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# Modulation of source-sink physiology through film antitranspirant induced drought tolerance amelioration in *Brassica napus*

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**Running title:** Drought and antitranspirant in flowering canola

**Abstract**

Increase in drought conditions during the oilseed rape (OSR) reproductive phase are predicted to occur more often in the temperate zone, leading to significant yield losses. Crop management solutions such as film antitranspirant (AT) applied at key drought-sensitive growth stages on both wheat and oilseed rape have recently been shown to alleviate drought-induced yield losses. However, there is a lack of information regarding potential AT effectiveness to reduce drought damage on OSRplants at different soil moisture regimes. Therefore, two similar experiments were performed in a computer-controlled glasshouse/phenotyping centre to investigate the physiological responses of OSR to well-watered (WW), moderate water stress (MWS), water stress (WS) and severe water stress (SWS) conditions. Stress treatments were imposed at the initiation of flowering and treated with an AT or water onto the leaf-canopy. Stress limited the gas-exchange and increased leaf temperature, leaf-to-air temperature, bud-to-air temperature and ABA concentrations which increased with stress intensity in all tissues analysed. Yield components were significantly reduced by WS and SWS treatments when compared to the WW plants. Application of AT counteracted the detrimental effect of WS and SWS by decreasing water use over the first few days of stress application thus improving relative water content and leaf water-use efficiency, decreasing ABA accumulation in leaf and all the reproductive organs analysed (buds, flowers and pods) and avoiding bud-to-air temperature increases. AT application sustained pod formation and seed production under WS but only seed production under SWS conditions. These data suggest that leaf-canopy application of AT at key phenological stages under particular magnitudes of soil moisture deficit may sustain OSR reproduction and reduce yield losses.

## Introduction

Drought is considered one of the main detrimental factors in crop productivity and the magnitude of dry events may increase with climate change (Parmesan and Yohe 2003; Cattivelli et al. 2008). Therefore, understanding of crop physiological mechanisms behind the drought response and subsequent exploitation of crop management tools along with crop genetic improvement are urgently required to meet the future challenge of producing higher agricultural output with fewer water resources (Wallace 2000).

It is well recognized that crop productivity is mainly reduced when drought occurs over key sensitive phenological stages (i.e. reproductive periods) (Saini and Westgate 1999). Anthesis is a drought-sensitive stage in all the major food-crops such as wheat, maize and rice (e.g. Weerasinghe et al. 2015, Chapman and Edmeades, 1999; Boonjung and Fukai, 1996 respectively). Oilseed rape (OSR, *Brassica napus* L.) is considered one of the most drought sensitive crops during anthesis; several studies report reductions in the physiological performance leading to a significant drop in the reproductive efficiency and thus yield (Gammelvind et al. 1996; Mogensen et al. 1997; Faralli et al. 2016). There is evidence that drought periods over flowering and mid-pod development stage can cause up to 40% of yield losses (Richards and Thurling, 1978; Champolivier et al. 1996).

A crop avoids tissue dehydration by minimising water loss and maximising water uptake (Chavez et al. 2003) and these are achieved by decreasing transpiration through stomata closure or by improving root characteristics to increase water uptake, respectively. Minimising water loss through stomatal closure is mediated by the plant hormone abscisic acid (ABA) (Finkelstein, 2013). Leaf ABA

accumulation leads to a substantial amount of water saved due to reduced transpiration but at the expense of photosynthetic efficiency (Finkelstein, 2013). ABA accumulation in plant tissues however has been related to other detrimental effects, in particular during plant reproduction. High ABA concentration in wheat spikelets has been directly related to a reduced seed set and final grain yield (Westgate et al. 1996). Similarly, droughted soybean showed a substantial increase in pod ABA concentration that was significantly correlated with reduced pod set (Liu et al. 2004). In OSR, while considerable effort has been focused on the leaf canopy response to drought, less attention has been paid to the reproductive organ responses to stresses despite it being generally recognised that OSR reproductive organs are often highly sensitive to water deprivation (Faralli et al. 2016; Guo et al. 2013; Mogensen et al. 1997). It has been recently postulated that in the *Brassicaceae* family, buds showed a lower stomata index and smaller stomata compared to leaves, and their water status is dependent on leaf gas-exchange and leaf water status through a source-sink "self-adjustment" (Guo et al. 2013). Therefore, bud/reproductive organ temperatures are important traits to understand crop drought response since, due to their small size, it is not possible to evaluate their transpiration rate with standard physiological techniques (Guo et al. 2013; Guo et al. 2015).

In this context, significant efforts investigating the use of crop management tools to minimise plant water loss have been made. It has been hypothesized that yield can benefit by an additional reduction in water loss over the most sensitive phenological stage to drought (Weerasinghe et al. 2015). In particular film-forming antitranspirant (AT) and metabolic compounds with antitranspirant activities have been recently tested. Application of AT reduced stomatal conductance *via* an ABA-independent mechanism (Faralli et al. 2016; Iriti et al. 2009) leading to significant

reduction in ABA concentration at the leaf and floral organ level under drought (Faralli et al. 2016). Application of AT during the wheat and OSR reproductive periods just prior to transient water shortage significantly improved plant water status following significant reductions in leaf water loss in both glasshouse (Abdullah et al. 2015; Faralli et al. 2016) and field (Patil and De, 1978; Weerasinghe et al. 2015) conditions. Recently, significant improvements in OSR reproductive organ water status have been reported after leaf-canopy AT treatments following leaf stomatal conductance reductions and hence leaf water status improvements under water deficit (Faralli et al. 2016). However, in OSR the specific correlations between plant gas-exchange, ABA accumulation, reproductive organs and leaf temperatures, and yield formation at different soil moisture deficits have not been extensively explored when compared to other major food crops such as wheat (Westgate et al. 1996) or soybean (Liu et al. 2004). Moreover, information regarding the effect of AT on the overall-plant physiological response to different drought intensities is sparse; to our knowledge, the effect of the AT leaf-canopy application on the relationship between leaf and reproductive organs under drought has never been explored.

Therefore two glasshouse experiments using a computer-controlled gravimetric-automated system for pot watering investigated this area. The aim of this study was to understand the physiological interactions between i) gas-exchange traits; ii) ABA concentration in leaf and reproductive organs; iii) leaf and bud temperatures; iv) water use; v) yield components of OSR plants subjected to four watering regimes over flowering with or without applications of AT.

## Materials and methods

*Plant material and experimental design*

In both of the experiments winter OSR seeds (cv. Excalibur, Dekalb, UK) were sown into seedlings trays filled with John Innes No. 2 compost (loam, peat coarse sand and base fertiliser, John Innes Manufacturers Association, Reading, UK) on the 20$^{th}$ December 2014 for Experiment I and the 3$^{rd}$ June 2015 for Experiment II. Seedlings at the fourth leaf stage were transferred into a cold room and vernalized at 4$^{o}$C for 8 weeks (16h / 8h light-dark photoperiod at ~200 µmol m$^{-2}$ s$^{-1}$ PAR). On the 16$^{th}$ February 2015 for Experiment I and on the 19$^{th}$ August 2015 for Experiment II the vernalized plants were moved inside the National Plant Phenomics Centre (NPPC, Institute of Biological, Environmental and Rural Sciences, Aberystwyth, UK). The same day the plants were transplanted into 3.5 L pots containing John Innes No. 2 compost and manually watered around the calculated field capacity value every two days. A liquid feed of Chempak 2 (high nitrogen, Thompson and Morgan) was applied just before the AT treatment and again at pod fill. The pots were moved at the bud emerging stage (GS 5.0) to the NPPC conveyor system. Plants were grown at 19.7 ± 4.7°C and 18 ± 0.6°C daily average temperature (Experiment I and Experiment II respectively), 41 ± 4.7% and 56.3 ± 4.3 relative humidity (Experiment I and Experiment II respectively) and an average daily photon flux density of 400 µmol photons m$^{-2}$ s$^{-1}$ from natural light supplemented by high pressure sodium lamps (16h / 8h light-dark photoperiod). The experiments were both arranged in a randomized complete block 4x2 factorial design with four levels of soil moisture [well-watered (WW), moderate water stressed (MWS), water stressed (WS) and severe water stressed (SWS)] and two levels of antitranspirant treatment (water only and water treated with 1% v/v Vapor Gard (Miller Chemical and Fertilizer LLC, Hanover, USA. a.i. di-1-*p* menthene 96%)) in six (Experiment I) and seven (Experiment II) blocks.

