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Terpenoid trans-caryophyllene inhibits weed germination and induces plant water status alteration and oxidative damage in adult Arabidopsis

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Abstract

trans-Caryophyllene (TC) is a sesquiterpene commonly found as a volatile component in many aromatic plants. Although the phytotoxic effects of TC on seedling growth are relatively explored, little information is available regarding the phytotoxicity of this sesquiterpene on weed germination or on adult plants. The phytotoxic potential of TC was assayed in vitro on weed germination and seedling growth to validate its phytotoxic potential on weed species. TC effects were assayed on the metabolism of adult Arabidopsis thaliana plants through two different methods of application, spraying and watering, in order to establish the primary affected organ and understand the unknown mobility of this compound. The results clearly indicate that TC inhibited both seed germination and root growth, as demonstrated by comparison of ED_{50} values. Moreover, although TC-sprayed adult Arabidopsis did not show any effect, TC-watered plants were strongly affected. The results suggest that root uptake was key to the effectiveness of this natural compound, and that its phytotoxicity on adult plants was mainly due to an alteration in plant water status accompanied by 17oxidative damage.

Introduction

Terpenoids are one of the largest families of plant secondary metabolites, with over 30,000 isolated compounds. This group is commonly present in higher plants, principally the Labiatae (K€ollner et al. 2004), and has mostly been studied in plants, animal cells and yeasts (Tarapore et al. 2013; Cimmino et al. 2014; Crandles et al. 2015). Constitutively produced in vegetative tissues and flowers (Flamini et al. 2003; Mulyaningsih et al.2010) or induced by stress conditions (Holopainen 2004; Niinemets et al. 2013), terpenoids are implicated in attracting pollinators and/or seed dispersers, in

direct/indirect herbivore and pathogen protection (Cheng et al. 2007; Huang et al. 2010), and in plant–plant communication (Hossaert-McKey 2013). Furthermore, monoterpenes and sesquiterpenes are known volatile constituents of essential oils, which confer a characteristic scent or taste to plants. Besides their ecological role, these compounds negatively affect metabolism of receptor plants. Several monoterpenes are known to inhibit seedling root and shoot growth, respiration and photosynthesis, decrease cell wall permeability, or interfere with hormone activity (Abrahim et al. 2000; Grana et al. 2013a). Among monoterpenes, citral was found to inhibit seed germination (Dudai et al. 2000), having herbicide potential for weeds (Gra~na et al. 2013b), together with anti-fungal (Zhou et al. 2014), antibacterial (Apolonio et al. 2014) and nematocidal (Mukherjee & SinhaBabu 2014) activities. Monoterpenes also inhibit microtubule polymerisation in plant cells(Chaimovitsh et al. 2010) through arresting cell division, causing root malformations mediated by an alteration in auxin and ethylene content in Arabidopsis thaliana (Gra~na et al.2013a). Among sesquiterpene lactones, artemisinin, dehydrozaluzanin C and isozaluzanin have been studied for their phytotoxicity (Dayan et al. 1999; Galindo et al. 1999; Mac_ıas et al.2000). In particular, artemisinin, mainly found in the Asteraceae, strongly affects root growth and cell division, and reduces photosynthesis and respiration (Dayan et al. 1999; Hussain & Reigosa 2015). Moreover, our group has demonstrated the phytotoxicity of several terpenoids isolated from the Mediterranean plant Calamintha nepeta, highlighting the importance of synergistic activity in phytotoxic responses (Araniti et al. 2013a). The strong biological activity of terpenes, together with their simple chemical structure, has increased interest in understanding their mode of action. The phytotoxic potential of terpenes and their relatively simple synthesis make them excellent candidates as templates for the production of new synthetic herbicides with new modes of action (Vaughn & Spencer 1993; Mac_ıas et al. 2000). Cinmethylin, a synthetic herbicide analogue to monoterpenoid 1,4-cineole, is the most well known example of their potential application in agriculture (Grossmann et al. 2012). trans-Caryophyllene (TC) is a bi-cyclic sesquiterpene actively produced by plants in response to herbivore damage (Kollner et al. 2008). Its synthesis is regulated by trans-caryophyllene synthase, which catalyses the conversion of farnesyldiphosphate to trans-caryophyllene (Cai et al. 2002). This secondary metabolite inhibits seedling growth of Brassica campestris and Raphanus sativus (Wang et al. 2009) and similar effects have also been reported for Achyranthes japonica (Miq.) (Kil et al. 2000). Recently, Araniti et al. (2013a) observed a strong dose-dependent effect of TC on shoot and root growth of A. thaliana seedlings. Although the phytotoxic effects of TC on seedling growth have been explored, little information is available on seed germination and adult plant responses to this sesquiterpene. Moreover, no information is available on the primary target within the plant or xylem/phloem mobility for TC. Therefore, in the present work, the phytotoxic potential of TC was assayed on the

metabolism of adult A. thaliana plants using two different methods of application, spraying or watering, in order to establish the primary affected organ and understand the mobility of this compound. Furthermore, to validate the phytotoxic potential of this molecule, in vitro bioassays on weed germination and root growth were carried out. This study will allow better understanding of the biological activity of TC and its potential use in weed management.

MATERIAL AND METHODS

Germination and root growth bioassays

Germination and root growth bioassays were carried out on four common weeds: barnyardgrass (*Echinochloa crusgalli* L. Beauv.), perennial ryegrass (*Lolium perenne* L.), redroot pigweed (*Amaranthus retroflexus* L.) and hairy crabgrass (*Digitaria sanguinalis* L. Scop.). Pre-germination treatments were applied in order to break seed dormancy and synchronize weed germination. Specifically, redroot pigweed and barnyardgrass seeds were soaked in water at 4 °C for 15 days, and hairy crabgrass seeds were placed under continuous light at 4 °C for 60 days. No pretreatments were needed for perennial ryegrass seeds. Seeds were surface sterilised with 15% (v/v) NaClO solution for 15 min and then flushed three times with distilled water. Successively, 10 seeds for each species were evenly distributed in Petri dishes (6 cm Ø) on a double layer of filter paper wetted with 2 ml of different TC solutions. Specifically, TC (Sigma Aldrich, Spain) was solubilized in EtOH (0.1%) and diluted in distilled water to the following concentrations 0 (control), 50, 100, 200, 400, 800, 1200 lM. The Petri dishes were then placed in a growth chamber at 25 °C (30 °C for redroot pigweed) and 70% relative humidity. All the experiments were carried out in dark conditions. Germinated seeds were counted at different times, depending on the tested species, and seeds were considered as germinated when the root protruded through the seed coat (1 mm). Using values from Chiapusio et al. (1997) at the end of these observations, total germination rate [GT (%)], average speed of germination (S) and speed of accumulated germination (AS) were calculated. Together with these calculations, a dose response curve was produced for the concentration of TC causing 50% (IC_{50}) and 80% (IC_{80}) inhibition of germination. To independently study the effects of TC on germination and root growth and prevent interference from the effects of TC on germination, pre-germinated seeds (five seedlings) with uniform root length were selected, placed in sterile Petri dishes and treated, as described, for 48 h (Hoagland & Williams, 2004). At the end of the experiment, images of roots, for each treatment and species, were captured with a scanner (Epson Expression 800; Regent Instruments, Quebec, Canada). Total root length (TRL) was measured using the WinRhizo Pro System software, version 2002a (Regent Instruments; Araniti et al. 2014) and a dose–response curve applied. The concentrations of TC causing 50% (IC_{50}) root growth inhibition were obtained. Experiments on adult plants

