A Thesis Submitted for the Degree of Doctor of Philosophy at

Harper Adams University

Film antitranspirant application to enhance spring drought tolerance of oilseed rape (*Brassica napus* L.)

Thesis submitted to the Harper Adams University for the degree of Doctor of Philosophy

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Abstract

Oilseed rape (OSR) is an important crop worldwide and drought occurring during reproduction is the main limitation on yield. Following the recent problems in breeding new drought tolerant OSR varieties, there is potential for agronomic exploitation of film antitranspirant (AT) application to avoid yield losses.

The main objectives of this study were i) to evaluate whether AT application during OSR reproductive stages may sustain yield under drought; ii) to elucidate the physiological mechanisms behind the potential AT-induced yield mitigation; iii) to explore the apparent physiological inconsistency between the old and the new work on AT.

In four glasshouse experiments and two field experiments drought conditions were applied at different phenological stages and OSR plants were sprayed with different AT chemicals. Three experiments were carried out on OSR seedlings in growth chambers under different atmospheric CO_2 concentration, drought conditions and sprayed or not with AT.

The data showed physiological amelioration and yield loss mitigation from application of 1% v/v (glasshouse) and 1 L ha^{-1} (field) of a commercially-available AT (Vapor Gard) just prior to flowering (GS 6.0) on droughted OSR. The AT application under drought triggered complex physiological mechanisms, collectively leading to a sustained photosynthetic rate, reductions in ABA concentration, improved leaf water status and therefore sustaining pod and, to a greater extent, seed production. In addition, the data show that increasing the atmospheric CO_2 concentration does not have an ameliorative effect on OSR seedling's drought tolerance. On the contrary, the experiments showed that in recent years the increased atmospheric CO_2 concentration may have counteracted the source-limiting effect of AT following increasing substrate available at the site of carboxylation, therefore leading to higher potential efficiency of AT chemicals under water-limited conditions.

To conclude, AT may deserve further investigation as a potential and flexible management tool to counteract drought damage to OSR yield.

Declaration

This thesis has been written by myself and describes the work carried out by myself unless otherwise stated. Information from other sources has been fully acknowledged and referenced in the text.

Michele Faralli

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This work is for Riccardo, brother and friend. You will always be by my side.

Ad maiora semper.

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Chapter 5

Chapter 1

Introduction to Oilseed rape, drought and antitranspirant

Oilseed rape (*Brassica napus* L.) production and economic importance

Oilseed rape (Brassica napus L.; genome AACC, 2n = 38) is a member of the Brassicaceae family (nomen conservandum: Cruciferae), known since the 13th century as an oil crop (Snowdon et al. 2007). Resulting from a natural crossing between Brassica oleracea L. (2n = 18) and Brassica campestris L. (2n = 20), it is an amphidiploid interspecific hybrid, probably originating in the Mediterranean region of southwest Europe (natural habitats of the two diploid parental species) (Snowdon et al. 2007). Oilseed rape is known by different names worldwide (canola, rapeseed, rape, rapa, colza), and is widely cultivated in the temperate zone (Fig. 1). Canada, Western Europe, China and India are the largest growers of oilseed rape (Faostat, 2013).

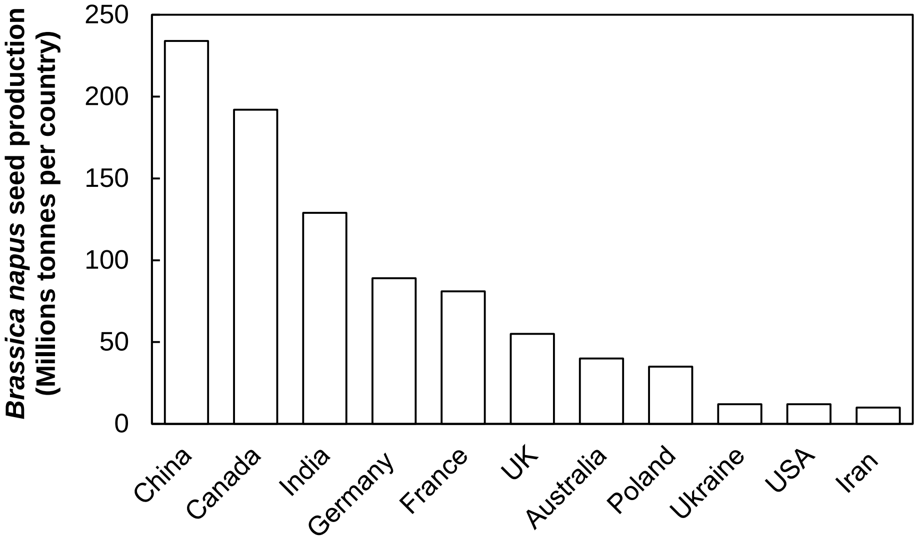

Figure 1. Oilseed rape production (Mt). (Data from Faostat, 2013)

In the past, oilseed rape was used mainly for industrial use, but over the last forty years interest in the crop has increased thanks to the development of low erucic acid varieties “0” and “00” (Downey, 1990). The first “0” variety, released in

Canada in the 1970s, contained 1% of erucic acid, while the previous rapeseed varieties had up to 50% (Stefansson and Hougen, 1964). Erucic acid (cis 13-docosenoic acid, 22:1n-9) in high doses can lead to cardiac problems and other related health problems. It is a long-chain fatty acid and its properties are optimal for industrial use: stability at high temperatures, high smoke point and fluidity even at low temperatures (Piazza and Foglia, 2001). The “00” varieties, released for the first time in 1974, are low erucic acid and low glucosinolate content varieties. Glucosinolates are sulphuric compounds that issue toxic by-products during monogastric animal digestion (Snowdon et al. 2007). The marketing of these new varieties increased the interest in growing rape, and it has become the second largest source of edible oil in the world after soybean (Aider, 2011). The 2013 world arable land area of rapeseed was approximately 36 million hectares (ha) (Faostat, 2013), which continues the positive trend of increasing area from 7 million ha in 1965 to 27 million ha in 2005 (Berry and Spink, 2006). The average yield in the world stands at 1.99 tonnes/ha, increasing every year. In the United Kingdom 715,000 ha of arable land are cultivated with oilseed rape (Faostat, 2013).

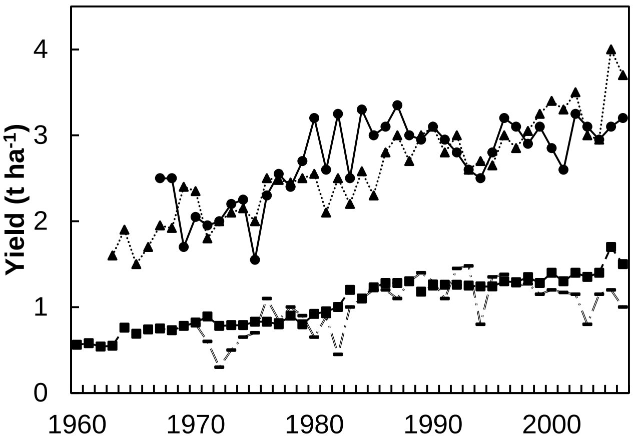

Figure 2. Oilseed rape seed yield trends: World (square symbols), Australia (lines symbols), Germany (triangle symbols) and UK (round symbols). Source: Berry and Spink (2006), Faostat Website (2013)

The rate of yield increase varies widely around the world. The average yield in the world increased from 0.6 t ha^{-1} in 1960 to 1.8 t ha^{-1} in 2012, with an average increase per year of 26 kg/ha. In some nations the average yield per year is continuing to increase (China, India, Germany, Canada), others such as UK (Fig. 2), France and Poland, appear to have halted or decreased their annual rate (Berry and Spink, 2006).

Botanical characteristics

The name of the Cruciferae family is derived from the typical flower aspect: four petals in cross form with four alternating sepals (Fig.3).

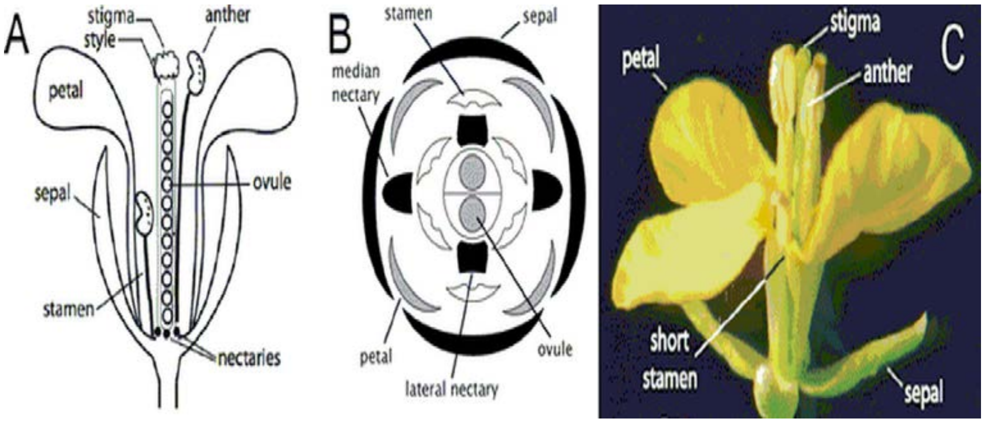

Figure 3. (A) Schematic longitudinal section of Cruciferae flower (B) Schematic cross-section of flower with relative location of floral organs (C) Whole Brassica flower (from Hampton et al. 2010)

Racemose inflorescences have indeterminate flowering, developing with an ascending process from the lowest bud on the main raceme. The yellow flower has two lateral short stamens and four median longer stamens. The ovary is superior and the pistil is made up of two fused carpels (Rendle, 1925). *Brassica napus* is a facultative cross-pollinating species, with a high self-pollination rate. The cross-pollination increases when pollinator insects are copious (Snowdon, 2007).

Oilseed rape is grown as a winter-sown crop in Europe and Asia. The spring form is preferred in Canada and Northern Europe because the long cold winter does not allow survival (Diepenbrock, 2000). The oilseed rape primary gene pool can be distinguished into two *Brassica napus* subspecies: *Brassica napus* ssp. *napobrassica* (Swedes oilseed rape) and *Brassica napus* ssp. *napus*. The latter includes winter and spring species and fodder rape forms (Snowdon, 2007). Spring oilseed rape varieties do not require low temperature to induce flowering while the winter varieties, sown in autumn, survive the cold season in leaf rosette form: the floral primordia is induced by cold temperatures in late autumn and the flowering process is triggered by the increasing photoperiod and temperature the following spring (Filek et al. 2007).

Phenology and growth stages

The development of a plant is defined as the orderly progress of defined stages, from germination to death (McMaster, 1997). The main climatic variables that are relevant to the life cycle of the plants are the temperature and the photoperiod (McMaster, 1997). In oilseed rape, the effects of these environmental factors have been extensively investigated both in winter and in spring varieties (Filek et al. 2007, Robertson et al. 2002).

In winter oilseed rape temperature plays an important role in the flowering process: in order to induce the reproductive stage, plants need to meet the cold requirements (vernalization), between 0 and 5°C for 30 days (Filek et al. 2007).

The life cycle of oilseed rape can be divided into nine stages: germination, leaf development, formation of the side shoots, stem elongation, inflorescence emergence, flowering, development of the pods, seeds ripening and plant senescence (BBCH canola growth stages, 1990).

After sowing, the reserves in the seeds are hydrolysed following imbibition processes. This leads to the formation of the cotyledons and its establishment depends on temperature and soil humidity (Marshall and Squire, 1996).

The vegetative stage, which begins following cotyledon formation, continues until flower formation. In this stage the differentiation of leaves (“rosette”) and, before spring, the internodes elongation (“stem elongation”) occurs. The “rosette” stage is a key factor for the protection of the autumn-induced floral primordium during the winter period (Filek et al. 2007).

At the end of the winter, with higher temperature (> 5°C) and with longer photoperiods (12 h), both stem elongation and inflorescence appearance begin: these stages develop simultaneously, and their phenological identification is sometimes inaccurate (Snowdon et al. 2007): the beginning of each stage is not dependent on the completion of the preceding stage (Snowdon et al. 2007), thus several growth stages tend to overlap. From the onset of budding each growth stage is commonly determined by examining the main flowering (terminal) stem.

The flowering starts with an ascending process from the lowest bud to the main raceme. At the end of the flowering stage the main phases are pod development and seed ripening: in these stages there is an increasing accumulation of oil in the seeds and a significant loss of water (Snowdon et al. 2007)

Two numerical keys were generated to determinate oilseed rape development stages (Harper, 1973, Weber and Bleiholder, 1990). In this work the BBCH-scale (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie growth scale) adapted from Meier (2001) will be used. The BBCH scale is centesimal, and divides the crop cycle in ten main stages (0-10) in turn divided in ten sub-stages (00-99) (Table.1)

Table1. Winter oilseed rape growth stages with codes (Meier (2001), BBCH canola growth stages, 2001)

Growth stage	Code(s)	Description
Germination	0.0-0.9	Dry seed to cotyledons emergence through soil surface
Leaf development	1.0-1.9	Cotyledons unfolded to 9 or more leaves unfolded
Formation of side shoots	2.0-2.9	No side shoots to 9 or more side shoots detectable
Stem elongation	3.0-3.9	“Rosette” stage to 9 or more extended internodes
Inflorescence emergence (bud stage)	5.0-5.9	Flower buds enclosed to “yellow bud” stage
Flowering	6.0-6.9	First flower open to majority of flower fallen (end of flowering)
Development of fruit (pod development)	7.0-7.9	10% of pods developed to al pods reached final size
Ripening (Seed development)	8.0-8.9	Green seeds to full ripening (seeds dark and hard)
Senescence	9.7-9.9	Plant dead and dry: ready to be harvested

Yield structure of oilseed rape

The yield structure of oilseed rape is a cumulative interaction between plant population density, number of pods per plant, number of seeds per pod and individual seed weight (Fig.4) (Diepenbrock, 2000). This flexible yield structure is based also on secondary components as seed yield per pod, number of seeds per plant, number of pods per area and seed yield per area (Diepenbrock, 2000).

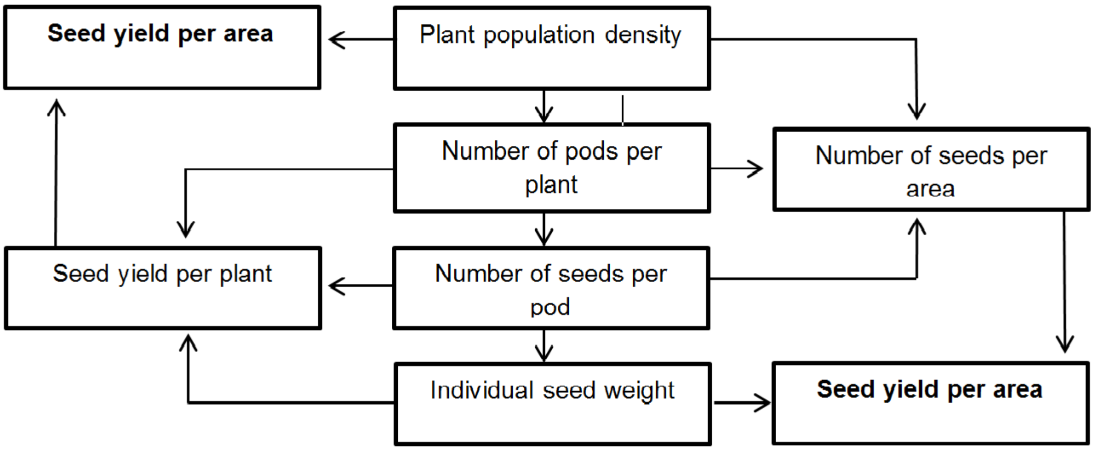

Figure 4. Primary components of the yield structure of winter oilseed rape. Adapted from Diepenbrock (2000)

The plant population density is an important factor for the yield of the crop as it is capable of influencing the physiology and the morphology of the whole yield structure (Diepenbrock, 2000). Although the density is depending on both biotic and abiotic stresses that are able to negatively influence the emergence, there is a general trend in decreasing the plant population (50 seeds/m^2) (Momoh and Zhou, 2001): high plant density (12.75 X 10^4 plants ha^{-1}) has been related to a decrease in branches, pod per branch and oil content with a non-significant increase in economic yield compared to the low plant density (9.75 X 10^4 plants ha^{-1}). In some cases it has been shown that decrease the seed rate at sowing could be effective in decreasing pests (Momoh and Zhou, 2001). The emergence inhomogeneity due to abiotic or biotic factors has been linked to a decrease in plant performance and canopy homogeneity, leading to lower yield (Sierts et al. 1987).

There is a strong and inverse relationship between number of pods per plant and number of plants per unit area (Diepenbrock, 2000) as, increasing plants' number, canopy light interceptor decreases and thus photoassimilates availability. Other factors affecting pod formation are the influence of abiotic stresses (Qaderi et al.

2006), the nutrient availability (Rathke et al. 2006) and the hormone metabolism and pattern (de Bouille et al. 1989). A limitation in nitrogen contribution and the delay in sowing seem to have similar effect on pod formation, reducing flower fertility, leaf area index and assimilate partitioning (Richards and Thurling, 1978).

The number of seeds per pod is negatively related to the number of pods: a decrease in pod number due to a delay in sowing or abiotic factors leads to an increase in seeds per pod (Diepenbrock, 2000). In oilseed rape, the number of seeds per pod is variable from the main to the lateral branches (Richards and Thurling, 1978).

Yield physiology of oilseed rape

The yield of oilseed rape is defined as the produced seed weight at 9% moisture content (Berry and Spink, 2006). Oilseed rape yield potential, defined as the yield of a cultivar when grown in environments to which it is adapted, with nutrients and water non-limiting and with pests, diseases, weeds, lodging, and other stresses effectively controlled, is estimated to be around 6.5 t ha^{-1}, while in UK the average yield hardly exceeds the 3.5 t ha^{-1} (Berry and Spink, 2006). According to Berry and Spink (2006) maximizing the yield of oilseed rape is possible by improving and/or sustaining two yield components i) seed yield per area and ii) individual seed weight.

The number of seed per area (sink) is determined during a crucial stage that starts at the beginning of flowering and lasting around 300 growing degree days after mid-flowering (Mendham et al. 1981). This 20-25 days window (depending on the environmental conditions) is the most important phase for pod and then seed abortion and thus the light radiation intercepted by photosynthetic organs (leaves, pods and stem) is of great importance (Mendham et al. 1981). The large number of flowers during this period has an impact on the canopy photosynthetic efficiency, due to a large amount of photosynthetic active radiation reflected (Berry and Spink, 2006). This detrimental effect is partially compensated by pod and stem assimilation (Fig.5) (Diepenbrock, 2000). However, the area generated by the pods (Pod area index, PAI) is inadequate to offset the leaf area (Leaf area index, LAI) deficiency leading to an insufficient area to support the pods development (Diepenbrock, 2000). It has been, in fact, extensively reported that the inverse relationships between seed per area, seed per pod and number per pod results in an optimum of 8000 pods/m^2: smaller canopies were too small to trap all the incident radiation, reducing seed number per area. However bigger canopies (over 8000 pods/m^2) resulted in i) high light reflectance of the thick flower layer and ii) lower radiation reaching the photosynthetic organs and thus decreasing seed per pod and seed per area (Berry and Spink, 2006). According to Fray et al. (1996), an apetalous oilseed rape was able to better sustain pod and seed set in the lower canopy compared to conventional varieties.

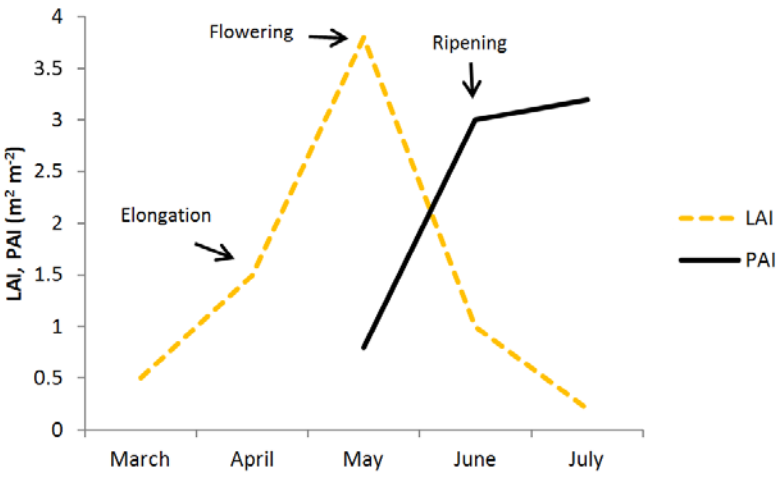

Figure 5 Development of spring PAI (Pod area index) and LAI (Leaf area index) in winter oilseed rape. Adapted from Diepenbrock (2000)

Seed weight is determined from mid-flowering to physiological maturity: the two yield components overlap their determination period and thus the late flowering stage is recognised to be a key factor on yield determination (Mendham et al. 1981). During the post-anthesis phase the green area index (GAI) turn in favour of the pods (60% of the total GAI), while leaves represent only a 20% (Grammelvind et al. 1996). However the photosynthetic capacity of the pod is estimated to be around 50% of the leaves (Grammelvind et al. 1996) and the seed filling process required a 45% more assimilates than the pre-anthesis biomass (Sinclair and de Wit, 1975): due to this, upper pods becomes easily light-saturated while lower-canopy pods received a small amount of light. This source-limiting factor reduces the radiation use-efficiency from 1.2-1.7 g/MJ (pre-anthesis) to 0.4-0.75 g/MJ (post-anthesis) according to Berry and Spink (2006). Achieving a uniform-distributed solar irradiation is the major objective for the seed weight yield component improvement, and erectophile pods (Fray et al. 1996) and un-lodged plants were able to better support seed filling (Baylis and Wright, 1990).

Agronomy and crop protection

Oilseed rape is mainly grown as a break crop in rotation with *Triticum aestivum* and *Hordeum vulgare* to control weeds and plant disease (Devos et al. 2005). In the UK, the winter varieties are mainly grown (HCGA, 2013). Farmers can choose between a large number of “pure line” and “hybrid” varieties. Optimum sowing date varies, depending on latitude and weather conditions. In England and Wales seeding between mid-August and mid-September is recommended, while in Scotland sowing cannot be delayed beyond the first days of September (HCGA, 2013). However, sowing must be early enough to allow the plant to reach the rosette stage (6-8 leaves) before winter, so as to resist frosts.

Oilseed rape can grow on a wide range of soils. Growth is restricted by poor drainage, soil compaction and soil pH less than 5.5 (HCGA, 2013).

During the vegetative growth, oilseed rape may be affected by a number of diseases and pests. Phoma leaf spot, stem canker, light leaf spot, sclerotinia stem rot, clubroot and verticillium wilt are the most common diseases. Slugs, cabbage stem flea beetle, peach-potato aphid, pollen beetle and seed weevil are the most common pests (Canola Council of Canada, 2013).

Oilseed rape requires high fertilization rate, especially of nitrogen, to ensure maximum light interception and thus crop photosynthetic efficiency. Harvesting begin when the seeds moisture content is below 14% (for winter varieties it occurs around the end of June). Rape seeds are dried and stored at 8-9% of moisture content (Canola Council of Canada, 2013).

Drought and oilseed rape

Brassica oilseed crops (i.e. *Brassica napus, Brassica juncea, Brassica rapa, Brassica carinata, Brassica campestris*) are the third largest vegetable oil crops in the world after soybean and oil palm (FAOSTAT, 2013) and their economic importance is rising worldwide. Among these species, *Brassica napus* L. (oilseed rape) is the most important species in the temperate zone (Snowdon et al. 2007). Therefore, the 2013 world arable land area of oilseed rape was approximately 36 million hectares (ha) (FAOSTAT, 2013), which continues the positive trend of increasing area from 7 million ha in 1965 to 27 million ha in 2005 (Berry and Spink, 2006). The average yield in 2013 fluctuated from 3.5 t ha^{-1}, in countries were winter varieties are adopted, to 1.5 t ha^{-1} in spring varieties-grown countries (Berry and Spink, 2006).

Environmental stresses are playing a major role in the reduction of the agricultural productivity (Cattivelli et al. 2008). Drought stress is threatening the production of the major crops worldwide such as wheat (Kettlewell, 2014), maize (Chapman and Edmeades, 1999), rice (Boonjung and Fukai, 1996) and potatoes (Deblonde and Ledent, 2001). Climate change has been related to increased drought events over the crop cycle and the unpredictability of the severity and the duration of these events are likely to condition the crop adaptation and production (Cattivelli et al. 2008).

Winter and spring oilseed rape (OSR) are mainly grown in temperate zones where an adequate amount of rainfall may be expected to exploit the yield potential of the elite breeding lines (Berry and Spink, 2006). However the prominent and steady trend in increased drought periods has underlined water-limited periods for winter OSR yield in the Mediterranean area (Papantoniou et al. 2013) and France (Champolivier and Merrien, 1996) and spring OSR yield in Canada (Wan et al. 2009) and Australia (Thurling 1991). A review by Berry and Spink (2006) suggested that even in the UK spring drought conditions may be one of the major factors involved in the yield restriction of OSR.

The aims of this review are to i) examine the present drought-limiting traits of OSR from a physiological point of view ii) compare the results of the major research on the yield decline of the crop under drought iii) compare the results with other crops iv) evaluate future objectives to improve the drought tolerance of the crop from a breeding and management point of view.

Drought tolerance of oilseed rape

Drought and crop phenology

Drought events are described as periods when the water uptake by the crop exceeds the water ensured by the rainfall (Cattivelli et al. 2008). All the crops respond to drought periods with complex strategies that involve molecular signals, hormones alteration and physiological and morphological modifications (Cattivelli et al. 2008). The drought resistance of plants can be divided into four basic types: drought avoidance (the capacity to avoid plant tissues and cells dehydration under drought stress), drought tolerance (the capacity to sustain function when the plant is dehydrated), drought escape (the “early flowering capacity” of the plants when drought events occur), and drought recovery (the capacity recovery of function upon dehydration).

OSR response to drought can be classified as “drought avoiding”, “pessimistic” or “near-isohydric” and thus a plant that tends to maximally preserve the water resources by triggering a relatively fast stomatal closure to maintain fairly constant water potential (Jensen et al. 1996). The magnitude of drought effects on OSR production depends on the phenological timing, duration and severity of the stress (Jensen et al. 1996). During the vegetative stages (emergence until bud emerging), OSR shows good recovery ability after drought stress periods (Thurling 1991). On the contrary, even short water deficits may lead to yield decline (Jensen et al. 1996, Champolivier et al. 1996) when they occur over the yield-determination periods (i.e. early flowering to mid pod development stages): in several report, water deficit during this time-frame can decrease seed dry matter production up to 40% (Champolivier et al. 1996, Istanbulluoglu et al. 2010).

The effect on yield and yield components

In recent years, the OSR yield has been subjected to strong increases due to the development of elite high-yield varieties and to the improved crop management (Snowdon et al. 2007). However, despite the steady yield increase in all the growing countries, there is an evident variability in the economic yield around the world within a range of ~4 t ha^{-1} (Germany, France) and ~1 t ha^{-1} (India) (Berry and Spink, 2006). This enormous variation is due to several factors such as species (*Brassica Juncea* lower yielding species compared to *Brassica napus*), reproductive cycle (winter forms higher yielding compared to spring forms) and environmental conditions (drought, heat, salinity). According to Berry and Spink (2006), the most common rapeseed grown in Europe and China should achieve the 6.5 t ha^{-1} economic yield. However, northern Europe growing countries showed an average yield of ~4 t ha^{-1} (Diepenbrock, 2000). One of the main limiting factors for yield is the water available over the reproductive stages (Richard and Thurling, 1978). Berry and Spink (2006) suggested that assuming a water availability of between 143 and 224 mm (depending on the soil properties), in the UK, OSR required an additional water supply from 91 to 172 mm between mid-March and July and that the average spring-rainfall (45-85 mm) may not be enough for yield exploitation. Experiments carried out in the north-east of Turkey (Tekirdag province), showed that OSR grown in a drought-prone environment (annual rainfall of 546 and 304 mm) results in a 4.80 t ha^{-1} seeds yield when an additional 250 mm (on average) of water was applied over flowering/pod development/seed ripening stages (Istanbulluoglu et al. 2010). In contrast the rain-fed OSR exhibited a 3.30 t ha^{-1} yield and strong increases in grain yield (~0.70 t ha^{-1}) were found when an average of ~60 mm was applied over flowering stage

(Istanbulluoglu et al. 2010). Flowering stage has been recognized as the key drought-sensitive stage in *Brassica napus* in both glasshouse (Champolivier et al. 1996; Sinaki et al. 2007; Gan et al. 2004) and field experiments (Wright et al. 1995; Jensen et al. 1996). Nevertheless, significant yield decreases were found when drought periods occur over bud emerging and pod development stages (i.e. the whole reproductive stage) (Champolivier et al. 1996; Gan et al. 2004).

Seed yield decreases due to drought periods were associated to soil type. Indeed sandy soils are more disadvantageous than loamy soils since the water content at seedling stage is lower (lower water holding capacity) and the soil water reduction over spring is more prominent (Jensen et al. 1998) .

The effect on oil content

The results on the effects of drought on oil content are contradictory. During the seed ripening period, water-limited periods affect the oil content (% per unit ground area) (Champolivier et al. 1996). However, according Champolivier et al. (1996) seed oil content was significantly affected by drought from flowering to seed ripening and a negative relation was found between oil and protein content. In contradiction, several reports suggests that the allocation of assimilates to the ovule at the early megaspore stage is of crucial importance for oil content (Henry and MacDonald, 1978). Thus a water deprivation during the bud emerging stage has been related to seeds with lower oil content (Krogman and Hobbs, 1975). Despite that, several reports suggest no significant differences in oil content despite lower but not significant value were often found (Champolivier et al. 1996). The strong genotypic variability in both oil content (Snowdon et al. 2007) and response to water deficit (Richards and Thurling, 1978) may explain the conflicting results that however may also depend on the stress application (glasshouse, field), stress duration and cross-stress application.

The effects of drought on drought-related physiological traits

Light interception and growth

The number of seeds per area (sink) is determined during a crucial stage that starts at the beginning of flowering and lasting around 300 growing degree days after mid-flowering (Mendham et al. 1981). This 20-25 days window (depending on the environmental conditions) is the most important phase for pod and then seed abortion and thus the light radiation intercepted by photosynthetic organs (leaves, pods and stem) is of great importance (Mendham et al. 1981). The large number of flowers during this period has an impact on the canopy photosynthetic efficiency, due to a large amount of photosynthetic active radiation reflected (Berry and Spink, 2006). This detrimental effect is partially compensated by pod and stem assimilation (Fig.5) (Diepenbrock, 2000). However, the area generated by the pods (Pod area index, PAI) is inadequate to offset the leaf area (Leaf area index, LAI) deficiency leading to an insufficient area to support the pods development (Diepenbrock, 2000). It has been in fact extensively reported that the inverse relationships between seed number per area, seed per pod and number per pod result in an optimum of 8000 pods/m^2: smaller canopies were too small to trap all the incident radiation, reducing seed number per area. At the same time bigger canopies (over 8000 pods/m^2) resulted in i) high light reflectance of the thick flower layer and ii) lower radiation reaching the photosynthetic organisms and thus decreasing seed per pod and seed per area (Berry and Spink, 2006).

According to Fray et al. (1996), apetalous OSR was able to better sustain pod and seed set in the lower canopy compared to conventional varieties.

Dry soil sensed by the roots triggers the ABA-mediated long-distance signal that promotes stomatal closure (Wilkinson et al. 2012). It results in a decreased CO_2 uptake by the plants in turn leading to smaller leaf and pod area and, with higher soil moisture deficit, lower leaf and pod number (Wilkinson et al. 2012, Qaderi et al. 2006). Experiments with seedlings showed that water deprivation significantly decreased stem height and leaf area accompanied by a significant depression in assimilation rate and transpiration (Qaderi et al. 2006). At field level water deprivation decreased the photosynthetic active radiation interception probably because of the limited gas-exchange (Andersen et al. 1996). During the flowering stage the impaired radiation quotient seems to be a major limitation (ca. 65% less under drought) for total yield biomass whereas light interception was strongly decreased over pod development stage (Andersen et al. 1996). Thus the sink limitation over flowering (due both to lower assimilates availability and/or damages at the pod/seed primordia due to low water potential) can be considered the main harmful factor for yield, leading to up to 65% of the assimilates fixed during the pod-development stage in the straw fraction (Diepenbrock, 2000).

Plant water status

Leaf relative water contents, leaf water potential, stomatal conductance and rate of transpiration are influenced by water deprivation. The optimization of carbon uptake *vs* water loss by plants has been recently corroborated since there is a clear non-linear relationship between the two variables with the water loss restricted before and more intensely than photosynthesis. *Brassica napus* has been classified as a “drought avoiding” crop (Jensen et al. 1996): ecologically, the “drought avoidance” crops or “pessimistic” (Jones et al. 1980) are the genotypes/families that under drought tend to maximize their water status by triggering a fast stomatal closure. Conversely the “optimistic” crops make a richer use of the water available via a poorer stomatal control (Jones et al. 1980). In turn, Tardieu and Simonneau (1998) classified “isohydric” and “anisohydric” adaptation to drought, with the isohydric plants capable in maintaining constant midday leaf water potential whilst in the anisohydric type leaf water potential decrease with the evaporative demand. Several studies showed that the stomatal behaviour could be extensively contradictory and OSR, despite “drought avoidance”, does not show isohydric behaviour but more near-anisohydric at leaf level (Jensen et al. 1996).