*Drought application, daily evapotranspiration and water use estimation.*

Before the drought treatment (hence, from GS 5.0 to GS 6.0, BBCH canola growth scale, green bud emerging and first flower open respectively) target watering was started to the plants by the automatic NPPC watering system ensuring full irrigation to all the plants (~ 2400 g of target weight, ~35% of volumetric water content). Drought was applied at GS 6.0 and applied over the whole flowering stage (until GS 6.9 BBCH canola growth scale, end of flowering - 10% of pods at final size). The four soil moisture treatments were determined based on John Innes No. 2 water retention curve: for John Innes No. 2 compost the permanent wilting point and the pot capacity were ~7% volumetric water content (VWC) and ~45% VWC respectively as reported by Faralli et al. (2016). The total available water content (AWC) in mL was then calculated as the difference between the weight of the pot at pot capacity and the previously evaluated weight (~400g) of an OSR plant at flowering stage (~2700 g in total) and the weight of the pot + plant at 7% VWC (~1650 g in total) by moisture probe (Time Domain Reflectrometry, TDR TRIME-FM, Envco, Auckland, New Zealand). Thus, the watering regimes were imposed as well-watered (WW - pot target weight 2630, ~ 950 mL AWC, ~40% VWC), moderate water stress (MWS - pot target weight 2430, ~700 mL AWC, ~30% VWC), water stress (WS - pot target weight 2130,~450 mL AWC VWC ~20%), and severe water stress (SWS - pot target weight 1830, ,~200 mL AWC, ~10% VWC). Plants were re-watered every day in the late afternoon (i.e. 7.00-8.00 PM) by the automatic NPPC watering system to reach the fixed target weight for each watering treatment. Total daily plant evapotranspiration (ET) was then calculated as the difference between the reached daily target weight of the pot and the weight of the pot after 24 hours. Plant water use (WU) was estimated by

including pots (n=3) with no plants with a gravimetric soil moisture similar to that of the four watering regimes applied (WW, MWS, SWS, WS). This allowed the daily evaporative loss from the compost ($SE_{vap}$) to be calculated in similar gravimetric fashion to the daily ET. These data were averaged across a group of compost-only pots and then subtracted from the plant data to provide an evaporative loss correction following the equation:

$$WU = ET - SE_{vap}$$

*Antitranspirant application*

The antitranspirant was applied in the early afternoon just prior to drought initiation (Days after spraying (DAS) 0). Timing of all measurements taken after AT was applied is referred to as DAS. The adaxial surface of the leaf-canopy was uniformly sprayed with either i) water (-AT) or ii) a solution of 1% v/v of Vapor Gard (+AT) in water by a hand sprayer (Peras 7, Hozelock Exel, Beaujolais – France) on the 24 of March 2015 for Experiment I and on the 9 of September 2015 for Experiment II (i.e. when the first flower in the main stem was open).

*Stomatal conductance, gas-exchange and chlorophyll fluorescence combined analysis*

In Experiment I, leaf stomatal conductance to water vapour ($g_s$) was analysed with a transient state diffusion porometer (AP4, Delta-T Devices, Cambridge, UK). The data were collected on DAS 1, 3 and 8 and the device was calibrated before every analysis by using the calibration plate provided. For each treatment (n=6) a measurement of the adaxial $g_s$ and abaxial $g_s$ was collected on the tagged first

fully expanded leaf of the top canopy at GS 6.0. Total $g_s$ ($g_{stot}$) was then calculated as adaxial $g_s$ + abaxial $g_s$. Data were collected between 09:00 and 12:00.

In Experiment II gas exchange analysis were carried out on the tagged first fully expanded leaf of the top canopy at GS 6.0 (as above) on DAS 2, 5, 8, 11, 14, 17 and 20 (n=4) using a WALZ GFS-3000 system (WALZ, Effeltrich, Germany) with a 4 $cm^2$ cuvette ensuring a saturating 1200 µmol $m^{-2}$ $s^{-1}$ PAR; the cuvette was provided with a dual LED/PAM (pulse amplitude modulation) fluorometer module. All the data were recorded after 3–4 min at 400 ppm $CO_2$ level, when steady-state photosynthesis was achieved. Intrinsic water-use efficiency (¡WUE) was then calculated as the ratio between the micromole of $CO_2$ assimilated ($A_{max}$) and the mole $H_2O$ loss (*gs*) through stomatal conductance. Data were recorded between 09:00 and 12:00. At the same time the actual photochemical efficiency of the photosystem II ($\Delta F/F_m'$) was calculated as follow:

$$\Delta F/F_m' = (F_m' - F_o')/ F_m'$$

Where $F_m'$ is maximal fluorescence of a light adapted leaf and $F_o'$ is minimal fluorescence of a light adapted leaf. $\Delta F/F_m'$ was then used to calculate electron transport rate (ETR) equation calculated as:

$$ETR = \Delta F/F_m' \times PPFD \times \alpha \times \beta$$

Where PPFD is the photosynthetic photon flux density, α is the assumed leaf absorbance (0.84) and β is the assumed partitioning of absorbed quanta between PSII and PSI (0.5) (Baker, 2008).

*Relative water content (RWC)*

Relative water content (RWC) was calculated according to Barr and Weatherley (1962) and Faralli et al. (2015). For Experiment II over DAS 2, 8, 12 and 16, one leaf disc (2.5 $cm^2$) per plant (n=4) was collected from the leaf positioned below the tagged leaf used for gas-exchange and placed in a 50 mL tube. The fresh weight was then recorded ($F_W$) with a balance (Mettler-Toledo XS 205 Dual Range, Columbus, USA) and the disks were soaked in distilled water in the dark at ~4°C over 4 hours (turgid weight, $T_W$). The dried disks (oven-dried at 80°C for 12 hours) were weighed the day after (dry weight, $D_W$). RWC (%) was then calculated as:

$$\mathrm{RWC}(\%) = \frac{\mathrm{F_W} - \mathrm{D_W}}{\mathrm{T_W} - \mathrm{D_W}} \mathrm{x}\ 100$$

*Infrared thermometer, thermal infrared imaging and near infrared analysis.*

In Experiment I and II, plants thermal images were collected with a VarioCAM HiRes 640x480 camera (spectral range 7.5 µm to 14 µm; Jenoptik, Germany), via control/image capture software IRBIS 3 plus (InfraTec GmbH, Dresden, Germany) on DAS 1, 3 and 8. To provide a uniform background (thus an easier image segmentation), images were taken whilst plants were stationary in front of a black plastic panel. The tripod-mounted camera captured portrait-orientated images of the plants from a distance of 1.2 m. Images were captured as 640×480 csv files, with a short string of metadata attached; these files were analysed using '20151028_heatmap_analyser.R', which segments the images against the background, and provides a series of images and temperature distributions for single plants and the population as a whole. For all the images and after segmentation, the average distribution of leaf canopy temperatures were pooled and used for statistical analysis.

Additionally in Experiment II, leaf temperature and bud temperature were collected with an infrared thermometer (Fluke 66, Fluke Corporation, WA, USA) with a minimum 2.5 mm diameter measurement area on DAS 1, 2, 4, 6, 8, 10, 12 and 14. Air temperature was collected with another digital thermometer with a five-second responsiveness time positioned at ~10 cm distance from the tissue analysed. The leaf adaxial surface temperature (n=4) was measured on the same tagged leaf used for gas-exchange whilst one 'ready to open' lateral bud at analogous leaf canopy height was analysed to detect bud temperature (n=4). For each leaf and bud temperature measurement, the ambient temperature was recorded by using the digital thermometer described earlier. The difference between leaf and ambient temperatures and bud and ambient temperatures was used to calculate $L_T$ (leaf temperature - ambient temperature) and $B_T$ (bud temperature - ambient temperature) respectively.

