6a	43.8c	4.6a	3.6b	4.0a	0.4a	1.6b
	5	18.3a	43.9c	5.4a	4.4a	3.5b	0.3a	1.9b
	7	14.3b	46.2b	5.1a	4.5a	3.8ab	0.3a	2.6a
	10	12.7b	46.3b	4.3a	4.8a	3.9ab	0.3a	2.4a
Early-red	Initial	5.8d	45.4a	3.4c	-	5.1a	0.5a	1.0b
	3	23.7d	41.5b	4.4b	2.2a	4.7a	0.5a	2.7a
	5	20.5b	41.7b	4.3b	2.7a	5.0a	0.4a	2.8a
	7	18.9c	41.6b	5.8a	2.8a	4.9a	0.4a	2.9a
	10	22.0a	40.9b	5.4a	3.0a	5.2a	0.5a	2.6a

Significant differences between treatments in a given column are indicated by different letters (P<0.05, Tukey's test after ANOVA).

[a] CIELab parameters.

[b] Deformation under compression load of 10 N (Instron testing machine).

[c] SSC = soluble solids concentration.

[d] TA = titratable acidity in % citric acid.

[e] Refer to the text for scale description.

Figure 1. Mortality (%, mean ± SEM) of *Tuta absoluta* during development from egg to adult in fruit from three different tomato cultivars (cvs. Syrem, Pitenza and Daniela) and on tomato plants (Control) at 25 ± 2 ºC, 70 ± 10% RH and 16:8 L:D photoperiod. Reduction of egg hatching and mortality during larvae stage corresponds to dotted and striped bars, respectively. 20 replicates per treatment and 5 eggs per replicate.

Figure 2. Mortality (%, mean ± SEM) of *Tuta absoluta* during development from second-third instar to adult in tomato fruit (cv. Pitenza) and on tomato plants (Control) at 25 ± 2 ºC, 70 ± 10% HR and 16:8 L:D photoperiod. Means followed by the same letter are not significantly different (Wilcoxon/Kruskal-Wallis test, $P<0.05$). 20 replicates per treatment and 3 larvae per replicate.

Figure 3. Reduction of egg hatching and mortality of larvae, pupae, and adults of *Tuta absoluta* (%, mean ± SEM) exposed to 25 ºC to an air atmosphere (Control, dotted bars) or a modified atmosphere of 98% CO_2 for 10 (grey bars) or 20 h (striped bars). Eggs were exposed on tomato leaflets in Petri dishes, pupae and adults in Petri dishes, and larvae on artificially infested tomato fruit (cv. Daniela). For each insect stage, bars with different letters are significantly different according to Tukey's test ($P<0.05$) after ANOVA.

Figure 4. Reduction of hatching (%, mean ± SEM) of eggs of *Tuta absoluta* exposed for up to 10 days to a cold temperature of 1 ± 0.5 ºC . Eggs were exposed on tomato leaflets in Petri dishes. The following probit line fitted to these data: $y= 2.58x – 5.414$ ($\chi^2 = 29.36$; d.f. = 7; LT_{50} = 125.41 h, 95% lower limit = 74.49 h, 95% upper limit = 195.50 h).

Figure 5. Reduction of egg hatching (%, mean ± SEM) of eggs of *Tuta absoluta* first exposed to 98% CO_2 at 20 ºC for 24 h (CO_2) followed by exposure to a temperature of 1 ºC for 0, 3, 5, 7, or 10 days. Eggs were exposed on tomato leaflets in Petri dishes. Bars with unlike letters are different according to Tukey's test (P<0.05) applied after ANOVA.