Arabidopsis thaliana L. (Heyn.) ecotype Columbia (Col-0) seeds were first sterilised by dipping in EtOH (50%) and successively in NaClO:Triton X-100 (0.5:0.01%) solution for 3 min. Then the sterilised seeds were carefully rinsed three times in sterile water and vernalised at 4 °C in 0.1% agar for 48 h in order to break dormancy and synchronize germination (Araniti et al. 2013a). Successively, seeds were sown in Petri dishes (25 cm Ø) containing agar medium (0.8%) enriched with macro- and micronutrients (Murashige–Skoog basal salt; Sigma-Aldrich) and sucrose (1%), pH 6.0. Petri dishes were then transferred into a growth chamber under 60 µlmolm-^{2}s-^{1} light, 16-h/8-h light/dark photoperiod, at 22 °C and 55% relative humidity for 15 days. After this period, uniform seedlings (30 per treatment) were individually transplanted into pots (5 cm x 6 cm, diameter x height) containing sterilised Perlite previously moistened with half-strength Hoagland nutrient solution. Perlite was used instead of soil to avoid degradation of allelochemicals, as reported previously (Mac_ıas et al. 2006). For seedling establishment, the pots were placed in a growth chamber with same growing conditions as described above for 7 days. During this time, seedlings were watered every second day with half-strength Hoagland solution (pH 6.0). TC treatment began when plants were 3 weeks old.

trans-Caryophyllene was solubilised in EtOH (0.1%) and diluted in half-strength Hoagland solution to reach final test concentrations: 0 (control), 450, 900 and 1800 µM. TC solutions were applied by watering the Perlite (25 ml_pot_1) every second day or by spraying the plants with TC solutions every day (15 ml.pot^{-1}), while the plants received Hoagland solution every second day for 21 days. Only 0.1% EtOH was included in the controls. After treatment, measurements were conducted both before and after harvesting, as indicated above.

Chlorophyll a fluorescence measurements

The chlorophyll a fluorescence emitted by plants (three per treatment) sprayed or irrigated with TC was determined as described in Gra~na et al. (2013b) with a Maxi-Imaging-PAM fluorometer (Walz, Effeltrich, Germany) every 2 days during the 21-day treatment. After each measurement of red (R) and near-infrared (NIR) emission for calculation of absorption (Abs) as 1 – R/NIR, the plants were kept in darkness (10 min). The leaves were then successively illuminated at 0.5 µlmolm^{-2}s^{-1} for measurement of F0, the minimum fluorescence of dark-adapted leaves; a saturating pulse of 2700 µlmolm^{-2}s^{-1} was used for measurement of Fm, the maximum fluorescence of dark-adapted leaves. After 5 min actinic illumination at 120 µlmolm^{-2}s^{-1} (with measurement of the corresponding fluorescence, Fs), samples received 20 s of 800-ms saturating pulses of 200 lmol_m_2_s_1 to assess Fm', maximum fluorescence of light-adapted leaves. These values were used to calculate parameters used for comparisons between treatments (Kramer et al. 2004; Klughammer & Schreiber 2008): the maximum quantum efficiency of dark-adapted photosystem II (Fv/Fm); maximum quantum

efficiency of illuminated photosystem II (Φ II); energy dissipation in the form of heat (Φ NPQ); non-regulated energy dissipation (Φ NO, fluorescence emitted); estimated electron transport rate (ETR); nonphotochemical quenching coefficient (qN); and the fraction of open photosystem II reaction centres (qL). The photosynthetic response was monitored for 5 min, and fifteen measurements were obtained for each parameter at each measuring time.

Leaf osmotic potential (_s)

The Ψs was measured on three leaves per treatment and replicate. Once harvested, leaves were inserted into an empty syringe and frozen at 20 °C. After 24 h, leaves were pressed into a syringe and, after discarding the first drop, the extract was collected and Ψs measured with a cryoscopic osmometer (Osmomat 030; Gonatec, Berlin, Germany). Leaf Ψs was expressed in MPa. Leaf area, fresh weight, dry weight and DW/FW ratio At the end of the experiment, three adult plants were collected, number of leaves counted and leaf area (LA, cm2) measured using a C1-202 area meter (CID, Vancouver, Canada). Shoots were weighed (FW) then oven-dried at 70 °C until constant weight for determination of the dry weight (DW). The DW/FW was also calculated.

Element analysis

After 21 days of treatment, dried shoots (3 mg) were analysed to determine C, H, N and S content using a Fisons Instruments EA1108 (Fisons, Ipswich, UK) elemental analyser with a detection limit of 10 ppm. The quantification of other compounds (Al^{3+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+}, K^{+}, PO_{4-} and Zn^{2+}) was carried out using inductively coupled plasma optical emission spectrometry in a Perkin Elmer Optima 4300DV Perkin Elmer, Waltham, MA, USA).

Total protein content

Protein content of A. thaliana leaves was determined according to Bradford (1976), using bovine serum albumin as standard. Protein content was expressed as micrograms per gram dry weight.

Lipid peroxidation

As described in Hodges et al. (1999), lipid peroxidation was indirectly determined by measuring the increase of malondialdehyde (MDA) in the harvested samples. An aliquot of 100 mg dried shoots was homogenised with 1 ml 80% ethanol then centrifuged at 3000 g and 4 °C for 10 min. The supernatant was incubated at 95 °C with 20% TCA containing 0.01% hydroxytoluenebutylate, with

or without 0.5% thiobarbituric acid (TBA) for 25 min. After incubation, the reaction was stopped in ice for 10 min. Subsequently, the sample was centrifuged at 3000 g at 15°C for 10 min and absorbance of the supernatant measured at 440 nm, 532 nm (specific absorbance MDA) and 600 nm (absorbance of non-specific MDA). The equivalents of MDA were calculated according to Hodges et al. (1999).

Proline content

Quantification of free proline was conducted according to Bates et al. (1973) after 21 days of treatment. An aliquot of 250 mg homogenised shoots was extracted with 5 ml 3% sulphosalicylic acid (w/v) and filtered through Whatman No 2 paper. The filtrate (2.5 ml) was reacted with 2 ml acid-ninhy-drin and 2 ml glacial acetic acid at 100 °C for 1 h; the reaction was then stopped in ice. Successively, the solution was extracted with 4 ml toluene and mixed vigorously for 20 s. The chromophore containing toluene was collected from the aqueous phase, warmed to room temperature and absorbance read at 520 nm, using toluene as blank. Proline content was determined from a standard curve and expressed as micromoles per milliliter per gram of dry weight.