*Sample collection and ABA tissue concentration analysis*

On DAS 3, 7 and 16 leaf and reproductive organs were excised with a scalpel (n=4), flash-frozen in liquid nitrogen and stored at -80°C. Reproductive organs sampled were ready to open buds on DAS 3, flower/pod on DAS 7 and pod on DAS 16. The samples were then freeze-dried and stored for ABA assay. ABA concentration ([ABA]) was measured with an enzyme linked immunosorbent assay (ELISA) (Cusabio Biotech Co. Ltd, Carlsbad, CA, USA). Samples were finely ground and ELISA was performed following the manufacture's procedure as reported by Faralli et al. (2016).

*Yield component analysis*

For both of the experiments plants were hand harvested at complete maturity and pods were counted to determine pods per plant. The harvested pods were oven-dried at 30°C for four days. Pods were then opened and the dried seeds were weighed (Balance: PCB 2500-2, Kern and Sohn GmbH, Balingen, Germany) to determine seed dry matter.

*Statistical analysis*

Watering data (daily ET, AWC, WU) are presented as means of the two experiments ± standard error (SE). Stomatal conductance from porometry (Experiment I), gas-exchange (Experiment II), thermal infrared analysis (Experiment I), leaf-to-air and bud-to-air (Experiment II), relative water content (Experiment II) and ABA concentration (Experiment II) data were subjected to a two-way analysis of variance (ANOVA) to assess antitranspirant (-AT and +AT) and watering regime (WW, MWS, WS, and SWS) effects. For yield components, similar observations and trends were recorded between the two experiments. However, the three-way ANOVA for yield components showed significant interactions between experiments and the other two factors (watering and antitranspirant) and an $F_{max}$ test revealed significant differences between the two sets of data. Thus, yield components (seed dry matter and pods per plant) data from the two experiments are presented separately. Data were checked for normality by examining residual plots. A Tukey's test ($P<0.05$) was used for means separation. To test the relationships between the data presented, linear regressions were used. All the statistical analyses were performed by using Genstat (17th edition, VSN International Ltd, UK).

**Results**

*Daily evapotranspiration, pot available water content and water use*

WW plants had a water availability of ~950 mL after re-watering in the late afternoon with an average ET of ~400 mL over flowering stage (Fig. 1A and 1B). Thus the plants were never subjected to stress, since the soil water potential at re-watering was never below -100 kPa according to the soil retention curve used with an average AWC of ~550 mL. The AWC after re-watering on MWS plants was ~700 mL with a daily ET just below the WW plants (~350 mL) and an AWC at re-watering of ~400 mL. The total ET of WS plants was significantly lower than that of the WW and MWS plants with an average value of ~250 mL over the stress. The AWC after the re-watering was ~450 mL whereas before the re-watering the AWC dropped to an average value of ~200mL that equates to a soil water potential value of -300 to -400 kPa. The SWS plants had an average AWC of ~200 ml after and ~0-50 ml before re-watering, with a daily ET fluctuating from 100 to 150 mL.

On WW plants, an AT application decreased the WU by 31 mL on average compared to the un-sprayed plants from DAS 0 to DAS 8 whereas the decrease was lower from DAS 9 to DAS 16 (8 mL) (Table 1). On MWS plants AT-treated plants exhibited a decrease in WU by 6 mL on average compared to the un-sprayed whereas in WS plants the AT reduced the WU by 10 mL from DAS 0 to DAS 8. In contrast, AT-treated SWS plants did not show any significant reduction in WU compared to the un-sprayed plants, with the latter displaying a lower WU (2 mL from DAS 0 to DAS 8 and 1 mL from DAS 9 to DAS 16).

*Leaf gas-exchange*

In Experiment I mean $g_{stot}$ in WW over DAS 1, 3 and 8 was 620 mmol $m^{-2}$ $s^{-1}$ and MWS, WS and SWS plants exhibited a reduction by 20%, 56% and 83%

308 respectively when compared with the WW plants (Fig. 2A). Application of AT
309 decreased $g_{stot}$ by 32%, 29%, 17% and 2% on WW, MWS, WS and SWS
310 conditions respectively. When compared to the WW plants, the stress increased
311 the leaf temperature by 3%, 8% and 13% on MWS, WS and SWS plants (Fig. 2B).
312 Although not significant, application of AT slightly increased leaf temperature when
313 +AT plants were compared to each relative control -AT. Abaxial $g_s$ in WW was 310
314 mmol $m^{-2}$ $s^{-1}$ on average and MWS, WS and SWS conditions decreased abaxial $g_s$
315 by 17%, 56% and 84% respectively when compared to the WW plants (Fig. 2C).
316 Abaxial $g_s$ was increased at all the watering regimes by AT application by 4%, 7%,
317 18% and 62% at WW, MWS, WS and SWS conditions respectively. Adaxial $g_s$ in
318 plants grown under WW conditions was 300 mmol $m^{-2}$ $s^{-1}$ (Fig. 2D). When
319 compared to the WW plants MWS, WS and SWS conditions decreased adaxial $g_s$
320 by 22%, 54% and 82% respectively. Application of AT decreased adaxial $g_s$ by
321 68%, 66%, 52% and 37% in plants grown under WW, MWS, WS and SWS
322 conditions respectively.

323 In Experiment II mean $CO_2$ assimilation rate of WW plants fluctuated from ~18-20
324 µmol $m^{-2}$ $s^{-1}$ at DAS 2 to ~12µmol $m^{-2}$ $s^{-1}$ at DAS 20 (Fig. 3A). Drought conditions
325 over the flowering stage reduced the $CO_2$ assimilation rate on MWS, WS and SWS
326 plants by 14.6%, 41.0% and 64.4% on average respectively compared to WW
327 (Fig. 3B, C and D). In WW plants, AT-treated plants showed lower assimilation
328 rate values compared to the un-sprayed ones. The reduction was steady over the
329 whole period of data-collection: on average, AT-plants experienced a loss in $CO_2$
330 assimilation capacity by 12.7% compared to the un-sprayed. MWS AT-treated
331 plants displayed a 10.1% reduction compared to the un-sprayed. On the contrary,
332 AT-treated WS plants, despite the initial 10.5% reduction in $CO_2$ assimilation

compared to the un-sprayed (DAS 2), exhibited a sustained higher value over the droughted period of 17.5% compared to the un-sprayed plants. No significant differences were found between un-sprayed and AT-treated plants at SWS.

Mean stomatal conductance of WW plants was ~570 mmol $m^{-2}$ $s^{-1}$ on the first 7 days of the flowering stage decreasing until an average value of ~400 mmol $m^{-2}$ $s^{-1}$ before GS 6.9 (Fig. 3E). Water deprivation affected *gs* on MWS, WS and SWS by 21.6%, 48.5% and 77.2% respectively compared to WW plants (Fig. 3F, G and H). Over the flowering period and on WW, MWS and WS plants, AT depressed *gs* by 28.4%, 15.6% and 24.1% on average compared to their respective un-sprayed control. Conversely, no significant reductions were found between AT-treated and un-sprayed plants at SWS water regime conditions.

Thus, the calculated ¡WUE was substantially increased over the first few days of water deficit on WS and SWS plants by an average of 21.3% and 73.7% respectively compared to the WW plants (Fig. 3Q and R). With respect to the WW plants, MWS stressed plants exhibited only a slight increase by 4.8% on average (Fig. 3O and P). Under WW conditions, AT-treated plants showed a slight (non-significant) increase in ¡WUE by 3.0% on average compared to the un-sprayed plants. Similar responses were found under MWS, where AT-treated plants exhibited an increased ¡WUE by 5.8% on average compared to the un-sprayed. In contrast, the AT application on WS plants showed a significant increase in ¡WUE over the whole experiment by an average of 53% compared to the un-treated plants. AT application on SWS plants decreased the ¡WUE by 7.8% with respect to the untreated plants.