Under stressful environments, OSR typically showed decreases in transpiration rate due to an expected stomatal closure as showed in Table 2. High temperatures in combination with water stress ensure higher transpiration rate than water stress alone followed by photosystem II (PSII) downregulation leading to strong effects on stem height (Qaderi et al. 2012). On the contrary, plants grown under high atmospheric CO_2 concentrations and drought exhibited lower transpiration rate than drought alone accompanied by lower leaf ABA concentration (Qaderi et al. 2006). This suggests that, even in OSR, the rising atmospheric CO_2 concentration may have a beneficial effect on drought tolerance by strongly improving water-use efficiency (WUE).

Table.2. Pot-grown and field-grown OSR physiological responses (A, CO_2 assimilation rate – E, transpiration) to drought and interactions with different environmental factors.

Plant	Stress	Treatments	A $\mu mol\ m^{-2}s^{-1}$	E $mmol\ m^{-2}s^{-1}$	Other parameters
Pot-grown Brassica napus L. (cv. 45H72 Pioneer) Qaderi et al. 2012	Water deficit (~wilting point) and high temperatures (28°C/24°C)	Well-watered, control temperatures	6.5	2.8	Stem height (cm, after stress) ~13
		Well-watered, high temperatures	5-5.5	3.8	Stem height (cm, after stress) ~11
		Droughted, control temperatures	4	1.9	Stem height (cm, after stress) ~6.5
		Droughted, high temperatures	3.3	2.5	Stem height (cm, after stress) ~5.8
Pot-grown Brassica napus L. (cv. 45H72 Pioneer) Qaderi et al. 2006	Water deficit (~wilting point), high temperatures, elevated atmospheric CO^2 concentration (740 ppm)	Well-watered, ambient CO^2	6.8	6	ABA concentration (ng g^{-1} DW^{-1}), ~100
		Well-watered, elevated CO^2	8.5	5	ABA concentration (ng g^{-1} DW^{-1}), ~100
		Droughted, ambient CO^2	5.9	4.9	ABA concentration (ng g^{-1} DW^{-1}), ~1500
		Droughted, elevated CO^2	7.2	3.5	ABA concentration (ng g^{-1} DW^{-1}), ~1100
Pot-grown Brassica napus L. (cv. 46A65 Pioneer) Sangtarash et al. 2009	Water deficit (~wilting point), ABA treatment (10 µg)	Well-watered, - ABA	8	6.8	Ethylene (pmol g FW^{-1} h^{-1}), ~70
		Well-watered, + ABA	7	5.3	Ethylene (pmol g FW^{-1} h^{-1}), ~90
		Droughted, - ABA	4.5	5	Ethylene (pmol g FW^{-1} h^{-1}), ~140
		Droughted, + ABA	5	4.9	Ethylene (pmol g FW^{-1} h^{-1}), ~160
Field-grown Brassica napus L. (cv.Ceres) Grammelvind et al. 1996	Field capacity irrigation and no irrigation	Irrigation Leaf 16 (top)	30-20	n.a.	Pod photosynthesis, 15-5
		Irrigation leaf 5 (bottom)	15	n.a.	n.a.
		Drought Leaf 16 (top)	30-15	n.a.	Pod photosynthesis, 12-3
		Drought leaf 5 (bottom)	15	n.a.	n.a.
Field-grown Brassica napus L. (cv. Global) Jensen et al. 1998	Field capacity irrigation and no irrigation: loam and sandy soil	Irrigation loam soil	30	2	LWP (MPa), - 0.8
		Irrigation sand soil	30	2	LWP (MPa), - 0.9
		Drought loam soil	25	1	LWP (MPa), - 1.2
		Drought sand soil	20	0.8	LWP (MPa), - 1.5

Photosynthesis

Photosynthesis is the most important and, at the same time, intricate process in plants and any reduction affects growth and economic yield of OSR (Jensen et al. 1998). Drought affects photosynthesis mainly via stomatal limitations (Flexas et al. 2006). In some cases however ribulose1,5-bisphosphate (RuBP) regeneration due to an impaired ATP synthesis has been reported (Tezara et al. 1999) In *Brassica napus* all the organs, although at different degrees due to variable stomatal distribution, participate to gas-exchange and thus carbon assimilation (Jensen et al. 1998; Jensen et al. 1996; Mogensen et al. 1997; Gammelvind et al. 1996). Leaf photosynthesis plays an important role until the late phase of the reproduction (Gammelvind et al. 1996); during pod-development and seed filling the latter becomes of less importance and the formed pods represent the key-organ for assimilation (Mogensen et al. 1997). Compared to other crops, during the pre-flowering stages, OSR showed a high photosynthetic capacity (~30-35 µmol CO_2 $m^{-2}s^{-1}$) (Jensen et al. 1996) with significant differences among the position of the leaf on the canopy (Gammelvind et al. 1996). Chlorophyll fluorescence analysis of leaves during the pre-flowering stage suggests that drought can decrease the maximum quantum yield of PSII after 8-10 days of stress (Muller et al. 2010). Since the high photosynthetic rate over the pre-flowering stage has the effect of creating the assimilate stores for the early establishment of the inflorescence (Jensen et al. 1998; Jensen et al. 1996), even early water deprivation may have a substantial effect on the yield output (Champolivier et al. 1996). To confirm that, suppression of the activities of vacuolar and extracellular invertases, important for sugar signaling pathways, osmotic functions and sustaining of sink growth were found when plants were stressed during bud emerging stage (Muller et al. 2010). During the crucial period of flowering, the leaf photosynthetic rate is decreased

(~15-20 µmol CO_2 $m^{-2}s^{-1}$) (Gammelvind et al. 1996; Jensen et al. 1996) due to i) the strong nitrogen use of the inflorescence (Jensen et al. 1998) and ii) the shading effects of the flowers that can reflect up to the 60% of the incoming light (Diepenbrock, 2000). Thus, as a crucial stage, flowering is also the phase where the plant can easily experience source-limitation. Water deficit over flowering significantly decrease CO_2 assimilation due to stomatal limitation, but over strong water deficit, mesophyll resistance and/or metabolic limitations can play important roles as reported by Jensen et al. (1996)

At the end of the flowering stage the photosynthesis from chloroplasts located in the pod wall layers dominates assimilate production for seed filling (Mogensen et al. 1997, Gammelvind et al. 1996). Often, water deficit may occur over the latest periods of reproduction (i.e. seed development) and this affects the seed enlargement and oil content and composition (Berry and Spink, 1996). The efficiency of non-matured pods in assimilating CO_2 is roughly half of the leaves (~10-12 µmol CO_2 $m^{-2}s^{-1}$), decreasing to ~5-6 µmol CO_2 $m^{-2}s^{-1}$ in matured pods (Mogensen et al. 1997). Stomatal closure triggered by water deficit affected pod assimilation by 38% but the seed filling was un-affected (Mogensen et al. 1997). As well as in other crops (wheat, soybean) this confirmed that maternal tissues can store assimilates over early stages (i.e. bud emerging-flowering) and mobilize them to maintain assimilate supply under restrictive conditions (Jensen et al., 1996). Thus, several reports support the hypothesis that late water deficits are of less severity for yield (Mogensen et al. 1997; Mendham et al. 1981; Jensen et al. 1996; Gammelvind et al. 1996). Moreover, the negligible differences in photosynthetic efficiency found between plants stressed in sandy and loamy soil suggests that seed filling is better source-protected under drought conditions (Mogensen et al. 1997).

Water-use efficiency

Water-use efficiency (WUE) is a trait of major importance to understand plant responses to water deficit. In the literature there are three major ways to measure the WUE including single-leaf gas exchange (Farquhar et al. 1989), carbon isotopic discrimination (Farquhar et al. 1989) and the agronomic evaluation of the biomass produced per unit of water used (Patil and De, 1978). Under water deficit stomata play a trade-off between CO_2 uptake and water loss that is depending not only on environmental conditions (CO_2, light, salinity, and drought) but also on internal physiological factors (i.e. sub-stomatal CO2 concentration (C_i), mesophyll conductance (g_m), water status) (Farquhar et al. 1989). As a drought avoidance crop, water stress induces a relatively fast stomatal closure optimising water status at the expense of CO_2 uptake (Jensen et al. 1996). Thus, since the relation between CO_2 uptake and stomatal conductance is not linear during the first period of moderate water deficit, a substantial increase in intrinsic water-use efficiency ($_i$WUE, the ratio between the CO_2 assimilated and the stomatal conductance) should be expected. In Qaderi et al. (2006 and 2012) OSR seedlings subject to water deprivation slightly increase (Qaderi et al. 2012) or not do change (Qaderi et al. 2006) their $_i$WUE despite the depression in CO_2 uptake. On the contrary in Hess et al. (2015) the “agronomic” WUE was increased by 27% when water was withheld compared to the control irrigated. Carbon isotope discrimination of OSR genotypes suggests that a substantial genotypic variation exists in *Brassica napus* germplasm for high WUE that deserves further investigation (Moroni et al. 2009).

Osmotic adjustment

Osmotic adjustment is the capacity of plants to accumulate solutes and sustain the metabolic activity in tissues by keeping the symplast volume constant while the apoplastic water fraction becomes reduced (Jensen et al. 1996). It is considered one of the crucial processes in plant adaptation to drought (Kumar and Singh, 1998). However, from an agricultural point of view, osmotic adjustment has not shown strong benefits in terms of yield in several crops (Farooq et al. 2009). On the contrary, in *Brassica* species, osmotic adjustment has been shown to be positively related with seed yield (Kumar and Singh, 1998) by maintaining turgor and turgor-associated processes even at low water potential. In particular, under water deficit conditions, high osmoregulation may maintain high stomatal conductance rate and transpiration hence assuring cooler canopies (Kumar and Singh, 1998). Persistent higher conductance can sustain photosynthetic rate and thus shoot growth and deeper roots growth (Kumar and Singh, 1998). This osmoregulation-dependent mechanism, under drought, allows higher water supply and ensures extra water resources for the essential physiological processes (Kumar and Singh, 1998). Nevertheless, reports on the osmoregulation capacity of *Brassica napus* suggest that solute accumulation is limited: under drought conditions, Good and Maclagan (1993) did not find osmoregulation in *Brassica napus* genotypes whereas in Jensen et al. (1996) the osmoregulation was limited. However, has been clearly reported that there is a substantial genetic variability in oilseed *Brassica* osmoregulation capacity (Kumar and Singh, 1998) and thus high chance to exploit the existing genetic resources. In Kumar and Singh (1998) high-osmotic adjustment *Brassica* oilseed genotype, showed higher stomatal conductance and transpiration cooling than low-osmotic genotypes under drought conditions and a significant correlation was found between osmotic adjustment and seed yield.

Root traits

Under mild drought stress, generally root growth is favoured rather than shoot growth through assimilates reallocation (Daie, 1996). Nevertheless, in severe stress conditions, decreases in root growth have been observed. According to Hess et al. (2015) OSR root system efficiency is comparable to that of wheat (i.e. root growth, distribution and root hydraulic conductivity per unit root length) with a similar efficiency in water uptake. Under drought conditions, root length densities significantly decreased for all the soil depth, suggesting a low capacity in assimilate reallocations to below-ground organs during drought (Hess et al. (2015). However, carbon isotope analysis, showed rhizogenesis adaptive mechanisms in OSR that allows high dry matter turnover and development of new drought-adapted root systems leading to high rehydration efficiency (Deleens et al. 1989). Vartanian et al. (1994) showed that in droughted *Arabidopsis*, the *ga5* line, a gibberellin biosynthetic mutant, had a highly rhizogenesis production, suggesting that gibberellin play an important role in *Brassicacae* roots drought adaptation

According to Berry and Spink (2006), to achieve the 9.2 t ha^{-1} yield potential estimated for the current elite varieties in the UK, root length density at depth should be a major trait to improve. This would allow plants to remove the spring water shortfall and avoids reproductive damages.

Reproductive processes

Plant reproduction in agriculture is of major importance, and it is underpins of the economical yield of crops (Jablonski et al. 2002). Environmental stresses affect most of the early reproductive processes of micro- and megasporogensis such as pollen and stigma viability, anthesis, pollination, pollen tube growth, fertilization, and early embryo development (Prasad et al. 2008). It is largely recognized that the female gametophyte shows a higher stress-tolerance than male organs, possibly due to an adaptive mechanism where pollen sterility and/or male sterility can be overcome by cross-pollination at field level (Saini, 1997).

The success of reproduction in OSR largely depends on environmental conditions (Diepenbrock, 2000). Among the various environmental stresses affecting the crop, drought stress showed direct and major impacts on OSR reproduction (Diepenbrock, 2000). In OSR the reproductive phase starts from GS 5.0 (bud emerging) and ends with seed ripening (GS 8.0) (Diepenbrock, 2000).

Over bud emerging stage, drought can significantly impair the activity of vacuolar and extracellular invertase enzymes (Muller et al. 2010). These enzymes are involved in sucrose hydrolysis into glucose and fructose monomers that are directly used in metabolic processes, in the regulation of sucrose partition between source and sink, in sink cell expansion, in seeds lipid storage and in many other sugar-based signal pathway and signal transduction (Koch, 2004). Thus, under drought, the decrease in invertase enzymes (in particular vacuolar invertase) accompanied by osmolyte accumulation and PSII efficiency reductions, was significantly correlated with the strong decrease in pod dry weight, suggesting the down regulation of sucrose portioning and hydrolysis before the flowering stage may lately affect pod expansion and thus seed production (Muller et al. 2010).

OSR naturally balances pod formation with abortion (Guo et al. 2015). During the drought-sensitive stage of flowering (GS 6.0), drought significantly increases the rate of aborted flowers (Guo et al. 2015). However, the physiological mechanism controlling reproductive failure in crops under drought stress is not well understood and substantial variability has been found between species and between genotypes (Prasad et al. 2008). Mainly, abortion in crops has been related to i) a direct decrease in water potential of the floral tissues (Liu et al. 2003) ii) reduction in carbohydrate and/or nitrogen flux supply (Andersen et al. 1996) iii) plant signal involving hormones, gene expression and transcriptional factors (Westgate et al., 1986) and iv) interactions between these factors (Liu et al. 2005). In contrast to other crops (i.e. soybean) Mogensen et al. (1997) showed that droughted OSR plants after anthesis exhibited a more negative pod/flower water potential value that however was not affecting seed enlargement and/or pod formation. Thus, in OSR there is evidence suggesting that carbohydrate supply could be the pivotal factor in abortion under drought stress (Mogensen et al. 1997; Diepenbrock, 2000). In absence of stress an *in vitro* analysis showed that OSR seed abortion was significantly related to assimilate supply (Bouttier and Morgan, 1992). Despite the natural source limitation occurring over flowering due to the shading effects of flowers and the additional drought-induced decrease in photosynthesis, it is generally recognized that small embryos required low photosynthates amounts due to their small sink (Prasad et al. 2008). Additional root-to-shoot or even leaf-to-reproductive organ signals are required to explain reproductive failure under drought.

Exogenous application of ABA impaired mitotic and meiotic processes and developing seeds under non-stressed conditions in maize, with similar responses to those of drought stress leading to lower seed-set and seed development (Mambelli and Setter, 1998). ABA application at different dose rates in *in vitro* grown OSR pods under low sucrose concentration (1%) affected pod dry weight and length but not the seed number at all the dose rates (Bouttier and Morgan, 1992). On the contrary, the ABA effect was negated at high sucrose concentration (Bouttier and Morgan, 1992). At the same time higher ABA concentrations compared to wild type were found in male sterile mutants of OSR, suggesting that an ABA-triggered or ABA-based mechanism is involved in reproductive organ development disruption (Shukla and Sawhney, 1994). Since ABA plays an important role in stress signal transduction to the whole plants (Wilkinson et al. 2012), and since in many crops its involvement in abortion have been well established (Liu et al. 2003; Westgate et al. 1986), further *in vivo* experiment of droughted OSR are required to understand the complex mechanism of reproduction failure under water deficit conditions and the cross-talk between ABA and carbohydrates supply.

Crop management under limited water availability

Crop management

Improving yield of crops under drought by crop management has been extensively studied giving interesting and promising results to be explored further.

Partial root-zone drying (PRD) has been studied in OSR (Wang et al. 2005). The studies showed mild effects (Mousavi et al. 2010) or even negative (Wang et al. 2005) suggesting that in OSR, a crop where vegetative and reproductive growth overlap, PRD is ineffective. Moreover, the practical application of PRD in the western OSR production areas may be particularly complex and expensive for a relatively low value crop which is often not irrigated.

The triazole, metconazole, an anti-gibberellin plant growth regulator, was successfully used to improve lodging and restrict the canopy accompanied by increasing lateral branching (Berry and Spink, 2009). As a second effect, triazole was successful at improving rooting due to the different assimilate partitioning between root and shoot. Further studies should be done in this direction to evaluate the potential of different chemicals at improving rooting that can allow plants to make a more wide use of the water available in deeper soil layers. (Berry and Spink, 2009)

ABA application was successfully used at 2 μmol L^{-1} to improve WUE in OSR (Jun et al. 2012). However, higher dose rates (100 and 200 μmol L^{-1}) depressed pod and seed number, possibly due to the decreased CO_2 available after stomatal closure and/or the counteractive effect of ABA on reproduction. Improving WUE via exogenous application of chemicals was successfully used on *Brassica campestris* by Patil and De (1976) and Patil and De (1978). Film-forming emulsion and kaolin were sprayed on the crop under dryland conditions increasing WUE and seed dry matter production by 22% and 14% with respect to the un-sprayed control, through a decrease in total evapotranspiration (Patil and De, 1978). The effectiveness of stomatal blocking and/or closing chemicals on improving drought tolerance of OSR has not been extensively studied, and thus further investigations must be done to exploit potential for use in crop management.

Antitranspirant: an overview

Antitranspirant compounds are a range of agrochemicals used in agriculture and, sprayed in emulsion with water onto the crops canopy, can reduce water losses from stomatal pores and cuticle (Solarova et al. 1981). There are three types of commercially available antitranspirants:

1. The reflectant (e.g. Kaolin) that are compounds widely used to decrease the impact of solar radiation on increasing leaf temperature. These compounds are largely used on fruit plants to decrease leaf or fruit temperature (Boari et al. 2015)
2. The inhibitors of stomata opening (e.g. ABA, phenyl-mercuric acetate, chitosan) that, however, are often expensive or poisonous.
3. The film-forming antitranspirants that sprayed in emulsion with water are a large range of chemical compounds (e.g. di-1-*p* menthene (Fig 6 A & B), poly-1-*p* menthene (Fig. 6 C), silicone, waxes, oils) that create a waterproof layer in leaf surface (Solarova et al. 1981).

Figure 6. A) di-1-*p* menthene chemical structure (Empirical formula: $C_{20} H_{34}$). B) The commercial available di-1-*p* menthene-based antitranspirant “Vapor Gard” (Miller Chemical, USA). C) The commercial available poly-1-*p* menthene-based antitranspirant/spreader/sticker “Nu-Film P” (Miller Chemical, USA)

Nearly fifty years ago, film-forming antitranspirants were considered a promising agronomic tool to preserve water in plants and thus avoid yield decreases under water deficit: their effectiveness was limited as their stomata-blocking property was strongly related to a drastic decrease in CO_2 assimilation (Solarova et al. 1981). These materials are applied to leaves in an emulsion with water and, by creating an elastic polymer film, are able to block stomata and hence decrease transpiration (Davenport et al. 1972; Kettlewell, 2014). Solarova et al. (1981), Davenport et al. (1974) and Gale and Hagan (1966) reviewed several experiments carried out between the early 1960's and 1970's. These reviews showed that film-forming treatments were able to improve plant water status by decreasing transpiration rate but at the expense of a decrease in CO_2 gained by the plant. Therefore, the film-forming compounds were merely but successfully used in crop protection to control powdery mildew and leaf rust on wheat (Ziv and Frederiksen, 1987) and to reduce grey mould (*Botrytis cinerea*) in potatoes, peppers, beans, tomatoes and cucumbers (Elad et al*.* 1990). In the last decade new work has shown that a film-forming treatment on droughted crops was able in many cases to sustain the carbon dioxide assimilation rate compared to the un-treated/water-stressed plant (Moftah and Al-Humaid, 2005, Abdullah et al. 2015). Moreover the film-forming compounds were capable of improving WUE in many crops: in essence the menthene based compounds (mainly used as film-forming antitranspirant in the last decade) were effective at depressing transpiration, but proportionally less effective at depressing carbon assimilation (Patil and De, 1976-1978; Palliotti et al. 2013; Moftah and Al-Humaid*,* 2005). In addition, the large amount of commercially available film-forming compounds showed different effectiveness decay and thus appropriateness for different water-limited requirements: in Anderson and Kreith (1978) Mobileaf and Wiltpruf silicone-based film-forming compounds were effective at decreasing transpiration for 10-14 days while in Palliotti et al. (2010) two pre-flowering 3% v/v di-1-*p* menthene applications on *Vitis Vinifera* were effective in depressing transpiration until harvest (around 90 days).

In this review the physiological response of different crops to film-forming antitranspirant treatments will be shown and discussed.

Droughted crops physiological responses to film-forming treatments

According to the biggest and most robust reviews on antitranspirants (Solarova et al. 1981, Davenport et al. 1974 and Gale and Hagan, 1966), the use of film-forming treatments is highly marginal on agriculture being strictly related to a decrease in CO_2 uptake by the plants. Most of the research carried out during the 1960’s and 1970’s used polymers/waxes/silicones at high dose rates, often sprayed on both the leaf surfaces, leading to a great number of stomata blocked. For instance, a 3 L/ha di-1-*p* menthene treatment sprayed on grapes at pre-flowering (Palliotti et al. 2010) and post-veraison (Palliotti et al*.* 2013) stages was adequate to create source-limitation and significantly decrease the bunch compactness, berry size and clusters sugar content. In Anderson and Kreith (1978), the efficiency of different film-forming antitranspirant compounds in decreasing transpiration of different herbaceous species was evaluated. The antitranspirants were able to depress transpiration, at the expense of the carbon assimilation: the two compounds were sprayed at the high 16.5% v/v concentration; it must be said that according to the commercial product recommendations, film-forming antitranspirant dose rate (di-1-*p* menthene, poly-1-*p* menthene) should not exceed the 1 L/ha (1% v/v up to 400 L water volume) for the most common crops (D. Scicchitano, Miller Chemical &Fertilizer, Hannover, USA, personal communication). Moreover, high dose rates are practically unsustainable at field scale due to the high costs of the compounds that are substantially used to mitigate the detrimental effect of water deficit on crops yield (Kettlewell, 2011). Many other factors such as timing of the treatment (i.e. drought-sensitive phenological stages), stomatal distributions on the abaxial-adaxial leaf surface and decay period of the treatment have been underestimated or even ignored in much of the research.

Plants water relations and transpiration

The main target to achieve with film-forming antitranspirant treatments is to save water in plants by avoiding high transpiration rate, and thus reducing the crop damage in arid or semi-arid environment. Film forming treatments have been successfully studied to increase stomatal resistance to water vapour by creating a physical barrier in a substantial amount of stomata (Solarova et al. 1981).

There is considerable evidence that plants benefit from an antitranspirant treatment at water-status level by decreasing the transpiration rate. Gale and Poljakoff-Mayber (1965) performed both field and controlled environment experiments to assess the effectiveness of different film forming compounds in reducing transpiration. In the field, all the compounds increased the leaf temperature due to the transpiration depression, showing evident differences between windy and windless days: no differences in leaf temperature were recorded with wind between sprayed and un-sprayed plants. In controlled environment conditions, the S-789 acetate acrylate ester compound, depressed the transpiration by 36% compared to the un-sprayed control accompanied by a 1-1.5°C increase in leaf temperature. A fan used to simulate windy conditions restored leaf temperature to the air temperature level, showing no differences between treated and un-treated plants and suggesting that transpiration may be only one of the factors in cooling plant leaves at field level. In Ludwig et al*.* (2009) Vapor Gard-treated (2% v/v) beans plants on both the leaf surfaces were placed onto analytical balances and the evaporative flux (each 30 seconds) jointly with thermal imaging were collected: the film-forming treatment was successfully able to decrease the plants' transpiration (and thus water use) and, at the same time, increasing leaf temperature by 4°C compared to the un-treated control. Weerasinghe et al. (2016) and Kettlewell (2014) clearly showed that a di-1-*p* menthene application on droughted wheat at the meiosis in the pollen mother cells key-stage was statistically significant and effective in improving the crop leaf water potential by significantly suppressing the stomatal conductance. Strong improvements on plant water status parameters were found also by Patil and De (1976 and 1978) in OSR with a significant increase in relative water content and leaf water potential on treated plants compared to the droughted un-treated. Davenport (1972) found that an application of a film-forming treatment on *Prunus persica* subjected to water stress was effective in raising the leaf water potential compared to the un-treated plants: despite the high antitranspirant dose rate were disrupting the CO_2 assimilation, growth was strictly dependent of cells expansion and turgor and thus growth was not inhibited.

Antitranspirant-treated leaves have displayed wider stomata guard cells apertures. In Iriti et al. (2009) Vapor Gard treated bean plants exhibited an increase in stomatal width by 23% compared to the un-treated control. In Davenport (1972), faba bean plants treated with a 1:5 antitranspirant/water ratio, increased the stomatal aperture by 3-fold in watered conditions whilst in droughted environment the aperture was increased by 4.5-fold compared to the control un-sprayed. As in Iriti et al. (2009), the sub-stomatal carbon dioxide concentration of the Vapor Gard-treated plants was just 8% lower compared to the control and thus the increasing stomatal width could not be correlated with the decreasing carboxylation but possibly to an increase in leaf water potential; this in fact may be related to an increasing water inflow at leaf level and to an increasing K^+ concentration following an increasingly negative electrical potential of the cells and a reduction in ABA-GE concentration at leaf level (Davenport, 1972; Iriti et al. 2009). Concurrently, under water deficit conditions, Pier and Berkowitz (1987) showed that wheat treated with K^+ ions in nutrient solution was effective at improving plant gas-exchange (and thus photosynthesis) possibly due to a pH stromal alteration following the exchange of the cytoplasm K^+ for the stroma H^+ ions. Hence, as a second outcome, under water deficit, a water-status-improved plant should display increase carbon assimilation.

Gas exchange, water-use efficiency and photosynthesis

As theorized by Gale (1967), assuming an antitranspirant with a same degree of permeability to H_2O and CO_2, under water stress conditions (low soil moisture or high evapotranspiration demand) the depression on transpiration should be theoretically more prominent than the CO_2 assimilation with respect to the untreated plants. Indeed, the CO_2 pathway, subjected to an extra resistance between sub-stomatal cavities and chloroplasts (the “mesophyll” resistance), should benefit by the application of an extra resistance (antitranspirant) under water deficit.

Davenport et al. (1974) reviewed different works carried out to explore the efficacy of film-forming treatments at improving drought tolerance; although there was evidence that the compounds decreased bean’s photosynthetic rate and yield under water deficit, the improvement at water status level was significant in many other plants (oleanders and orchard). It was concluded that the antitranspirant was an important tool to improve crops’ drought tolerance but their use was restricted at particular phenological stages where plant water status is more important than photosynthesis. The largest review on antitranspirants made by Solarova et al. (1981) concluded that the film-forming compounds restrict the carbon assimilation: only in 6 out of the 29 works reviewed was there an increase in CO_2 assimilated by the plants compared to the control (despite is not clear whether the control was a droughted control or a watered control) and in three of them the increase was associated with an increase in transpiration rate (thus a de-polymerization or a degradation of the sprayed compounds). Moreover in most of the researches reviewed the transpiration/carbon assimilation ratio (the contrary of the WUE parameters) was enhanced by the treatment suggesting a general decrease in WUE. Comparing the newest works (2000-2015) with the older ones (1960-1980) it is evident that the source limitation could be related to the high dose rate used. As reported above a 3% v/v solution of Vapor Gard was enough to create source limitation in *Vitis Vinifera* for more than 30 days and decreasing the transpiration by 60% compared to the control unsprayed: in Solarova et al. (1981) the average dose rate of the different sprayed antitranspirant compounds was 15.25% v/v.

Researches carried out by by Moftah and Al-Humaid, (2005), Prakash et al. (2000) and Gale (1967) showed an increase in carbon assimilation when film-forming antitranspirants were sprayed on droughted plants. There was evidence that the increase was related to an increase in chlorophyll *a* and *b* content at leaf level, fundamental for photosynthesis, being the pigments involved in the light-harvesting reaction at photosystems level, possibly due to the improved plant water status. Chlorophyll fluorescence analysis of well-watered grape (*Vitis vinifera* L.) plants treated with the di-1-*p* menthene compound to decrease sugar accumulation showed no significant differences in F_v/F_m ratio when sprayed at high dose rate (3 L/ha), suggesting absence of photo-inhibition at the photosystem II complex despite the depression in CO_2 assimilation; nevertheless the plastoquinone pool size on the reducing side of Photosystem II value was severely depressed, denoting a hypothetical lower efficiency of PSII on reducing the plastoquinone and hence a lower capacity on fixing CO_2 (Palliotti et al*.* 2013). Conversely, as reported by Latocha et al. (2009), *Actinidia* water-stressed genotypes showed higher F_v/F_m ratio value (photochemical efficiency of PSII), F_v/F_o ratio (related to the oxygen evolving complex activity), plastoquinone pool size and total chlorophyll content when treated with a pinolene-based (1% v/v) compound (di-1-*p* menthene) with respect to the unsprayed control: it was concluded that an antitranspirant treatment could prevent damage at light reaction level when sprayed on droughted crops, possibly due to the improvement at plant water status level.

Del Amor et al*.* (2010), investigated the effect of a 2.5% v/v foliar application of di-1-*p* menthene on droughted pepper seedling subjected to 380 and 2000 μmol mol^{-1} of CO_2; at strong water deficit imposition (8 days) and ambient CO_2 (380 μmol mol^{-1}), the metabolic limitation of photosynthesis was predominant and the film-forming treatment was able at significantly increasing the F_v/F_m ratio of the droughted plants compared to the un-treated. At 2000 μmol mol^{-1} of CO_2

atmospheric concentration and under water deficit the di-1-*p* menthene treated plants showed a significant increase in carbon assimilated with respect to the un-treated control, suggesting that elevated atmospheric CO_2 counteracted the increasing stomatal resistance to the carbon pathway of di-1-*p* menthene treated plants; the research showed the importance of the film-forming treatment on future climate changes (increasing CO_2 and drought) and their potential as a crop management method for atmosphere-controlled greenhouses.

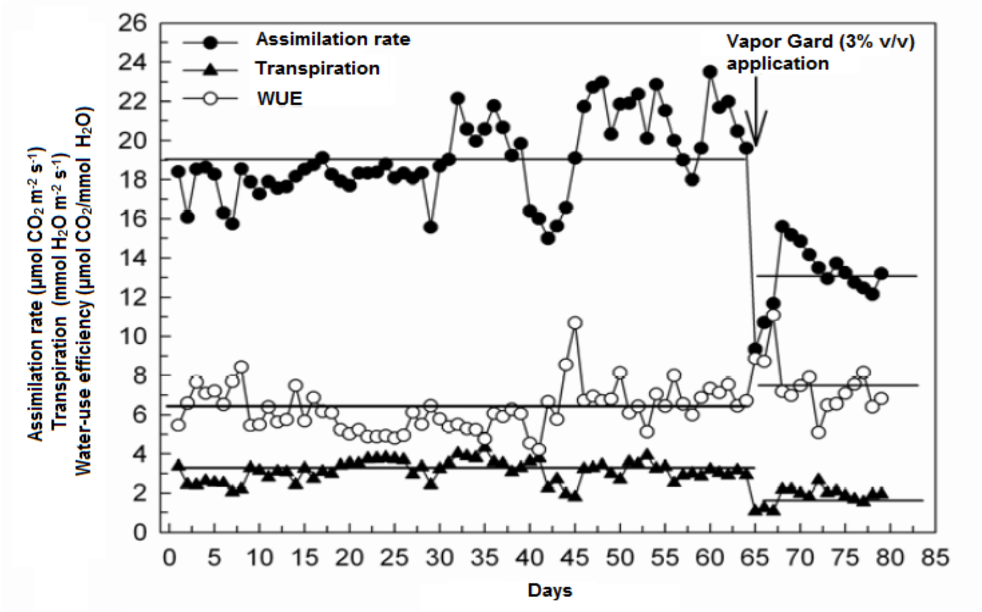

Figure 7. Canopy gas-exchange pattern of *Vitis vinifera* (cv. “Sangiovese”) grown in 60 L pots and treated with Vapor Gard (di-1-*p* menthene) at 3% v/v in early September. Horizontal lines represent the average value for each parameter (assimilation rate, transpiration and WUE) before and after the Vapor Gard application. Reproduced from Miller Chemical Trial report 2007-2009

On the other hand, the evident increase in instantaneous water use efficiency (iWUE) (Palliotti et al*.* 2013, Moftah and Al-Humaid*,* 2005) has been related with a high permeability to CO_2 of some compounds (especially the pinolene-based): it clearly showed that the degree of transpiration suppression, due to a film-forming treatment, is higher than the suppression in carbon dioxide assimilation (Figure 7).