Photosynthetic pigment quantification

The total chlorophyll a (Chla), chlorophyll b (Chlb) and carotenoids (Ct) was analysed and calculated according to Wellburn (1994). A total of 100 mg dried shoots per replicate and treatment was extracted with 1.5 ml methanol, and extracts centrifuged at 170 g for 5 min. Successively, 500 µl supernatant were mixed with 500 ll methanol and absorbance of extracts determined at 470, 653, 666 and 750 nm. Pigment content was expressed as micrograms per gram of dry weight and evaluated according to the equations of Wellburn (1994).

Statistical analysis

A completely randomised design was applied in all the experiments, with four replicates for germination and root growth experiments and three replicates for all the other treatments. Data were evaluated for normality with the Kolmogorov-Smirnov test, and tested for homogeneity of variances with Levene's test. The statistical significance of differences among group means was estimated by ANOVA, followed by Least Significant Difference tests (LSD) in the case of homoscedastic data, and with Tamhane's T2 test in the case of heteroscedastic data ($P < 0.05$).

Seed germination [GT (%)] and root growth (TRL) data were fitted through nonlinear regression to determine the IC_{50} index (dose causing 50% inhibition; Araniti et al. 2012). Differences between IC50 values were estimated by ANOVA followed by LSD in the case of homoscedastic data, and by Tamhane's T2 test in the case of heteroscedastic data ($P < 0.05$).

RESULTS

Germination and root growth bioassays

Measurement of germination in response to increasing doses of trans-caryophyllene suggested strong phytotoxicity of this molecule, which was able to negatively affect all germination parameters in all tested species evaluated. In particular, barnyardgrass displayed 30% inhibition with 400 μM TC, reaching 93% at 1200 μM (Table 1). Perennial ryegrass, redroot pigweed and hairy crabgrass showed 44%, 63% and 71% inhibition, respectively, at the highest concentration (1200 μM; Table 1). This difference in response was also confirmed by comparison of IC_{50} values, which provided the following hierarchy of sensitivity: barnyardgrass (680 μM) > hairy crabgrass (770 μM) > redroot pigweed (909 lM) > perennial ryegrass (not detectable) (Table 1). In terms of germination dynamics of the four weeds, both speed of germination and accumulated germination were sensitive to TC even at the lowest concentrations (50 and 100 μM). In particular, the most sensitive was barnyardgrass (50 lM) followed by hairy crabgrass (100 lM), redroot pigweed (100 lM) and perennial ryegrass (200 lM; Table 1). Root growth (total root length, TRL) was more sensitive to TC than germination, as all the tested weeds showed significant inhibition after TC treatment (Table 2). Hairy crabgrass was the most sensitive, with dose-dependent TRL inhibition and 92% inhibition at the highest TC concentration (1200 μM). Conversely, barnyardgrass was the least sensitive, with significant inhibition of 58% at 1200 lM TC. Perennial ryegrass and redroot pigweed were moderately affected by TC. Perennial ryegrass only showed a significant reduction of root growth (55% and 65%) at the stronger concentrations (800 and 1200 lM; Table 2), whereas redroot pigweed showed 50–68% inhibition when treated with concentrations from 200 μM to 1200 μM TC (Table 2). A comparison of the IC50 of TRL indicated that hairy crabgrass (214.9 μM) was the most sensitive to TC, followed by redroot pigweed (331.5 μM), perennial ryegrass (689.1 μM) and barnyardgrass (1194.1 μM; Table 2).

Experiments on adult plants

The effect of sprayed or watered TC on adult A. thaliana was evaluated over 21 days of treatment. Although sprayed plants did not show any significant effect in any parameter here considered (data not shown), TC-watered plants were strongly affected by this secondary metabolite, not only with a severe and significant reduction in growth and development, but also characteristic morphology after the treatment. TC-watered plants were smaller, had less leaves and more chlorotic areas than control plants at the end of the experiment, and the rosettes showed characteristic counter-clockwise growth (Fig. 1), with a highly consistent fixed orientation (Fig. 1) at the highest TC concentration. Plants watered with 900 and 1800 μM TC showed a significant reduction in leaf number after 9 days of

treatment (Fig. 2). The number of leaves recorded from day 9 until the end of the experiment showed a significant reduction for all the concentrations applied, being most drastic for plants watered with 1800 lM TC. This reduction was accompanied by a significant decrease in leaf area (LA) of about 17% and 73%, at 900 and 1800 μM TC, respectively, at the end of the experiment (Fig. 2). A weak but positive effect was observed on leaf osmotic potential (Ψs) at the highest concentrations (900 and 1800 μM), with significant increases of 5% and 14%, respectively, compared to controls (Table 3). Moreover, watering TC treatment caused a significant reduction compared to controls in both FW and DW of shoots (74% and 69%, respectively) at the highest concentration (1800 μM; Table 3), with a significant increase in DW/FW of about 19% compared to controls (Table 3). Watered TC plants showed a significant reduction in leaf protein content at all tested concentrations of 42–47% compared to controls (Table 3). Conversely, MDA content, as an indirect measure of lipid peroxidation, showed a strong and significant increase of 53% and 99% compared to controls with 900 and 1800 μM TC, respectively (Table 3). A similar effect was observed in free proline, which increased (3.95-fold) for the highest treatment (1800 μM; Table 3). Furthermore, TC also affected the concentration of several nutrients in watered adult Arabidopsis (Table 4). In particular, Na^{+} and Cu^{2+} content significantly increased with increasing TC concentration. Conversely, Mn^{2+} and Zn^{2+} content was negatively affected at all concentrations applied. On the other hand, Ca^{2+} and Mg^{+} fell significantly at 900 and 1800 μM TC, whereas Fe^{3+} fell significantly only at the highest concentration. P and Al^{3+} content increased significantly at the lowest concentrations, decreasing thereafter. C, N and H content was not affected (Table 4). Regarding chlorophyll a fluorescence, TC affected both photochemical and non-photochemical quenching of Arabidopsis. In particular, TC-watered plants showed a highly significant reduction in the photochemical quenching ΦII from day 17 onwards in response to the highest TC concentration (Fig. 3). Conversely, the lowest TC concentration caused a stimulation of this parameter after 21 days of treatment (Fig. 3). The ΦNPQ fell significantly even after 11 days of treatment, especially with 900 and 1800 μM TC (Fig. 3). A similar trend was served for qN and qL, coefficients of non-photochemical and photochemical quenching, respectively (Fig. 4). In particular, similarly to ΦNPQ, qN fell significantly from day 11 of treatment, which was maintained until the end of the experiment, although the sensitivity of this index was stronger than ΦNPQ for 900 μM TC-treated plants. Also, the qL coefficient was similar to ΦII, displaying a strong reduction from day 17 onwards (Fig. 4). In contrast, emission of fluorescence (ΦNO) was strongly increased for 1800 μM TC-treated plants even during the first week of treatment, and this persisted until the end of the experiment (Fig. 3). Furthermore, TC-watered plants showed a very significant reduction in maximum efficiency of PSII (Fv/Fm) at the highest TC concentration (1800 lM), from day 15 onwards, whereas the electron transport rate (ETR) fell significantly from day 17 to the end

of the experiment (Fig. 5). Finally, photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were significantly inhibited in 1800 μM TC-watered plants, while plants treated with the lowest concentration (450 μM) were not affected, and 900 μM TC induced a significant increase in chlorophyll a and carotenoids when compared to the controls (Fig. 2).