Declines in ETR were evident in WS and SWS plants (20.8% and 21.4% on average compared to the WW) whilst in MWS plants no significant ETR downregulations were found compared to WW plants (Fig. 3I, L, M and N). AT application on WW and MWS plants showed reduced ETR values compared to the un-sprayed control by 7.9 and 8.1% respectively. Conversely, AT-treated plants subjected to WS and, to a lesser extent, SWS watering regimes exhibited higher ETR values compared to un-sprayed controls. Particularly, AT-treated WS plants showed an increase by 10.2% on average, whereas a significant increase in ETR at SWS was observed only in the last period of stress (i.e. DAS-11 to DAS 14)

*Leaf Relative water content*

Over the experiment, leaf RWC of WW plants exhibited a persistent reduction from ~ 95% at DAS 2 to ~89% at DAS 16 (Fig. 4A). Under droughted regimes (WS and SWS), significant reductions in RWC were observed throughout the experiment starting at DAS 2 ($P<0.001$, $P=0.003$, $P<0.001$ and $P<0.001$ for DAS 2, 8, 12 and 16 respectively) (Fig. 4C and D). Thus under WS and SWS, for all the DAS, RWC was significantly lower than that of the WW plants. Conversely, under MWS no significant reductions were observed with respect to the WW plants (Fig. 4B).
AT application on WW and MWS plants did not statistically affect the RWC compared to the un-sprayed plants. In contrast, AT-treated WS plants on DAS 2, 8 and 12 exhibited significant higher RWC with respect to the un-sprayed WS plants. Similarly, under SWS, significantly higher values were observed on AT-treated plants with respect to the un-sprayed at all the DAS.

*Leaf and bud infrared thermometer*

WW plants maintained a relatively large negative $L_T$ value (-2.25°C) over the experiment with a slightly less negative $B_T$ value (-1.7°C) (Fig. 5A and B). Compared to WW plants, MWS plants had higher $L_T$ and significantly higher $B_T$. Compared to WW plants, WS and SWS plants had significantly higher $L_T$ and $B_T$ with the latter close to 0°C (air temperature). With respect to the un-sprayed plants, WW and MWS AT-treated plants exhibited a significant lower $L_T$ value at most DAS. In contrast no significant differences were found in $L_T$ between AT-treated and un-sprayed plants from WS and SWS watering regimes. $B_T$ was not significantly affected by AT in WW and MWS plants despite the fact that lower negative values were observed compared to the un-sprayed plants in MWS plants. WS plants showed significantly less negative $B_T$ values when AT-treated throughout the stress imposition. Plants subjected to SWS exhibited less negative $B_T$ values on average when AT-treated but the value was not statistically significant. $L_T$ and $B_T$ were significantly correlated (linear regression, $R^2$=0.98 for -AT and polynomial regression, $R^2$=0.99 for +AT) (Fig. 5C).

*ABA concentration*

Leaf [ABA] in WW plants was 332.5 ng $g^{-1}$ DW, 372.6 ng $g^{-1}$ DW and 194 ng $g^{-1}$ DW at DAS 3, 7 and 16 respectively (Fig. 6A, C and E). With respect to the WW, leaves of MWS plants showed an increase in [ABA] by 16 %, 42% and 51% at DAS 3, 7 and 16. In contrast WS plants showed a significant 2-fold [ABA] increase at DAS 3 compared to WW plants and a 4-fold increase at DAS 7 and 16. On SWS plants [ABA] was 4-fold higher than that of WW plants at DAS 3 increasing to 15-fold and 12-fold higher on DAS 7 and DAS 16 respectively.

WW plants bud, flower and pod [ABA] was constantly 2-fold higher than that of the leaf (Fig. 6 B, D and F). Similar higher bud and flower [ABA] compared to the leaf were found in MWS, WS and SWS plants, with a steady 2/3-fold higher value. Pod [ABA] of MWS and WS was only 1.5-fold higher than that of the leaves whilst under SWS stress condition a ~25% increase in leaf [ABA] compared to pod [ABA] was observed. MWS plants exhibited an average increase in bud, flower and pod [ABA] of 50% compared to the WW plants whereas the increase in WS was 3-fold, 5-fold and 4-fold respectively. With respect to the WW plants, SWS plants exhibited a 6-fold increase in bud [ABA] a 4.5-fold increase in pod [ABA] and a 7-fold increase in flower [ABA].

In WW and MWS plants no statistically significant differences were observed between –AT and +AT plants in any tissues at any assessment timing except for the leaf at DAS 3 where MWS+AT exhibited a significant decrease in [ABA] compare to the MWS-AT. In contrast, AT application significantly decreased leaf [ABA] compared to the –AT at DAS 3 and DAS 7 as well as [ABA] in flowers and pods. Despite not being statistically significant, AT reduced bud and leaf [ABA] at DAS 16 on WS plants, compared to –AT plants by 33% and 47% respectively. SWS+AT plants showed significantly lower [ABA] compared to SWS-AT plants for all the tissues at each assessment (DAS).

*Yield components*

Plants grown under WW condition had a seed dry matter production of ~16.6 g and ~350 pods per plant on average (Figure 7A, B, C and D). When grown under MWS, WS and SWS conditions plants showed an average decrease of 10%, 24% and 36% in seed dry matter and 9%, 37% and 53% in pods per plant. AT

application on WW plants decreased seed dry matter and pods per plant by on average 5% and 9% respectively and while the effect on pods per plant was significant in both the experiments, seed dry matter was statistically reduced only in Experiment I. AT application under MWS, however, did not show any effect on pods per plant whilst in Experiment I, a significant increase by 6% was found when compared to the MWS-AT plants. AT application in WS plants increased both seed dry matter and pods per plant by ~12% on average in both experiments. However, in Experiment I no statistically significant differences were recorded between WS-AT and WS+AT plants for pods per plant. No significant effects of AT were found under SWS conditions on pods per plant. Conversely, when compared to the SWS-AT plants, an average 12% increase in seed dry matter was recorded, significant ($P<0.001$) in Experiment I only.

## Discussion

*The physiological effects of different drought intensities during reproduction*

Water availability over the plant reproduction stage is a key factor for OSR productivity. All the physiological traits examined were significantly down-regulated from the imposition of MWS. Indeed, the physiological decline over the different stress treatments led to a lowered seed production that increased with the severity of the stress treatment. From a stomatal-response point of view, OSR shows a “pessimistic” or “isohydric” response and the results are in accordance with Jensen et al. (1996). There were significant declines in evapotranspiration and WU from MWS, suggesting fast root-shoot [ABA] signaling resulting in stomatal closure. The significant reduction in stomatal conductance from MWS resulted in no significant differences in leaf RWC between WW and MWS. In our experiments

leaf [ABA] was non-linearly and negatively correlated with leaf RWC ($R^2$= 0.58). This suggests that, since OSR exhibits low osmotic adjustment capacity (as reported by Jensen et al. 1996), the “pessimistic” response may be beneficial only when stress is moderate but increasing the magnitude of stress can lead to concomitant decreases in plant water status and $CO_2$ uptake. Therefore, under WS and SWS the reduction in $A_{max}$ was highly significant when compared to the WW plants leading to a slight increase in ¡WUE and a significant non-linear relationship between $g_s$ and $A_{max}$ ($R^2$= 0.63).

Stomatal closure following ABA accumulation significantly increased $L_T$ and $B_T$. In our experiment the two values were less negative and of similar magnitude. In contrast, Guo et al. (2013 and 2015) showed *Brassica rapa* buds with lower water loss and lower temperatures under stress compared to the leaves. This may indicate that the higher drought tolerance of some *Brassica rapa* genotypes compared to *Brassica napus* may be due to the lower sensitivity of reproductive organs to water shortage (e.g. lower stomatal sensitivity to ABA and/or higher osmotic adjustment). Indeed OSR reproduction depends on several factors and hormones and the water status of the reproductive organs may play a pivotal role (Faralli et al. 2016; Mogensen et al. 1997). As expected, increasing soil moisture deficit decreased the leaf RWC and in turn promoted ABA accumulation in the leaf, bud, flower and pod for all the DAS analysed. These results are similar to those of Qaderi et al. (2006) and Faralli et al. (2016). In MWS plants however ABA accumulation was not accompanied by a significant decrease in RWC, suggesting the efficiency of the “isohydric” strategy to cope with moderate water shortage as shown earlier. Strong correlations were found between OSR leaf [ABA] and reproductive organs [ABA] ($R^2$= 0.69) (reproductive organs [ABA] was in turn

475 correlated with seed dry matter production, $R^2$=0.96) confirming leaf-to-
476 reproductive organ ABA translocation, possibly both dependent on root-to-shoot
477 xylem transport (Liu et al. 2004). Significant correlations were also found between
478 $B_T$ (hence transpiration) and $L_T$ (as shown in Fig. 5C), between RWC and $L_T$
479 ($R^2$=0.70) (due to ABA accumulation) and in turn $B_T$ with bud/flower/pod [ABA]
480 ($R^2$=0.63); this overall picture of the link between the leaf and reproductive organs
481 supports the idea of strong source-sink connections in OSR under stress that
482 could potentially be exploited for further breeding programmes focusing on OSR
483 reproductive stage drought tolerance.