According to the above literature, film-forming compounds could suppress carbon assimilation and thus assimilate production and translocation, but strong evidence suggested that the high dose rates were involved: the new research discussed showed that despite the stomatal-blocking properties of the treatments, the improvement at photosynthesis is conceivable due to i) the permeability to CO_2 of the compounds ii) the protection of the photosynthetic apparatus under drought stress (i.e. increasing the stomatal limitation of photosynthesis, the metabolic limitation is prevented by the improvement at plant water status level).

Hormones and roots development

An underestimated effect of film-forming antitranspirant treatments on plants is their ability to decrease the abscisic acid concentration in leaves concurrently with a transpiration reduction as reported by Iriti et al. (2009) on beans; un-sprayed leaves showed an average ABA content of 0.218 µg g^{-1} while a Vapor Gard application (2% v/v) decreased by 4-fold the ABA amount (0.058 µg g^{-1}). It must be said that a main goal of the genetic engineering breeding program for drought tolerance is to increase ABA accumulation/sensitivity on different crops to promote stomatal closure (and thus improve WUE) by up-regulating the *NCED* pool genes encoding for ABA-synthesizing pathway enzymes (Wan et al*.* 2009). The main obstacles are i) the strong reduction in carbon gained during the whole cycle of the crop (Ji et al*.* 2011) and ii) the potential ABA-induced damages during reproductive stages (as extensively reported by Westgate et al*.* 1986 and Ji et al*.* 2011). These problems were partially overcome by adopting drought-inducible promoters that can trigger the ABA synthesis/sensitivity only under water deficit conditions (Xiao et al*.* 2009), showing in any case limitations due to cross-talk between hormones (cytokinins and ethylene) and other signal molecules. As reported before, there is evidence regarding the effectiveness of the antitranspirant treatments in modulating gas-exchange and improving water status of droughted crops: moreover the ABA decrease at leaf level could be an additional value, leading to a lower ABA translocated to the floral organs. As extensively reported by Liu et al*.* (2003) in *Glycine max* L., there is a strong and inverse relation between pod set and ABA content in floral organs and root and/or leaf produced ABA translocated to the reproductive units, which along with a decrease in water potential probably plays a major role in pod abortion. Thus, the capacity of film-forming treatment to decrease ABA concentration possibly via reduction of ABA signaling is an additional effect that deserves in-depth further studies.

ABA has been shown to be positively involved in adaptive “deeper” root elongation and proliferation under water deficit conditions by limiting ethylene synthesis and production (Spollen et al*.* 2000). In an experiment conducted in pepper seedlings, Goreta et al. (2007) showed that the application of different film-forming and metabolic treatments was able to affect the physiology of the plants under water deficit. ABA was the most effective in decreasing stomatal conductance of the plants and thus improving relative water content and leaf water potential; conversely film-forming antitranspirant treatments (Vapor Gard, Anti-stress and Transfilm) successfully increased the relative growth rate of the plants: in particular, the di-1-*p* menthene (Vapor Gard) showed the highest increase in root length and root weight. This could be linked to suppression in roots- produced ABA translocation to the above-ground organs through the xylem vessels following suppression of the transpirational stream. It is conceivable that a film-forming treatment could be effective in down-regulating the drought-inducible chemical signals (e.g. pH) due to the water status improvement (and thus ABA synthesis and translocation). There are strong correlations between drought tolerance and root depth in crops: as reported by Blum (2009), the rice cultivars that were well adapted in water-limited environments, exhibited a deep root system, allowing plants to explore large portion of soil.

Film antitranspirant effects on major food crops

Effects on droughted wheat (***Triticum aestivum*** and ***Triticum durum***)

Drought periods occurring during the wheat reproductive stages lead to a strong decrease in yield: it has been postulated that up to 70% of the wheat producing areas are situated in arid and semi-arid conditions (Hongbo et al. 2005). Film-forming antitranspirant application has been previously studied to avoid yield losses and improve drought tolerance of wheat.

According to Mokhtari et al. (2006) a linseed oil application at 7.5% concentration was significantly effective in decreasing *Triticum durum* leaf transpiration and thus increasing stomatal resistance, although with different degree between the four varieties tested. The carbon isotope discrimination analysis, linearly related with the plants water-use efficiency (as previously reported by Farquhar et al. 1982) showed that, as expected, plant discriminate against ^{13}C during photosynthesis (caused by the preference of ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme for $^{12}CO_2$ over $^{13}CO_2$ (Farquhar et al. 1982)) and the linseed oil was effective during the water stress period at increasing the $\delta^{13}C$ value, and thus the predicted WUE. Mokhtari et al. (2006) concluded that antitranspirants reduced CO_2 uptake under well water condition but not under stressed environment, suggesting their use to improve *Triticum durum* drought tolerance during reproductive stages.

Pinolene-based compounds were successfully studied by Kettlewell et al. (2010) and Weerasinghe et al*.* (2010) to avoid yield decrease of droughted *Triticum aestivum.* In Weerasinghe et al*.* (2010), a 2.5 L/ha di-1-*p* menthene treatment on droughted wheat at different phenological stages (BBCH GS 33, GS 39, GS 41 and GS 59) was effective in increasing grains ear^{-1} leading to non-significant differences between the control watered and the droughted sprayed treatments. Moreover the di-1-*p* menthene treatment significantly depressed transpiration without depressing net photosynthesis and significantly increased the pollen viability of the treated plants.

Effects on droughted maize (*Zea Mays* L.)

Maize (*Zea mays* L*.*) is the second source of food in the world after rice: its sensitivity to water stress period during anthesis is considered the largest limitation for the crop, leading to silking interval disruption and thus yield reductions (Westgate and Boyer, 1986).

Fuehring and Finkner (1983) investigated the effect of a Folicote (a hydrocarbon film material) application on droughted corn over nine field experiments. The film-forming compound was sprayed at different dose rate from 0.93 L/ha to 4.67 L/ha and at different water volumes (168 to 458 L/ha). The field experiments were carried out in New Mexico (USA) and the plants, treated after the complete leaf emergence or before tassel emergence, were naturally subjected both to water stress and high temperatures. The sprayed plants showed a significant yield increase on the order of 11 to 17% at the point of the maximum dose response quadratic curve (2 L/ha for both surfaces sprayed and 3 L/ha for only adaxial surface sprayed). The consistent yield increase over different climatic conditions and soil moisture after a so large range of application, showed the potential and the flexibility of the treatment in increasing the yield of droughted maize. Similar results were found by Shekour et al. (1987) where the di-1-*p* menthene based Vapor Gard was effective in improving maize water status, increasing leaf area, dry weight and total dry matter with respect to the control un-sprayed.

Effects on other major food crops

The effectiveness of a film-forming antitranspirant in increasing drought tolerance has been largely explored in other major crops. In potatoes, a di-1-*p* menthene application seven days after full bloom reduced water uptake by 20-40% leading to increases in yield compared to the un-treated and higher soil moisture levels (Lipe and Thomas, 1980). At field level, an application of 2% v/v concentration Folicote before vine kill, was effective on increasing yield by 2352 kg/ha compared to the un-treated (Lipe and Thomas, 1980). Similar results were found in Lipe and Wendt (1978) were antitranspirant was able to extend the period of irrigations. In soybean (*Glycine max L.*) antitranspirant treatments were able to increase yield under water deficit conditions, significantly increasing seed yield compared to the un-treated control (Javan et al. 2012). A Folicote (2% v/v) application before booting stage on droughted sorghum was effective in increasing the grain production by 17% (Fuehring, 1973).

Effects on droughted *Brassica campestris* L.

Patil and De (1976, 1978) carried out two investigations to test the effectiveness of antitranspirant treatments in increasing drought tolerance of *Brassica campestris*. Three different chemicals were used: a stomata-closing treatment (PMA, phenyl-mercuric acetate), a reflectant treatment (kaolin) and a film-forming treatment (Mobileaf). The physiological responses to the different treatments at 75% soil moisture deficit were studied: the film-forming treatment was the most effective at increasing the relative water content (RWC) and dry matter production compared to the control (un-watered). In this experiment, both the leaf surfaces were sprayed with a high concentration of the film-forming antitranspirant (1:8 Mobileaf-Water ratio).

Patil and De (1978) also reported two field experiments to evaluate both physiological responses and yield components following the antitranspirant application. The antitranspirants were applied at the initiation of flowering stage. Plants' relative water content was highly improved by all the treatments but the most effective was the film-forming (1:10 Mobileaf-Water ratio) compared to the metabolic and the reflectant treatment. The WUE (dry matter production/water transpired) was significantly improved by antitranspirant due to a decrease in transpiration and an increase in total dry-matter production. Mobileaf sprayed at flowering stage showed a significant increase in seed yield in both years: total yield (t ha^{-1}) was increased by 23.53% and 18.19% in the two years compared to the un-sprayed un-watered treatment. No significant variations in oil content were observed. These two research papers are the only available literature focusing on the use of film-forming antitranspirant on droughted oilseed rape.

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General objective of the study

The literature review clearly shows that drought-induced oilseed rape yield reductions are a major problem and crop improvement and crop management tools are strongly required.

On the basis of the above evidence it was hypothesised that the yield of droughted oilseed rape can benefit from a film-forming antitranspirant application over the most drought-sensitive phenological stages due to improvements at plant water status level. Also it was hypothesized that the increasing atmospheric CO_2 concentration may counteract the source-limiting effect of the film-forming treatment leading to a substantial higher efficiency of the compounds under stress conditions. Despite the work described above on the use of film-forming treatment on droughted *Brassica campestris*, there is a limited knowledge on the physiological mechanisms behind the yield increase and, to our knowledge no studies were carried out on droughted *Brassica napus*. Thus, the general objective of this work was to evaluate the potential of the film-forming treatment at increasing the yield of droughted oilseed rape and validate it as a possible crop management tool.

The remaining chapters (i.e. 2-5) of the thesis are written as a series of papers. Thus each chapter is presented with an abstract, an introduction and a general discussion. The referencing style of each chapter is in accordance with the target-journal guidelines. The specific objectives for each chapter are explained below.

Specific objectives

Chapter 2: Canopy application of film antitranspirants over the reproductive phase enhances yield and yield-related physiological traits of water stressed oilseed rape (*Brassica napus* L.).

(Published in *Crop and Pasture Science*).

The objective in **Chapter 2** was to investigate a substantial number of drought-related physiological traits in sprayed and un-sprayed oilseed rape plants with two selected film-forming compounds (Nu-Film P and Vapor Gard, known to have different long-lasting effectiveness) and subjected to watered and stressed watering regimes. In particular, it was hypothesised that a uniform adaxial-surface application of a film forming treatment may depress leaf stomatal conductance leading to a significant alteration in stress response mechanisms (i.e. stomatal opening, photosynthetic efficiency, water-use efficiency, ABA concentration, tissues temperatures and overall plant water status) leading to a higher reproduction efficiency and lower drought-derived damage during the key-yield determining flowering stage.

Chapter 3: Modulation of oilseed rape (Brassica napus L.) source-sink physiology at different watering regimes through film antitranspirant evidenced drought tolerance ameliorations that are dependent on the stress magnitude.

(Submitted to *Journal of Agronomy and Crop Science*).

The objective in the **Chapter 3** was to evaluate the efficiency of Vapor Gard (the most effective compound in Chapter 2) at avoiding drought-induced yield losses over four soil moisture regimes. Two experiments were carried out in oilseed rape over the flowering stage at four different watering regimes. The National Plant Phenomics Centre facilities (IBERS, Aberystwyth University) were used after the project approval at the EPPN. Thus, the aim was to investigate the interactions between yield components with ABA concentration of leaf and reproductive organs, leaf-to-air and leaf-to-bud temperature, gas exchange, chlorophyll fluorescence traits and plant water status of oilseed rape plants and to relate them to reproduction efficiency.

Chapter 4: Dynamic regulation of leaf gas-exchange through antitranspirant in *Brassica napus* seedlings subjected to reduced water availability depends on CO_2 concentration *via* effects on mesophyll function.

(Submitted to *Plant, Cell and Environment*).

In **Chapter 4**, the objective was to evaluate whether film antitranspirant efficiency is dependent on the atmospheric CO_2 concentration. Mainly, in this chapter has it been hypothesized that, since the literature review is showing an increasing number of successful applications of antitranspirants at reducing drought damage in the last years, the increasing atmospheric CO_2 concentration has played a role at reducing the leaf source-limiting effect of the chemicals. The experiments in Chapter 4 were carried out at the Institute of Bio- and Geosciences (IBG-2) of Jülich Forschungszentrum (Germany). A Short-Term Scientific Mission through the COST application was accepted on September 2015 giving funds available from January 2016 and April 2016. Thus three experiments were carried out at three different atmospheric CO_2 concentrations in a controlled environment chamber simulating the CO_2 concentration of 1960, ~2012-2016 and the predicted 2050

(300, 400 and 700 ppm respectively). Oilseed rape seedlings were arranged in a factorial design and droughted/watered and sprayed/unsprayed with a film antitranspirant. Analysis of leaf gas-exchange, plant water status, chlorophyll fluorescence, ABA concentration and biomass accumulation were carried out along with a combined gas-exchange/chlorophyll fluorescence protocol for mesophyll conductance estimation.

Chapter 5: In-field application of film antitranspirants shows potential yield protection from drought in *Brassica napus* L.

(To be submitted to *Crop and Pasture Science*).

The effectiveness of film-forming applications at mitigating drought-induced yield reductions on field-grown oilseed rape was investigated in **Chapter 5**. In **2014-2015,** the hypothesis that the application of two film-forming treatments improves yield under water deficit conditions at different phenological stages was tested. Conversely, in **2015/2016** the hypothesis that the efficiency of a film-forming treatment at increasing the yield of droughted oilseed rape is dependent on the dose rate was tested over flowering and pod development stages.

Chapter 2

Faralli M, *Grove IG, Hare MC, Boyle RD, Williams KS, Corke FMK, Kettlewell PS*. 2016 **Canopy application of film antitranspirants over the reproductive phase enhances yield and yield-related physiological traits of water stressed oilseed rape (*Brassica napus* L.).** *Pasture and Forage Science, 67 (7). pp. 751-765.*

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Abstract

Oilseed rape (*Brassica napus* L.) yield is strongly decreased by water deficit and crop management solutions are urgently required following the emergent difficulties in breeding for drought tolerant varieties. Film-forming antitranspirants (polymers) are agrochemicals that, applied onto the crop canopy, mechanically block the stomata and decrease canopy transpiration. In this work, the drought-protection efficacy of an adaxial-surface application at the flowering stage of two film-forming treatments (poly-1-*p* menthene and di-1-*p* menthene) was investigated in pot-grown, droughted oilseed rape over two glasshouse experiments. Over the drought period, the two compounds reduced leaf stomatal conductance (P<0.001), and as the soil moisture deficit increased, sustained carbon assimilation and improved water use efficiency with differing efficacy. Following the antitranspirant treatments, ABA concentration in leaves and reproductive organs was severely reduced and this was accompanied by significant improvements in leaf and flower/pod water potential. Drought significantly decreased by 39% on average the seed dry matter production of oilseed rape plants. The treatments significantly increased the seed dry matter by 13% and 17% respectively on average with respect to the un-sprayed droughted plants as a result of a significant increase in pods per plant by 11 and 13% respectively. These results suggest that film-forming compounds may be a useful crop management tool to avoid severe drought-induced yield losses in oilseed rape by improving water-use efficiency and plant water status and thus alleviating ABA signalling under water deficit.

Introduction

Climate change is increasing the need to maintain agricultural production under water-restricted conditions (Cattivelli *et al*. 2008). This steady trend of increased drought periods is motivating researchers to find new technologies and agronomic tools to avoid yield reductions in water-limited environments. Oilseed rape (*Brassica napus* L.) is the third largest source of vegetable oil in the world (FAOSTAT 2014). Hess *et al*. (2015) reported that oilseed rape is more drought sensitive than other crops such as wheat, and drought stress occurring at the reproductive stages is considered one of the main detrimental factors, reducing by 40-50% the total crop productivity (Champolivier and Merrien 1996). The sink size of the crop (hence, seed yield) is determined over a crucial period between flowering stage and mid-pod development stage (Mendham *et al*. 1981, Berry and Spink 2006). During this time-frame, adverse environmental conditions can substantially limit pod and seed production (Diepenbrock 2000).

Improving the water use efficiency (WUE) of crops and thus drought tolerance by stimulating stomatal closure through genetic modifications has been extensively used in a broad set of crops (Wan *et al*. 2009). In oilseed rape, significant yield improvements under moderate water deficit were found by Wang *et al.* (2005) where down-regulation of the α and β farnesyltransferase subunits showed higher yielding plants compared to the wild type through a reduced stomatal conductance over the drought period. In Georges *et al*. (2009), transgenic oilseed rape lines transformed to over-express the phosphatidylinositol-specific phospholipase C enzyme, involved in phosphatidylinositol-specific signal transduction pathway and thus responsible for stimulating intracellular Ca^{2+} release, exhibited early maturation, lower abscisic acid (ABA) and ABA catabolic derivate concentration and an improvement in instantaneous WUE. Despite these efforts, however a drought tolerant oilseed rape variety is still far from being achieved (Wan *et al*. 2009).

Under water deficit, stomatal closure triggered by the abscisic acid-mediated long-distance signals network helps the plants to avoid turgor loss at the expense of CO_2 uptake (Wilkinson *et al*. 2012). Stomatal closure, to attain WUE improvement, could be artificially triggered by exogenous ABA or ABA-dependent chitosan (Iriti *et al.* 2009) applications. However for large-scale farming, chitosan exhibited limited efficacy, i.e. could be useful only for occasional and short dry periods as reported by Iriti *et al.* (2009), and ABA is too expensive. Furthermore, ABA may show harmful effects on reproductive development. High ABA concentrations over the reproductive stages have been related to pod abortion in soybean (Liu *et al*. 2003), lower seed set in wheat (Westgate *et al*. 1996), and pollination and bud development disruption in oilseed rape (Shukla and Sawhney, 1994). Thus an ABA-independent stomatal conductance suppression that improves WUE and avoids ABA-mediating mechanisms which cause reproductive abortion and/or affect xylem ABA translocation to the reproductive organs may be preferred for crop management under drought conditions.

Film-forming antitranspirants (AT) are polymers that when sprayed in an emulsion with water onto the crop canopy are able to mechanically block the stomata (Kettlewell 2014) over a period between 10 (Anderson and Kreith 1978) and 30 days (Palliotti *et al*. 2013), depending on their chemical composition. Their efficiency in decreasing stomatal conductance (Kettlewell 2014), enhancing WUE under both non-stressed (Palliotti *et al*. 2013) and stressed (Moftah and Al-Humaid 2005) conditions and improving plant water status (Davenport 1972, Kettlewell 2014) have been well established. Previous work showed yield improvement under water-limited conditions when film-forming treatments were applied on wheat (Kettlewell *et al.* 2010). Indeed, a di-1-*p* menthene application over meiosis stage in pollen mother cells, improved pollen viability and grains per ear of droughted wheat through a reduction in transpiration rate, suggesting that the water saved was enough for the successful development of the reproductive processes (Kettlewell 2014). Similar yield-responses of droughted wheat treated with AT were recently shown by Abdullah *et al.* (2015). Furthermore, Iriti *et al*. (2009) showed that ABA concentration in leaves of AT-treated bean plants was significantly lower with respect to the un-treated control, suggesting the existence of an adaptive mechanism primed by a stomata blocking treatment. Despite the fact that when sprayed at high concentrations AT may create significant source-limitation by reducing CO_2 diffusion inside the sub-stomatal cavity (Anderson and Kreith 1978), field trials over two years showed that an AT application at the initiation of flowering (hence, at the beginning of the drought sensitive stage) on droughted *Brassica campestris* was able to increase the seed yield of the crop by 22% compared to the untreated control (Patil and De 1978). To our knowledge, however, the efficiency of ATs and their impact on the physiological mechanisms and processes have never been studied on droughted *Brassica* species.

In our work, two experiments were carried out i) investigating the effectiveness of two different film-forming compounds at increasing the yield of droughted oilseed rape and ii) understanding the physiological mechanisms involved in antitranspirant-related yield increase. Over the two experiments the hypothesis that the yield of droughted oilseed rape benefits from an additional decreased leaf conductance over flowering stage following AT application was tested. In addition, in Experiment I, leaf temperature was collected to evaluate a possible detrimental impact of an extra resistance at leaf level. In Experiment II the CO_2 assimilation, plant water status, stomatal behaviour, ABA concentration and their correlation with yield components of droughted oilseed rape were analysed.

Materials and methods

Plant material and experimental design

In Experiment I oilseed rape seeds (cv. Excalibur, DK seeds) were sown into six seedling planter trays (each with 24 pots with a volume of 100 mL) filled with John Innes No.2 loam-based potting compost (John Innes Centre, Norwich, UK) on the 1st of July 2014 inside the Harper Adams University (HAU) glasshouse (18±4°C average daily temperature, 70±10% relative humidity and an average daily photon flux density of 400 µmol m^{-2} s^{-1} from natural light supplemented with tungsten lamps measured with a PAR sensor installed in the CIRAS cuvette), and after twenty days, transferred into a cold room at 4°C for 8 weeks of vernalization (8 h/16 h light-dark photoperiod). Seedlings at the fifth true leaf stage (GS 1.5, Biologische Bundesanstalt, Bundessortenamt and Chemical Industry, BBCH canola growth scale) were transplanted on the 1st of October into 5 L pots (one plant per pot) containing John Innes No. 2 compost (~2000 g at 22 ± 1% volumetric water content [VWC] analysed with a soil moisture probe [ML2X theta probe, Delta-T-device, Cambridge, UK]) and placed again inside the glasshouse in the same conditions as above. The plants at rosette stage were then moved to the National Plant Phenomics Centre (NPPC) glasshouse on the 12th October 2014 and manually watered approximately to saturation every two days. Plants were grown in the glasshouse at 18±4°C temperature, 60±10% relative humidity and an average daily photon flux density of 400 μmol photons m^{-2} s^{-1} from natural light supplemented with incandescent lamps (16h / 8h light-dark photoperiod).

In Experiment II oilseed rape seeds (cv. Excalibur, DK seeds) were sown on the 15th of October 2014 inside the HAU glasshouse and, after twenty days (i.e. at fifth true leaf stage), moved into a polytunnel to vernalize over three months in natural winter conditions (minimum -4.5°C, maximum 19.0°C, average temperature 7.7°C). Plants at rosette stage were transplanted on the 16st of January 2015 into 5 L pots (one plant per pot) using John Innes No.2 compost and placed inside a glasshouse. All the pots were filled with compost at the same weight (2500 g at 22 ± 1% volumetric water content [VWC] analysed with a soil moisture probe [ML2X theta probe, Delta-T-device, Cambridge, UK]). Plants were grown in the greenhouse at 18±4°C temperature, 70±10% relative humidity and an average daily photon flux density of 400 μmol photons m^{-2} s^{-1} from natural light supplemented by incandescent lamps (16h / 8h light-dark photoperiod). A trickle irrigation system was used to ensure uniform irrigation at ~90% of field capacity of the compost. Before and immediately after the drought imposition (Days after spraying, [DAS] -3 and DAS 25 respectively) plants were fertilized by applying 0.5 g of N, 0.30 g of P and 0.30 g of K per pot. Powdery mildew was controlled by applying azoxystrobin (as Amistar Pro 1%, Syngenta, Basel, Switzerland) on DAS -5 and DAS 30.

Both experiments consisted of a 2 x 3 factorial design with two watering regime levels (well-watered, WW and water stressed, WS) and three antitranspirant treatments (no antitranspirant [- AT], 1% v/v Nu-Film P [NFP, a.i. poly-1-*p* menthene 96%] and 1% v/v Vapor Gard [VG, a.i. di-1-*p* menthene 96%]) in three randomised blocks for Experiment I and six blocks for Experiment II.

Drought imposition

The available water content (AWC) in mL of the pots was calculated by plotting a volumetric water content (VWC) - pot weight curve: three pots (filled with ~2500 g of compost at 22 ± 1% VWC) were water-saturated and then dried over ten days at 30°C. The VWC by soil moisture probe (ML2X theta probe, Delta-T-device, Cambridge, UK) and the weight by balance (0.1 g resolution, PCB 2500-2, Kern and Sohn GmbH, Balingen, Germany) were recorded daily. For John Innes No. 2 compost the permanent wilting point and the pot capacity were ~7% VWC and ~45% VWC respectively according to Saeed *et al.* (2008, unpublished data). The total AWC in mL was then calculated as the difference between the weight of the pot at pot capacity (~2700g in Experiment I and ~3350 g in Experiment II) and the weight of the pot at 7% VWC (~1600 g in Experiment I and ~2000 g in Experiment II) by moisture probe

In Experiment I, one week before the application of the ATs, the water use (WU) of each plant was calculated by weighing the pots each day using a balance integrated in the LemnaTec conveyor system (LemnaTec GmbH, Germany). From the WU data, watered plants were set to target weights of 2700g (well-watered, WW) and droughted plants were set to 2000g (~ 400 ml AWC, droughted, WS) at GS 6.0 (first flower open), from the 10th to the 24th of November (Fig. 1A). The pots were automatically weighed by the system at 07:00, 12:00, 19:00 and 24:00. At 19:00 plants were re-watered according to the target weights calculated in the week before the treatment application and from the AWC-weight curve. Soil evaporation was not taken into account. Air temperature was recorded by data logger (Priva group, De Lier, Netherlands) over the experiment (Fig. 1C).

In Experiment II, drought was imposed for 16 days (from GS 6.0 to GS 7.1, i.e. from the first flower open until the pods on the main raceme were completely formed) by removing the trickle irrigation. The pot AWC was then calculated as Eq. 1: [AWC= Pot weight – (pot weight at permanent wilting point + plant weight)]. Three spare plants were used to evaluate plant fresh weight at GS 6.0 (~300 g). During the stress treatment pots were weighed by the 0.1 g resolution balance every afternoon (15:00-16:00) (Fig. 1B). In late afternoon (17:00-18:00), the watered pots (WW) were re-watered to pot capacity if necessary while the droughted pots (WS) were filled to 500 mL of AWC by using Eq. 2 (water to add= 500 mL – Pot AWC [mL], weighed by balance). At 17 DAS trickle irrigation was re-applied to the WS plants. To evaluate the impact of the increasing plant weight on the re-watering process, the soil moisture probe was used to monitor daily the effective VWC of the pots. Air temperature was recorded by data logger (DS1921G Thermochron iButton, Maxim Integrated, San Jose, CA, USA) over the experiment (Fig. 1D).

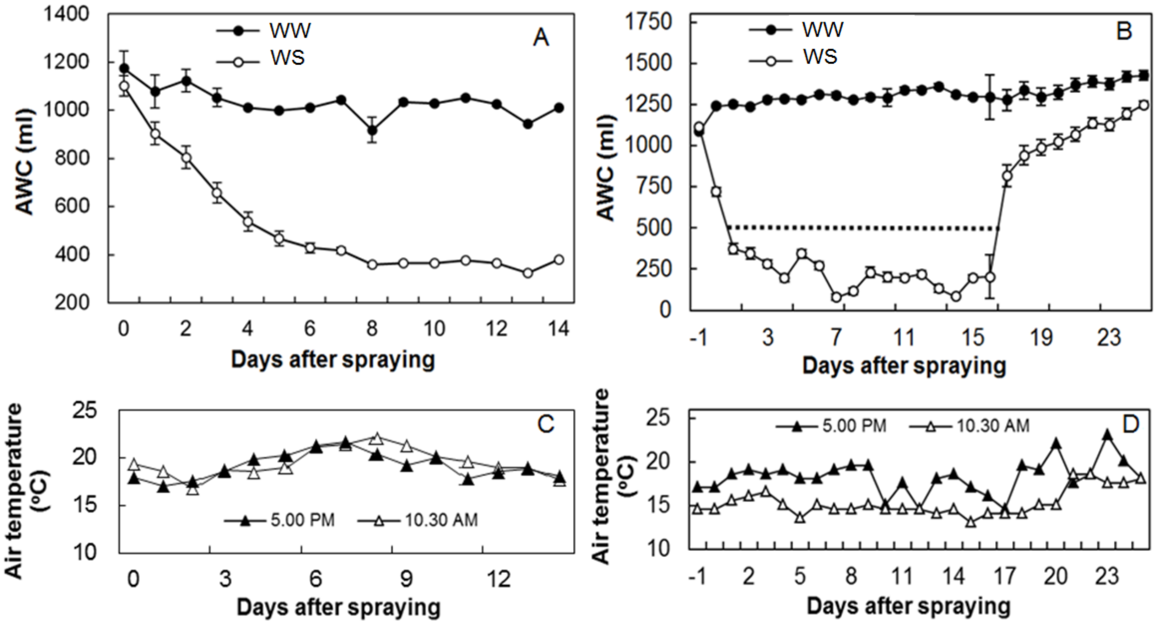

Figure 1. Pot available water content (AWC, mL) and air temperature over time for Experiment I (A, C respectively) and Experiment II (B, D respectively). Drought was applied at days after spraying (DAS) 0 on both the trials and removed at DAS 14 in Experiment I and at DAS 16 in Experiment II. In B, WS values represent the data before the afternoon re-watering and the dotted line represents the mean AWC values of the WS plants after the re-watering. Error bars=standard error (S.E.) (*n*=9).

Antitranspirant applications

Antitranspirants were applied just prior to the drought application on the same day in the early afternoon. The adaxial surface of the leaf was uniformly sprayed with either i) water (- AT), ii) a solution of 1% v/v of Nu-Film P (NFP) or iii) a solution of 1% v/v of Vapor Gard (VG) in water (20 mL of VG or NFP in 2000 mL of water ensuring 200 L ha^{-1} water volume at ~150-200 kPa) by a hand sprayer (Peras 7, Hozelock Exel, Beaujolais, France) on the 10th of November 2014 for Experiment I and on the 16th of February 2015 for Experiment II, i.e. GS 6.0 (first flower in the main stem open).

Gas-exchange analysis

In Experiment I, stomatal conductance of water vapour (g_s) was measured using a transient state diffusion porometer (AP4, Delta-T Devices, Cambridge, UK). The data were collected 3, 7, 9 and 14 days after spraying (DAS). The device was calibrated before every use by using the calibration plate provided. Three measurements of the abaxial g_s and adaxial g_s surface were collected from three fully expanded leaves on the top canopy per plant and the mean was used for statistical analysis. Total g_s (g_{stot}) was then calculated as adaxial g_s + abaxial g_s. Data were collected between 09:30 and 12.00.

In Experiment II the light-saturated CO_2 assimilation (A_{max}, µmol CO_2 m^{-2} s^{-1}) was measured on the first fully expanded leaf of the top canopy (*n*=6) using a CIRAS portable photosynthesis system (PP system, MA, USA) with a 2.5 cm^2 cuvette ensuring a saturating 1200 µmol m^{-2} s^{-1} PAR; all the data were recorded after 3–4 min at 380 ppm CO_2, when steady-state photosynthesis was achieved. Data were recorded between 09:30 and 12.00 the day before the spray application and 2, 5, 7, 9, 13, 15, 17, 20 and 22 DAS.

At the same time and on the same leaf, the adaxial (*n*=6) and the abaxial (*n*=6) leaf g_s was measured as described for Experiment I. A good correlation was found between values measured with the CIRAS and the porometer (R^2=0.53). Data were collected the day before the spray application and 1, 2, 3, 5, 7, 9, 11, 13, 15, 17, 20, 22, 24 and 25 DAS. Total stomatal conductance (g_{stot}) was then calculated as adaxial g_s + abaxial g_s (*n*=6).The intrinsic water-use efficiency ($_i$WUE) was then calculated as $A_{\mathrm{max}}/g_{\mathrm{stot}}$ (*n*=6).

Thermal image collection and analysis

Thermal images were collected during Experiment I with a VarioCAM head HiRes 640 x 480 pixel quality (spectral range 7.5 μm to 14 μm) camera (Jenoptik AG, Germany). Images (one per plant) were manually collected from the side on the 13th of November (3 DAS) and adjusted to around 40°-45° angle at 7, 9 and 14 DAS. These were collected between 16:00 and 17:00 inside a LemnaTec chamber installed in the conveyor system. The images were collected in the afternoon to avoid differences in temperature and light irradiance inside the data-collection chamber. At the same time, pictures were collected with a Basler (2454 x 2056) (Basler AG, Germany) camera (spectral range 3.8 μm to 7 μm) from both the side and top view. These data were collected around 18:00 before re-watering over the whole experiment.