DISCUSSION

Germination and root growth

Identified in the n-hexane fraction obtained from methanolic extract of aerial parts of several wild Mediterranean species, such as Artemisia arborescens (Araniti et al. 2013c) and Calamintha nepeta (Araniti et al. 2013b,d), trans-caryophyllene has high phytotoxic potential to these species. Its phytotoxicity was also observed in seedlings of the model species A. thaliana, where, alone or in combination with other molecules, it strongly affected both root and shoot growth (Araniti et al. 2013a). However, evaluating TC phytotoxicity on weed species and understanding the mechanism of action could be important for its future use as a bioherbicide in weed control strategies. Hence, the phytotoxic effects of TC were here evaluated on seed germination and root growth of four of the most common weeds, barnyardgrass, perennial ryegrass, redroot pigweed and hairy crabgrass. As already observed for molecules and/or plant extracts with allelopathic activity (Valera-Burgos et al. 2012), TC responses were species-specific and dose-dependent. The differences in seed size, coat permeability and/or absorption mechanisms among plant species could account for this difference in sensitivity (Pellissier 2013). In particular, Usuah et al. (2013) observed that very small seeds were generally more sensitive to allelochemicals, and seed size defined the concentration needed to affect germination. McCalla & Norstard (1974) defined germination as the most sensitive vegetative stage to phytotoxins, since a short period of inhibition or stimulation strongly compromised plant ability to compete with neighbours (Loydi et al. 2015). In general, the GT (%) index showed a similar response pattern in most species, decreasing with increasing concentration, except in perennial ryegrass, which was the least sensitive to TC. Moreover, germination speed clearly fell after treatment with low concentrations of TC (50–100 μM), causing a delay in germination for almost all species examined. Grana et al. (2013b) observed similar effects on different weeds treated with citral. This phenomenon could have an important ecological role, since it might be a strategy for competition in natural and agricultural ecosystems (Gibson et al. 2002), and/or be employed for weed management, reducing the vigour of weeds during the initial stages of crop growth (Hedge & Miller 1990; Liebman & Davis 2000). Furthermore, the delay could compromise early stages of the seedlings, their survival and the subsequent reproductive phase. Several factors could be responsible for germination delay: (i)

volatilisation or degradation of molecules and consequent reduction in phytotoxic potential (Mac_ıas et al. 2008); (ii) an osmotic effect caused by high concentrations of molecules and/or extracts, as observed in alfalfa by Chon et al. (2004); (iii) a direct and/or indirect effect on key germination enzymes such as a- and b-amylase (Rice 1984); (iv) an alteration to hormones such as ABA, which is involved in seed dormancy and considered a potent inhibitor of germination (Lara-N_u~nes et al. 2015); and (v) an anti-hormone effect, as observed for several terpenes (Kruse et al. 2000). Moreover, in agreement with several authors (Chon et al. 2002; Araniti et al. 2012) and comparing germination and root length IC50 values, root growth appeared to be more sensitive to TC than germination. The high metabolic rates of rootgrowth make it highly susceptible to environmental stresses such as allelochemicals in the soil (Cruz-Ortega et al. 1998). Furthermore, TC treatment had a strong phytotoxic effect on root length of all weeds except barnyardgrass. In fact, root growth of barnyardgrass appeared to be TC-insensitive, confirming its resistance to chemical compounds (Araniti et al. 2013b). In contrast, only germination of barnyardgrass was very sensitive to TC treatment, showing the importance of independently measuring germination and root length in phytotoxin-treated seedlings. Phytotoxicity in adult plants Although there were no effects on adult plants sprayed with TC, plants watered with the higher TC concentrations showed a strong decrease in FW, DW, number of leaves and leaf area, indicating a global reduction in plant development. In TC-watered plants there was a decrease in protein content, accompanied by an increase in leaf osmotic potential, lipid peroxidation and proline content, which was five times higher than in controls. These effects were also associated with a strong alteration in several essential elements involved in plant metabolism, as also reported by S_anchez-Moreiras et al. (2011) for Arabidopsis after treatment with BOA (2-3H-benzoxazolinone). Taken together, the results here obtained suggest that TC induced an alteration in water status followed by oxidative damage, as also observed by Moran et al. (1994) for drought stress in pea plants. Gra~na et al. (2015) found similar results for A. thaliana treated with citral. This hypothesis was also supported by altered leaf morphology (spiralisation) after exposure to the highest TC concentration. Blancaflor & Hasenstein (1995) and later Shoji et al. (2006) suggested this could be due to damage to cortical microtubules caused by intracellular osmotic imbalance (Poschenrieder et al. 1989; Watanabe et al. 2000). The increase in DW/FW ratio, reduction in DW, increment in foliar Ψp, strong increase in proline and Na+ content in treated plants, known to strongly compromise cortical microtubule organisation (Shoji et al. 2006), further support this hypothesis. The strong accumulation of proline in TC-watered plants could be related to its function as an osmo-regulator synthesised in response to water stress: (i) to compensate for deficient water potential for the homoeostasis of metabolic functions (Hellmann & Estelle 2002; Bacelar et al. 2009; Farkhondeh et al. 2012); (ii) to help cells maintain structural integrity of cytoplasmic proteins under