484 Yield component analysis showed a significant reduction in seed dry matter
485 production and pods per plant in WS and SWS plant. Pods per plant and seed dry
486 matter were similarly sensitive to water deprivation, leading to similar percentage
487 losses with increasing drought intensities. In OSR, seed yield is determined from
488 the initiation of flowering to mid-pod development (Mendham et al. 1981). Thus
489 while the pods on the main stem are already formed, lateral buds are still opening
490 and hence both pod and seed yield components determination is disrupted by
491 stress over flowering (Mendham et al. 1981). Seed dry matter and pod number
492 were well correlated with leaf RWC ($R^2$= 0.91 and $R^2$= 0.78 respectively) as well
493 as with $B_T$ temperatures ($R^2$= 0.72 and $R^2$= 0.81 respectively) suggesting that leaf
494 and reproductive organ water status is an important trait together with gas-
495 exchange (assimilates availability) for stress determination over reproduction.
496 However, significant correlations were found between [ABA] in the reproductive
497 organs and pod ($R^2$= 0.83) and seed dry matter production ($R^2$= 0.96) as
498 described above, suggesting potential involvement of ABA in reproductive
499 physiology under drought as previously reported for wheat (Westgate et al. 1996).

Indeed further investigations are required to evaluate whether a genotypic variability for the above traits is present in the current OSR varieties and thus whether potential reproductive stage drought tolerance is available.

*The effect of AT on mitigating drought damage on OSR*

Previous work on AT application showed similar gas-exchange results after AT application (in particular Vapor Gard) in well-watered *Vitis vinifera* L. (Palliotti et al. 2013) and *Phaseolus vulgaris* L. (Iriti et al. 2009). The detrimental effects on $A_{max}$ in WW and MWS plants are symptoms of the AT-derived stomatal occlusion that restricted the diffusion of $CO_2$ into the intracellular airspace of the adaxial-sprayed leaf side. In the present work, however, AT-treated WS plants showed a significant sustained assimilation rate when compared to the -AT plants which is consistent with the data of Abdullah et al. (2015). Indeed AT application shifts the non-linear $A_{max}$-to-$g_s$ correlation by sustaining $A_{max}$ and reducing $g_s$ ($R^2$= 0.43). This behaviour dramatically increased leaf $_i$WUE by avoiding the drought-induced decline of $A_{max}$ without negatively affecting photochemistry (Iriti et al. 2009). To confirm this, in our experiments $F_v/F_m$ was never reduced by AT application when compared to each relative -AT control. Previous work speculated that the sustained $A_{max}$ under stress following AT application was due to the significant improvement in plant water status (Abdullah et al. 2015; Faralli et al. 2016). In the present work AT reduced WU over the first days of stress and improved RWC in particular under WS conditions leading to a higher capability of fixing $CO_2$ possibly following i) the higher water resources available and ii) a higher abaxial $CO_2$ uptake due to stomatal opening compensation (Faralli et al. 2016). Moreover in our experiments, plants were positioned ~50 cm from each other inside the glasshouse. Thus, it can be speculated that a field-OSR canopy may benefit more

525 from AT due to high plant density that allows lower soil evaporation and thus a
526 hypothetical higher water-saving effect. Kettlewell (2011), derived a soil moisture
527 deficit threshold for AT application in wheat and suggested that the threshold may
528 vary depending on wheat and AT prices. However, it is generally recognised that
529 wheat has an anisohydric response to water stress, thus no AT-efficiency
530 limitations from stomatal closure should occur. Our data show that, at the gas-
531 exchange level and in an isohydric crop such as OSR, AT efficiency is dependent
532 on the magnitude of the drought-induced stomatal closure and application under
533 MWS or SWS conditions may not give significant effects.

534 Application of AT reduced $g_s$ and slightly increased leaf temperature for all the
535 watering regimes in both the experiments, but however with lower efficiency when
536 ABA-induced stomatal closure occurred. It has been previously reported that AT
537 application decreased $g_s$ without increasing leaf temperature (Faralli et al. 2016;
538 Palliotti et al. 2013) and the experiments confirm this even in SWS conditions. In
539 this context, AT application plays a significant role in minimising the detrimental
540 effects of water stress on reproduction. First, in these experiments AT was applied
541 onto the leaf-canopy and the reproductive organs (buds and flowers) were not
542 treated. The results suggest that AT prevented the drought-induced increase in
543 [ABA] in all the tissues analysed under water deficit conditions. Hence, the water
544 saved in the pot following AT application had a significant role at reducing xylem
545 ABA signaling. To confirm that, temperature analysis showed a reduction in $B_T$
546 suggesting a higher bud transpiration rate under stress conditions if leaf canopy
547 water status is maintained (in our experiments, $g_s$ and leaf temperature showed a
548 strong correlation, $R^2$= 0.98). However, only WS plants were subjected to this

549 beneficial property of AT presumably because of the ameliorated leaf gas-
550 exchange.

551 No significant reduction in $L_T$ values were found under WS+AT and SWS+AT
552 plants when compared to the WW+AT. Since $g_s$ was reduced, an increase in $L_T$
553 was expected due to a reduction in transpiration and thus reduced leaf cooling.
554 However, there is evidence that the leaf heat balance is dependant not only on
555 transpiration but also on plant water status. In Cohen et al. (2005) strong
556 correlations were found between CWSI (crop water stress index measured
557 through thermal imaging) and LWP. In our experiments, the improved RWC may
558 have counteracted the reduction in evapotranspiration leading to no significant
559 reduction in $L_T$ values (thus similar leaf temperatures) between +AT and -AT plants
560 under WS and SWS conditions.

561 Application of AT reduced the yield components of WW plants and only mild
562 effects on MWS plants despite higher seed dry matter production values being
563 recorded for MWS+AT in Experiment I. AT application at 2% v/v on WW *Vitis*
564 *Vinifera* reduced the leaf $CO_2$ assimilation rate, in turn reducing assimilate
565 availability for berry ripening (Palliotti et al. 2013). Similarly, the lower $CO_2$
566 assimilation rate found under WW+AT conditions when compared to WW-AT
567 suggests that AT may decrease the amount of assimilates translocated from the
568 source to the sink, thus reducing the carbohydrate available for seed development
569 under optimal conditions for plant growth. Significant increases, however, with
570 respect to the -AT plants were found under WS and SWS conditions confirming
571 the capacity of the AT to sustain yield under drought in OSR (Faralli et al. 2016,
572 Patil and De 1978). However, in SWS plants, only seed dry matter was sustained
573 following AT application (Experiment I) while in WS plants both pods (Experiment

II) and seed dry matter (Experiment I and II) were enhanced suggesting different AT-mechanisms under the two watering regimes conditions. First, AT canopy application maintained more negative $B_T$ in WS plants but not in SWS plants. Therefore, it is possible that low bud water status is the main factor affecting pod formation possibly by reducing fertilization and/or harming pollen tube growth and thus leading to flower abortion (Guo et al. 2013). This may explain the effect of AT in sustaining pod number under WS but not under SWS. Second, [ABA] in WS+AT and SWS+AT plants was lower than that of the -AT plants. Since in both WS and SWS seed dry matter was sustained compared to their relative -AT, high [ABA] may have significantly disrupted seed set in late pod formation. ABA appears to act as the modulator of ACC levels, thus of ethylene, perhaps leading to increased seed abortion (Gómez-Cadenas et al. 2000) and there is strong evidence that ABA can directly harm seed formation in several crops (as shown by Yang et al. (2001) in rice and by Liu et al. (2004) in soybean). Westgate et al. (1996) suggested that maintenance of high shoot water status under drought reduces the effect of soil water deficit on grain set by reducing the accumulation of [ABA]. Weldearegay et al. (2012) showed that ABA accumulation in wheat spikelet was three-fold higher under stress in the genotype showing higher WU (thus lower soil moisture available over the stress treatment) and this was related to a lower seed set. This work corroborates the hypothesis that maintaining low WU (AT or high transpiration-efficiency genotypes) over key drought-sensitive periods may be beneficial for grain yield production in crops. Thus, minimising ABA signaling under drought may alleviate a detrimental direct effect of the hormone in seed development in OSR.