For thermal image analysis, the objective was to separate the area of interest (leaves) from other objects included in the images. Image temperature was standardized between 15 °C and 20 °C using R (R Development Core Team 2013) to remove temperature background. A MatLab matrix (MathWorks Inc., Natick, Massachusetts, USA) was used to display the pixels calculated from matrix Z and to fill the areas between the pixels using constant colours corresponding to the current figure temperature. After the image conversion, the temperatures of 25 randomly selected pixels per leaf on three leaves per replicate representing the leaves used for g_s analysis were extracted and an Excel file was created for statistical analysis. The mean of the 75 pixels per plant was used for statistical analysis.

Water potential and stomatal analysis

On DAS 1, 7 and 14, plants from Block 1, 2 and 3 of Experiment II were used for leaf water potential (*L*ψ) and flower/pod water potential (*F*ψ) analysis. Between 12:00 and 14:00, three leaves or flowers/pods (without petals, as previously reported by Mogensen *et al.* (1997)) were excised with a scalpel from three plants for each treatment (*n*=3) and water potential was immediately analysed by a Scholander pressure chamber (SKPM 1405/50, Skye Instruments Ltd, UK). The tissues were analysed on the cut end of the petiole 1 cm from the base (leaf or flower/pod) (Mogensen *et al.* 1997). The water potential value (MPa) was collected by using a magnifying lens to see when water was exuding from the cut surface.

After water potential analysis, the first fully expanded leaf of the top canopy of plants (Block 3, 4 and 6) was used for stomatal imprinting. Dental putty (President Plus-light body; Coltène/Whaledent Ltd, Burgess Hill, West Sussex, UK) impressions were taken on DAS 1 and 7 on both the adaxial and abaxial surface of the leaf. The hardened material was removed from the leaf after approximately two minutes. Nail varnish peels were then produced from the impressions and these were transferred onto Polysine microscope slides (SLS; Hessle, North Humberside, UK). The stomatal width (the distance between the two guard cells) was measured using a microscope (Olympus CX31RBSF, Olympus Co., Shinjuku, Japan) fitted with a camera (Infinity 2, Lumenera Co, Nepean, Canada) and image processing equipment (Infinity analyse, Lumenera Co., Nepean, Canada). On each slide, 30 stomata were measured at 400x magnification. Stomatal number was also counted on 1 cm^2 area (*n*=3).

Plant dry matter analysis

On DAS 16, plants from Block 1, 2 and 3 of Experiment II were harvested to evaluate above ground biomass dry weight and root dry weight (*n*=3). Plants were separated into above ground biomass (stem, braches, pods and flowers) and roots by using pruning shears. Roots were then washed with tap water to carefully remove all the remaining soil. The collected tissues were then oven-dried at 70°C for 48 hours. The dry weight (DW) was recorded using the 0.1 g precision resolution balance and the ratio of root to shoot dry weight was then calculated.

ABA assay

At 12:00, the upper canopy leaves (DAS 3 and DAS 16) the buds/flowers (DAS 3) and the flowers/pods (DAS 16) from the main stem of the Experiment II plants were collected from Block 1, 2 and 3 for ABA assay (*n*=3). Tissues were excised with a scalpel, placed in 15 mL tubes and immediately flash-frozen in liquid nitrogen. The samples were then freeze-dried and stored in a -20°C freezer for ABA assay.

ABA concentration was measured with an enzyme linked immunosorbent assay (ELISA) (Cusabio Biotech Co. Ltd, Carlsbad, CA, USA). Briefly, freeze-dried samples (~0.4 g) were ground and extracted overnight at 4°C by adding 5 mL of extraction buffer (Cusabio Biotech Co. Ltd, Carlsbad, CA, USA). The supernatant was then collected and ELISA was performed according to the company procedure (code CSB-E09159Pl, http://www.cusabio.com, Cusabio Biotech Co. Ltd, Carlsbad, CA, USA).

Yield component analysis

For both experiments at complete maturity (13th of January 2015 for Experiment I, 6th of May 2015 for Experiment II), plants were hand harvested and pods were counted to determine pods per plant. Harvested above ground biomass and pods were oven-dried at 30°C for four days. Pods were then opened and dried seeds and remaining plant material weighed by balance (PCB 2500-2, Kern and Sohn GmbH, Balingen, Germany). Dried seeds were counted by seed counter (Henry-Simon KL8 CountMaster, UK) and the number of seeds per plant recorded. Harvest index (HI), seeds per pod and 1000-seed weight yield components were calculated as described by Diepenborck (2000). Seeds were then crushed and 1 g of dried seed per treatment was used for soxhlet (Soxtec Ht 1043, Tecator Ltd, Denton, UK) extraction using petroleum ether. Oil concentration was then calculated as (weight of fat / sample weight) x 100.

Statistical analysis

Watering data from Experiment I and II are presented as means ± standard error (SE) calculated as the standard deviation divided by the square root of the sample size . Stomatal conductance, leaf temperature, gas-exchange, water potential, stomatal width, dry matter production, ABA concentration and the pooled yield components of the two experiments data were subjected to a two-way analysis of variance (ANOVA) to assess antitranspirant (no antitranspirant, 1% NFP and 1% VG) and watering regime (WW and WS) effects. Data were checked for normality by examining residual plots. A Tukey's test ($P < 0.05$) was used for means separation. Regression was used to test the relationships between the data. All the statistical analyses were performed by using Genstat (16th edition, VSN International Ltd, UK)

Results

Leaf temperature and stomatal conductance

Unsprayed plants at pot-capacity showed an average g_{stot} of 402 mmol m^{-2} s^{-1} (Fig. 2A) and a mean temperature of 16.4 °C (Fig. 2B). An antitranspirant application on WW plants increased leaf temperature by 0.2 °C for NFP and 0.7 °C for VG on average when compared to the –AT by decreasing g_{stot} by 9% and 30% respectively on average. WS plants showed a mean temperature of 1.3°C higher than WW plants accompanied by a 65% significant reduction in g_{stot}. An antitranspirant application on WS plants increased leaf temperature to an average temperature of 17.9 °C for NFP and 18.2 °C for VG and reduced g_{stot} by 11% and 50% respectively with respect to the WW-AT plants.

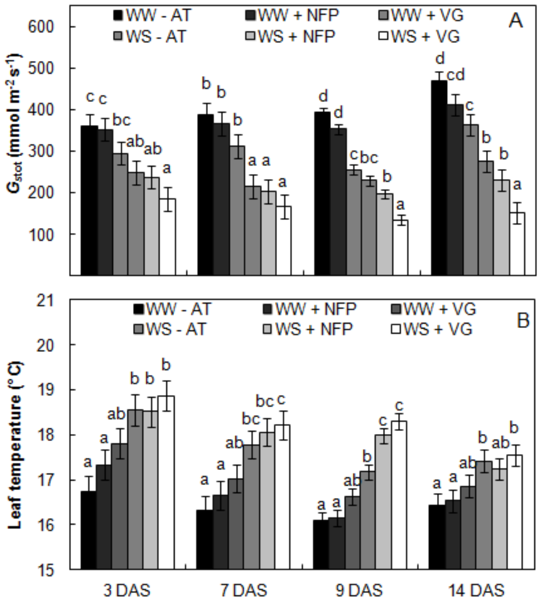

Figure 2. Effect of –AT (no antitranspirant), NFP (Nu-Film P 1% v/v) and VG (Vapor Gard 1% v/v) applications under WW (well-watered) and WS (droughted) conditions on A) Total stomatal conductance (g_{stot}) of oilseed rape plants and B) Leaf temperature of oilseed rape plants on days after spraying (DAS) 3, 7, 9 and 14. Data from Experiment I. Columns with the same letter are not significantly different according to Tukey's test at $P \leq 0.05$. Data are means (n=3) ± standard error of the differences of the mean (SED)

Gas exchange

Water stress at flowering stage reduced g_{stot} by ~40% in Experiment I and by ~50% in Experiment II on average compared to the WW plants ($P<0.001$ for all the DAS) (Fig. 2A and Fig. 3 A and 3B). Vapor Gard (VG) significantly decreased the g_{stot} in both WW and WS plants showing longer long-lasting effectiveness compared to NFP with a steady reduction in g_{stot} over both experiments. Nu-Film P showed smaller reductions in g_{stot} compared to VG (Fig. 2A and Fig. 3A and 3B) and these were not significant under WS throughout the experiments. The adaxial and abaxial surfaces clearly showed different responses to water-limitation: in Experiment II, water stress decreased the g_s on the abaxial surface by ~70% on average compared to the WW plants, while on the adaxial surface it decreased g_s by ~45% (Fig. 3C and E) and similar results were observed in Experiment I ($P<0.001$ for all the DAS on the abaxial surface, data not shown). In both of the experiments, the abaxial g_s analysis showed no significant differences between –AT, NFP and VG treatments as shown in Fig. 3C and 3D. However, although not significant, there was an indication of slightly higher values following the VG and NFP treatment under WS compared to the -AT and this was accompanied by significantly higher values during the first days of the recovery period. In contrast, the adaxial g_s values showed the conductance-reduction ability of the two film-forming treatments: sprayed on the WW plants, both NFP and VG significantly decreased the adaxial g_s for 13 and 22 days respectively, with a lower g_s value for the VG compared to the NFP plants (Fig. 3E and 3F). Vapor Gard treatment under WS showed a significant reduction in adaxial g_s during the whole water-limitation period in both of the experiments. In a similar way, though to a lesser extent, NFP was significantly able to reduce adaxial g_s but only for a relatively short period (DAS 5) in both of the experiments.

Oilseed rape plants experienced a strong reduction in CO_2 uptake when water-limited. The decrease in CO_2 assimilation was accompanied by a decrease in g_{stot} (Fig. 3 and 4). In Experiment II, both compounds applied to the WW plants significantly decreased the A_{max} over the experimental period (Fig. 4A and 4B). In contrast, both NFP and VG plants showed higher CO_2 uptake during the WS period.

Water-limited plants showed non-significant but higher $_i$WUE values compared with the WW plants (Fig. 4C and 4D). Vapor Gard treatment showed a significant increase in $_i$WUE when sprayed on WS oilseed rape plants compared with the -AT (Fig. 4D) whilst NFP did not significantly increase $_i$WUE (Fig. 4C). Furthermore, WW + VG plants gave an indication of higher $_i$WUE values with respect to WW-AT plants.

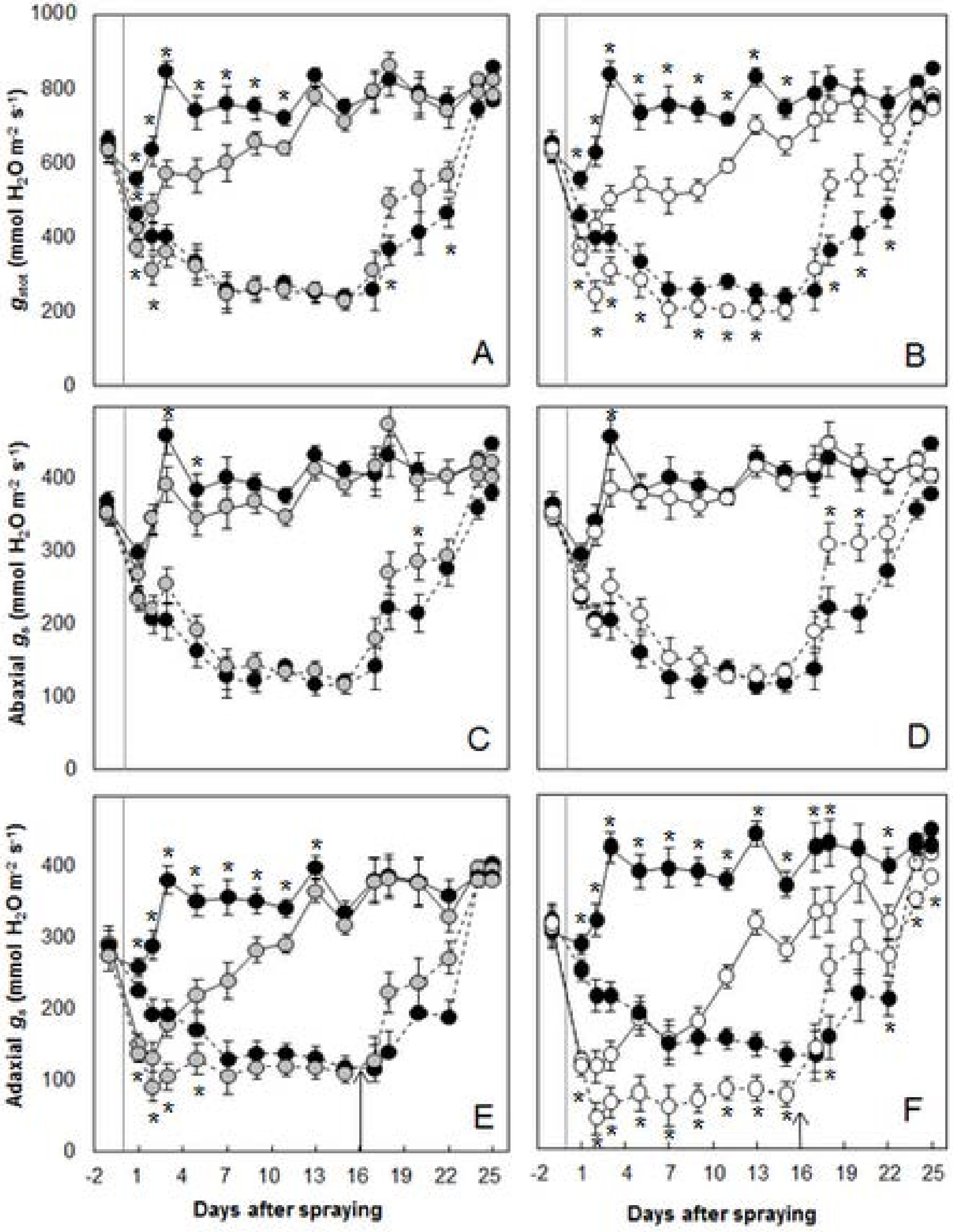

Figure 3. Total g_s (g_{stot}), abaxial g_s and adaxial g_s trend of A, C and E) WW-AT (—●—), WS-AT (--●--),WW+NFP (—●—), WS+NFP (--●--) and B, D and F) WW-AT (—●—), WS-AT (--●--), WW + VG (—○—), WS+VG (--○--) oilseed rape plants over flowering stage (WW – well-watered, WS – droughted, -AT – no antitranspirant, NFP – Nu-Film P 1% v/v, VG – Vapor Gard 1% v/v). The vertical line represent the day at which drought and spray were applied. Plants were re-watered at days after spraying (DAS) 16 (arrows). Asterisks represent significant differences between –AT and the respective +AT value (top for WW and bottom for WS) according to Tukey's test. Error bars= standard error of the differences of the mean (SED) (*n*=6 until DAS 16, from DAS 16 *n*=3). Data from Experiment II

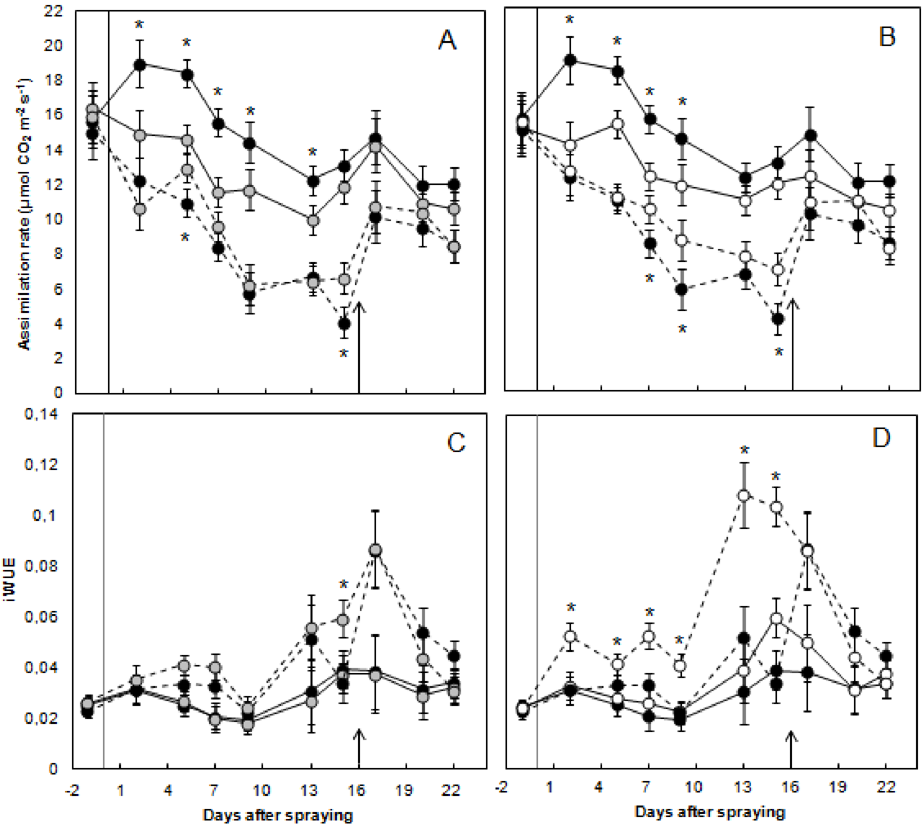

Figure 4. Light-saturated CO_2 assimilation rate trend of A) WW-AT (—●—), WS-AT (--●--), WW+NFP (—●—), WS+NFP (--●--) and B) WW-AT (—●—), WS-AT (--●--), WW+VG (—○—), WS+VG (--○--) and intrinsic water-use efficiency (${}_{i}$WUE, calculated as A_{max}/g_{stot}) of C) WW-AT (—●—), WS-AT (--●--), WW+NFP (—●—), WS+NFP (--●--) and D) WW-AT (—●—), WS-AT (--●--), WW+VG (—○—), WS+VG (--○--) of oilseed rape plants over flowering stage (WW – well-watered, WS – droughted, -AT – no antitranspirant, NFP – Nu-Film P 1% v/v, VG – Vapor Gard 1% v/v). Asterisks represent significant differences between –AT and the respective +AT value (top for WW and bottom for WS in A and B and top for WS in C and D) according to Tukey's test. The vertical line represent the day at which drought and spray were applied. Plants were re-watered at days after spraying (DAS) 16 (arrows). Error bars= standard error of the differences of the mean (SED) (*n*=6 until DAS 16, from DAS 16 *n*=3). Data from Experiment II

Leaf and flower/pod water potential

In WW plants *L*ψ and *F*ψ fluctuated around an average value of -0.3 and -0.5 MPa respectively from DAS 1 to DAS 14 (Fig. 5). With respect to the WW plants, drought significantly decreased the *L*ψ and *F*ψ to an average value of -1.2 and -

1.9 MPa respectively during DAS 7 and 14. Under WW conditions, the NFP and VG sprayed plants showed no significant differences to the control. In contrast, WS+VG plants had significantly higher *L*ψ and *F*ψ at DAS 7 and DAS 14 with respect to the WS-AT plants. NFP was able to increase *L*ψ and *F*ψ at DAS 7, with no significant differences at DAS 14 compared to the -AT.

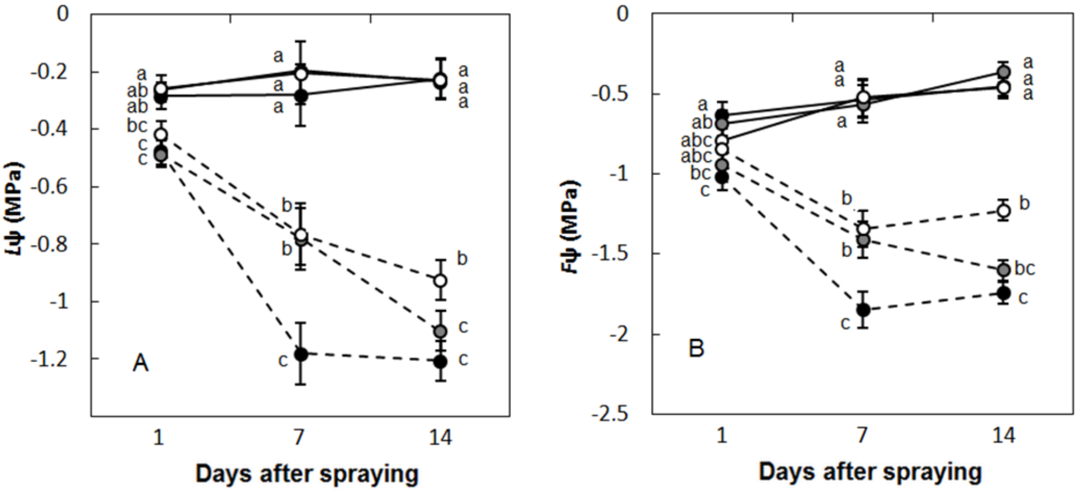

Figure 5. Leaf (*L*ψ, A) and flower/pod (*F*ψ, B) water potential of WW-AT (—●—), WW+NFP (—◍—), WW+VG (—○—), WS-AT (--●--), WS+NFP (--◍--), WS+VG (--○--) oilseed rape plants (WW – well-watered, WS – droughted, -AT – no antitranspirant, NFP – Nu-Film P 1% v/v, VG – Vapor Gard 1% v/v). Data points with the same letter are not significantly different according to Tukey's test at *P*≤ 0.05. Error bars= standard error of the differences of the mean (SED) (*n*=3). Data from Experiment II

Stomatal analysis

Stomata were unequally distributed between the surfaces with ~40% of total stomata number on the adaxial and 60% on the abaxial surface (data not shown). Microscope analysis of the stomatal width at DAS 1 showed significant increases in the adaxial stomatal apertures of the NFP and VG plants compared to both the WW-AT and WS-AT (Fig. 6A). At DAS 7 WS was effective in decreasing both the abaxial and the adaxial stomatal apertures by 55% relative to the WW plants (Fig. 6B). In WW pots and compared to the WW -AT plants, VG increased stomatal width at DAS 7, while the effect was not observed in NFP plants. No differences were observed in abaxial stomatal apertures under the WW regime. Conversely under WS both NFP and VG significantly increased adaxial stomatal aperture width (Fig. 6). Under WS, abaxial stomatal aperture was wider with VG than the untreated control (-AT), whereas NFP was not significantly different from either.

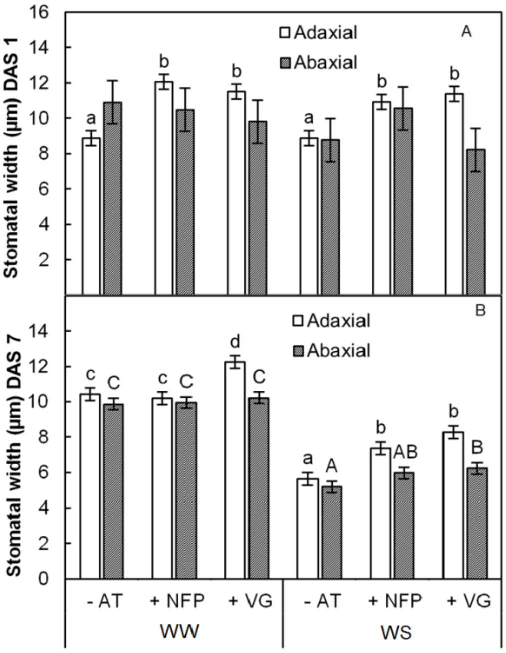

Figure 6. Stomatal widths of oilseed rape plants treated with -AT (no antitranspirant), NFP (Nu-Film P 1% v/v) and VG (Vapor Gard 1% v/v) under WW (well-watered) or WS (droughted) conditions for 16 days during flowering stage. A) days after spraying (DAS) 1 B) DAS 7. Columns with no letters (lowercase - adaxial, capital –abaxial) are not significantly different according to Tukey's test at $P \leq 0.05$ (n=30). Error bars= standard error of the differences of the mean (SED).

Plant dry matter

Water stress significantly decreased the plant above ground DW compared to the WW plants. In WW plants VG application significantly decreased the above-ground DW. On the contrary no differences in above-ground DW were found in NFP-treated plants with respect to the -AT (Table 1). Under drought, the two products (NFP and VG) increased the above-ground DW by 18% and 15% respectively compared to the WS –AT.

In contrast, root DW was not significantly affected by either drought or antitranspirant application.

The root/shoot ratio was significantly affected by the water limitation with an increase compared to the WW plants. Nu-Film P application did not affect root/shoot ratio in either of the water regimes. Vapor Gard application did not change the root/shoot ratio when sprayed on WS plants but significantly increased the root/shoot ratio on WW oilseed rape plants relative to the -AT due to a significant decrease in above-ground DW and a non-significant increase in root DW.

Table 1. Effect of –AT (no antitranspirant), NFP (Nu-Film P, 1% v/v) and VG (Vapor Gard, 1% v/v) applications on plant above-ground dry weight (DW), root DW and root/shoot ratio of oilseed rape plants grown in WW (well-watered) and WS (droughted) conditions over flowering stage. Means within a column followed by the same letter are not significantly different according to Tukey's test at *P*≤ 0.05 (*n*=3, d.f.=17). Data from Experiment II

Treatment	Above-ground DW (g)	Root DW (g)	Root/shoot ratio
WW – AT	53.33^c	32.32^a	0.61^a
WW + NFP	49.94^c	34.02^a	0.75^{ab}
WW + VG	43.69^b	41.16^a	0.92^b
WS – AT	34.32^a	31.90^a	0.92^b
WS + NFP	42.18^b	34.83^a	0.82^{ab}
WS + VG	39.67^b	35.89^a	0.90^b
ANOVA			
SED	1.49	3.58	0.06
Watering regimes *P*	<0.001	0.451	0.008
Antitranspirants *P*	0.007	0.079	0.019
Watering regimes x Antitranspirants *P*	<0.001	0.472	0.012

ABA concentration

Leaf ABA concentration in WW plants was 319 ng g^{-1} DW on DAS 3 (Fig. 7A) and 360 ng g^{-1} DW on DAS 16 (Fig. 7C) which was significantly lower than that of the WS plants (646 ng g^{-1} DW on DAS 3 and 1391 ng g^{-1} DW on DAS 16) (Fig. 7A and 7C respectively). A NFP and VG application on WW plants showed statistically non-significant changes from the WW-AT plants, despite the lower values (288 ng g^{-1} DW and 250 ng g^{-1} DW on DAS 3 and 355 and 265 ng g^{-1} DW on DAS 16 respectively). On WS plants, both NFP and VG significantly decreased the ABA concentration relative to the -AT control by 66% and 74% respectively on DAS 16 whilst on DAS 3 the reduction was ca. 2-fold.

The flower/pod ABA concentration of WW plants was found to be ca. 2-fold higher than that of the leaf on both DAS 3 and DAS 16 (Fig. 7). The WS plants showed a strong increase in ABA concentration by 3-fold on DAS 3 and by 4-fold on DAS 16 compared to their relative WW control. A NFP application on WW plants did not change the ABA concentration while in WS plants a significant 4-fold reduction was found compared to the -AT plants on DAS 3 and a 2-fold reduction on DAS 16. At the same time the ABA concentration was decreased by VG on WS plants by 4-fold relative to the WS–AT plants on both DAS 3 and DAS 16.

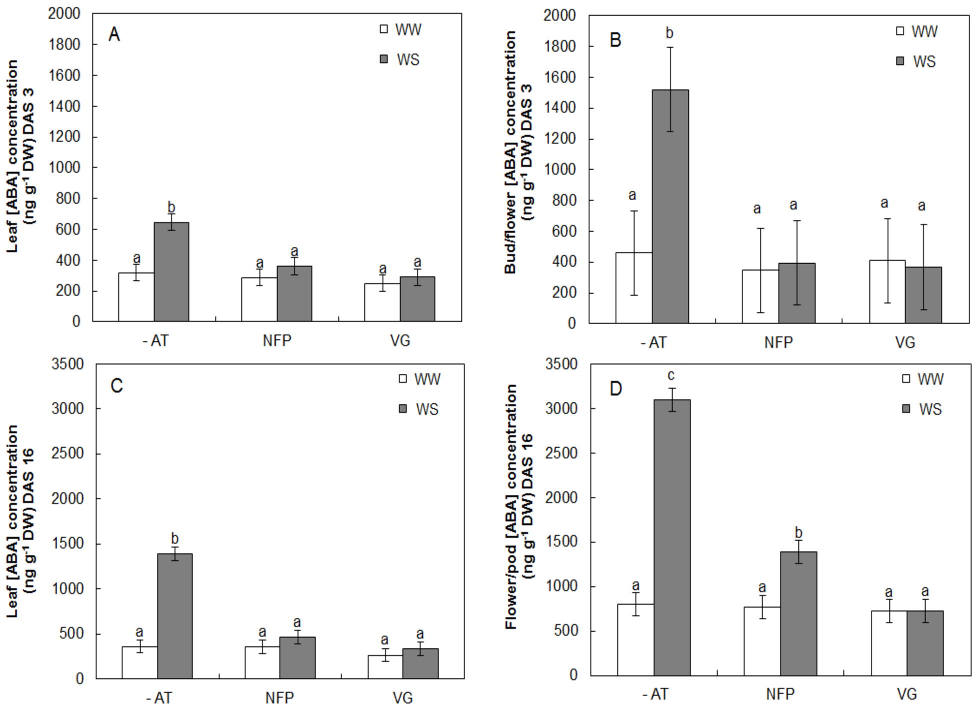

Figure 7. Endogenous ABA concentration in leaf (A) and bud/flower (B) sampled at days after spraying (DAS) 3 and in leaf (C) and flower/pod (D) sampled at DAS 16 of oilseed rape plants sprayed with -AT (no antitranspirant), NFP (Nu-Film P 1% v/v) and VG (Vapor Gard 1% v/v) and subjected to WW (well-watered) and WS (droughted) conditions. Columns with the same letter are not significantly different according to Tukey's test at $P \leq 0.05$ ($n=3$). Error bars= standard error of the differences of the mean (SED). Data from Experiment II

Yield components

Water deficit over the reproductive stages decreased the seed dry matter, pods per plant, seeds per pod, and the harvest index by 39% ($P<0.001$), 34% ($P<0.001$), 3% ($P=0.002$) and 22% ($P<0.001$) respectively with respect to the WW plants. The changes in 1000-seed weight were negligible. Oil content was not significantly affected by either drought or antitranspirant application in both of the experiments (data not shown).

On WW plants, both NFP and VG application significantly decreased pods per plants (Table 2). However, seed dry matter production and the other yield components were not affected. NFP application on WS plants showed a trend at increasing seed dry matter, pods per plant and harvest index by 13%, 11%and 9% with respect to the WS-AT. On the contrary, 1000-seed weight was significantly increased by 11%.

With respect to the WS-AT, WS-VG plants had higher seed dry matter and pods per plant by 17% and 13%respectively. Among the physiological traits analysed, seed dry matter showed significant relations with *F*ψ (Fig. 8C) as well as pods per plant (Fig. 8D). Furthermore, *F*ψ was well correlated with *L*ψ (Fig. 8A).

Table 2**.** Effect of –AT (no antitranspirant), NFP (Nu-Film P, 1% v/v) and VG (Vapor Gard, 1% v/v) applications on yield components of oilseed rape plants grown in WW (well-watered) and WS (droughted) conditions over flowering stage. The data presented are averages of two experiments. Means within a column followed by the same letter are not significantly different according to Tukey's test at *P*≤ 0.05 (*n*=6, d.f.=35).