water deficiency (Sharmila & Saradhi 2002); and/or (iii) to protect cell membranes from oxidative damage (Hellmann & Estelle 2002; Xiong & Zhu 2002; Kishor et al. 2005; Hasanuzzaman et al. 2014). Actually, changes in water status are involved in oxidative stress in TCwatered plants. The increase in malondialdehyde, an end product of lipid peroxidation and indicative of cell damage; the decrease in protein content, probably due to the strong Zn^{2+} content reduction, an essential element for protein biosynthesis (Eide 2006); and degradation of photosynthetic pigments sustain this hypothesis (Gra~na et al. 2013b, 2015). Further, several elements affected by TC could be responsible for lipid peroxidation onset. In particular, at all TC concentrations, there was a marked increase in Cu^{2+} content. Althoughm Cu^{2+} is an essential element for plants, a cellular increment in its concentration could interfere with numerous physiological processes in leaves through inhibitory effects on photosynthetic electron transport and degradation of the chloroplast inner structure and pigment content (Ciscato et al. 1997; Horvath et al. 1998; Navari-Izzo et al. 1998; Quartacci et al. 2000). This was also accompanied by a reduction in K^{+} content related to increased membrane permeability under lipid peroxidation (Janas et al. 2010), and by a reduction in Ca^{2+} content, which limits protection from degradation for membrane lipids (Schmitz-Eiberger et al. 2002). Moreover, the results are in accordance with Ercisli et al. (2005), who observed a significant reduction in Fe3+, K^{+}, Ca^{2+} and Mn^{2+} content after juglone treatment. Chlorophyll a, chlorophyll b and carotenoids increased in 900 µM TC-treated plants according to Gra~na et al. (2013a) after citral treatment. Ramel et al. (2012) demonstrated that carotenoids are strongly involved in plant defence against singlet oxygen toxicity, playing a pivotal role in protection against photoinhibition (Havaux & Kloppstech 2001). Interestingly, the increase in carotenoids in TC-treated plants may contribute to prevent photodegradation of chlorophyll a, as suggested by the high levels of Chl a and Chl b. In contrast, leaf photosynthetic pigments strongly decreased at the highest TC concentration (1800 lM), suggesting damage due to this stress. These results would support the reduction in ΦII, ETR and Fv/Fm in TC-watered plants exposed to the highest concentration. This reduction was also observed after exposure to cinnamic acid in Lolium perenne, Dactylis glomerata and Rumex acetosa leaves (Hussain & Reigosa 2011), and in Cucumis sativa (Ye et al. 2004), suggesting that the efficiency of open reaction centres was strongly affected by TC. According to Shangguan et al. (2000), altered water status in TC-watered plants could have led to a decrease in non-photochemical quenching (NPQ), which is the most efficient photoprotective response in plants, able to dissipate as heat the surplus light required in order to prevent ROS production (Inderjit & Duke 2003; Eberhard et al. 2008). In particular, after 6 days of treatment, there was a strong reduction in ΦNPQ and qN at the highest TC concentration. These results suggest that TC decreased NPQ capacity. The simultaneous

increase in ΦNO could indicate the plant's inability to dissipate excess energy through regulated mechanisms of energy emission through fluorescence. According to Demmig-Adams & Adams (1996) and Young et al. (1997), the inability of a plant to implement the processes of energy dissipation in the form of heat, at all TC concentrations tested, could be due to a reduction in carotenoid content. Demmig-Adams et al. (1990) suggested that a reduction in carotenoids could be due to inability to implement the xanthophyll cycle. Hence, the reduction in carotenoids may be the cause of the increased fluorescence emission (Choudhury & Behera 2001) and pronounced reduction in PSII efficiency due to physical damage at the level of the PSII complex antenna, with consequent reduced values of Fv/Fm in plants treated with 1800 lM TC (Sipos & Prange 1986; Georgieva 1999).

CONCLUSIONS

trans-Caryophyllene affected not only germination and root elongation of the studied invasive weeds, but also interfered with adult plant metabolism and growth, altering water and oxidation status. Moreover, the results further suggest that foliar treatment did not affect plants, while its effectiveness was through root absorption. Such knowledge may prove useful for its potential future application in weed management and/or as a backbone for the production of new herbicides.

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Table 1: Total germination index [GT (%)] and related ED_{50} values, average speed of germination (S) and speed of accumulated germination (AS) of crops and weeds exposed to different *trans*-Caryophyllene concentrations (0–1200 µM).

	Germination indexes							
	Crops				**Weeds**			
	Z. mays	***L. sativa***	***R.sativus***	***X Triticosecale***	***A. retroflexus***	***D. sanguinalis***	***E. crus-galli***	***L. perenne***
G_T (%)								
0 µM	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
50 µM	98.2^{a}	98^{ab}	100^{a}	98.2^{ab}	101.4^{a}	90.8^{ab}	97.2^{a}	100.4^{a}
100 µM	60.2^{b}	98^{ab}	90^{a}	96^{ab}	100.1^{a}	79.7^{ab}	99.1^{a}	98.8^{a}

200 µM	56.2^{b}	86^{b}	96^{a}	81.6^{b}	90.5^{a}	77^{b}	80.5^{ab}	97.6^{a}
400 µM	33.1^{c}	40^{c}	52^{b}	54.9^{c}	60.4^{b}	81.3^{ab}	74.5^{b}	97.3^{a}
800 µM	22.9^{c}	0^{d}	26^{c}	48.9^{c}	63^{b}	36.5^{c}	48.9^{c}	79.2^{b}
1200 µM	0^{d}	0^{d}	12^{c}	0^{d}	44^{c}	29.8^{c}	6.8^{d}	59.2^{c}
ED_{50}	**477.2^{c}**	**394.6^{d}**	**465.7^{c}**	**506.9^{bc}**	**909^{a}**	**770.5^{b}**	**680^{b}**	**ND**
S								
0 µM	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
50 µM	99.2^{a}	89.5^{b}	98.1^{a}	100.3^{a}	97.3^{a}	85.7^{ab}	73.8^{b}	95.7^{ab}
100 µM	66.9^{b}	86.8^{b}	63.1^{b}	82.7^{b}	89.4^{a}	77.9^{b}	74.1^{b}	94.5^{ab}
200 µM	56.2^{b}	71.6^{c}	47.4^{b}	62.4^{c}	73.6^{a}	75.6^{b}	62.9^{b}	86.7^{b}
400 µM	35.5^{c}	25.6^{d}	25.3^{c}	38.1^{d}	49.2^{b}	71.3^{b}	59.5^{b}	69.5^{c}
800 µM	18.2^{d}	0^{e}	12.4^{cd}	33.8^{d}	41.7^{b}	37.9^{c}	34.5^{c}	56.9^{d}
1200 µM	0^{e}	0^{e}	5.7^{d}	0^{e}	29^{c}	30.8^{c}	4.9^{d}	45.7^{d}
AS								
0 µM	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
50 µM	100.6^{a}	82.7^{ab}	97.5^{a}	102.7^{a}	94.8^{a}	84.4^{ab}	60.6^{b}	90.7^{a}
100 µM	74.3^{b}	77.9^{b}	54.7^{b}	67.4^{b}	80.5^{b}	79^{b}	60.1^{b}	89.6^{ab}
200 µM	56.1^{bc}	57.4^{c}	28.6^{c}	41.3^{c}	57.1^{c}	76.8^{b}	54.7^{b}	77.5^{b}
400 µM	38^{d}	11.5^{d}	14.9^{d}	18.2^{d}	39.7^{cd}	65.5^{bc}	52.9^{bc}	49.3^{c}
800 µM	12.9^{d}	0^{e}	7.1^{e}	16.2^{d}	19.2^{d}	40.2^{c}	25.6^{c}	40.8^{c}
1200 µM	0^{e}	0^{e}	3.3^{e}	0^{e}	13.3^{d}	32.6^{c}	3.4^{d}	37.6^{c}

Different letters along columns, or along ED_{50} row, indicate statistically significant differences with $P < 0.05$ (Tukey test); (N = 4).

Table 2: Total Root Length (TRL) and related ED_{50} values of crops and weeds exposed to different *trans*-Caryophyllene concentrations (0–1200 µM).