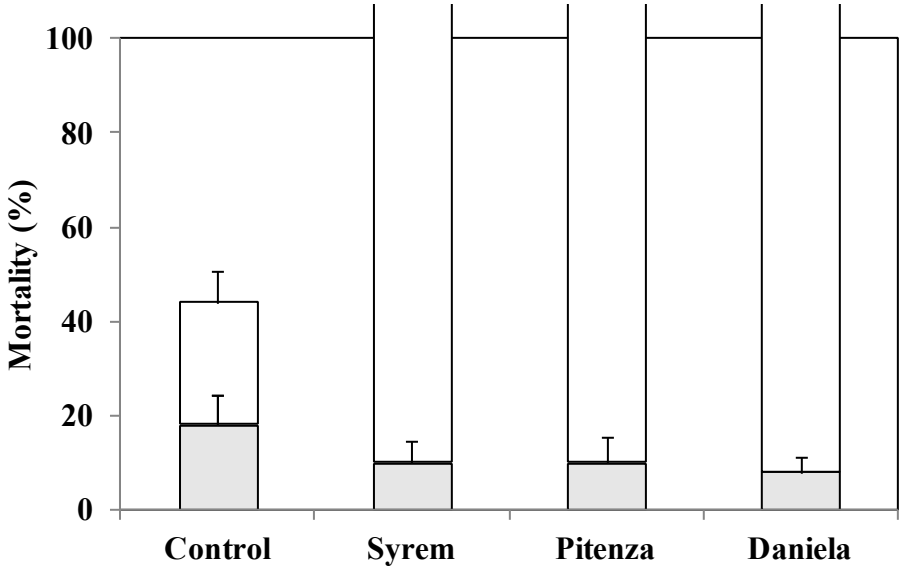

Fig. 1

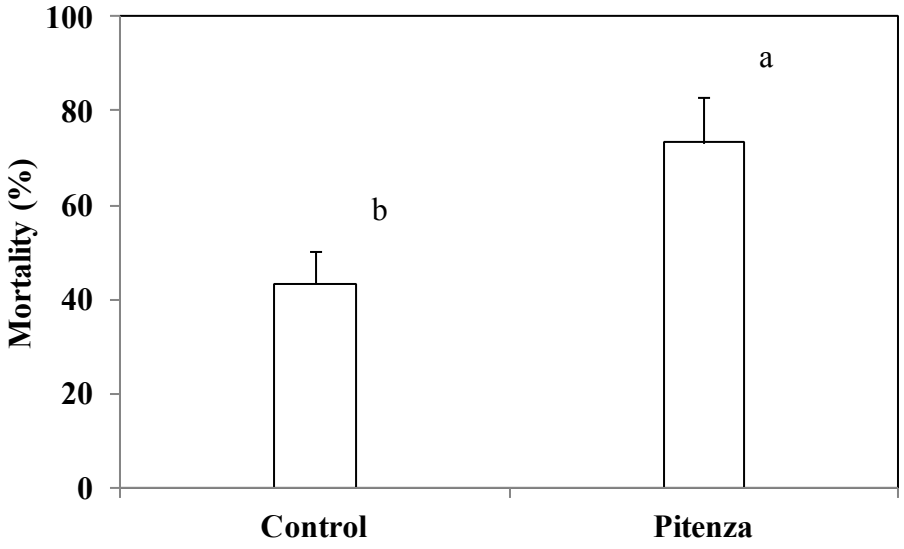

Fig. 2

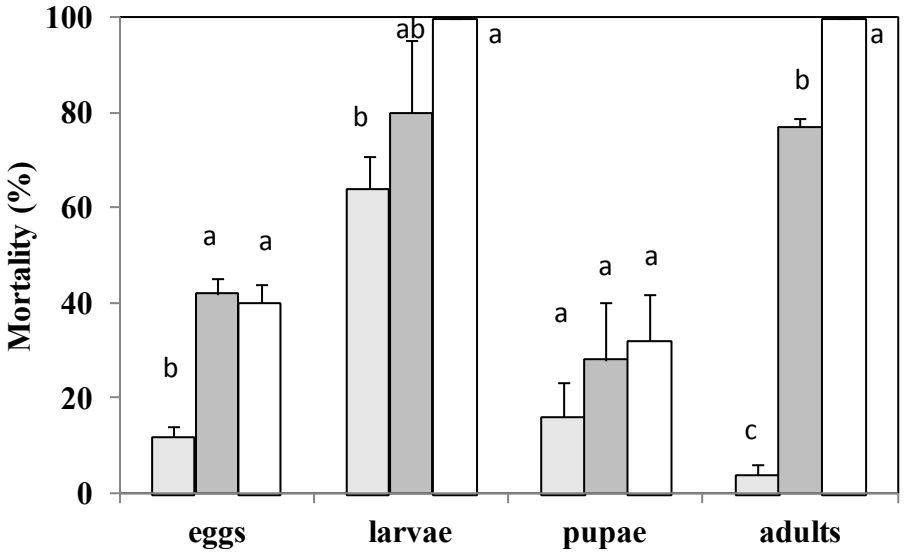
100
80
60
40
20
0
Mortality (%)
b
a
a
b
ab
a
a
a
a
c
b
a
eggs
larvae
pupae
adults