Treatment	Seed dry matter (g)	Pods per plant	Seeds per pod	1000-seed weight	Harvest index
WW – AT	22.49^{c}	367.00^{d}	11.13^{ab}	5.41^{ab}	25.52^{c}
WW + NFP	20.92^{c}	331.80^{c}	11.83^{ab}	5.14^{a}	24.61^{bc}
WW + VG	22.47^{c}	325.70^{c}	12.88^{b}	5.26^{a}	24.69^{bc}
WS – AT	13.55^{a}	242.20^{a}	10.85^{ab}	5.39^{ab}	19.80^{a}
WS + NFP	15.49^{ab}	269.70^{ab}	9.96^{a}	6.02^{c}	21.75^{ab}
WS + VG	16.29^{b}	276.30^{b}	10.38^{a}	5.88^{bc}	20.65^{a}
ANOVA					
d.f.	35	35	35	35	35
SED	0.60	11.5	0.76	0.17	1.14
		P-values			
Antitranspirant	0.008	0.871	0.357	0.26	0.765
Watering regime	<0.001	<0.001	0.002	<0.001	<0.001
Experiment x Antitranspirant	0.005	0.588	0.845	0.227	0.454
Experiment x Watering regimes	<0.001	0.003	0.54	<0.001	<0.001
Antitranspirant x Watering regimes	0.001	<0.001	0.133	0.004	0.231
Experiment x Antitranspirant x Watering regimes	0.237	0.406	0.631	0.132	0.45

Discussion

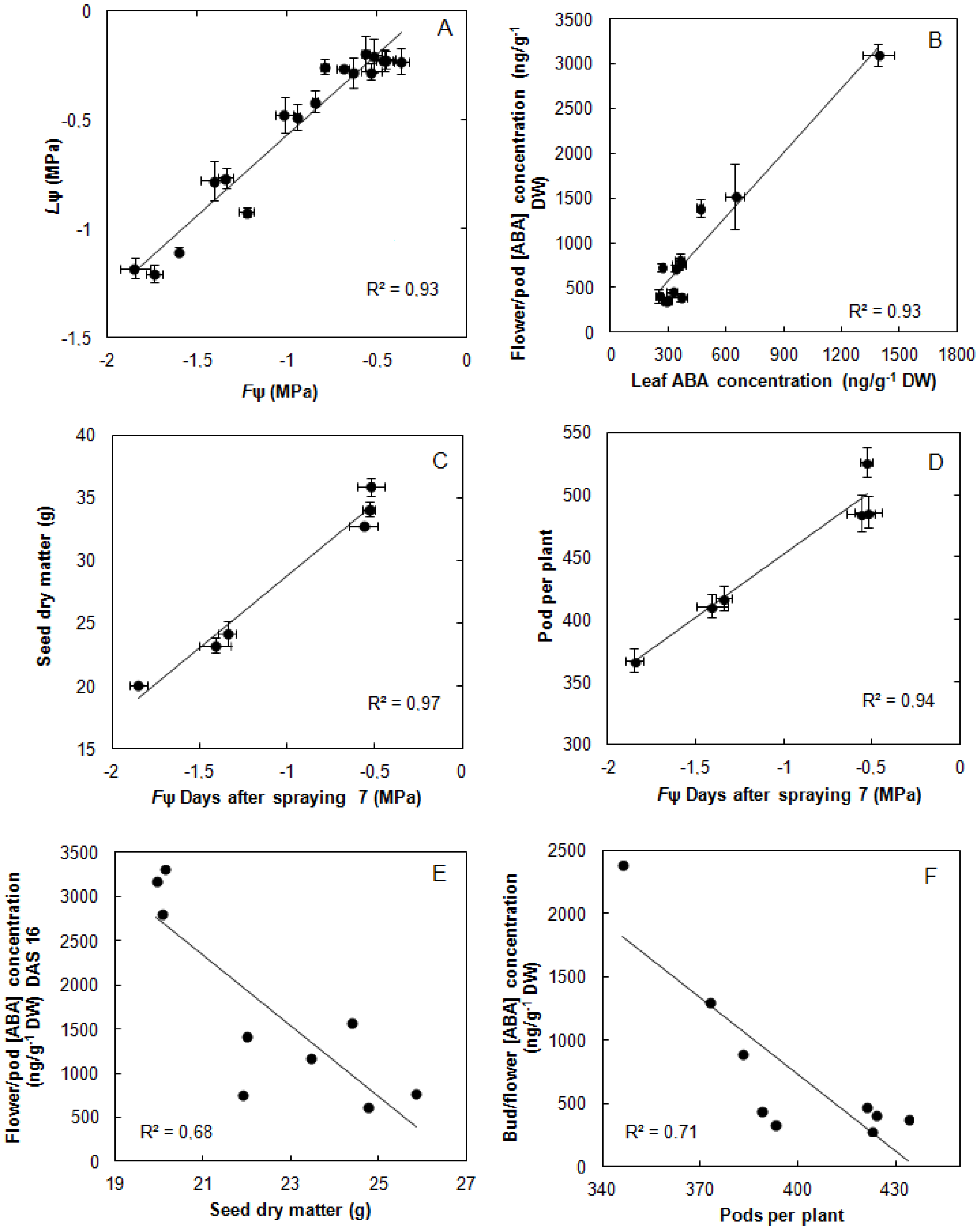

Figure 8. Relationship between leaf water potential (*L*ψ) and flower/pod water potential (*F*ψ) (A), flower/pod ABA concentration and leaf ABA concentration (B), flower/pod water potential (*F*ψ) and seed dry matter (C), flower/pod water potential (*F*ψ) and pod per plant (D), flower/pod ABA concentration and seed dry matter under water stress (E), bud/flower ABA concentration and pods per plant (F). Points are single plant data in E and F whilst in A, B, C, D points represent means. Lines were fitted using linear regression. Data from Experiment II. Bars in A, B, C and D indicated standard error of the differences of the mean (SED) (*n*=3).

Gas-exchange and leaf temperature

Film forming antitranspirants, unlike the metabolic compounds (e.g. ABA or chitosan), depress leaf gas-exchange by mechanically blocking the stomata (as extensively shown in Weerasinghe 2013). In our experiments, both VG and NFP were able to suppress g_{stot}, with a stronger and longer-lasting effect of VG (di-1-*p* menthene) compared to NFP (poly-1-*p* menthene): similar long-lasting effectiveness of VG was found by Palliotti *et al.* (2013). The g_{stot} suppression of the treatments was purely derived from a suppression of the adaxial surface g_s (i.e. where the compound was sprayed to simulate a field treatment) while under WS, a small increase in abaxial g_s was also found. Under WS, the abaxial g_s was less affected by drought than the adaxial, suggesting different mechanisms involved in stomatal control and/or sensitivity of guard cells to ABA and Ca^{2+}as previously reported by Wang *et al.* (1998). The lower effect of both of the ATs on gas-exchange characteristics under WS compared to WW environments found in Experiment II suggests that as stomata close, the induced gas-exchange suppression by the treatments may therefore lose efficiency. Leaf temperature was significantly (P<0.001) and negatively (r=-0.99) correlated with the g_{stot} indicating that ATs raised leaf temperature. However, the range of leaf temperatures observed did not affect the normal physiological processes of the plants. Similar results were found by Gale and Poljakoff-Mayber (1965). In the field, leaf temperature is controlled by a large number of environmental (e.g. wind) and biological factors and transpiration is only part of the leaf heat balance. The results from Palliotti *et al*. (2013) and Gale and Poljakoff-Mayber (1966) support this suggestion. Further work is needed to evaluate the interactions between drought, heat and ATs.

The improvement in $_i$WUE followed by a sustained A_{max} value under WS found in VG treated plants was previously described by other authors. Canopy gas-exchange of *Vitis vinifera* treated with 2% v/v VG confirmed the capacity of the product to increase plant $_i$WUE (Palliotti *et al*. 2010). In Moftah and Al-Humaid *(*2005) and Latocha *et al.* (2009), photosystem II stability and/or carbon assimilation of different crops were sustained after VG application prior to water deficit imposition suggesting that i) the product could be more permeable to CO_2 rather than H_2O or that ii) ATs could avoid drought-induced photoinhibition due to the improvement of the plant water status. Moreover, as reported by King *et al.* (1967), where excision of the ear from wheat plants decreased photosynthetic rate, sink strength controls the carbon assimilation of the source-leaf due to the source-sink interrelationships. Thus, lower pod abortion and higher seed set may lead to higher assimilate demand by the sink following the film-forming treatment. Whether the sustained A_{max} is due to the wider abaxial stomata opening or to metabolic factors needs further investigation. Abdullah *et al*. (2015) argued that the sustained carbon assimilation over the drought period following a Vapor Gard application on wheat may be due both to the higher soil moisture conservation of the sprayed plants and to a possible higher carbon recycling/re-fixation allowed by the higher plant water status. The faster and more efficient gas-exchange recovery of the VG and NFP treated plants after the stress imposition compared to the -AT ones may be evidence that metabolic factors could be involved in reducing photochemistry efficiency and/or carbon fixation in the presence of strong water deficit.

Plant water status and stomatal opening

Reproductive structures often have a high sensitivity to water deprivation (Prasad *et al.* 2008, Liu *et al*. 2003). It has been previously shown that a more negative water potential has a direct effect on the functions and structures of the reproductive organs of soybean and wheat (Liu *et al.* 2003, Westgate *et al*. 1996). In maize, low water potential down-regulated the transcription of acid-invertase genes in turn reducing carbohydrate metabolism in the ovaries (Andersen *et al*. 2002). In our experiments, *L*ψ was significantly correlated with *F*ψ, and thus the extra resistance from application of polymer at leaf level (NFP and VG) significantly improved the water status of the reproductive structures. Similarly, in Kettlewell (2014) a 1.25 v/v di-1-*p* menthene application on droughted wheat improved leaf water potential by 25% compared to the un-sprayed plots. Since the stomata under a film-forming treatment are more open, the increase in *L*ψ is conceivable as previously reported by Davenport *et al.* (1972) and Iriti *et al*. (2009). In addition the higher water potential found in NFP (DAS 7) and VG (DAS 7 and 14) treated plants under WS in both leaves and pods was closely correlated with the increased seed dry matter production of the plants (R=0.60 and R=0.48 on DAS 7 and 14 respectively). The stomatal opening was affected differently by the two compounds (NFP and VG) with the VG (high stomatal conductance suppression) which showed wider stomata compared with NFP (low stomatal conductance suppression) thus reflecting their capacity to improve *L*ψ. The guard cells distance could also be used as an alternative tool to screen efficiency of film-forming compounds in improving plant water status, if a rapid technique for assessment could be devised. The results suggested that AT's, by increasing the stomatal resistance to water vapour, were able to save water for the normal biological processes over the reproductive stages as previously reported in wheat by Kettlewell (2014).

ABA concentration

Similar ABA concentrations in tissues of watered and droughted oilseed rape were found by Qaderi *et al*. (2006). The small decrease in ABA concentration found in WW VG and NFP treated plants on DAS 3, is in contrast to the 4-fold decrease found by Iriti *et al*. (2009) in WW bean plants. However, in our experiments the sampling was done on different days compared to Iriti *et al*. (2009) and a strong diversity in terms of xylem sap alkalinisation (hence ABA concentration) has been previously shown in a large number of plant species by Sharp and Davies (2009). The strong decrease in leaf ABA concentration on both DAS 3 and DAS 16 under WS suggests that by maintaining higher soil moisture due to the decreased g_s, ATs may attenuate ABA signalling and thus ABA xylem translocation.

It has previously been shown that ABA is not produced in the reproductive organs under water deficit (Liu *et al*. 2003) and Morgan and King (1984) suggested that ABA accumulation in the spikes of droughted wheat is linearly related to leaf turgor decrease and leaf ABA concentration. Westgate *et al*. (1996) used root pressure chambers to maintain high tissue water potential under drying soil. There was evidence that by maintaining high leaf and shoot water status, the effect of water deficit in wheat grain set was reduced due to less ABA accumulated (only 3-fold compared to the control unlike a 15-fold accumulation in stressed un-pressurized plants). In our study leaf and flower/pod ABA concentrations were significantly and linearly correlated (Fig. 8B), reinforcing the hypothesis that leaf ABA could be a major source of the ABA translocated to the reproductive organs leading to detrimental effects on yield. Consequently the AT treatments, by decreasing leaf ABA were capable of depressing flower/pod ABA. However, the decline in ABA concentration of the AT-treated plants under WS (restored to similar values to the WW) does not match the significant less negative values in *L*ψ and *F*ψ. VG and NFP applications may have i) a strong impact in cell turgor maintenance leading to the lower leaf ABA accumulation (and thus translocation) and ii) an impact on the soil moisture available leading to a suppression in ABA signalling. Whether this is only due to lower ABA translocation, lower *in loco*-leaf production or higher ABA catabolism deserves in-depth analysis.

The flower/pod ABA concentration was consistently higher than that of the leaf. Hence the root-produced and the xylem-translocated ABA could contribute to ABA accumulation in the reproductive organs under water-limited conditions. In addition, as previously reported by de Bouille *et al.* (1989), low ABA concentrations could play a role in oilseed rape flower/pod development.

In oilseed rape, there is a lack of studies related to seed or pod abortion following ABA accumulation. Shukla and Sawhney (1994) in a semi-*in vivo* study showed that pollen viability and bud length were dramatically decreased with a strong increase in ABA concentration (10^{-3} M). Bouttier *et al*. (1992) showed that *in vitro* ABA addition (10^{-5} M) to pod cultures in the absence of stress decreased seed number per pod, pod length and pod dry weight. It is not clear to what extent ABA concentration plays a role in pod or seed set; however, the strong depression of the concentration following the NFP and VG treatments in WS conditions and the significant increase in seed dry matter and pods per plant suggest that, at high concentrations, an ABA-mediated abortion mechanism affecting pod and/or later seed formation may have occurred in oilseed rape over WS periods. Our results indeed suggest that under drought conditions there are significant and negative correlations between pods per plant and bud/flower ABA concentration at DAS 3 (Fig. 8F) and seed dry matter and flower/pod ABA concentration at DAS 16 (Fig. 8E).

Plant dry matter

The increased root to shoot ratio in WS plants compared to WW plants confirms the plant's capacity under drought stress to re-partition assimilates. Other work has shown that stressed plants, via root/shoot chemical signals, tend to increase root proliferation to improve water and/or nutrient uptake (Deleens *et al.* 1989) at the expense of the shoot biomass accumulation. In *Brassica napus*, under water deficit, roots became the main sink for assimilates leading to long-term adaptive strategies such as root rhizogenesis (Deleens *et al.* 1989). In our study, however, AT-treated plants showed higher above-ground DWs and a trend (*P*=0.079) at increasing root DWs, suggesting higher photoassimilate availability over the drought stress period potentially because up-regulation of ABA under stressful environments can reduce shoot/leaf expansion (Wilkinson *et al*. 2012) and/or via enhanced root hydraulic conductivity. Since the AT-treated plants showed a significant decrease in ABA concentration compared to the -AT, the increase in plant dry matter is conceivable. Moreover, as reported by Davenport *et al.* (1972), cell growth and expansion is dependent on both carbohydrate availability and water status, thus an improvement in $L\psi$ may explain the increase in above ground DW. Under stressed conditions, consistent with our work, higher DW values after a film-forming treatment on droughted peppers were found by Goreta *et al.* (2007). The increased root/shoot ratio in VG-treated plants under WW regimes suggests the existence of a root-to-shoot source-sink alteration triggered in the plant after the treatment: indeed the data shows a trend (*P*=0.079) at increasing the root proliferation and a significant decrease of the above-ground DW accumulation that deserve further investigations.

Yield components

In both of the experiments, the reduction in pods per plant due to the NFP and VG application on WW plants is an indication of the source-limitation imposed by the two film-forming compounds, which, despite the increase in $_i$WUE, depresses A_{max}. However the seed dry matter was not affected and the increase in seeds per pod in VG-treated plants suggests that plants were able to recover after the flowering stage antitranspirant-induced source-limitation. This hypothesis is also supported by the lower above-ground dry matter production of the VG-treated plants, despite there being no difference in harvest index. Therefore, it is possible that more assimilates were available and/or translocated to the seeds. Similar results were found by Berry and Spink (2009) in several field experiments where a triazole (metconazole) treatment in earlier growth stages (GS 5.0) enhanced seed set probably by both suppressing stem extension (thus more assimilates available) and branch length (thus more light intercepted by the lower canopy pods). However, while the metconazole mechanism is due to the inhibition of gibberellin biosynthesis (Berry and Spink 2009), the AT canopy regulation, at this stage, could only be explained by the induced source limitation.

Water deprivation over GS 6.0 and GS 7.1 significantly decreased the seed dry matter production of oilseed rape by 39%. The decrease in seed yield was associated with a reduction in seeds per plant (data not shown) and, although to a lesser extent, to lowered pods per plant yield. Our results are in agreement with the data from Champolivier and Merrien (1996). Since pod number and seed yield showed a strong positive correlation (Richards and Thurling 1978) and their determination seems to be fully defined just after anthesis (Habekotte, 1993), a carbohydrate limitation over the flowering stage due to less carbon assimilated appeared to be the major factor involved in pod abortion and limitation of the sink size as postulated by Habekotte (1993) and by Bouttier *et al*. (1992).

VG application improved the yield components of the crop as a result of the number of the physiological traits improved over the flowering stage. A film-forming compound (Mobileaf, a film-forming wax emulsion) sprayed at the initiation of flowering on droughted *Brassica campestris* increased seed dry matter production by ~20% with respect to the un-treated droughted control (Patil and De 1978). However, in our experiments, while NFP resulted in a significant increase in 1000-seed weight with a trend in increasing pods per plant, seed dry matter and harvest index, VG showed increases in pod per plant, seed dry matter, and an indication of an increase in the harvest index without significantly enhancing the 1000-seed weight. NFP reduced gs_{tot} less and over a shorter period compared with VG suggesting that the protection from drought was applied just over the first days of stress (thus over the pod formation period on the main stem). Conversely, increased 1000-seed weight could be related to the intrinsic compensative capacity of oilseed rape and the physiological amelioration of NFP under WS may have made a significant contribution. In contrast, VG, by ensuring a protection over the whole period, resulted in a higher sink size. This led to higher seed dry matter that depended only on the higher number of pods formed. This suggests that VG was not able to increase seed per pod yield component. This can be due to a possible lack of assimilates available that were not enough to entirely fill all the formed pods despite the VG-induced physiological amelioration under WS.

To conclude, we demonstrate that, as for other crops, oilseed rape yield is strongly affected during flowering stage by a synergistic effect of hydraulic and hormone signals, in turn related to a lack of assimilate availability due to the decrease in photosynthetic efficiency. According to our experiments, a film forming application at 1% v/v on WS oilseed rape canopy just after the GS 6.0 stage improved the physiological and the yield responses of the plant to WS by triggering a more complex mechanism than has been previously postulated (Solarova *et al.* 1981). The sustained carbon assimilation recently showed also by Abdullah *et al.* (2015) in wheat and the water potential improvement strongly correlated with the decrease in ABA concentration over the reproductive period are the main physiological traits involved in the yield decline avoidance under water deficit following a film-forming treatment. Moreover, significant modifications in root/shoot ratio were found. Vapor Gard seems to be more efficient for long drought periods (i.e. 20-25 days) whereas NFP could be a good candidate to overcome short water-deficit imposition (i.e. 7-10 days). However, at this stage, care must be taken in data interpretation as water deficit imposed in pots is much stronger and develops much more rapidly than the stress generally occurring in the field and the AT application was done just prior to the drought imposition and thus the optimal conditions for a film-forming treatment.

There is a lack of management strategies to avoid water deficit damage in oilseed rape. Accordingly, further investigation is required to assess the effectiveness of the treatments to improve oilseed rape responses under water-limited environment at the field level.

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Chapter 3

Faralli M, *Grove IG, Hare MC, Alcalde-Barrios A, Williams KS, Corke FMK, Kettlewell PS.* 2017.**Modulation of *Brassica napus* source-sink physiology through film antitranspirant induced drought tolerance amelioration that is dependent on the stress magnitude.** Journal of Agronomy and Crop Science, 203(5), pp.306-372.

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Abstract

Increase in drought conditions during the oilseed rape 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 OSR plants at different soil moisture regimes. Therefore, two similar experiments were performed in a computer-controlled glasshouse/phenotyping centre to investigate the physiological responses of winter oilseed rape 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 gas-exchange, increased leaf temperature, leaf-to-air temperature, bud-to-air temperature and ABA concentrations 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 under particular time-frame and magnitudes of soil moisture deficit may allow oilseed rape to sustain reproduction and avoid yield losses.

Introduction

Drought is considered one of the main detrimental factors in crop productivity and the magnitude of dry events may be subjected to significant increases 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 water scarcity appears over key-sensitive phenological stages (i.e. reproductive periods) (Saini and Westgate 1999). Anthesis is a high 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). In oilseed rape (OSR, *Brassica napus* L.) drought periods over flowering and mid-pod development stage can lead up to 40% of yield losses (Richards and Thurling, 1978; Champolivier et al. 1996). OSR is considered one of the most drought sensitive crops during anthesis; several studies report significant 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).

Crop’s drought-avoidance strategy avoids tissue dehydration by i) minimising water loss and ii) maximising water uptake (Chavez et al. 2003). These two goals are achieved by i) decreasing transpiration through stomata closure or by ii) 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 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 following the first step of the crop’s adaptation under drought (minimizing 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 chemical (AT) and metabolic compounds with antitranspirant activities have been recently tested with satisfactory results. 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 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 seedling trays filled with John Innes No. 2 compost (John Innes Manufacturers Association, Reading, UK) on the 20th December 2014 for Experiment I and the 3rd June 2015 for Experiment II. Seedlings at the fourth leaf stage were transferred into a cold room and vernalized at 4°C for 8 weeks (16h / 8h light-dark photoperiod at ~200 µmol m^{-2} s^{-1} PAR). On the 16th February 2015 for Experiment I and on the 19th 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 to saturation every two days. The pots were then 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) target watering was applied 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 (BBCH canola growth scale, first flower open) and over the whole flowering stage (until GS 6.9 BBCH canola growth scale, end of flowering - 10% of pods reached the 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 (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 subtracting from the daily ET the daily soil evaporation (SE_{vap}). Briefly, compost-only pots (n=3) were added to the experiments with a gravimetric soil moisture similar to that of the four watering regimes applied (WW, MWS, SWS, WS). This allowed the 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:

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

Where ET was the daily ET for each treatment and SE_{vap} was the daily soil evaporation for each watering regimes calculated as above.

Antitranspirant application

The antitranspirant was applied in the early afternoon just prior to drought initiation (Days after spraying 0, DAS 0). 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 24th of March 2015 for Experiment I and on the 9th of September 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 9:00 and 12:00

In Experiment II gas exchange analysis was 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 ($_i$WUE) was then calculated as the ratio between the micromole of CO_2 assimilated (A_{max}) and the mole H_2O loss (g_s) 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:

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

where 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). On 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, DAS 7 and DAS 16, leaf (n=4) and reproductive organs (ready-to-open bud on DAS 3, flower/pod on DAS 7 and pod on DAS 16, n=4) were excised with a scalpel, flash-frozen in liquid nitrogen and stored at -80°C. 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 (13th of January 2015 for Experiment I, 6th of May 2015 for Experiment II), 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 regimes (WW, MWS, WS, and SWS) effects. Similar observations and trends were recorded in yield components between the two experiments. However, the three-way ANOVA 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 daily 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/-400 kPa. The SWS plants had an average AWC after and before re-watering of ~200 mL and ~0-50 mL respectively with a daily ET fluctuating from 100 to 150 mL.

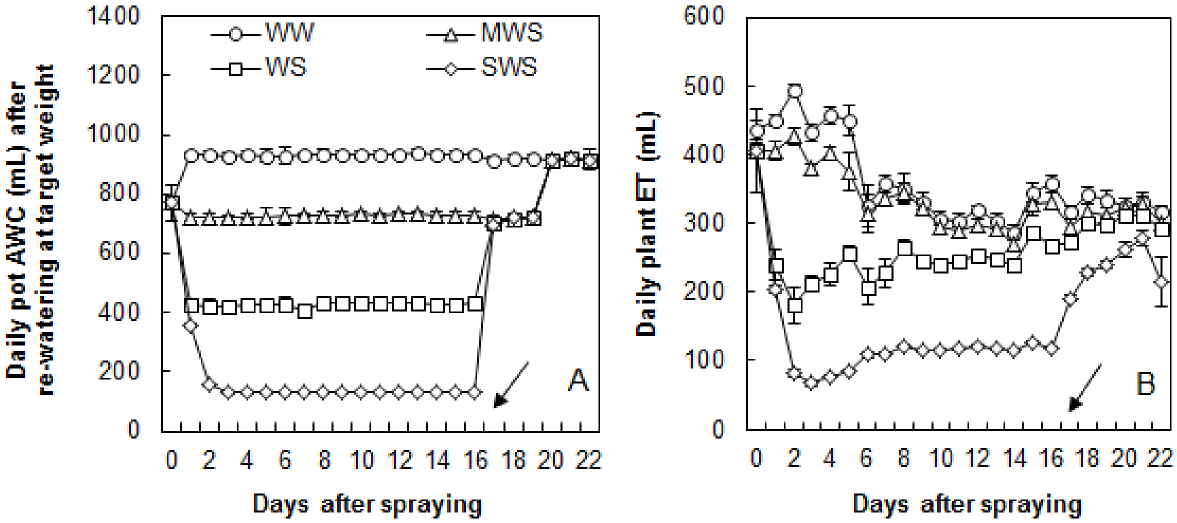

Figure 1. Example of daily pot available content (AWC, A), and daily pot evapotranspiration (ET, B) trends of oilseed rape plants subjected to well-watered (WW), moderate water stress (MWS), water stress (WS) and severe water stress (SWS) watering regimes over flowering stage at days after spraying 0 (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.

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).

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 the 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)

Leaf gas-exchange

In Experiment I mean g_{stot} in WW over DAS plants 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% respectively when compared with the WW plants (Fig. 2A). Application of AT decreased g_{stot} by 32%, 29%, 17% and 2% on WW, MWS, WS and SWS conditions respectively. When compared to the WW plants, the stress increased the leaf temperature by 3%, 8% and 13% on MWS, WS and SWS plants (Fig. 2B). Although not significant, application of AT slightly increased leaf temperature when + AT plants were compared to each relative control -AT except for SWS. Abaxial g_s in WW was 310 mmol m^{-2} s^{-1} on average and MWS, WS and SWS conditions decreased abaxial g_s by 17%, 56% and 84% respectively when compared to the WW plants (Fig. 2C). Adaxial g_s in plants grown under WW conditions was 300 mmol m^{-2} s^{-1} (Fig. 2D). When compared to the WW plants MWS, WS and SWS conditions decreased adaxial g_s by 22%, 54% and 82% respectively. Application of AT decreased adaxial g_s by 68%, 66%, 52% and 37% in plants grown under WW, MWS, WS and SWS conditions respectively.

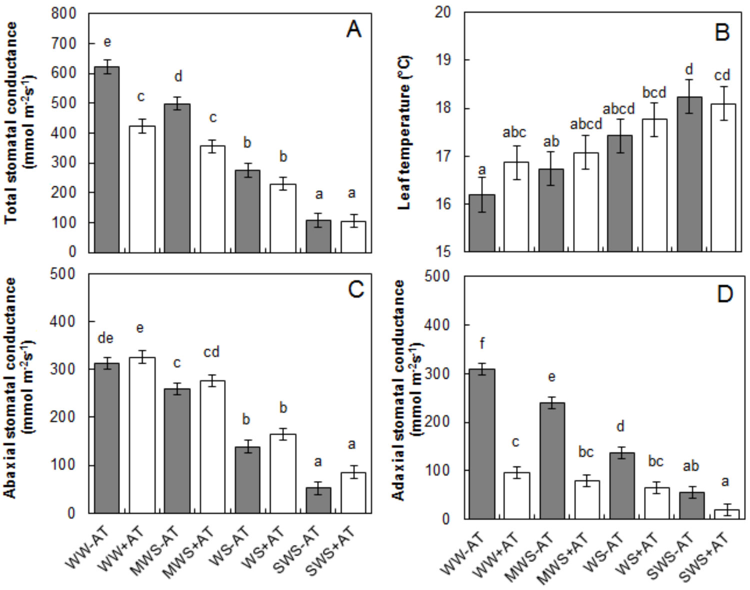

Figure 2. Total stomatal conductance (g_{stot}, mmol $m^{-2}s^{-1}$, A), abaxial stomatal conductance (abaxial g_s, mmol $m^{-2}s^{-1}$, B) and adaxial stomatal conductance (abaxial g_s, mmol $m^{-2}s^{-1}$, C) data of oilseed rape plants subjected to WW, MWS, WS and SWS watering regimes over flowering stage and treated with water (-AT) or 1% v/v Vapor Gard (+AT) at days after spraying 0 (DAS 0). Data are means (n=6, collected at DAS 1, 3 and 8 and averaged) ± standard error of the differences of the mean (SED). Columns different letters are significantly different according to the Tukey's test ($P<0.05$). Data from Experiment I.

In Experiment II mean CO_2 assimilation rate of WW plants fluctuated from ~18-20 µmol m^{-2} s^{-1} at DAS 2 to ~12µmol m^{-2} s^{-1} at DAS 20 (Fig. 3A). Drought conditions over the flowering stage reduced the CO_2 assimilation rate on MWS, WS and SWS plants by 14.6%, 41.0% and 64.4% on average respectively compared to WW (Fig. 3B, C and D). In WW plants, AT-treated plants showed lower assimilation rate values compared to the un-sprayed one. The reduction was steady over the whole period of data-collection: on average, AT-plants experienced a loss in CO_2 assimilation capacity by 12.7% compared to the un-sprayed. MWS AT-treated plants displayed a 10.1% reduction compared to the un-sprayed. On the contrary, AT-treated WS plants, despite the initial 10.5% reduction in CO_2 assimilation compared to the un-sprayed (DAS 2), exhibited a sustained value over the droughted period by 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 $_{i}$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 (data not shown). With respect to the WW plants, MWS stressed plants exhibited only a slight increase by 4.8% on average. Under WW conditions, AT-treated plants showed a slight (non-significant) increase in $_{i}$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 $_{i}$WUE by 5.8% on average compared to the un-sprayed. In contrast, the AT application on WS plants showed a significant increase in $_{i}$WUE over the whole experiment by an average of 53% compared to the un-treated plants. AT application on SWS plants decreased the $_{i}$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 respectively 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 their relative un-sprayed control. 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)

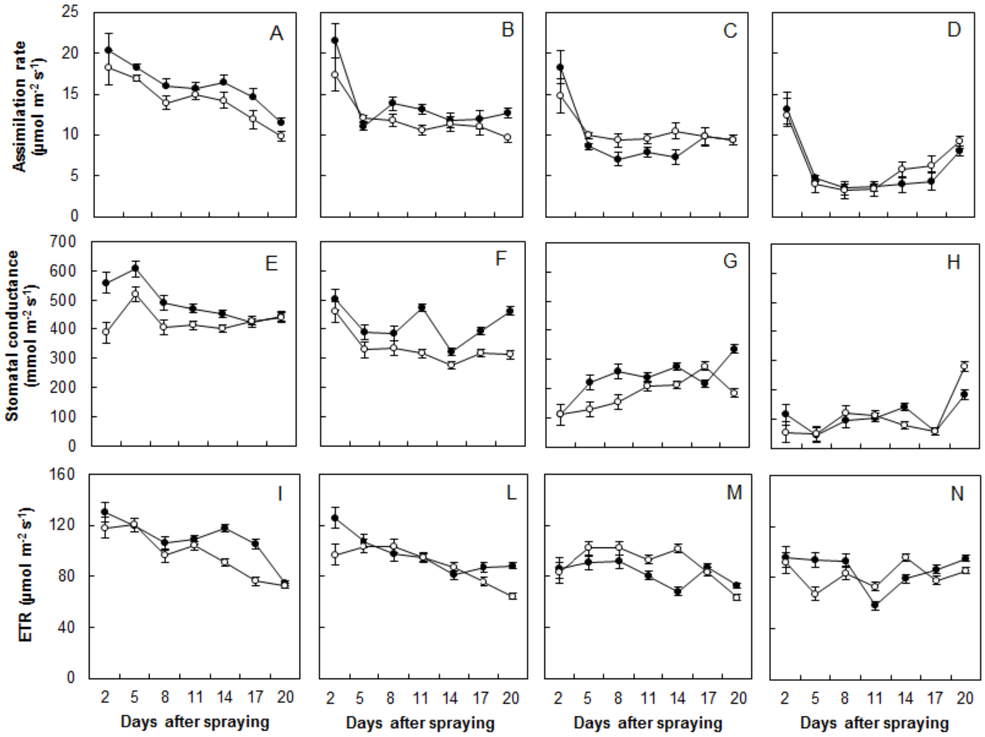

Figure 3. CO$_2$ assimilation rate, stomatal conductance and electron transport rate (ETR) trends of oilseed rape plants subjected to WW (A, E, I), MWS (B, F, L), WS (C,G, M) and SWS (D, H, N) watering regimes over flowering stage and treated with water (close circles) or 1% v/v AT (open circles) at 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.

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 from the two-way ANOVA) (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 significantly 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 on DAS 2, 8 and 12.

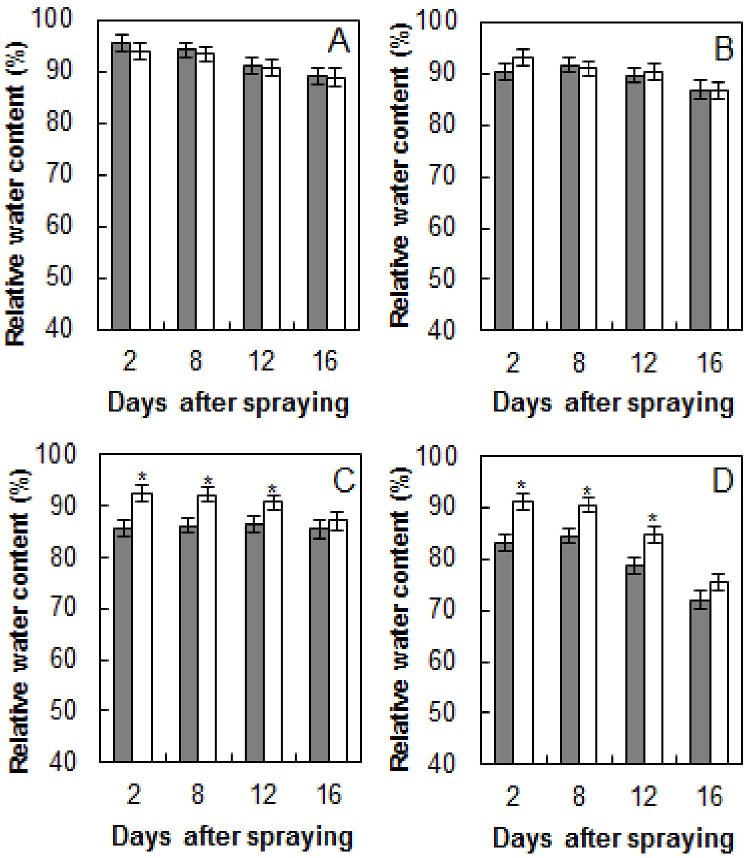

Figure 4. Leaf Relative water content (%) of oilseed rape plants subjected to WW (A), MWS (B), WS (C) and SWS (D) watering regimes respectively over flowering stage and treated with water (close bars) or 1% v/v AT (open bars) at days after spraying 0 (DAS 0). Plants were re-watered at DAS 16. Data are 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.