	Total Root Lenght							
	Crops				**Weeds**			
	Z. mays	***L. sativa***	***R.sativus***	***X Triticosecale***	***A. retroflexus***	***D. sanguinalis***	***E. crus-galli***	***L. perenne***
0 µM	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
50 µM	76^{b}	54^{b}	87^{ab}	58^{ab}	80^{ab}	71^{ab}	100^{a}	83^{a}
100 µM	62^{bc}	50^{b}	67^{abc}	46^{bc}	99^{a}	81^{ab}	84^{ab}	96^{a}
200 µM	55^{bc}	28^{c}	53^{bc}	40^{bc}	50^{b}	43^{bc}	80^{ab}	67^{ab}
400 µM	56^{bc}	19^{d}	42^{c}	9^{c}	37^{c}	39^{bc}	83^{ab}	67^{ab}
800 µM	46^{c}	15^{e}	33^{c}	8^{c}	33^{c}	14^{c}	66^{ab}	45^{b}

1200	39^{c}	17^{ed}	24^{c}	8^{c}	32^{c}	8^{c}	42^{b}	35^{b}
ED_{50}	**209.1^{d}**	**69.4^{f}**	**270^{cd}**	**128.5^{e}**	**331.5^{c}**	**214.9^{d}**	**1194.1^{a}**	**689.1^{b}**

Different letters along columns, or along ED_{50} row, indicate statistically significant differences with $P < 0.05$ (Tukey test); (N = 4).

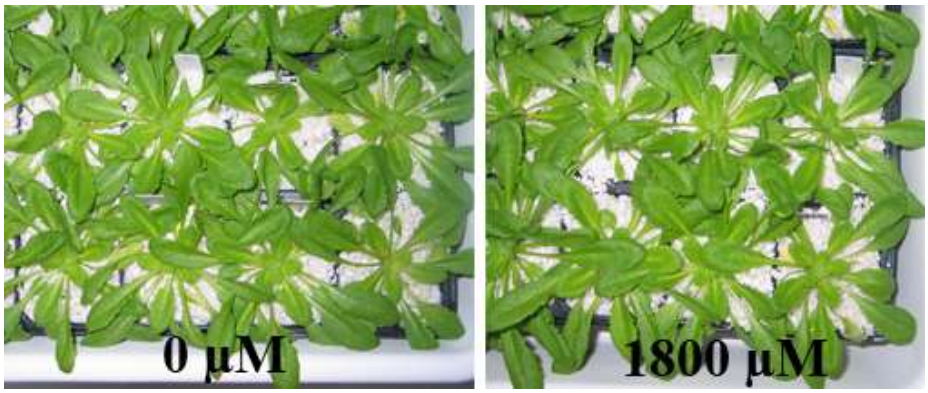


Figure 1: Arabidopsis adult plants (A, control) at the end of the spraying treatment (B) with *trans*-caryophyllene.

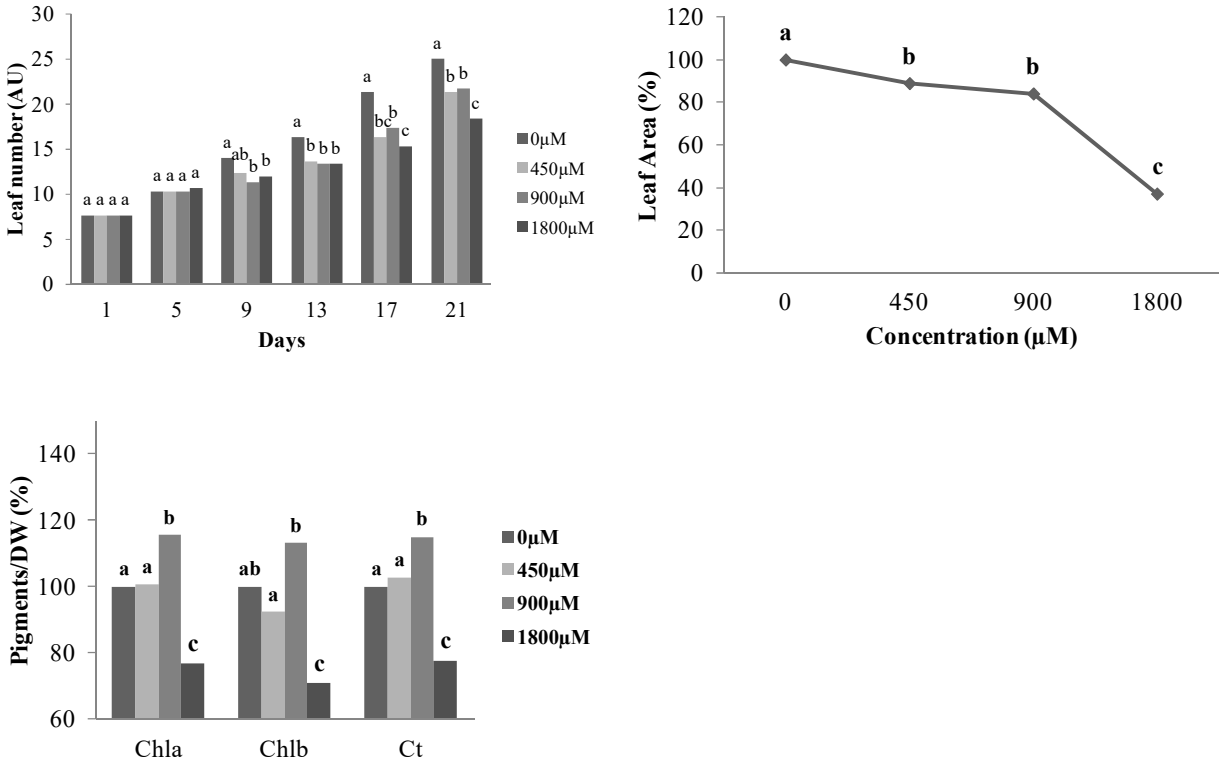


Figura 2: Leaf number, leaf area and pigment content of *A. thaliana* adult plants sub-irrigated with 0, 450, 900, 1800 µM *trans*-Caryophillene, for 21 days. Except for Leaf number [Arbitrary Units (AU)], all data are expressed as percentage of the control. Different letters along the bars and along the curve indicate statistical differences with $P<0.05$ (Tukey's test), N=3.