Collectively, in the context of the OSR reproductive physiology our results show that maintaining high leaf canopy water status by reducing leaf transpiration and reducing ABA signaling helps reproductive organs to maintain water use and avoid grain yield losses. It is proposed that OSR improvement for drought tolerance over the reproductive phase should focus on high WUE canopy and low bud temperatures (thus high buds water-use). This may lead to a i) maximisation of pod formation following the maintenance of high reproductive organs water status and a sustained transpiration-derived cooling under stress and to a ii) sustained seed set and development due to the reduction of drought-induced ABA accumulation.

In this context, the ameliorative effect of AT leaf-canopy application plays an important role in minimising seed yield lost due to water deprivation. However, significant differences in yield and physiological responses were found between AT application and watering regimes. Indeed only under WS conditions was AT application beneficial in maintaining high plant water status, minimising water loss, sustaining $CO_2$ assimilation, lowering ABA signaling and sustaining yield. This may be a useful indication for further in-field exploitation of the AT and its application under MWS and SWS may not give significant cost/effective benefits. However, while this may be true for crop with “isohydric” response to drought (e.g. OSR), AT may not have any restrictive-efficiency in “anisohydric” crops due to lower stomatal control under water stress conditions. Further work with AT in the field level and a screening evaluation of OSR genotypes with the above characteristics would be of major importance to meet the challenge of the global food security under climate change.

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## Figures and Tables

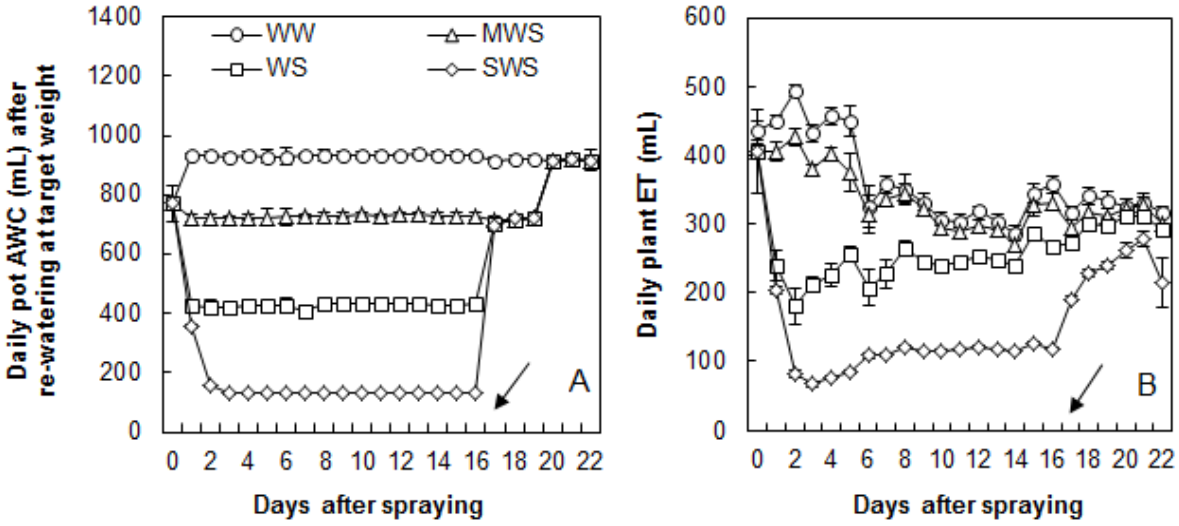
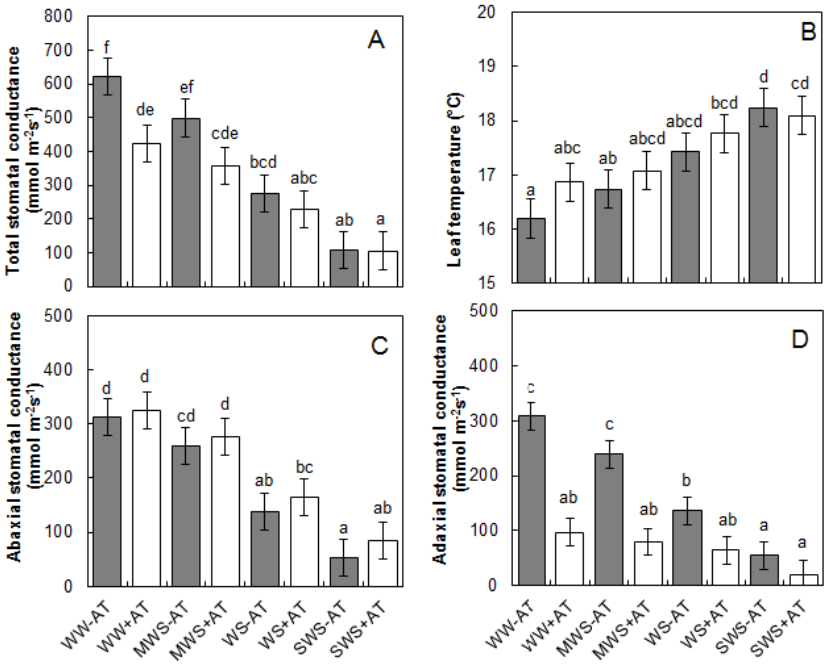



Figure 1. Example of daily pot available content (AWC, A), and daily pot evapotranspiration (ET, B) trends of oilseed rape plants subjected to well-watered (WW), moderate water stress (MWS), water stress (WS) and severe water stress (SWS) watering regimes over flowering stage at days after spraying 0 (DAS 0). Plants were re-watered at DAS 16 (arrows). Data are means (n=7) ± standard error of the mean (SEM). Data from Experiment II.

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774 Figure 2. Total stomatal conductance ($g_{stot}$, mmol $m^{-2}s^{-1}$, A), leaf temperature (°C,
775 B), abaxial stomatal conductance (abaxial $g_s$, mmol $m^{-2}s^{-1}$, C) and adaxial stomatal
776 conductance (abaxial $g_s$, mmol $m^{-2}s^{-1}$, D) data of oilseed rape plants subjected to
777 WW, MWS, WS and SWS watering regimes over flowering stage and treated with
778 water (-AT) or 1% v/v Vapor Gard (+AT). AT was applied at days after spraying 0
779 (DAS 0). Data are means (n=18, collected at DAS 1, 3 and 8 and pooled) ±
780 standard error of the differences of the mean (SED). Columns with different letters
781 are significantly different according to the Tukey's test ($P$<0.05). Data from
782 Experiment I.