Leaf and bud infrared thermometer

WW plants maintained a very 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). With respect to WW plants, MWS affected L_T and a significantly lower negative value was found on B_T. Significantly less negative L_T and B_T values compared to WW plants were found in WS and SWS plants with the latter showing a very close value to 0°C (air temperature) for both L_T and B_T. With respect to the un-sprayed plants, WW and MWS AT-treated plants exhibited a significant lower L_T value in most of the 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. With respect to the un-sprayed 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 (R^2=0.98 for -AT and R^2=0.99 for +AT) (Fig. 5C).

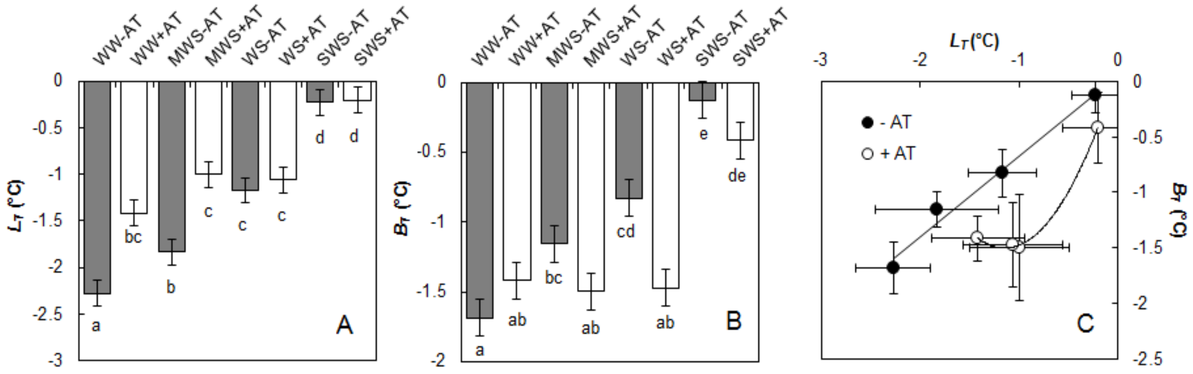

Figure 5. Leaf-to-air temperature (L_T, A), bud-to-air (B_T, B) temperature and their correlation of oilseed rape plants subjected to well-watered (WW), moderate water stress (MWS), water stress (WS) and severe water stress (SWS) watering regimes over flowering stage and treated with water (-AT) or 1% v/v Vapor Gard (+AT) at days after spraying 0 (DAS 0). Data are means (n=8, collected at DAS 1, 2, 4, 6, 8,10, 12 and 14 and averaged across DAS) ± 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.

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 ca. 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 ca. 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 (DAS) 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] compare to the –AT at DAS 3 and DAS 7 as well as [ABA] in flowers and pods of WW and SWS plants. Despite not being statistically significant, AT reduced bud and leaf DAS 16 [ABA] 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).

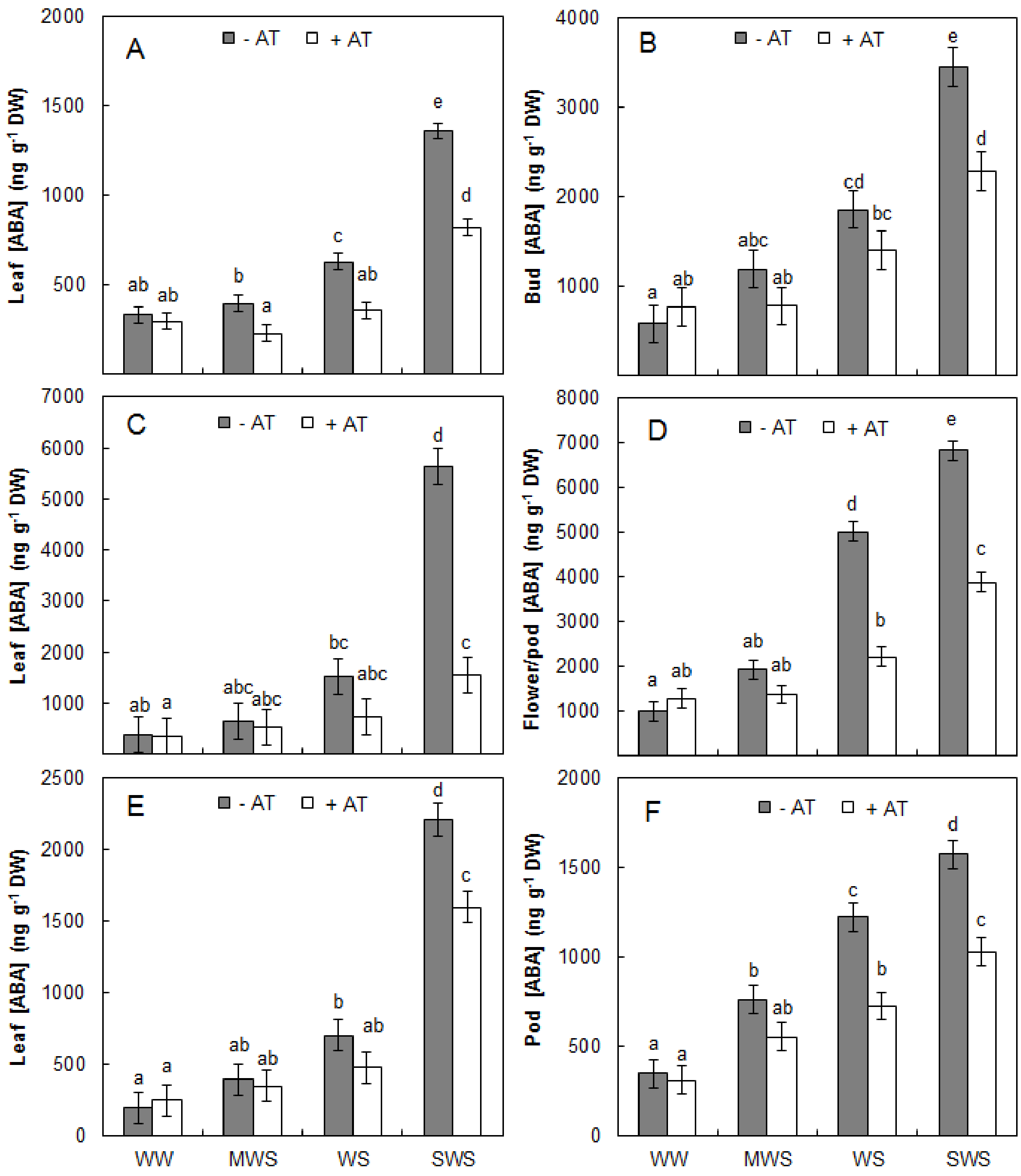

Figure 6. ABA concentration of oilseed rape plants subjected to WW, MWS, WS and SWS watering regimes over flowering stage and treated with water (close columns) or 1% v/v AT (open columns) at days after spraying 0 (DAS 0). Samples were collected at DAS 3 (A and B, leaf and bud respectively), DAS 7 (C 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.

Yield components

Plants grown under WW condition showed 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 that however was significant in Experiment I only.

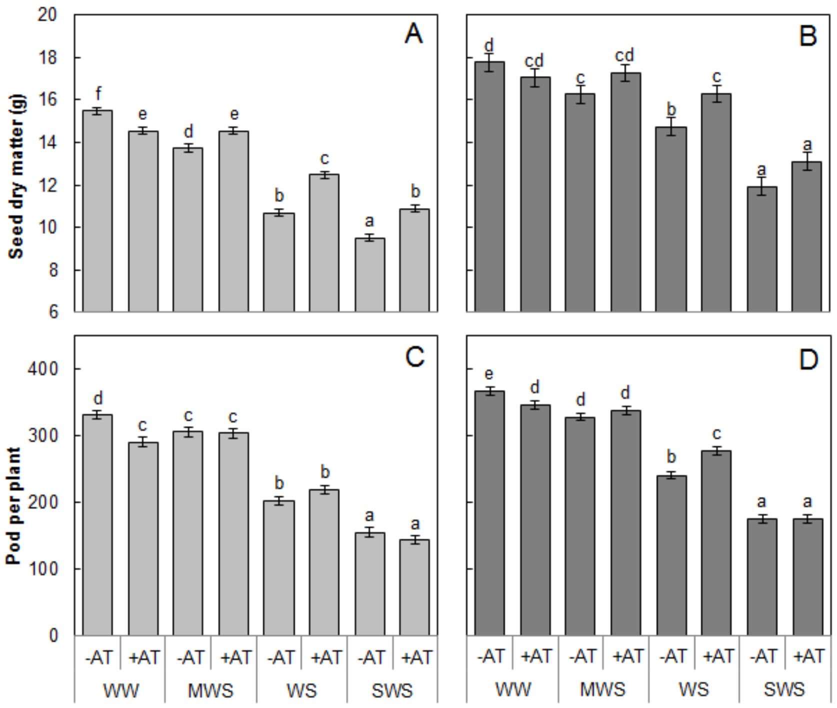

Figure 7. Seed dry matter (A - Experiment I; B, Experiment II) and pods per plant (C - Experiment I; D, Experiment II) yield components of oilseed rape plants subjected to WW, MWS, WS and SWS watering regimes over flowering stage and treated with water or 1% v/v AT at days after spraying 0 (DAS 0). Data 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).

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). Evapotranspiration and WU data indicated significant declines from MWS, suggesting fast root-shoot [ABA] signaling resulting in stomatal closure. The reduction in stomatal conductance which was significant 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, data not shown). 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 $_i$WUE and a significant non-linear relationship between g_s and A_{max} (R^2= 0.63, data not shown).

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, in Guo et al. (2013 and 2015), *Brassica rapa* buds showed 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 is depending on several factors and hormones and reproductive organs water status 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, data not shown) (reproductive organs [ABA] was in turn correlated with seed dry matter production, R^2=0.96, data not shown) confirming leaf-to-reproductive organ ABA translocation, possibly both dependent on root-to-shoot xylem transport (Liu et al. 2004). Significant correlations were also found between B_T (hence transpiration) and L_T (as shown in Fig. 5C), between RWC and L_T (R^2=0.70) (due to ABA accumulation) and in turn B_T with bud/flower/pod [ABA] (R^2=0.63, data not shown): this overall picture of the link between the leaf and reproductive organs supports the idea of strong source-sink connections in OSR under stress that could potentially be exploited for further breeding programmes focusing on the OSR reproductive stages drought tolerance.

Yield component analysis showed a significant reduction in seed dry matter production and pods per plant in WS and SWS plant. However, following SWS a strong compensation of the 1000-seed weight was found (data not shown) suggesting that, since WS plants were not subjected to significant 1000-seed weight increase, the compensation is triggered by the plants only over a threshold of drought-derived seed loss. Pods per plant and seed dry matter were similarly sensitive to water deprivation, leading to similar percentage losses with increasing drought intensities. In OSR seed yield is determined from the initiation of flowering to mid-pod development (Mendham et al. 1981). Thus while the pods on the main stem are already formed, lateral buds are still opening and hence both pod and seed yield components determination is disrupted by stress over flowering

(Mendham et al. 1981). Seed dry matter and pod number were well correlated with leaf RWC (R^2= 0.91 and R^2= 0.78 respectively, data not shown) as well as with B_T temperatures (R^2= 0.72 and R^2= 0.81 respectively, data not shown) suggesting that leaf and reproductive organ water status is an important trait together with gas-exchange (assimilates availability) for stress determination over reproduction. However, significant correlations were found between [ABA] in the reproductive organs and pod (R^2= 0.83, data not shown) and seed dry matter production (R^2= 0.96) as described above, suggesting potential involvement of ABA in reproductive 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 in 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 A_{max}-to-g_s correlation to the left by sustaining A_{max} and reducing g_s (R^2= 0.43, data not shown). 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 from AT due to high plant density that allows lower soil evaporation and thus a hypothetical higher water-saving effect. Kettlewell (2011), derived a soil moisture deficit threshold for AT application in wheat and suggested that the threshold may vary depending on wheat and AT prices. However, it is generally recognised that wheat has an anisohydric response to water stress, thus no limitations of the efficiency of AT should occur from stomatal closure. Our data shows that, at the gas-exchange level and in an isohydric cop such as OSR, AT efficiency is dependent on the magnitude of the drought-induced stomatal closure and application under MWS or SWS conditions may not give significant effects.

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

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

Application of AT reduced the yield components of WW plants and had only mild effects on MWS plants despite higher seed dry matter production values being recorded for MWS+AT in Experiment I. AT application at 2% v/v on WW *Vitis*

Vinifera reduced the leaf CO_2 assimilation rate, in turn reducing assimilate availability for berry ripening (Palliotti et al. 2013). Similarly, the lower CO_2 assimilation rate found under WW+AT conditions when compared to WW-AT suggests that AT may decrease the amount of assimilates translocated from the source to the sink, thus reducing the carbohydrate available for seed development under optimal conditions for plant growth. Significant increases, however, with respect to the -AT plants were found under WS and SWS conditions confirming the capacity of the AT to sustain yield under drought in OSR (Faralli et al. 2016, Patil and De 1978). However, in SWS plants, only seed dry matter was sustained 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 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 pods 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]. In Weldearegay et al. (2012), 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 period 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 shows that maintaining high leaf canopy water status by reducing leaf transpiration and reducing ABA signaling helps reproductive organs to have a more luxurious use of the water resource available and avoid grain yield losses. Speculating on that, hypothetical OSR improvement for drought tolerance over reproduction should be focusing on high WUE canopy and low buds 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 AT application was indeed helpful 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 the 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” crop in which a lower stomatal control under water stress conditions occur. 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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Chapter 4

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Dynamic regulation of leaf gas-exchange through antitranspirant in *Brassica napus* seedlings subjected to reduced water availability depends on CO_2 concentration *via* effects on mesophyll function.

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Abstract

The productivity of many important crops is significantly threatened by water shortage. It has been reported that the predicted elevated atmospheric CO_2 along with crop management solutions may play a significant role in reducing the negative impact of drought by limiting stomatal water losses. We examined the effects of all combinations of three different CO_2 concentrations (historical ~300 ppm, ambient ~400 ppm and elevated ~700 ppm), water stress and a foliar adaxial film-forming antitranspirant application on stress-related physiological traits of oilseed rape (*Brassica napus* L.) seedlings. Increasing CO_2 levels stimulated photosynthesis, increased leaf water-use efficiency of the well-watered plants and alleviated the detrimental effect on CO_2 uptake due to stomatal occlusion due to the film-forming chemical. Water stress severely decreased all the physiological parameters at all CO_2 concentrations. In contrast, antitranspirant application under drought showed significant improvements in water status and gas-exchange by alleviating the decrease of mesophyll conductance. Our results suggest that the beneficial effect of CO_2 enrichment for young plants and the reduction of leaf water loss under drought was offset by a comparatively larger oilseed rape leaf area leading to similar water transpired during each CO_2 treatment. Thus, in view of the predicted elevated atmospheric CO_2 environment, additional water-loss reduction through antitranspirant application or enhanced plant water-saving strategies may have a significant impact on the gas-exchange properties of oilseed rape seedlings under drought. It is hypothesized that the above improvement will occur following the increased substrate availability for Rubisco carboxylation allowing enhanced CO_2 uptake when plant water status is maintained. This work shows

that the effectiveness of crop management regimes can be altered by climate change encouraging further work in this field.

Introduction

The concentration of atmospheric CO_2 ([CO_2]) has been increasing steeply from 1958 (~316 ppm) to 2016 (>400 ppm) (NOAA Mauna Loa Observatory, Hawaii) and the Intergovernmental Panel on Climate Change has predicted a [CO_2] in 2050 between ~550 and ~700 ppm. From an agricultural point of view, the elevated [CO_2] has been related to direct effects on crops' physiological processes (Leakey *et al*. 2012). Indeed, C_3 crops (Fitzgerald *et al*. 2016; O'Leary *et al*. 2015) and, to a lesser extent C_4 crops (Ruiz-Vera *et al*. 2015), exhibited i) higher photosynthetic rates due to an increased Rubisco carboxylation activity and a decreased oxygenation reaction; ii) a lower stomatal conductance (g_s) following a depolarization of the membrane potential of the guard cells accompanied by an increase of the outward activity of K^+ channels and iii) a subsequent increase in guard cell Ca^{2+} concentration and a S type anion channel activities enhancement (Leakey *et al*. 2012, Ainsworth & Rogers, 2007). These physiological changes have been related to an ameliorated response of plants to drought by an improvement of water-use efficiency at the canopy level (Leakey *et al*. 2012). It is well-recognized that drought is the main factor affecting global crop production and food security (Cattivelli *et al*. 2008) and climate change is predicted to significantly increase the magnitude of water shortage and negatively affect the yield of crops (Parmesan & Yohe, 2003).

In this context, oilseed rape (OSR, *Brassica napus* L.) is becoming one of the most popular oilseed crops in the world. Since 1960, this crop has been subjected to intensive breeding programmes for genetic improvement focusing on oil quality and pest resistance (Snowdon *et al*. 2007). These programs resulted in a narrow genetic diversity and a relatively small phenotypic variation of the elite high-yielding varieties currently available (Guo *et al*. 2015). Drought periods over the most drought-sensitive phenological stages (anthesis until mid-pod development) can lead up to 40% of seed yield losses (Richards & Thurling, 1978) and, at present, breeding for drought tolerant varieties does not appear to have yet yielded the expected results (Zhang *et al*. 2014).

In the 1960s, leaf application of stomatal-blocking or stomatal-closing compounds to reduce leaf water loss and improve plant water status was extensively studied but in many cases reported effects were not statistically significant or were contradictory (Solarova *et al*. 1981). By closing or sealing part of the stomata, these chemicals had a strong negative effect on carbon assimilation both under stressed and non-stressed conditions leading to impaired photosynthesis. The majority of these studies were carried out when Rubisco was significantly substrate-limited (from 1960 to 1970, [CO_2] ~300-350 ppm) (as reviewed by Solarova *et al*. 1981). In recent years however significant increases in leaf water-use efficiency (A_{max}/g_s) under drought following antitranspirant application have been reported together with positive yield responses (e.g. Faralli *et al*. 2016; Weerasinghe *et al*. 2016; Abdullah *et al*. 2015; Kettlewell, 2014). Thus it is possible to hypothesize that the increased atmospheric [CO_2] may have played a role in reducing the detrimental effects on photosynthesis of stomatal-blocking compounds due to a higher [CO_2] available at the site of carboxylation. Therefore, the likely reduction of g_s should have lower impact on leaf carbon assimilation when CO_2 supply to Rubisco is comparatively less limited.

Antitranspirants can be divided into two main groups: metabolic compounds that are mainly abscisic acid (ABA)-dependent (e.g. chitosan) and film-forming compounds (AT) that exhibited an ABA-independent mechanism of action (Iriti *et al*. 2009). The ATs have been related to lower ABA accumulation in plant tissues (Iriti *et al*. 2009; Faralli *et al*. 2016) under both stressed and non-stressed conditions. These findings were previously explained by i) a reduced ABA-signaling following the higher water saved in the soil (Iriti *et al*. 2009; Faralli *et al*. 2016) and ii) a lower ABA *in situ* production in leaves due to an overall improved plant water status (Iriti *et al*. 2009; Faralli *et al*. 2016).

Several reports suggest that drought conditions and ABA accumulation reduce mesophyll conductance (g_m), significantly limiting the supply of CO_2 to the chloroplasts (Sorrentino *et al*. 2016). There is strong evidence that reduced CO_2 diffusion from the atmosphere to the site of carboxylation (due to both stomatal closure and reduced mesophyll conductance) is the main cause of decreased photosynthesis under most water stress (WS) conditions (Tomás *et al*. 2014). The mesophyll tissue represents a critical component of the drought stress response of plants and significant intra- and interspecific genetic variability in g_m has been previously reported (Tomás *et al*. 2014; Flexas *et al*. 2014). Concurrent decreases in g_s and g_m were often observed under drought and elevated [CO_2] (Flexas *et al*. 2014, Flexas *et al*. 2015). Because there is a strong inter-dependence between carbon assimilation and g_s a major target to improve A_{max}/g_s is to avoid decreases in g_m (Tomás *et al*. 2014). It is well recognized that high plant A_{max}/g_s at leaf level under water deprivation can be achieved by increasing g_m, and thus significant relationships are expected between the g_m/g_s ratio (resistance to CO_2) and A_{max}/g_s in plants where the resistance to H_2O loss is decreased without reducing CO_2

assimilation (Galmes *et al*. 2011). At the same time, since exogenous application of ABA reduced $g_{m,}$ both physical and biochemical factors may be involved in the decreased conductance to CO_2 under drought at the mesophyll site (Sorrentino *et al*. 2016). Therefore, a film-forming AT application may have significant effects at canopy level on plant drought tolerance (e.g. A_{max}/g_s) when [CO_2] is less limited. We hypothesize that this effect may occur by i) maintaining good plant water status without significantly impairing the CO_2 assimilation rate and by ii) sustaining g_m due to a reduced ABA accumulation.

While a few papers are available addressing the interactive responses of OSR to elevated [CO_2], drought, and other environmental stresses (e.g. Qaderi *et al*. 2006), much less attention has been paid to the physiological and morphological consequences of the increase [CO_2] levels from an agronomic perspective. Therefore, in this study we compared the AT efficiency at historical, ambient and projected [CO_2]. We conducted three experiments in the same controlled environment room at different CO_2 concentrations (~300 ppm – historical 1950/1960 [CO_2], $_h$[CO_2]; ~400 ppm – ambient [CO_2], $_a$[CO_2]; ~700 ppm – elevated [CO_2], $_e$[CO_2]) and all three comparing the same combinations of watering regimes and AT.

The aims were to i) explore the physiological effects of these [CO_2] on winter OSR seedlings growth and development: ii) to evaluate the interactions between the different [CO_2] and drought; iii) to investigate whether the source-limitation induced by an AT application may be counteracted by the elevated [CO_2] in both water-stressed and non-stressed conditions, and iv) to characterize and evaluate the relationships between physiological traits involved in the response of OSR to different [CO_2], drought and AT combinations.

Materials and methods

Plant materials and experimental layout

Winter oilseed rape (*Brassica napus* L.) seeds (cv. Excalibur, Dekalb, UK) were sown on 8th January 2016 for Experiment I, 20th January 2016 for Experiment II, and 9th February 2016 for Experiment III in individual pots (pot width 11 cm, pot height 13 cm, total volume ~1500 cm^3) containing the same amount (650 g + 25g of pot weight) of a peat, sand, and pumice plant cultivation substrate (SoMi 513, Dachstauden; Hawita, Vechta, Germany) characterized with a water retention curve as described by Barboza-Barquero *et al*. (2015). Plants were germinated in a growth chamber at 20°C day / 15°C night, 12 / 12 photoperiod, 60% RH and ambinet [CO_2]. PAR was provided by neon lamps at ~200 $\mu mol\ m^{-2}\ s^{-1}$ at shoot apex measured with a LI-190R PAR sensor (LI-COR, Lincoln, Nebraska USA). Plants were watered daily with rain water to maintain the optimum soil moisture conditions for plant germination and growth (around 90% of available water content, AWC, according to a water retention curve, as described in Barboza-Barquero *et al*., 2015). Three seeds were sown in each pot and for all the experiments two emerged seedlings were removed after 4 days from emergence. At the 2nd leaf stage (GS 1.2 BBCH growth scale, 19th January for Experiment I, 7th February for Experiment II and 25th February 2016 for Experiment III) seedlings were placed in a controlled environment chamber (Jülich Forschungszentrum, IBG-2 Plant Science, walk-in custom-made chamber) at 20°C day / 15°C night, 16/8 photoperiod, 60% RH and a VPD of 0.94 kPa. PAR was provided with HPI-T-plus lamps (400W/645 E40, Philips, Netherlands) at an average of ~500 µmol m^{-2} s^{-1} at plant level with a minimum value of 460 µmol m^{-2} s^{-1} and a maximum of 540 µmol m^{-2} s^{-1}.

Each experiment consisted of 120 plants arranged in five randomized blocks with four treatments and six replicates in each block. The treatments constituted a 2 x 2 factorial design and the two factors were watering regime (well-watered - WW and droughted - WS) and spray application (water only (-AT) and di-1-*p* menthene (+AT)). Treatments were applied when plants reached the 4th leaf stage based on visual observations (GS 1.4, BBCH oilseed rape growth scale) (days after treatment 0, DAT 0 - 25th January for Experiment I, 12th February for Experiment II and 1st March 2016 for Experiment III).

AT (Vapor Gard - VG, a.i. di-1-*p* menthene 96%) was uniformly applied at 1% v/v concentration onto the adaxial surface of the seedlings with a hand sprayer (Foxy plus, Birchmeier, Stetten, Switzerland). Plants without AT were sprayed with an equivalent water volume (-AT).

According to the soil retention curve of the SoMi substrate, target weight at "pot capacity" (100% of available water content) was set to 1070 g of pot weight (including 25 g of pot weight and 20 g of plant fresh weight at the 4th leaf stage) and the permanent wilting point was set at 600 g. Thus the total AWC of the pots was accounted to be 425 ml. Hence, well-watered pots (WW, n=6) were weighed daily with a MC1 balance (Sartorius, Göttingen, Germany) and then rewatered to avoid any decrease in soil moisture according to the equation:

$$\text{Eqn (1) } \text{Water to add} = \text{“pot capacity” weight} - \text{pot weight}$$

Watering was withheld to water stressed pots (WS) the same day of the AT application and over the whole experimental period (12 days). Pot saucers were used to avoid any water uptake (Fig. S1A, S1B and S1C). WS plants reached the soil permanent wilting point between DAT 7 and DAT 8.

For each individual experiment, the chamber was set at a different [CO_2]: Experiment I 400 ppm, $_a$[CO_2]; Experiment II 700 ppm, $_e$[CO_2]; and Experiment III 300 ppm, $_h$[CO_2]. A respirator (Fig. S2) made up of a tube connected with a plastic mask on one side (respirator) was developed and used in the growth chamber to avoid undesired [CO_2] fluctuations due to experimenter breathing during data collection and plant manipulation. The average [CO_2] for each experiment was 402.6 ± 10.1 (SD) for Experiment I, 700.9 ± 9.7 for Experiment II and 285.5 ± 14.3 for Experiment III (Fig. S1D)

Growth analysis and relative water content.

On DAT 1, 4, 8 and 12, plants (n=6) from each treatment were harvested for all the experiments. RWC was calculated according to Barr & Weatherley (1962) and was determined as described by Faralli *et al.* (2015). Briefly, between 12:00 and 13:00, one piece of leaf tissue of the fourth leaf (~2 cm^2) was collected using a scalpel for each plant (n=6) . These samples were collected in a central region of the lamina between the main vein and the leaf margin avoiding lateral veins. The fresh weight was then recorded (F_W) and the samples were soaked in distilled water in Petri dishes in the dark and at ~4°C during four hours. The turgid weight (T_W) was recorded, the dried disks (oven-dried at 80°C for 24 hours) were weighed the day after, and the dry weight was recorded (D_W). RWC (%) was then calculated according to:

Eqn (2) $\mathrm{RWC}(\%) = \frac{\mathrm{F_W - D_W}}{\mathrm{T_W - D_W}} \times 100$

After that, leaf number (LN) was counted and plants were excised at the base of the stem and leaf and stem fresh weight were recorded (Mettler Toledo XS 205 Dual Range, Columbus, USA). Total leaf area (LA) was recorded with a leaf area meter (LI-3100C area meter, Li-Cor, Lincoln, NE, USA). Samples were dried in an oven (Nabertherm P330, Nabertherm GmbH Lilienthal/Bremen, Germany) over 24 hours at 65°Cand leaf, stem and total shoot dry weight were recorded. Specific leaf area was calculated as: SLA (g m^{-2}) = leaf DW / leaf area.

Gas-exchange

Gas-exchange and chlorophyll fluorescence were simultaneously measured with a Li-Cor 6400 (Li-Cor, Lincoln, NE, USA) with an integrated fluorescence leaf cuvette (LI-6400-40; Li-Cor) on DAT 1, 4, 8 and 12 for all the experiments, between 09:00 and 12:00. To minimize potential leaf position and developmental age effects, all the gas-exchange measurements were taken on the 4th fully expanded leaf of six randomly selected plants for each treatment. In the Li-Cor cuvette, all the parameters (leaf CO_2 assimilation at saturating light - A_{max}, stomatal conductance - g_s and the ratio between sub-stomatal CO_2 and the ambient [CO_2] - C_i/C_a) were collected at 400 ppm CO_2 (Experiment I), 700 ppm CO_2 (Experiment II) and 300 ppm CO_2 (Experiment III). Cuvette temperature was maintained at 25°C, photosynthetically active photon flux density (PPFD) was 1200 µmol m^{-2} s^{-1} (saturating PPFD for OSR seedlings, previously evaluated by light curves, Fig. S3), with a 10:90 blue:red light and a flow rate of 300 µmol s^{-1}. Intrinsic water-use efficiency was calculated as A_{max}/g_s. The ratio (A_{max}/g_s) is considered to be more reliable than the water-use efficiency calculated from CO_2

assimilation to transpiration rate, since it is not affected by the VPD inside the leaf chamber (Webster *et al*. 2016). The data were collected after the leaf achieved a steady-state, and minimum fluorescence (F_s) and maximum fluorescence (F_m') were recorded by ensuring a light-saturating pulse of 8000 µmol m^{-2} s^{-1}. The actual photochemical efficiency of the photosystem II ($\Delta F/F_m'$) was calculated according to Baker (2008) as follow:

$$\text{Eqn (3) } \Delta\mathrm{F}/\mathrm{Fm}' = \frac{\mathrm{Fm}' - \mathrm{Fs}}{\mathrm{Fm}'}$$

and the electron transport rate as:

$$\text{Eqn (4) } \mathrm{J} = \Delta\mathrm{F}/\mathrm{Fm}' \text{ x PPFD x } \alpha \text{ x } \beta$$

where α is the leaf absorbance (0.84) and β is the partitioning coefficient of absorbed quanta between PSII and PSI (0.5) (Baker, 2008).

We also evaluated the response of g_m to the applied treatments ([CO_2], water stress and antitranspirant). Thus, respiration in the light (R_d) and the apparent CO_2 photo-compensation point (C_i*) were determined according to the method of Laisk (1977) as described in von Caemmerer (2000). A-C_i (assimilation rate to sub-stomatal CO_2 concentration) curves were measured using the gas-exchange system Li-6400 at three different PPFD (50, 250 and 500 µmol m^{-2} s^{-1}) and at six different CO_2 levels ranging from 300 to 50 mmol CO_2 ppm. The intersection point of the three A–C_i curves fitted with regression was used to determine C_i^* (x-axis, 23±4.24) and R_d (y-axis, 0.7±0.28). C_i^* was used as a proxy for the chloroplastic CO_2 photocompensation point (Γ^*) for all the treatments, as reported by Galmes *et al*. (2011). All the other leaf gas-exchange parameters were determined simultaneously with measurements of chlorophyll fluorescence using the gas-exchange system Li-6400 with an integrated fluorescence chamber head as described above (gas exchange section). The equation from Harley *et al*. (1992) was used to make estimations of mesophyll conductance (g_m):

$$\text{Eqn (5) } g_m = \frac{\mathrm{A}}{\mathrm{C_i} - \frac{\Gamma^* \, [\mathrm{J}+8\,(\mathrm{A}+\mathrm{R_d})]}{\mathrm{J}-4\,(\mathrm{A}+\mathrm{R_d})}}$$

where A, C_i and J values are taken from gas-exchange measurements at saturating light and Γ^* and R_d were estimated using the Laisk (1977) method (see above).