Fig. 3

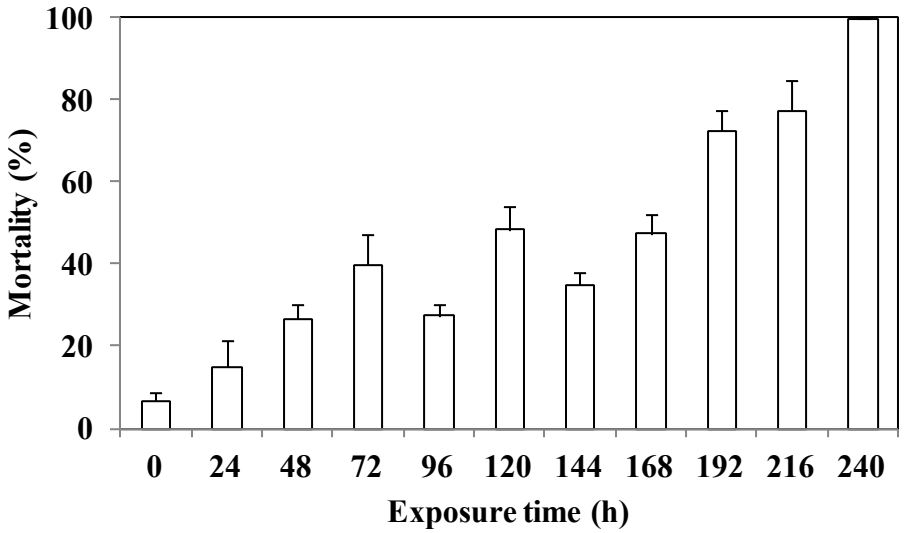
100
80
60
40
20
0
Mortality (%)
0
24
48
72
96
120
144
168
192
216
240
Exposure time (h)

Fig. 4

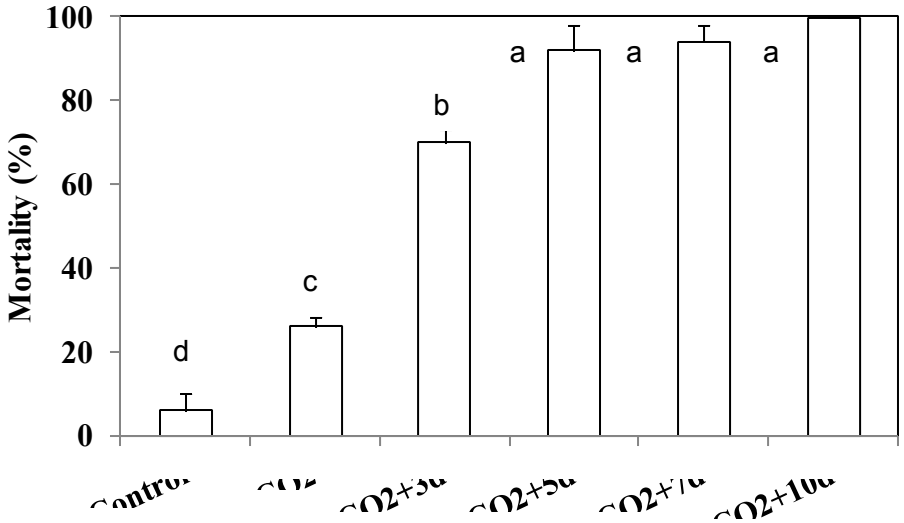

Fig. 5