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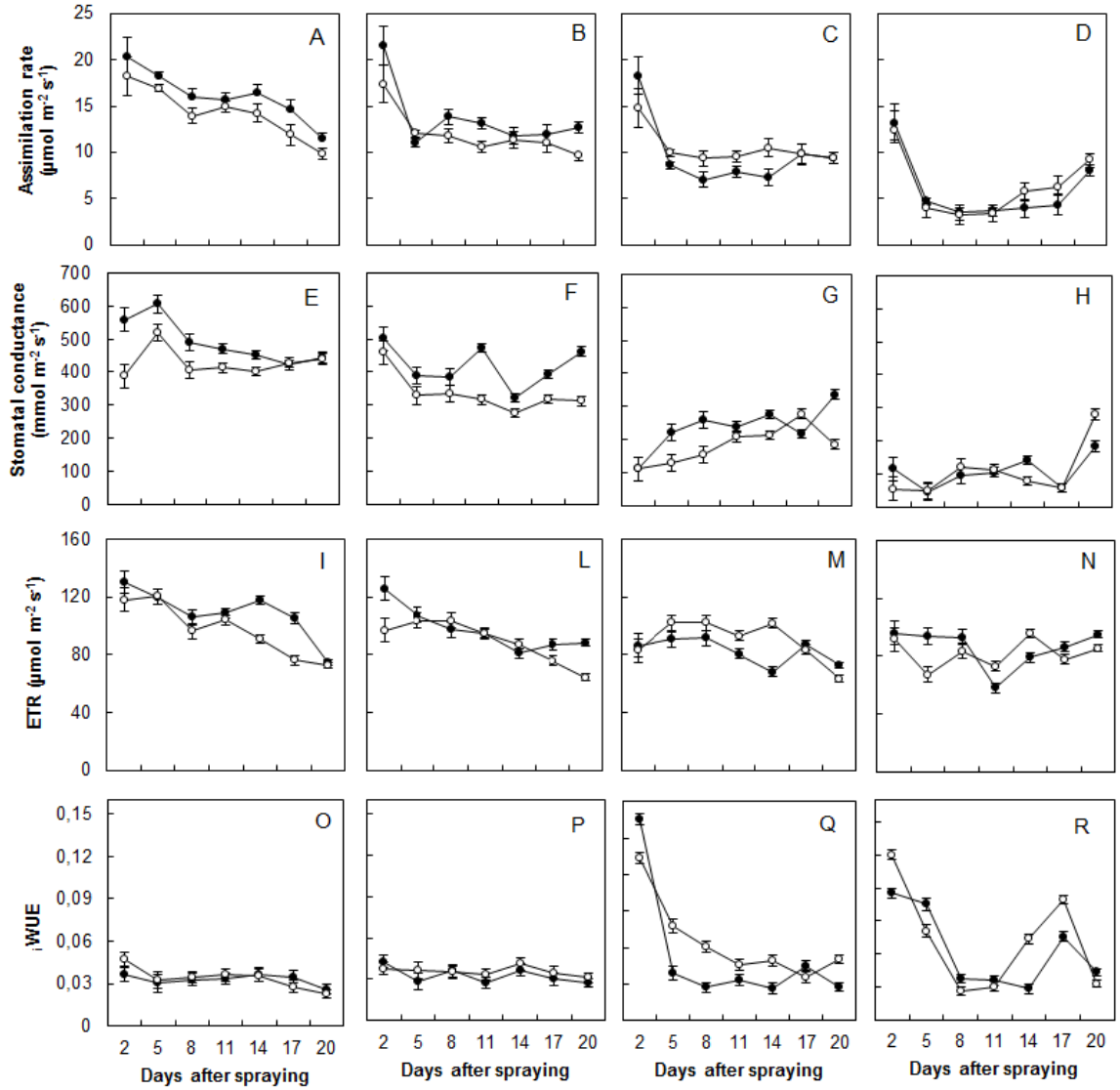


Figure 3. $CO_2$ assimilation rate, stomatal conductance, electron transport rate (ETR) and intrinsic water use efficiency (¡WUE) trends of oilseed rape plants subjected to WW (A, E, I,O), MWS (B, F, L, P), WS (C,G, M, Q) and SWS (D, H, N, R) watering regimes over flowering stage and treated with water (close circles) or 1% v/v AT (open circles). AT was applied at days after spraying 0 (DAS 0). Plants were re-watered at DAS 16. Data are means (n=4, subjected to a two-way ANOVA for each DAS) ± standard error of the mean (SEM). Data from Experiment II.

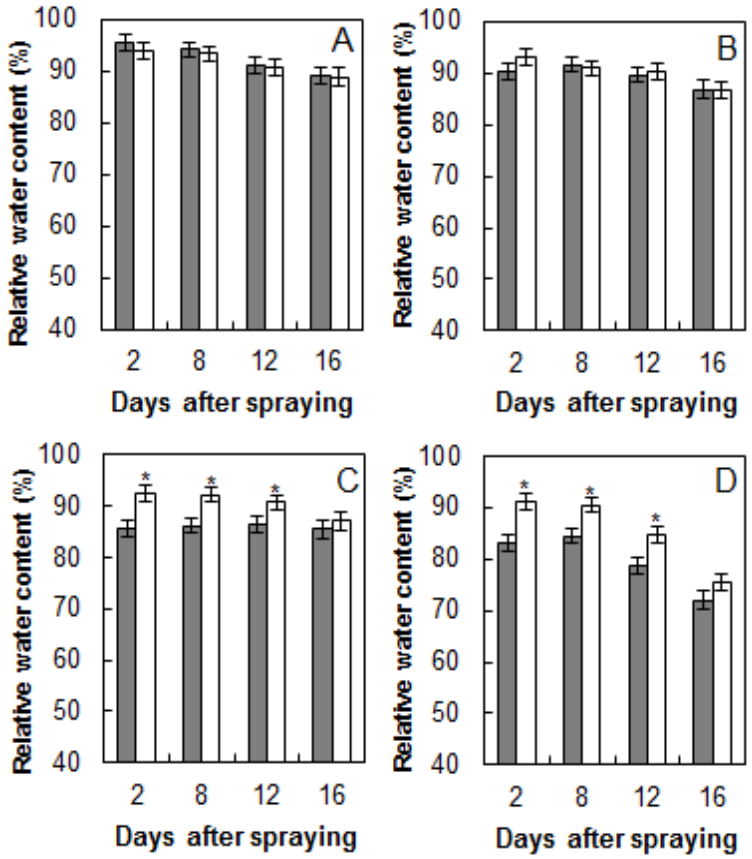


Figure 4. Leaf relative water content (RWC, %) of oilseed rape plants subjected to WW (A), MWS (B), WS (C) and SWS (D) watering regimes respectively over flowering stage and treated with water (close bars) or 1% v/v AT (open bars). AT was applied at days after spraying 0 (DAS 0). Plants were re-watered at DAS 16. Data are means (n=4, subjected to a two-way ANOVA for each DAS) ± standard error of the differences of the mean (SED). Significant differences between means of the -AT and +AT are highlighted with asterisks.

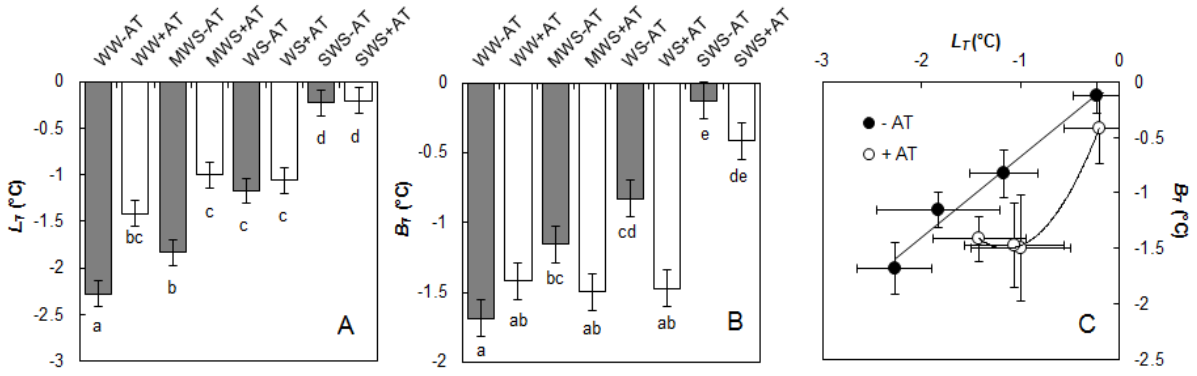


Figure 5. Leaf temperature - ambient temperature ($L_T$, A), bud temperature - ambient temperature ($B_T$, B) and their correlation of oilseed rape plants subjected to well-watered (WW), moderate water stress (MWS), water stress (WS) and severe water stress (SWS) watering regimes over flowering stage and treated with water (-AT) or 1% v/v Vapor Gard (+AT). AT was applied at days after spraying 0 (DAS 0). Data are means (n=32, subjected to a two-way ANOVA) ± standard error of the differences of the means (SED). Different letters represent significant differences according to the Tukey's test ($P$<0.05). In C, data points are means ± SD and lines were fitted with regression. Data from Experiment II.