Rapid light response curves

A portable MINI-PAM system (WALZ, Effeltrich, Germany) was used for chlorophyll *a* fluorescence analysis. The automated rapid light response curves (RLRC) used here provided changes in photosynthetic electron transport (ETR), non-photochemical quenching (NPQ) and light adapted yield of photosystem II ($\Delta F/F_m'$). The PPFD profiles in the RLRC usually ranged from 0 to 1200 µmol photons m^{-2} s^{-1} in nine steps (0, 100, 150, 200, 300, 450, 600, 900 and 1200) with a step width of 30 seconds each. On DAT 3, 7 and 11 between 09:00 and 12:00 a light adapted protocol was used (30 seconds of acclimation). Because in the growth chamber PAR was provided at 500 µmol m^{-2} s^{-1}, prior to each measurement, plants were shaded for 30 seconds by using a cardboard box, with an open side for data collection. Despite the samples were potentially not dark-adapted (therefore, not all the reaction centres were completely open), this method is considered a good approach to evaluate physiological mechanisms in response to drought stress (Valentini *et al*. 1995). An estimation of the $\Delta F/F_m'$ was measured by applying a saturating flash (800 ms at around 4000 µmol photons m^{-2} s^{-1}). RLRC were then constructed by exposing the leaf to eight steps of increasing PPFD. For each measurement care was taken not to change the ambient conditions and to ensure the same distance from the leaf to the light source. Six replicates of each treatment were randomly performed in turn for the light adapted protocol using the 4th fully expanded leaf. Then $\Delta F/F_m'$ to PPFD, ETR to PPFD and NPQ to PPFD curves were obtained using the Walz Wincontrol software. The light response of ETR curves was constructed using the model of Eilers & Peeters (1988). The model allowed the estimation of the maximum ETR (ETR_{max}), which is the asymptote of the curve, the maximum light use efficiency (α), which is the slope at the beginning of the curve, and the light saturation coefficient (EK = ETR_{max}/α). For this purpose, the WALZ "curve-solver" software was used (WALZ, Effeltrich, Germany) that obtains the best fit by varying the so-called free parameters (the a, b, and c) and thus calculating the ETR_{max} value for each curve.

ABA analysis

On DAT 7 and for each experiment, leaf tissues from each treatment (n=6) and from the fourth leaf (as above for RWC) were collected with a scalpel, placed in flask tube and immediately flash-frozen in liquid nitrogen. Samples were then stored at -70°C in a freezer and subsequently dried by freeze-drier. Finely ground samples were then analysed for ABA concentration ([ABA]) with an enzyme-linked immunosorbent assay (ELISA) following the protocol described by Faralli *et al*. (2016). Briefly, freeze-dried samples (~0.4 g) were ground and extracted overnight at 4°C by adding 5 mL of extraction buffer (Cusabio Biotech Co. Ltd, Carlsbad, CA, USA). The supernatant was then collected and ELISA was performed according to the company procedure (code CSB-E09159Pl, http://www.cusabio.com, Cusabio Biotech Co. Ltd, Carlsbad, CA, USA).

Statistical analysis

Available water content data from the three experiments are daily means (n=6) ± standard error (SE). Carbon dioxide treatments are means (n=1440) ± standard deviation (SD). With the exception of [CO_2], the parameters inside the growth chamber (i.e. RH, temperature, light and VPD) were very similar or identical during the three experiments. Also, since the growth conditions of the three experiments were very similar and no morphological differences were recorded between the three sets of plant material, a hypothetical time-specific effect affecting the CO_2 treatments was not considered a possibility. Therefore all the physiological traits were analysed with a three-way ANOVA (CO_2 x watering regimes x antitranspirant, Table S1) in GenStat 17th Edition (VSN International, UK). However, since the three experiments were formally independent, the homogeneity of variance for each three-way ANOVA was checked by visually examining the distribution histogram of the residuals and the fitted vs. residuals plot. While the distribution was acceptable for all the traits and DAT, in five cases (RWC DAT 12, Leaf Area DAT 8, Amax DAT 12, A_{max}/g_s DAT 1 and [ABA]) the fitted vs. residuals plot showed heteroscedasticity. These data were transformed to a natural logarithmic scale and in all cases (except [ABA]) the heteroscedasticity was reduced and the distribution was improved. Subsequently the log-transformed data were subjected to three-way ANOVA and the *P*-values for all the interactions were recorded. In all the cases logarithmic transformation had a negligible effect on *P*-values suggesting that the data heteroscedasticity was not significantly affecting the data.

Additionally, F_{max} test was performed to evaluate the variance comparability between the three groups of data (300, 400 and 700 ppm [CO_2]) by running for each trait and for each DAT a two-way ANOVA (watering regime x antitranspirant). The ratio of the greatest and smallest variance was compared with F_{max} table according to the Hartley's homogeneity of variance test (15 d.f. and 4 treatments) showing in 10 out of 37 three-way ANOVAs significant differences (however only between $P<0.05$ and $P<0.01$). These significances in variance were completely removed by log transformation. As above, the ANOVAs showed minimal differences between the original data and the data subjected to logarithmic transformation. Therefore, due to the above evidence, the data are statistically analysed and presented in their original form without logarithmic transformation and thus including [CO_2] factor in the ANOVA model. However, since for [ABA] WW and WS treatments showed two distinct distributions, the data were analysed and presented separately for WW and for WS. All the mean values were compared using a Tukey's *post-hoc* test ($P<0.05$).

Results

Growth analysis

Leaf number (LN) significantly increased over time in WW plants grown at all the [CO_2] (from 4 leaves at DAT 1 to 7.5-8 leaves at DAT 12) and no significant differences were found between the different [CO_2] treatments (data not shown). AT did not affect LN when plants were grown in WW conditions. WS significantly decreased LN compared to the WW plants when grown at $_h$[CO_2] and $_a$[CO_2] since DAT 4. However $_e$[CO_2] showed a reduction in LN under WS only from DAT 8. AT did not affect LN when plants were grown in WS conditions.

Over the twelve days of the experiments, WW plants grown in $_a[CO_2]$ had a shoot DW (Fig. 1B) that was significantly higher than that of the plants grown in $_h[CO_2]$ by 34% on average (Fig. 1A) (*P*<0.001 for DAT 4, 8 and 12). Increases in shoot DW of 20% on average were observed in plants grown at $_e[CO_2]$ (Fig. 1C) with respect to the plants grown under $_a[CO_2]$. Significant reductions in shoot DW were observed under WS on DAT 8 and DAT 12 at all the $[CO_2]$ when compared to the WW plants. However, WS plants grown under $_e[CO_2]$ exhibited a higher DW compared to $_h[CO_2]$ and $_a[CO_2]$ by 42% and 33% on DAT 4, 40% and 26% on DAT 8 and 37% and 27% on DAT 12, respectively. AT application on WW and WS plants did not significantly affect the shoot DW at any $[CO_2]$.

Leaf area (LA) of WW plants grown under $_a[CO_2]$ significantly increased with time from 190 cm^2 on DAT 1 to 800 cm^2 on DAT 12 (Fig. 1E). Under $_h[CO_2]$, WW plants exhibited a similar significant increase in LA that was however lower than that of the plants grown at $_a[CO_2]$ (Fig. 1D) (from 190 cm^2 on DAT 1 to 590 cm^2 on DAT 12). $_e[CO_2]$ stimulated LA of WW plants leading to an average LA of 1190 cm^2 on DAT 12 (Fig. 1F). For DAT 4 and DAT 8, however, no significant differences in LA were found between plants grown at $_e[CO_2]$ and $_a[CO_2]$. AT application on WW plants did not significantly affect the LA of plants at any $[CO_2]$. WS had a significant effect on LA at all the $[CO_2]$ and the decrease was significant since DAT 4 in $_e[CO_2]$ and $_a[CO_2]$-grown plants and since DAT 8 at $_h[CO_2]$. AT in WS plants did not affect LA at any $[CO_2]$ with the exception of $_e[CO_2]$ on DAT 12.

Specific leaf area (SLA) decreased over time on WW plants grown at all the $[CO_2]$ and no significant differences were recorded between -AT and +AT plants. WS significantly decreased SLA values at all the $[CO_2]$ ($P<0.001$ since DAT 4) with respect to the WW plants and no significant differences were observed between -AT and +AT plants.

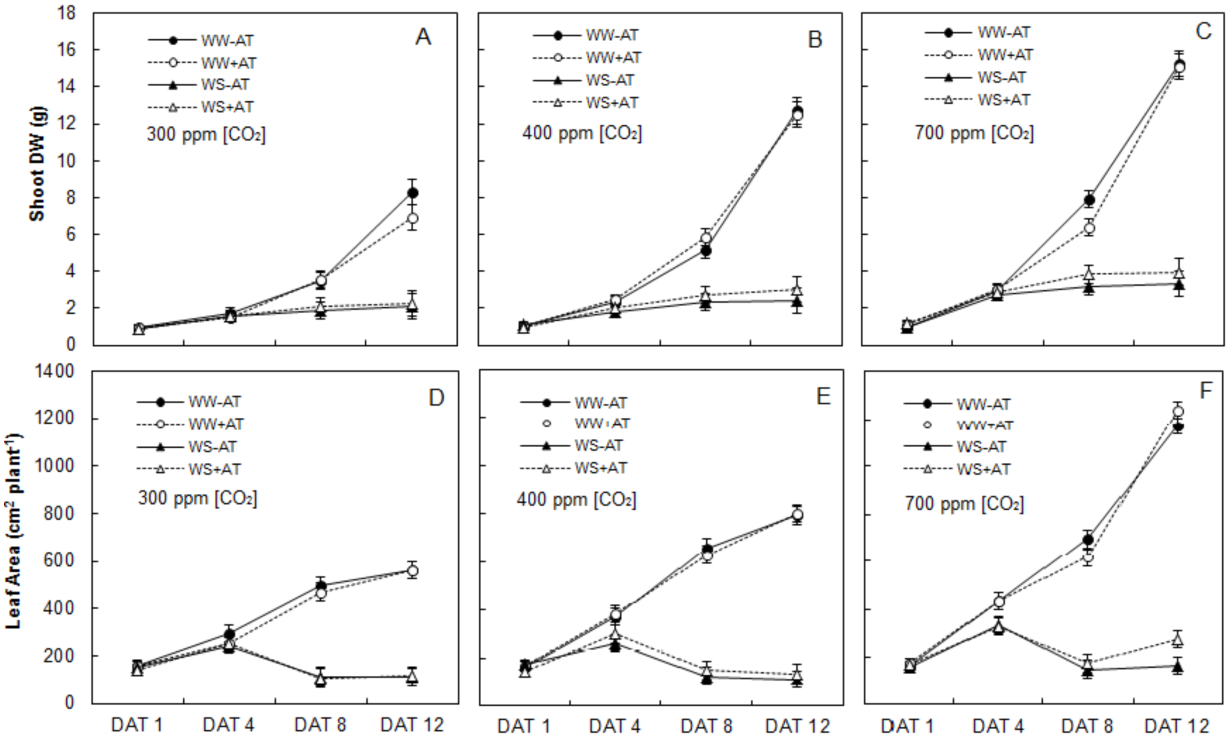

Figure 1. Development of shoot dry weight (DW, g) and total leaf area (LA, cm^2 $plant^{-1}$) of winter oilseed rape plants grown at historical (A and D), ambient (B and E) and elevated (C and F) atmospheric CO_2 concentration under well-watered (WW) and water-stress (WS) conditions and sprayed with a 1% v/v concentration of Vapor Gard (+AT) or water (-AT). Data were collected at days after treatment application (DAT) 1, 4, 8 and 12. Data are means (n=6) ± SE.

Plant water status

The leaf RWC of WW plants fluctuated from 90% to 91% on average and no significant differences were observed between the three $[CO_2]$ (Fig. 2A, B and C). AT application on WW plants did not significantly affect the leaf RWC with respect to the -AT plants. Under WS conditions, leaf RWC significantly decreased from DAT 4 at all the $[CO_2]$ ($P<0.001$ at all the DAT). RWC of $_{e}[CO_2]$ plants under WS conditions was significantly higher (RWC 70%, $P=0.018$) than $_{a}[CO_2]$ (RWC 62%)

and ${}_h[CO_2]$ (RWC 67%) at DAT 8 whereas the drop in RWC was similar during the other DAT. With respect to the -AT plants, leaf RWC was significantly sustained in +AT plants grown in ${}_e[CO_2]$ and ${}_a[CO_2]$ under WS at DAT 4, 8 and 12 (P=0.008, <0.001 and <0.001 for DAT 4, DAT 8 and DAT 12 respectively). On the contrary under ${}_h[CO_2]$, a significant increase in RWC compared to the -AT was observed at DAT 12 only.

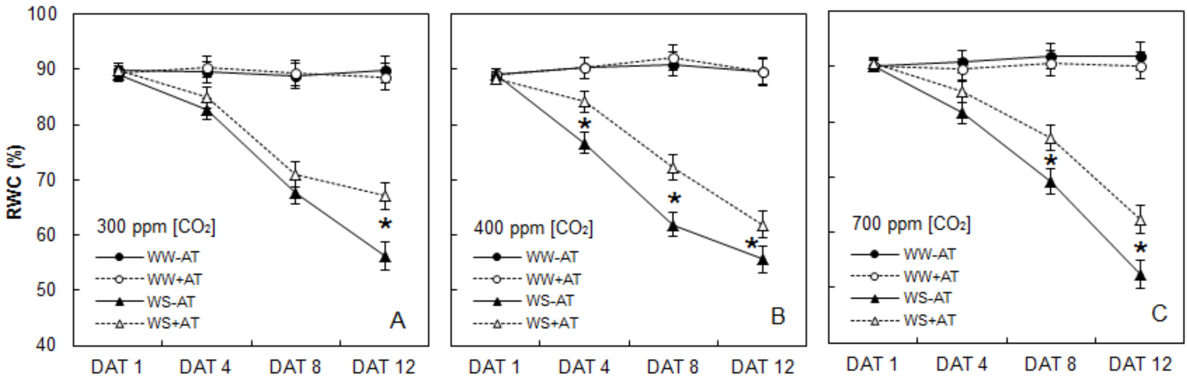

Figure 2. Development of leaf relative water content (RWC, %) of winter oilseed rape plants grown at historical (A), ambient (B) and elevated (C) atmospheric CO_2 concentration under well-watered (WW) and water-stress (WS) conditions and sprayed with a 1% v/v concentration of Vapor Gard (+AT) or water (-AT). Data were collected at days after treatments application (DAT) 1, 4, 8 and 12. Asterisks represent significant differences between –AT and the respective +AT value of WS plants. Data are means (n=6) ± SE.

Gas-exchange

WW plants grown under ${}_a[CO_2]$ showed an average A_{max} of 26 µmol m^{-2} s^{-1} that was 22% lower than that of the plants grown in ${}_e[CO_2]$ (Fig. 3B and C). On the contrary, ${}_a[CO_2]$ exhibited an increase in A_{max} by 12% on average with respect to the ${}_h[CO_2]$ (Fig. 3A and B). Compared with the -AT plants, AT application on WW plants decreased A_{max} by 15%, 9% and 6% under ${}_h[CO_2]$, ${}_a[CO_2]$ and ${}_e[CO_2]$, respectively. Plants under WS conditions exhibited a reduction in A_{max} compared to the WW plants that was significant from DAT 4 for all the $[CO_2]$ (P<0.001). No significant differences were found between WS-AT and WS+AT under $_h[CO_2]$. Conversely, AT application on WS plants sustained A_{max} compared to the WS-AT plants over DAT 4 and 8 under $_a[CO_2]$ and, to a greater extent, under $_e[CO_2]$.

WW plants grown under $_a[CO_2]$ exhibited a g_s of 0.67 mol mm^{-2} s^{-1} on average, showing a decreasing slope over the last days of the experiment (Fig. 3E). With respect to the $_a[CO_2]$, a 7% increase on average in g_s was observed in $_h[CO_2]$-grown plants (Fig. 3D), without showing any g_s reduction over time. On the contrary, g_s was significantly reduced over time in $_e[CO_2]$ plants and by 40% on average with respect to the $_a[CO_2]$ (Fig. 3F). WS conditions reduced g_s by 64%, 55% and 40% under $_h[CO_2]$, $_a[CO_2]$ and $_e[CO_2]$ respectively. With respect to the -AT plants, AT application on WW plants decreased g_s by 26%, 20% and 10% under $_h[CO_2]$, $_a[CO_2]$ and $_e[CO_2]$ respectively. Under WS, AT application reduced g_s by 21%, 23% and 25% on average under $_h[CO_2]$, $_a[CO_2]$ and $_e[CO_2]$ respectively when compared to the -AT plants.

WW plants grown under $_h[CO_2]$ and $_a[CO_2]$ showed a relatively stable A_{max}/g_s over time with an average value of 31 µmol mol^{-1} and 40 µmol mol^{-1} respectively (Fig. 3G and H). On the contrary, $_e[CO_2]$-grown plants showed a significant increase in time from DAT 1 to DAT 12 leading to an average value of 90 µmol mol^{-1} (Fig. 3I). With respect to the WW-AT, significant increases in A_{max}/g_s were observed in WS-AT plants under $_h[CO_2]$, $_a[CO_2]$ and $_e[CO_2]$ by 42%, 31% and 8%. Despite no significant differences for each DAT between -AT and +AT, AT application in WW plants improved leaf A_{max}/g_s by 16%, 14% and 3% on average under $_h[CO_2]$, $_a[CO_2]$ and $_e[CO_2]$, respectively. Improvements in A_{max}/g_s were recorded in WS+AT compared to the -AT plants by 14%, 23% and 25% under $_h[CO_2]$, $_a[CO_2]$ and $_e[CO_2]$, respectively.

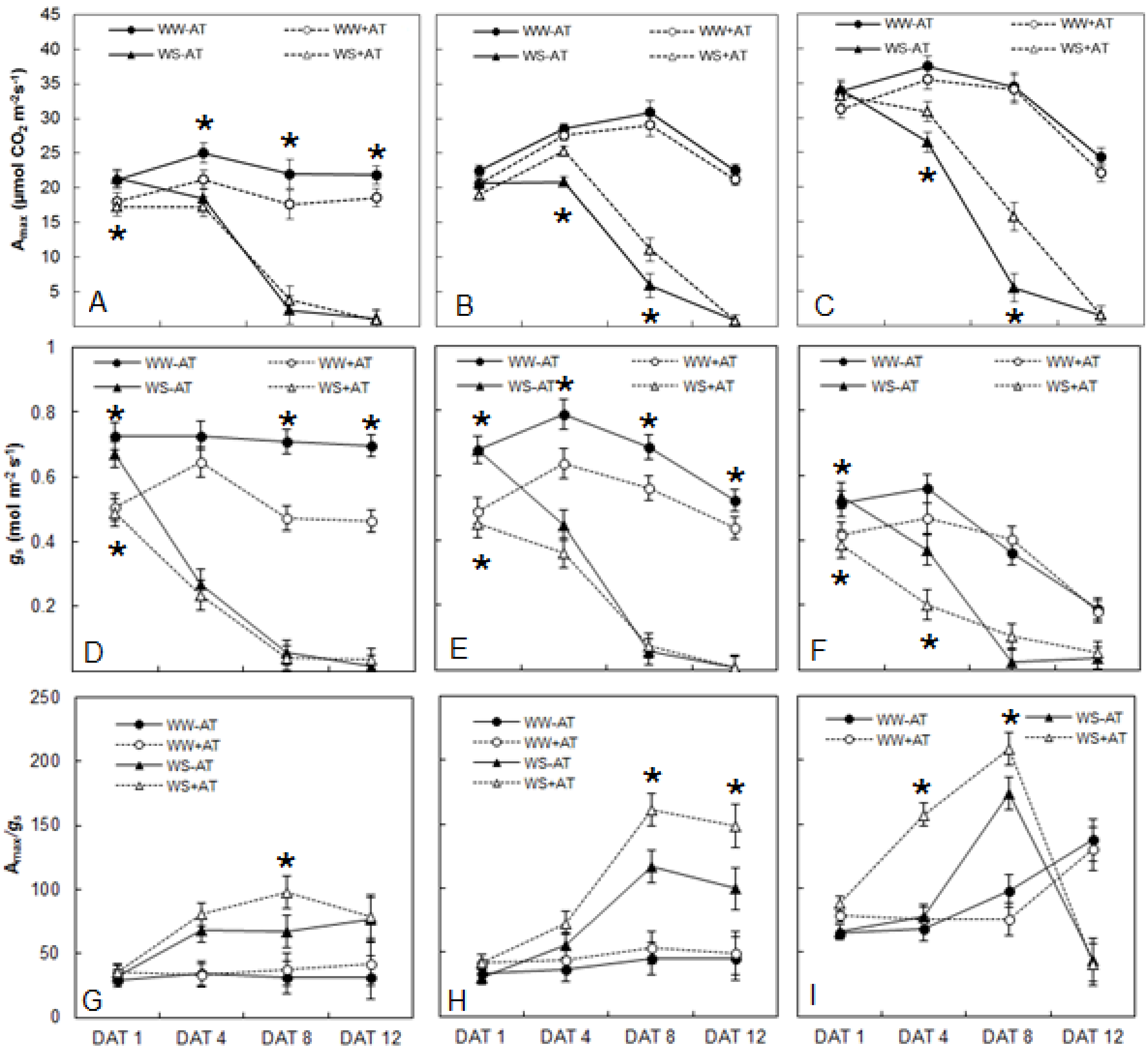

Figure 3. Development of the leaf CO_2 assimilation rate at saturating light (A_{max}, µmol $m^{-2}s^{-1}$), leaf stomatal conductance (g_s, mol $m^{-2}s^{-1}$) and intrinsic water-use efficiency (A_{max}/g_s, µmol $m^{-2}s^{-1}$ mol $m^{-2}s^{-1}$) of winter oilseed rape plants grown at historical (300 ppm, A, D and G), ambient (400 ppm, B, E and H) and elevated (700 ppm, C, F and I) atmospheric CO_2 concentration under well-watered (WW) and water-stress (WS) conditions and sprayed with a 1% v/v concentration of Vapor Gard (+AT) or water (-AT). Asterisks represent significant differences between –AT and the respective +AT value of WW and WS plants. Data were collected at days after treatments application (DAT) 1, 4, 8 and 12. Data are means (n=6) ± SE.

Mesophyll conductance

The pooled data of g_m over the experiments are shown in Figure 4. WW plants showed an average g_m of 0.18, 0.15 and 0.11 mol m^{-2} s^{-1} when grown at $_h[CO_2]$, $_a[CO_2]$ and $_e[CO_2]$, respectively ($P<0.001$) (Fig. 4A, B and C). AT did not have a statistically significant effect on g_m when applied on WW plants despite lower values recorded at all the $[CO_2]$. Water stress decreased g_m by 37, 45 and 48% when compared with WW plants grown at $_h[CO_2]$, $_a[CO_2]$ and $_e[CO_2]$, respectively ($P<0.001$). With respect to the WS-AT plants, WS+AT plants grown under $_h[CO_2]$ showed decreases in g_m by 2%. Conversely, at $_a[CO_2]$ and $_e[CO_2]$, the AT treatment under WS conditions increased g_m by 27 and 30% respectively when compared to the WS-AT plants (Watering regimes x antitranspirant $P=0.014$). In Figure 4D the correlation between g_m and g_s is shown in -AT and +AT plants. -AT plants exhibited a steep decrease in g_m following the g_s reduction due to WS application ($R^2=0.83$). In +AT plants despite lower g_m and g_s values in WW conditions when compared to the -AT, the relationship ($R^2=0.53$) showed a less steep slope due to the reduction in g_s and the sustained g_m (Fig. 4D). Similarly, correlations between g_m and A_{max} (Fig. 4E) and between g_m and RWC (Fig. 4F) show that +AT plants evidenced a different and less steep curve pattern, owing to sustained g_m values.

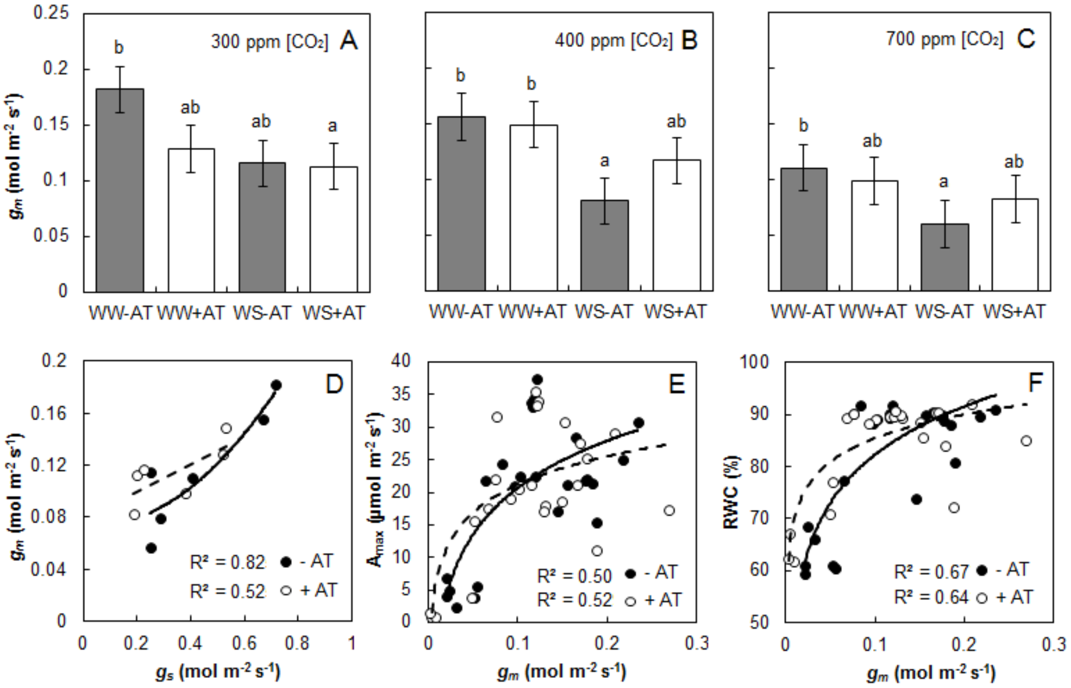

Figure 4. Mesophyll conductance (g_m, mol m^{-2} s^{-1}) pooled data of winter oilseed rape plants grown at historical (A), ambient (B) and elevated (C) atmospheric CO$_2$ concentration under well-watered (WW) and water-stress (WS) conditions and sprayed with a 1% v/v concentration of Vapor Gard (+AT) or water (-AT). Data were collected at days after treatments application (DAT) 1, 4, 8 and 12. Data are means (n=24) ± SED from three-way ANOVA. Columns with same letters are not statistically different according to Tukey's test (P<0.05). D) Relationship between g_m and g_s, E) relationship between A$_{max}$ and g_m and F) relationship between RWC and g_m. Points represent means between -AT and +AT plants (n=6 in D and n=24 in E and F). Lines were fitted by using linear regression for +AT and polynomial regression for -AT in D and by fitting a logarithmic model on both - AT and + AT in E and F.

Chlorophyll fluorescence and ETR$_{max}$

Under WW conditions no differences were found between plants grown under $_h$[CO$_2$] and $_a$[CO$_2$] in ETR$_{max}$ (Fig. 5A and B). On the contrary an average 13.7% ETR$_{max}$ increase was found in $_e$[CO$_2$] when compared to the $_h$[CO$_2$] and $_a$[CO$_2$] (Fig. 5C). Under WW conditions and with respect to the -AT plants, AT application did not statistically affect ETR$_{max}$. The ETR$_{max}$ value was significantly reduced by WS for all the [CO$_2$] from DAT 7. However, the CO$_2$ x watering regimes interaction was not significant for any of the [CO$_2$], suggesting similar ETR$_{max}$ reductions for all the [CO_2]. With respect to the control WW, ETR_{max} experienced a reduction by ~50% on DAT 7 and by~75% on DAT 11. There were no significant differences in ETR_{max} between +AT and -AT plants under WS except for DAT 7 at $_{a}$[CO_2] where ETR_{max} was significantly sustained (P=0.018) by the AT application.

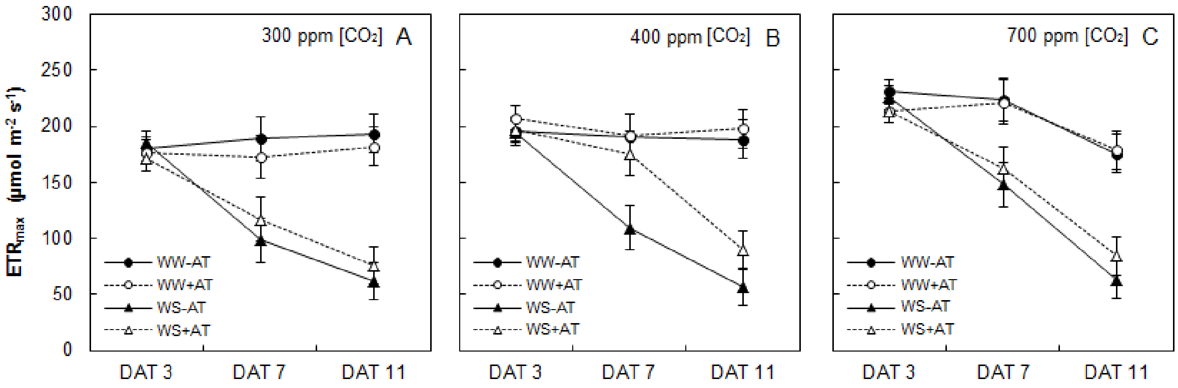

Figure 5. Development of the relative electron transport rate (ETR, μmol $m^{-2}s^{-1}$ mol $m^{-2}s^{-1}$) of winter oilseed rape plants grown at historical (A), ambient (B) and elevated (C) atmospheric CO_2 concentration under well-watered (WW) and water-stress (WS) conditions and sprayed with a 1% v/v concentration of Vapor Gard (+AT) or water (-AT). Data were collected at days after treatments application (DAT) 3, 7 and 11. Data are means (n=6) ± SE.

ABA concentration

Well-watered plants showed an average leaf [ABA] of 650, 420 and 160 ng g^{-1} DW when grown at $_{h}$[CO_2], $_{a}$[CO_2] and $_{e}$[CO_2] respectively (Fig. 6A). WW plants grown under $_{e}$[CO_2] have a significantly lower [ABA] than that grown under $_{h}$[CO_2]. No differences were found between WW-AT and WW+AT and under all the [CO_2]. Decreased water availability significantly promoted leaf ABA accumulation (Fig. 6B). With respect to their relative WW-AT control, [ABA] was increased by 8, 10 and 20-fold in plants grown under $_{h}$[CO_2], $_{a}$[CO_2] and $_{e}$[CO_2], respectively. Nevertheless, when compared to the WS-AT plants grown under $_{h}$[CO_2], [ABA] in $_{a}$[CO_2] and $_{e}$[CO_2]-grown plants was 8% and 13% lower, respectively. AT application on WS plants grown under $_a[CO_2]$ and $_e[CO_2]$ decreased [ABA] by 10 and 11%, but not under $_h[CO_2]$

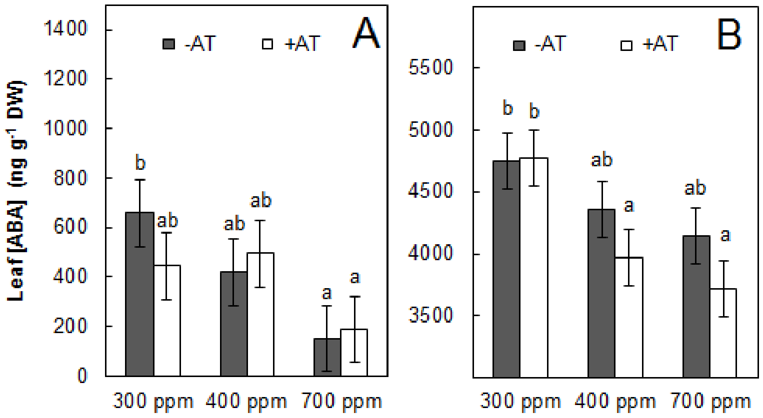

Figure 6. Leaf ABA concentration ([ABA]) data of winter oilseed rape plants grown at historical, ambient and elevated atmospheric CO_2 concentration under well-watered (WW, A) and water-stress (WS, B) conditions and sprayed with a 1% v/v concentration of Vapor Gard (+AT) or water (-AT). Samples were collected at days after treatments application (DAT) 7. Data are means (n=6) ± SED. Columns with same letters are not statistically different according to Tukey's test (*P*<0.05)

Discussion

$[CO_2]$ levels influenced biomass accumulation and leaf gas-exchange

Shoot DW and LA were significantly enhanced by $_e[CO_2]$ application and the magnitude of plant biomass accumulation decreased when the $[CO_2]$ was lowered (i.e. $_a[CO_2]$ and to a greater extent $_h[CO_2]$). In our experiments, $_e[CO_2]$ mainly stimulated shoot DW by increasing LA rather than LN. In Qaderi *et al.* (2006) CO_2 enrichment increased both LN and LA. However, unlike the winter cultivar used in the experiments presented here, in Qaderi *et al.* (2006) a spring cultivar was used suggesting possible intraspecific differences in assimilates allocation during the vegetative stage. In support of this interpretation, in our experiments the shoot biomass allocation was ~70% for leaf and ~30% for stem (similarly distributed at all the [CO_2]), whereas in Qaderi *et al.* (2006) the spring cultivar biomass allocation was ~50% for leaf and ~50% for stem. Therefore, further investigations should compare the response to elevated [CO_2] of spring and winter cultivars to elucidate intraspecific and genotypic variation in biomass accumulation at various [CO_2].