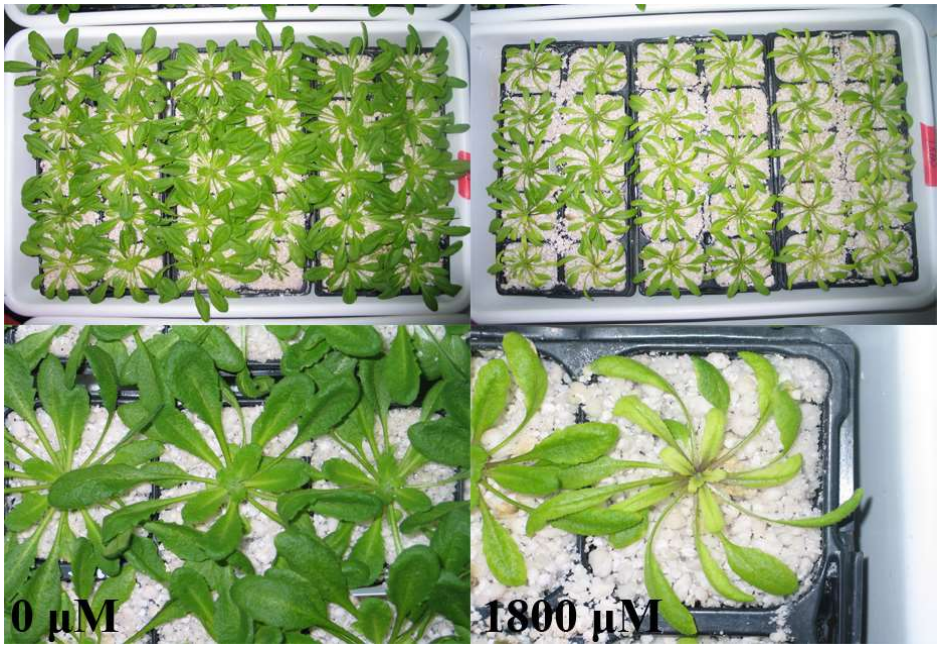


Figure 3: Particular of leaf morphology of *A. thaliana* plants treated for 21 days, through irrigation, with *trans*-Caryophillene.

Table 3: Values of fresh weight (FW), dry weight (DW), DW/FW, protein content (TP) lipid peroxidation (MDA), proline and osmotic potential (Ψπ) in adult plants of *A. thaliana* sub-irrigated with *trans*-Caryophillene (0, 450, 900, 1800 µM) for 21 days.

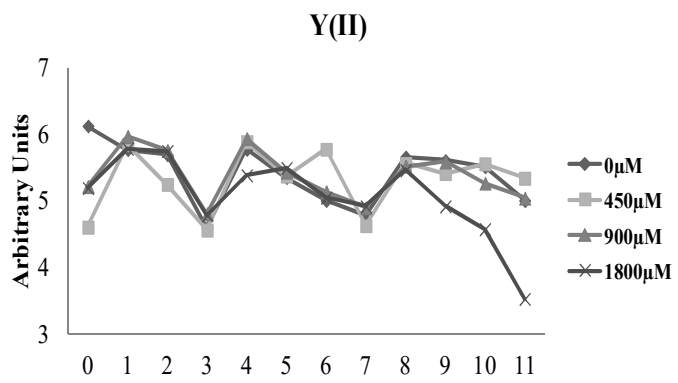
Concentration (µM)	***FW***	***DW***	***DW/FW***	***TP***	***MDA***	***Proline***	***Ψπ***
0	100[a]	100[a]	100[a]	100a	100a	100a	100[a]
450	73.85[b]	76.68[a]	105.52[a]	58.98b	124.70a	219.13a	100,8[a]
900	82.04[ab]	83.68[a]	102.21[a]	54.00b	153.09b	97.05a	104,7[b]
1800	26.70[c]	31.87[b]	119.38[b]	53.32b	199.50c	495.24b	114,3[c]

Data are expressed as percentage of control. Different letters along the column indicate statistical differences with $P<0.05$ (Tukey's test), N=3.

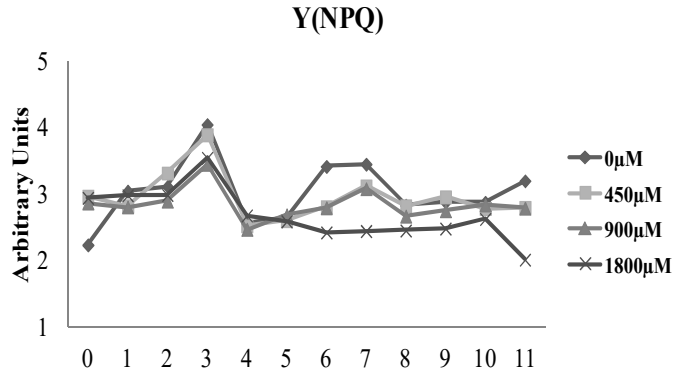
Table 4: Values of macro and micronutrient concentrations in adult plants of *A. thaliana* sub-irrigated with *trans*-Caryophillene (0, 450, 900, 1800 µM) for 21 days.

Elements	Concentration (µM)			
	0	**450**	**900**	**1800**
C	100[a]	99.59[a]	101.17[a]	105.18[a]
H	100[a]	96.42 [a]	98.25 [a]	104.49 [a]
N	100[a]	96.75[a]	93.59[a]	101.46[a]
P	100[a]	102.08[a]	104.23[b]	90.95[c]
K	100[a]	99.12[a]	104.17[a]	92.75[b]
Al	100[a]	132.35[b]	146.38[b]	71.85[c]
Ca	100[a]	101.20[a]	88.35[b]	52.58[c]
Cu	100[a]	164.53[b]	155.80[b]	263.65[c]
Fe	100[a]	109.35[a]	106.80[a]	67.72[b]
Mg	100[ab]	102.46[a]	97.23[b]	98.32[b]
Mn	100[a]	81.06[b]	49.75[c]	28.05[d]
Na	100[a]	107.50[b]	109.44[b]	153.54[c]
Zn	100[a]	91.31[b]	78.18[c]	37.94[d]

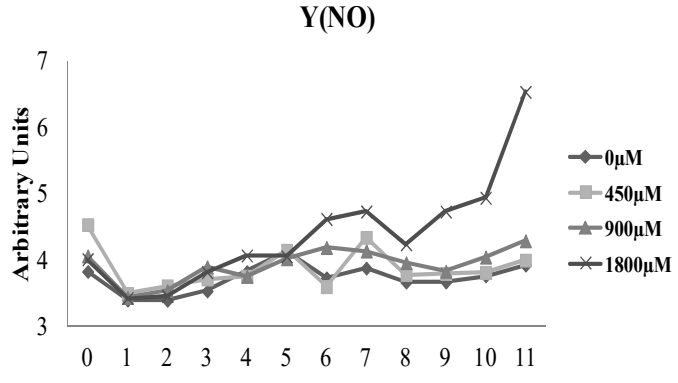
Data are expressed as percentage of control. Different letters along the column indicate statistical differences with $P<0.05$ (Tukey's test), N=3.