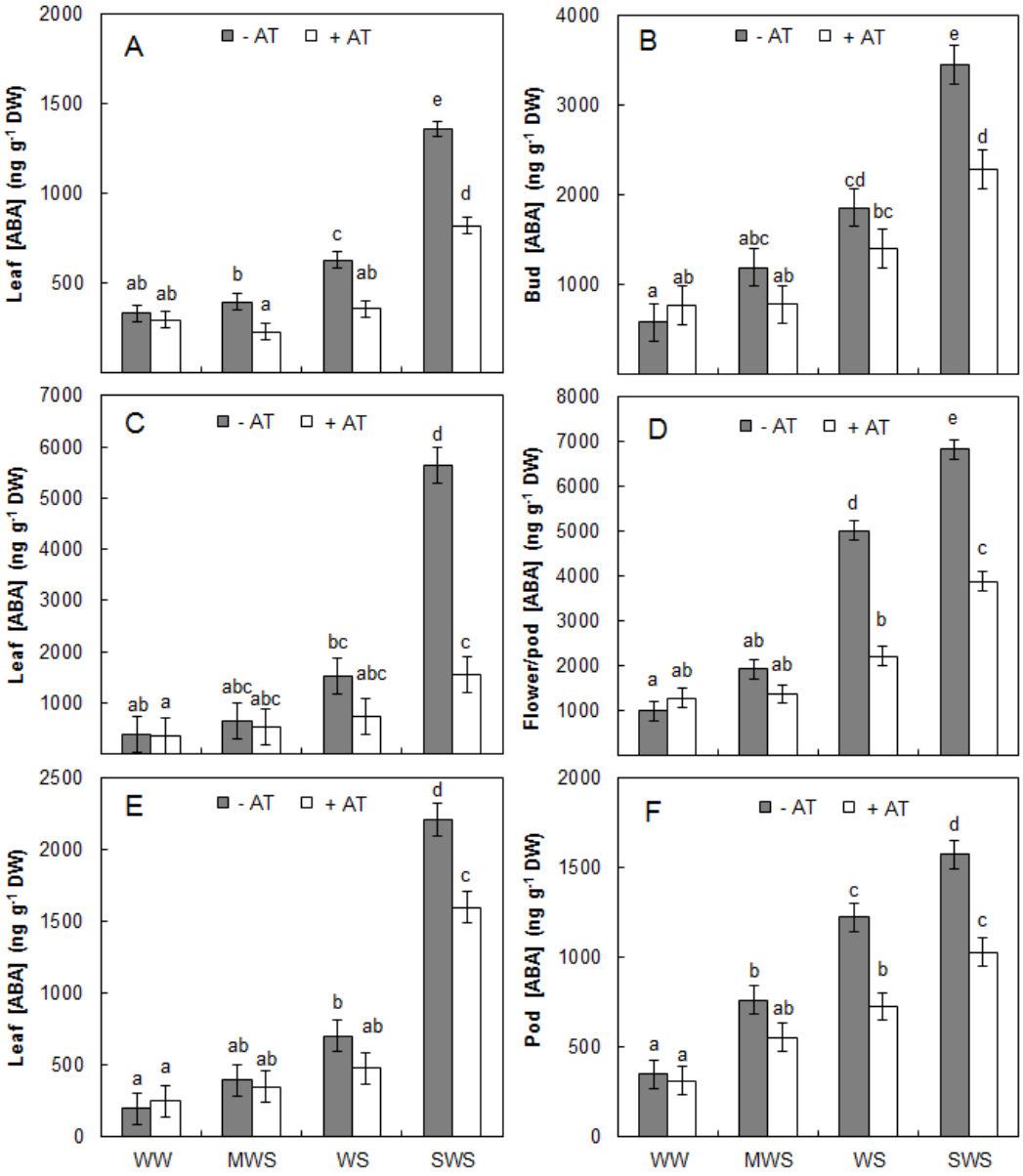


Figure 6. ABA concentration ([ABA]) of oilseed rape plants subjected to WW, MWS, WS and SWS watering regimes over flowering stage and treated with water (close columns) or 1% v/v AT (open columns). AT was applied at days after spraying 0 (DAS 0). Samples were collected at DAS 3 (A and B, leaf and bud respectively), DAS 7 (C and D, leaf and flower respectively) and just before re-watering at DAS 16 (E and F, leaf and pod respectively). Data are means (n=4), subjected to a two-way ANOVA for each DAS ± standard error of the differences of the mean (SED). Different letters represent significant differences according to the Tukey's test ($P$<0.05). Data from Experiment II.

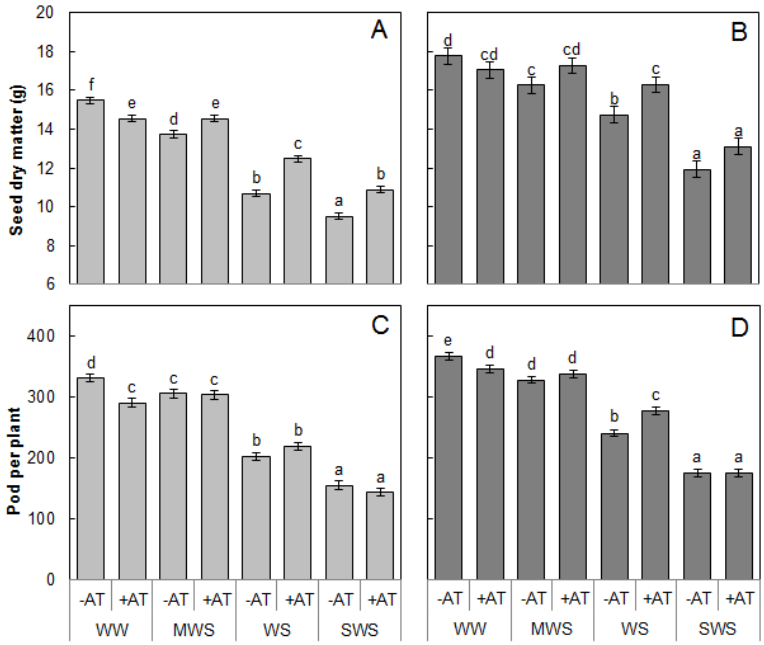


Figure 7. Seed dry matter (A - Experiment I; B, Experiment II) and pods per plant (C - Experiment I; D, Experiment II) yield components of oilseed rape plants subjected to WW, MWS, WS and SWS watering regimes over flowering stage and treated with water or 1% v/v AT. AT was applied at days after spraying 0 (DAS 0). Data are means (n=6 for Experiment I and n=7 for Experiment II, subjected to a two-way ANOVA) ± standard error of the differences of the mean (SED). Different letters represent significant differences according to the Tukey's test ($P$<0.05).

Table 1 Average plant water use (mL, WU) of oilseed rape plants subjected to well-watered (WW), moderate water stress (MWS), water stress (WS) and severe water stress (SWS) watering regimes over flowering stage and sprayed with Vapor Gard (+AT) or water (-AT). Asterisks represent statistical significant differences between –AT and +AT plants regardless soil moisture regime. Data are means (n=13) of Experiment I and II ± standard error of the mean (SEM)

| | From DAS 0 to DAS 8 | | | From DAS 9 to DAS 16 | | |
|---|---|---|---|---|---|---|
| | -AT | +AT | +AT effect on WU | -AT | +AT | +AT effect on WU |
| WW | 300 ± 9.5 | 269 ± 12.5 | 10.4 % (31 mL)* | 264 ± 8.1 | 256 ± 12.6 | 3.1 % (8 mL) |
| MWS | 269 ± 10.3 | 263 ± 10.4 | 2.3 % (6 mL) | 266 ± 8.8 | 264 ± 9.5 | 0.8 % (2 mL) |
| WS | 175 ± 7.6 | 165 ± 6.6 | 5.8 % (10 mL)* | 202 ± 4.1 | 198 ± 4.4 | 2.0 % (4 mL) |
| SWS | 80 ± 4.9 | 78 ± 3.9 | 2.5 % (2 mL) | 78 ± 1.4 | 77 ± 1.5 | 1.3 % (1 mL) |