Y(II)	450µM	900µM	1800µM
T0	- - -	- - -	- - -
T1			
T2	- - -		
T3			
T4			- - -
T5			
T6	+++		
T7			
T8			
T9			- - -
T10			- - -
T11	+++		- - -

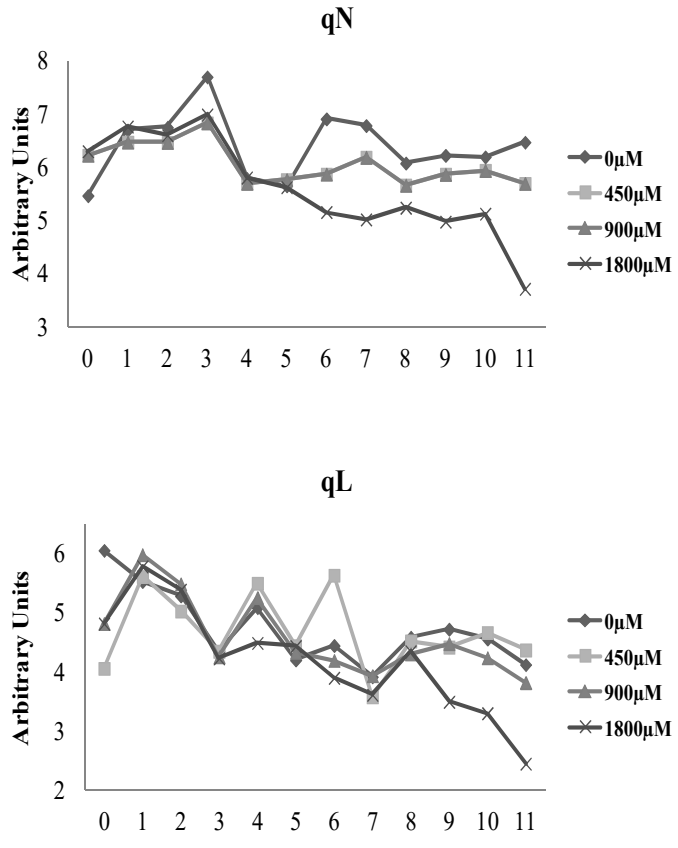


Y(NPQ)	450µM	900µM	1800µM
T0	+++	+++	+++
T1			
T2	+	-	
T3		-	-
T4			
T5			
T6	- - -	- - -	- - -
T7	- - -	- - -	- - -
T8		- - -	- - -
T9		- - -	- - -
T10			- - -
T11	- - -	- - -	- - -



Y(NO)	450µM	900µM	1800µM
T0	++		
T1			
T2	+		
T3			
T4			+
T5			
T6		+	+
T7			+
T8			+
T9			+++
T10			+++
T11		+++	+++

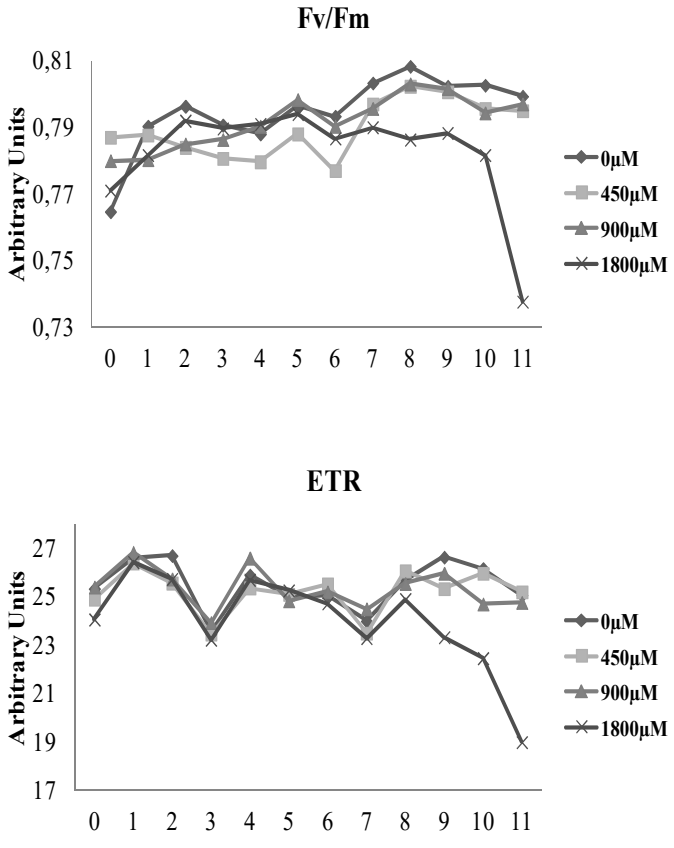
Fig. 4. Values of the effective photochemical quantum yield at PSII (YII), the quantum yield of light-induced nonphotochemical quenching (YNPQ) and the quantum yield of all photosynthetically active photon fluxes other than YNPQ and _II (YNO) in whole Arabidopsis plants after treatment with 0, 1, or 3mM 2(3H)-benzoxazolinone (BOA). Whole plants were measured and the values integrated afterwards. Fifteen measures were obtained for each parameter at each measuring time, which gave a kinetic plot for each parameter along the time. The integral value of the area was obtained from for each parameter at every time. Table shows the statistical significance of positive (+) or negative (−) differences with respect to untreated plants: + or −$p < 0.05$; ++ or − − $p < 0.01$; +++ or − − − $p < 0.001$. After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated by analysis of variance followed by least significant difference tests for homoscedastic data, and by Tamhane's T2 test for heteroscedastic data.



qN	450µM	900µM	1800µM
T0	++	++	++
T1			
T2		-	
T3		--	--
T4			
T5			
T6	-	-	-
T7	-	-	-
T8		---	---
T9		---	---
T10		---	---
T11	---	---	---

qL	450µM	900µM	1800µM
T0	---	---	---
T1			
T2			
T3			
T4			--
T5			
T6	+++		---
T7			
T8			
T9			---
T10		---	---
T11	+++	---	---

Fig. 5. Values of non-photochemical quenching parameters qN and qL in whole Arabidopsis plants after treatment with 0, 450, 900 and 1800 µM trans-caryophyllene. Whole plants were measured and the values integrated afterwards. Fifteen measures were obtained for each parameter at each measuring time, which gave a kinetic plot for each parameter along the time. The integral value of the area was obtained from for each parameter at every time. Table shows the statistical significance of positive (+) or negative (–) differences with respect to untreated plants: + or –, $p < 0.05$; ++ or − − $p < 0.01$; +++ or − − −$p < 0.001$. After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated byanalysis of variance followed by least significant difference tests for homoscedastic data, and by Tamhane's T2 test for heteroscedastic data.



Fv/Fm	450µM	900µM	1800µM
T0	+++	+++	
T1		-	-
T2	--	--	
T3			
T4	--		
T5			
T6			
T7	--		
T8			--
T9			--
T10			--
T11			---

ETR	450µM	900µM	1800µM
T0			--
T1			
T2	-	-	-
T3			
T4			
T5			
T6			
T7			
T8			
T9			-
T10		-	-
T11			-

Fig. 6. Values of maximum quantum efficiency of dark-adapted PSII (Fv/Fm) and apparent electron transport rate (ETR) in whole Arabidopsis plants after treatment with 0, 450, 900 and 1800 µM trans-caryophyllene. Whole plants were measured and the values integrated afterwards. Fifteen measures were obtained for each parameter at each measuring time, which gave a kinetic plot for each parameter along the time. The integral value of the area was obtained from for each parameter at every time. Table shows the statistical significance of positive (+) or negative (–) differences with respect to untreated plants: + or –, $p < 0.05$; ++ or − − $p < 0.01$; +++ or − − −$p < 0.001$. After Kolmogorov–Smirnov testing for non-normality and Levene's testing for heteroscedasticity, the statistical significance of differences among group means was estimated byanalysis of variance followed by least significant difference tests for homoscedastic data, and by Tamhane's T2 test for heteroscedastic data.