When grown under $_e$[CO_2], WW plants exhibited a significant decrease in g_s (and transpiration rate) leading to an overall increase in A_{max}/g_s ratio over time when compared to $_a$[CO_2] and $_h$[CO_2]. However, the larger plant size that can be achieved at elevated CO_2 is related to higher water use (hence transpiration) (Xu *et al.* 2013; Polley *et al*. 2008; Nowak *et al*. 2004). Our results shows that in winter OSR the standardized transpiration over time for LA is increased under $_a$[CO_2] (1106 ± 105 mmol H_2O) when compared to the $_h$[CO_2] (713 ± 105 mmol H_2O), but not under $_e$[CO_2] (852 ± 105 mmol H_2O) suggesting that the short-term acclimation to $_e$[CO_2] is highly beneficial in the WW plants even if the LA is dramatically increased.

In our experiments, WW plants grown under $_a$[CO_2] and, to a greater extent, $_e$[CO_2] showed declines in g_s over time. It is possible that, since we collected gas-exchange on the 4th leaf, the reduction was due to the production of newly formed and more functional leaves (i.e 5th-to-8th leaf) as already shown in Albert *et al*. (2012). This may have led to a substantial decrease over time of the gas-exchange efficiency of the 4th leaf. However, the strong reduction of g_s under $_e$[CO_2] may indicate acclimation of g_s to $_e$[CO_2] as proposed by Ball *et al*. (1987) that may deserve further studies.

Previous work on spring OSR at elevated [CO_2] (~770 ppm) showed stimulation of photosynthesis by ~22% and reduction in g_s by ~17% (Qaderi *et al.* 2006).

Similarly, in our experiments the $_e[CO_2]$ plants exhibited higher A_{max} and ETR_{max} when compared to $_a[CO_2]$ and, to a greater extent, $_h[CO_2]$. In C_3 plants, photosynthesis under enriched CO_2 conditions is enhanced mainly due to the increased Rubisco efficiency and the simultaneous decrease in the affinity of the enzyme for oxygen (Leakey *et al.* 2012). The fact that under $_e[CO_2]$ plants showed an increased ETR_{max} followed by a decrease in NPQ may be an indication that, although rubisco should not be co-limited by RuBP regeneration at 700 ppm $[CO_2]$, the increase in A_{max} may also be due to an improved quantum efficiency of the CO_2 fixation. In our experiments, $_e[CO_2]$ at 700 ppm stimulated A_{max} with respect to $_a[CO_2]$ less (~22% compared to $_a[CO_2]$) than in other C_3 crops such as durum wheat (~37%; Aranjuelo *et al.* 2015) and rice (~40%, Vu *et al.* 1997). Interspecific long-term acclimation of photosynthesis to elevated $[CO_2]$ due to Rubisco down-regulation has been previously shown (Leakey *et al.* 2012; Xu *et al.* 2013). In our experiments at DAT 12 the A_{max} of $_e[CO_2]$ plants was similar to that of $_a[CO_2]$ suggesting strong photosynthetic acclimation of winter OSR during the vegetative stage. During the vegetative stage, OSR exhibited very high photosynthetic rates and thus elevated N requirements. Therefore it is possible that the increased leaf area of pot-grown plants at $_e[CO_2]$ led to a fast reduction in Rubisco activity in turn due to a depression in area-based leaf N content. Further work should be done to understand whether the short-term photosynthetic acclimation under elevated $[CO_2]$ is due to a decreased Rubisco activity, to a decrease in leaf Rubisco content or to different N utilization and/or allocation of the crop and whether changes are dependent on the phenological stage.

Previous work showed that g_m decreased with the increase of the $[CO_2]$ (Flexas *et al.* 2014) and in our experiments consistent results were found for winter OSR.

There are, however, still many uncertainties about the mechanism relating g_m to $[CO_2]$, mainly due to possible artefacts derived from the gas-exchange/fluorescence estimation method (Singsaas *et al.* 2003). Leaf anatomical changes (e.g. thickness) may be related to decreases in g_m (Flexas *et al.* 2014). However, no differences were found in SLA between the different $[CO_2]$ treatments suggesting that although our experiments were conducted over a relatively short period, leaf structure may only be minimally involved. According to Sorrentino *et al.* (2016) ABA plays a major role at modulating g_s and g_m. In our experiment [ABA] was reduced in WW plants after increasing the $[CO_2]$. Similarly, in Teng *et al*. (2006), Arabidopsis plants grown under 700 ppm $[CO_2]$ exhibited a significant decrease in [ABA] when compared to the plants grown under ~370 ppm $[CO_2]$ followed by an increase in other growth-promoters hormones (e.g. auxins, cytokinins and gibberellins). Therefore it is possible that $[CO_2]$ modulates g_m even in OSR following an altered hormonal pattern and also, as reported by Krieg (1986) and then Kitao *et al.* (2015), following leaf starch accumulation under $_e[CO_2]$ in turn reducing the CO_2 diffusion in the chloroplasts due to an increased pathway around the large starch grains. Our data do not provide a clear insight of the possible factors involved in g_m alteration under different $[CO_2]$ and specific experiments should be developed.

$[CO_2]$ levels do not affect the whole-plant response to drought conditions.

Under WS conditions, similar reductions in shoot DW, LA and SLA were recorded in the different $[CO_2]$. However, conversely to the RWC trait, in shoot DW and LA, the CO_2 X watering regimes factor was significant in DAT 8 and 12. At the soil moisture deficit applied, plants did not accumulate biomass since DAT 8 at all the

$[CO_2]$. Thus, since A_{max} was significantly higher in DAT 1 and 4 increasing the $[CO_2]$, the higher shoot DW and LA of $_e[CO_2]$ WS-plants with respect to the $_h[CO_2]$ and $_a[CO_2]$ can be probably related to the higher A_{max} values over the first part of the stress (when the water in the pots was still available) rather than to an elevated CO_2-induced improvement in water-use efficiency.

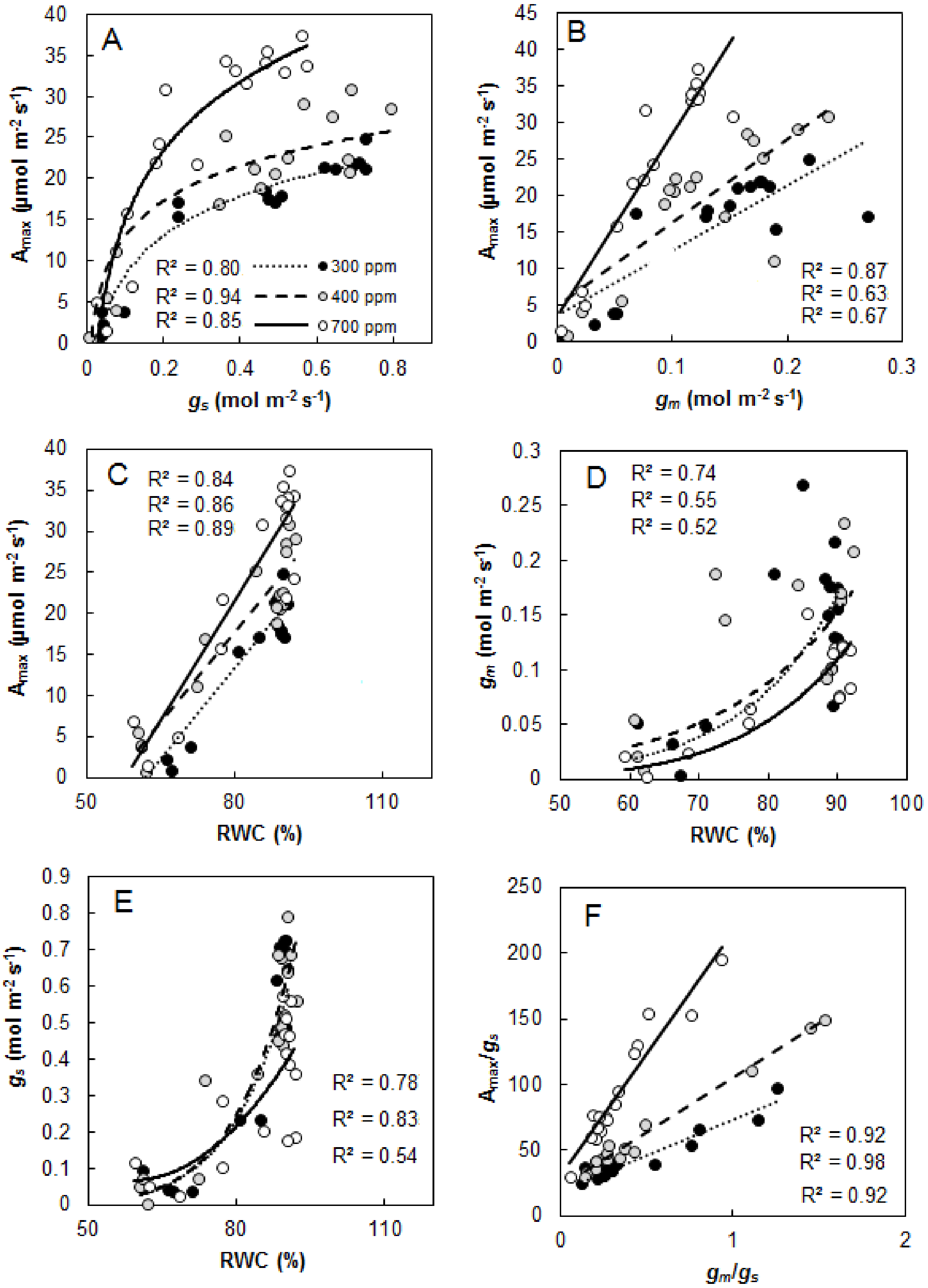

Figure 7. Correlations between A) CO_2 assimilation rate at saturating light (A_{max}) and stomatal conductance (g_s), B) A_{max} and mesophyll conductance (g_m) C) A_{max} and leaf relative water content (RWC), D) g_m and RWC, E) g_s and RWC, F) A_{max} /g_s ratio and g_m /g_s ratio of winter oilseed rape plants grown at 300ppm (black symbols), 400ppm (grey symbols) and 700ppm white symbols) atmospheric CO_2 concentration under well-watered (WW) and water-stress (WS) conditions and sprayed with a 1% v/v concentration of Vapor Gard (+AT) or water (-AT). Data points are means. Lines were fitted using linear regression for B,C and F, polynomial regression for D and E and exponential curve for A .

It has been proposed that by maintaining low g_s, elevated atmospheric [CO_2] may ameliorate plant water status under stress (Leakey *et al*. 2012). Polley *et al*. (1999) suggested that the effects of elevated [CO_2] on osmotic adjustment are minimal. Therefore, the maintenance of high plant water status in plants grown at elevated [CO_2] under stress is primarily a result of stomatal control. However, and contrary to expectation, in our experiments no differences were found in RWC between [CO_2] treatments. In our experiments the larger leaf area produced under $_e$[CO_2] led to a similar transpiration rate standardized for the LA (~200 mmol $m^{-2}s^{-1}$ for all the [CO_2] under stress) thus offsetting the water-loss reduction following the $_e$[CO_2]-induced stomatal closure. In addition, since the pots were not sealed (thus simulating a field scenario where a substantial amount of water was evaporated from the soil before the plants reached the “rosette” canopy), the beneficial effect of $_e$[CO_2] was minimized leading to similar WS regimes for all the [CO_2] as shown in Fig. S1.

In our experiments g_s was significantly reduced at all the [CO_2] even when elevated RWC values were recorded, showing a non-linear relationship between the two traits (Fig. 7E). A_{max}-to-g_s curves show that in the first part of soil drying, g_s is reduced more than A_{max} leading to higher A_{max}/g_s values for all the [CO_2] treatments (Fig. 7A). However, despite the curve plateau being higher at $_e$[CO_2], the shape of the A_{max}-to-g_s curves were similar for all the [CO_2], possibly because the increased leaf area resulted in similar detrimental effects on A_{max} between the [CO_2] under stress. Because the diffusion of CO_2 into the leaves is restricted when stomata progressively close to minimize water loss via transpiration, from DAT 4 and for all the [CO_2], A_{max} was reduced when compared to the WW plants and the magnitude of this reduction was linearly correlated with the decrease in RWC (Fig.

7C). Additionally, a strong correlation was found between A_{max} and g_m for all the [CO_2] corroborating the hypothesis that the mesophyll governs, together with the stoma, the provision of CO_2 to the chloroplasts (Fig. 7B). According to Chaves *et al.* (2009) the changes in mesophyll conductance under drought conditions may be due to leaf shrinkage, biochemistry alteration and modifications in membrane permeability. Indeed g_m was correlated with RWC (Fig. 7D), but strong differences were found between [CO_2] and the reduction was much higher under $_e$[CO_2] than in $_h$[CO_2]-grown plants. It has been shown that CO_2 is a regulator of the expression of several photosynthetic genes: in Van Oosten *et al.* (1994), elevated [CO_2] significantly repressed the nuclear genes involved in carbon metabolism leading to plant sugar accumulation in conditions of reduced sink activity. Flexas *et al.* (2006) showed that in tobacco the plasma membrane aquaporin gene NtAQP1 was involved in g_m regulation and modifications of the C_i triggered different leaf photosynthetic capacity. Therefore it is highly possible that [CO_2] can be directly involved in g_m modulation under drought. Similarly, ETR_{max} was significantly reduced from DAT 7, suggesting that strong stress intensities trigger PSII down-regulation and in turn NPQ processes in OSR seedlings. Thus, both stomatal and non-stomatal limitations of A_{max} may affect the photosynthetic performance of OSR under severe drought conditions with similar magnitudes at the different [CO_2].

The CO_2 x watering regimes interaction was statistically significant from DAT 4 for g_s. In $_e$[CO_2]-grown plants the reduction in g_s following water shortage was significantly lower than that of the $_a$[CO_2] and $_e$[CO_2]-grown plants. At the same time, [ABA] was lower in the leaf of plants grown under $_e$[CO_2] when compared to the $_h$[CO_2]. ABA is a ubiquitous hormone in plant response to water deficit and under drought all the plant tissue exhibited ABA accumulation via both xylem root-to-shoot transport and leaf *in situ* production (Zhang *et al.* 2006). An increase in ABA accumulation is expected with increasing drought intensities and indeed WS significantly promoted ABA accumulation in leaves. However, since [ABA] was lower in leaves of $_e[CO_2]$ when compared to those of $_h[CO_2]$-grown plants, possible combined effects of [ABA] and $[CO_2]$ may modulate the OSR response to drought.

AT efficiency is dependent on $[CO_2]$ and on drought intensities

The effect of AT in counteracting drought stress is well documented (e.g. Abdullah *et al*. 2015; Faralli *et al*. 2016; Solarova *et al*. 1981) and consistent with our $_a[CO_2]$ and $_e[CO_2]$ data. To our knowledge, no work has been published on the response of winter OSR plants to drought and historical, ambient and projected $[CO_2]$. Previously published analysis of AT performance (Solarova *et al*. 1981) has likely contributed to a limited use of these chemicals in the last three decades. In del Amor *et al*. (2010), elevated $[CO_2]$ (2000 ppm) and AT application significantly sustained CO_2 assimilation rates of pepper seedlings under moderate-to-severe drought conditions. In our experiments, application of AT resulted in a decrease in ET and thus AT-treated plants experienced a small but higher soil moisture in the pots when compared to the WS-AT plants. It was hypothesized that by maintaining a high transpiration rate, the $_h[CO_2]$ plants should have exhibited a prominent reduction in gas-exchange following AT application and thus significant improvements in plant water status. Although the interaction between watering regimes and antitranspirant was significant following DAT 4, in $_h[CO_2]$-grown plants no beneficial effects of the AT on g_s (except for DAT 12) were found. Adaxial application of AT leads to abaxial compensation of g_s (Faralli *et al*. 2016), and although g_s was not analysed on the two leaf surfaces, the higher stomatal apertures under $_h[CO_2]$ may have resulted in a higher abaxial compensation and thus more water used under WS conditions. Indeed lower g_s reductions were found in WS plants following AT application in plants grown at $_h[CO_2]$ when compared to $_a[CO_2]$ and $_e[CO_2]$. As explained above, the reduction in RWC of the WS+AT plants was much lower under $_a[CO_2]$ and $_e[CO_2]$ than $_h[CO_2]$. Under $_a[CO_2]$ and $_e[CO_2]$ the higher RWC values were recorded on DAT 8 and DAT 12, when however the differences in g_s between WS-AT and WS+AT were minimal. Although in these experiments the whole-plant transpiration was not analysed, it is plausible that the water saved in the pots from DAT 0 to DAT 7-8 (when plant reached permanent wilting point, thus a severe drought stress) following AT application, mitigated the RWC reduction leading to higher plant water status over the last part of stress from DAT 8 to DAT 12 (severe to terminal stress).

AT application in WW plants reduced A_{max}, g_s and g_m at all the $[CO_2]$ leading to a higher A_{max}/g_s ratio. The data are consistent with other work indicating the source-limiting effect of AT following direct stomatal occlusion (Palliotti *et al*. 2013). However plant biomass and fluorescence traits were not negatively affected possibly because of the direct amelioration of the A_{max}/g_s ratio. The AT source-limitation (reduction of A_{max} and g_m) under WW plants was indeed significantly different at different $[CO_2]$ and at $_e[CO_2]$ it was almost negligible. Consistent with our results, in del Amor *et al*. (2010) AT limited CO_2 uptake of pepper seedlings by 25% when grown under 380 ppm $[CO_2]$ whereas the reduction was much lower (7%) when grown under 2000 ppm despite a similar reduction in g_s (between 20 and 25%).

The beneficial effects at gas-exchange level of an AT application under drought have been recently shown on oilseed rape (Faralli *et al.* 2016) and wheat (Abdullah *et al.* 2015). AT mitigated the photosynthetic down-regulation under drought by reducing water use in the first part of the stress and thus improving plant water status. In our experiments three main parameters (RWC, A_{max} and g_m) were enhanced by AT application with significant stronger effects at increasing [CO_2]. Similarly in del Amor *et al.* (2010), A_{max} was sustained in AT-treated water-stressed pepper seedlings grown under 2000 ppm [CO_2], but not at ambient (380 ppm) [CO_2]. In our experiments significant increases in A_{max}/g_s ratio were recorded that were linearly correlated to g_m/g_s for the different [CO_2] (Fig. 7F). These data suggest that AT improved A_{max} supporting a "water saving" strategy as already reported by Abdullah *et al*. (2015). Also, the drought-induced mesophyll down-regulation was not exacerbated, possibly by avoiding leaf shrinkage or biochemical alteration (higher RWC). It has been previously reported that aquaporins may play a role in modulating mesophyll functions. As reported by Shatil-Cohen *et al.* (2011), application of ABA decreased leaf water potential and hydraulic conductivity due to aquaporins deactivation. Our data may indicate that the sustained mesophyll conductance of WS+AT plants only in situations of maintained RWC (hence $_a$[CO_2] and $_e$[CO_2]) may be an indication of possible further links to be explored between plant water status, aquaporins and leaf-response alterations following different [CO_2] levels. To confirm that, the J/A_{max} ratio was reduced in WS+AT by 4-fold in $_e$[CO_2] when compared to the $_h$[CO_2] suggesting higher capacity of CO_2 fixation following AT application in contrast to previous reports of work conducted when the atmospheric [CO_2] was lower (Solarova *et al.* 1981). Therefore, AT application under elevated [CO_2] may not create any source-limitation, but could have an ameliorative effect under drought since the improved plant water status counteracts mesophyll resistance in circumstances of higher CO_2 available at the site of carboxylation.

Significance for agriculture exploitation

Although our work has been carried out during an early stage of OSR development, there is a large number of useful information regarding the potential AT agricultural exploitations and OSR responses in a drought x CO_2 environment.

In an agronomic context AT has been used to decrease sugar accumulation in grapes (Palliotti *et al.* 2013) since, high dose rates of AT application significantly reduced the CO_2 diffusion to the sub-stomatal cavity. It is possible that at higher [CO_2], the AT efficacy at reducing photosynthetic rate may be counteracted by the increased CO_2 available at the site of carboxylation. Indeed in our experiments the largest source-limiting effect of AT in WW plants was recorded at $_h$[CO_2]. For this reason, AT may be useful in agricultural areas where a fast growth of the canopy is required with low water use to maximize soil coverage and avoid evaporation (e.g. drought-prone areas where spring crops are grown). In addition, further research should focus on the use of AT on glasshouse-grown crops to improve water-use efficiency where atmospheric CO_2 enrichment is already used (e.g. tomatoes).

Based on our results it is possible to speculate that under elevated CO_2 conditions, plants with lower stomata number or with higher stomata sensitivity to [ABA] (e.g. Wang *et al.* 2005) may have a significant advantage in adapting to drought conditions. In particular, OSR is considered a drought-sensitive crop over the reproductive stages (Faralli *et al*. 2016; Jensen *et al*. 1996). Since it has been proposed that OSR has a very minimal osmotic adjustment under drought (Jensen *et al*. 1996), “water saving” strategies over the reproductive stage (from flowering until pod development) may give significant advantages for yield protection under drought. In Wang *et al*. (2005) down-regulation of the *ERA1* gene that encodes for the β-subunit of farnesyltransferase enhanced the sensitivity of stomata to ABA, leading to reductions in leaf transpiration (Wang *et al.* 2005) and yield protection from drought (Wang *et al*. 2009). Our data suggests therefore that further increase in leaf water-use efficiency can occur under $_e[CO_2]$ thus increasing the possibility to exploit these tools without severely affecting CO_2 uptake but even sustaining the photosynthetic rates under water-limited conditions if plant water status is maintained. Further investigation in this direction is needed to gain additional knowledge on the interspecific responses of several crops to elevated $[CO_2]$ and drought environments and to study possible further management tools, biotechnological approaches and/or genotypic variation following the above responses.

Conclusions

The work presented here shows improvements in the physiological performance of winter OSR seedlings in elevated $[CO_2]$, mainly by stimulating photosynthesis and thus increasing leaf area. Strong differences were found between plants grown at 300 and 400 ppm suggesting that, despite in this study a modern-breed variety has been used, OSR was significantly source-limited in the first years of its agricultural production (~1960). Further investigations should focus on understanding the basis of crop CO_2 adaptation by comparing the responses of modern and old varieties to different CO_2 levels and possibly the interactive effects with environmental stresses. Increasing the [CO_2] decreased plant water loss but no significant beneficial effects were found under drought possibly due to the larger leaf area gained over the first few days of stress (when water was available) leading to similar total ET between the [CO_2] treatments. Higher [CO_2] reduced the source-limiting effect of AT on WW plants possibly because high [CO_2] allows higher rubisco fixation even when a substantial amount of stomata are blocked. The beneficial effects of AT under drought conditions was increased under elevated [CO_2] due to the synergistic effect of the additional stomatal resistance on RWC, A_{max} and g_m under drought. Thus the effect of AT under drought is dependent on the [CO_2] and further studies should be focused on the interactions between AT with drought, CO_2 and other major agricultural-related stressors, in particular heat. This work shows that the effectiveness of crop management regimes can be altered by rising [CO_2], and justifies further multifactorial experiments to evaluate crop management x environment interactions previously studied under ambient or even lower [CO_2].

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Supplementary Material

Supplementary Table 1. Analysis of variance (*P*-values) for [CO_2], watering regimes and antitranspirant application and their interactive effects on relative water content (RWC), plant dry-weight (shoot DW), Leaf area, CO_2 assimilation rate at saturating light (A_{max}), stomatal conductance (g_s), water use efficiency (A_{max}/g_s), maximum electron transport rate (ETR_{max}), leaf ABA concentration (three-way ANOVA), leaf ABA concentration of well-watered plants (CO_2 x antitranspirant), leaf ABA concentration of droughted plants (CO_2 x antitranspirant) and mesophyll conductance (g_m) of winter oilseed rape plants grown at 300ppm, 400ppm and 700ppm atmospheric CO_2 concentration under well-watered (WW) and water-stress (WS) conditions and sprayed with a 1% v/v concentration of Vapor Gard (+AT) or water (-AT). Values significance is highlighted with asterisks (*P*<0.05)

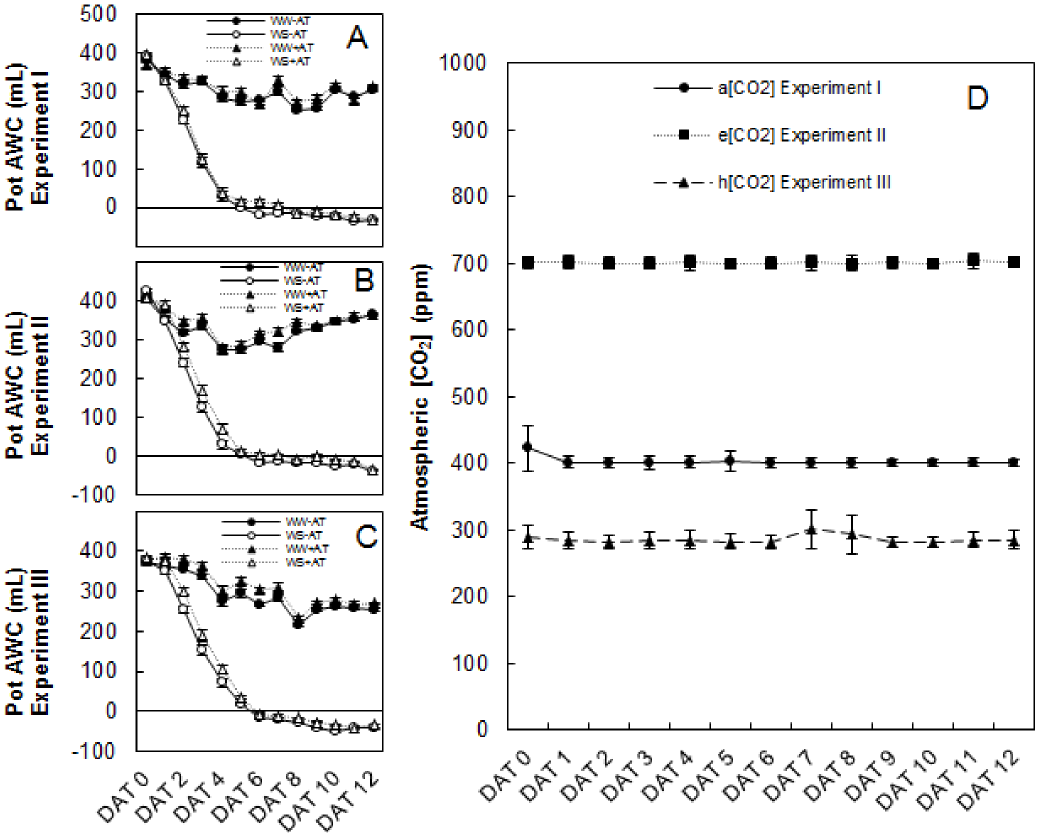
	RWC (%)				A_{max}			
	DAT 1	DAT 4	DAT 8	DAT 12	DAT 1	DAT 4	DAT 8	DAT 12
ANOVA Factor	*P*-values (d.f.=71)				*P*-values (d.f.=71)			
CO_2	0.064	0.182	0.016*	0.501	<.001*	<.001*	<.001*	0.008*
Watering regimes	0.802	<.001*	<.001*	<.001*	0.445	<.001*	<.001*	<.001*
Antitranspirant	0.967	0.008*	<.001*	<.001*	<.001*	0.778	0.025*	0.011*
CO_2 x Watering regimes	0.869	0.12	0.018*	0.065	0.043*	0.063	0.001*	0.071
CO_2 x Antitranspirant	0.84	0.384	0.217	0.792	0.030*	0.003*	0.004*	0.607
Watering regimes x Antitranspirant	0.606	0.005*	<.001*	<.001*	0.919	<.001*	<.001*	0.015*
CO_2 x Antitranspirant x Watering regimes	0.677	0.278	0.245	0.464	0.610	0.333	0.401	0.720

	Shoot DW				g_s			
	DAT 1	DAT 4	DAT 8	DAT 12	DAT 1	DAT 4	DAT 8	DAT 12
ANOVA Factor	*P*-values (d.f.=71)				*P*-values (d.f.=71)			
CO_2	0.219	<.001*	<.001*	<.001*	<.001*	<.001*	<.001*	<.001*
Watering regimes	0.662	0.053	<.001*	<.001*	0.254	<.001*	<.001*	<.001*
Antitranspirant	0.514	0.621	0.68	0.865	<.001*	<.001*	0.064	<.001*
CO_2 x Watering regimes	0.829	0.358	<.001*	<.001*	0.71	<.001*	<.001*	<.001*
CO_2 x Antitranspirant	0.171	0.686	0.105	0.386	0.086	0.26	<.001*	0.009*
Watering regimes x Antitranspirant	0.8	0.662	0.081	0.076	0.6	0.739	<.001*	<.001*
CO_2 x Antitranspirant x Watering regimes	0.64	0.961	0.019*	0.815	0.497	0.233	0.008*	0.004*

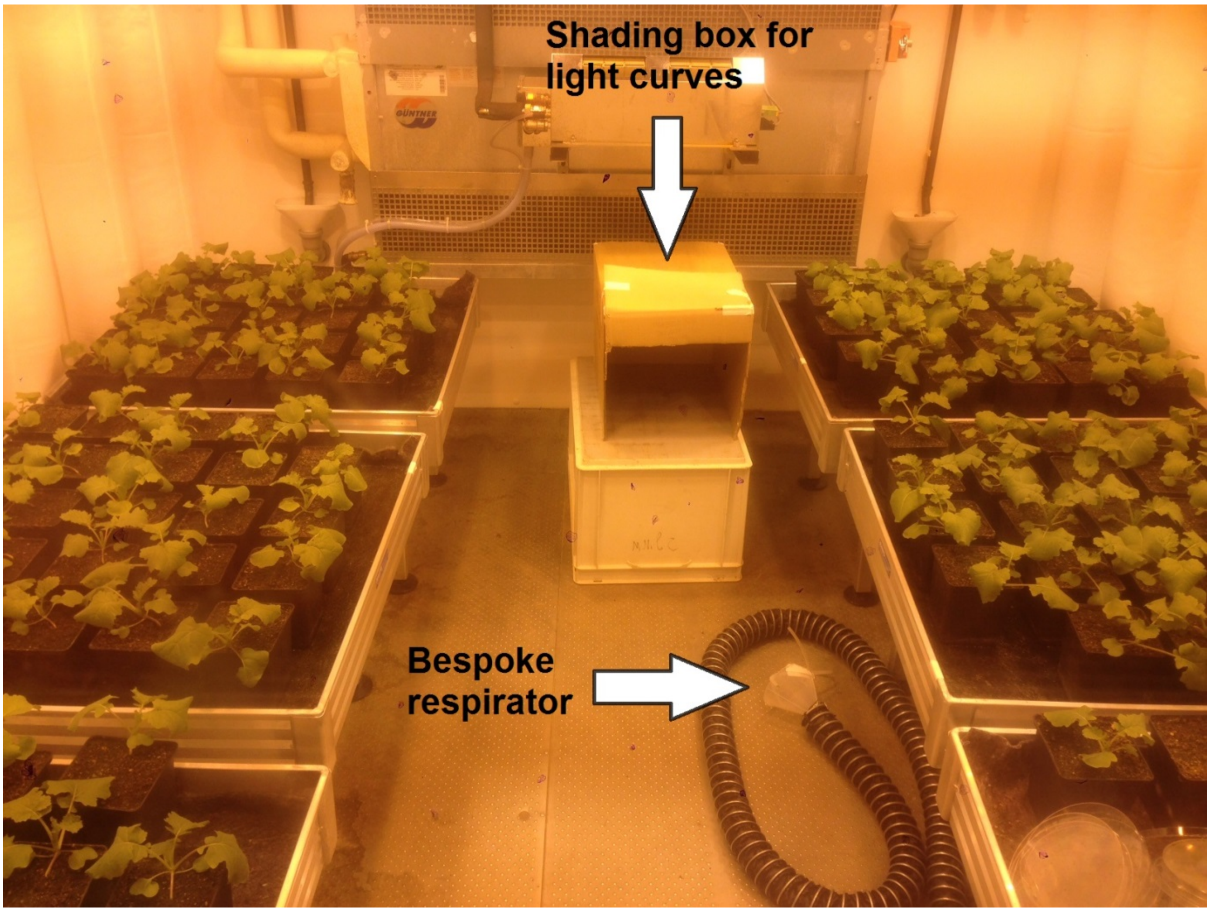
	Leaf Area				A_{max}/g_s			
	DAT 1	DAT 4	DAT 8	DAT 12	DAT 1	DAT 4	DAT 8	DAT 12
ANOVA Factor	*P*-values (d.f.=71)				*P*-values (d.f.=71)			
CO_2	0.333	<.001*	<.001*	<.001*	<.001*	<.001*	<.001*	<.001*
Watering regimes	0.367	<.001*	<.001*	<.001*	0.505	<.001*	<.001*	0.956
Antitranspirant	0.831	0.955	0.33	0.013*	<.001*	<.001*	0.753	0.944
CO_2 x Watering regimes	0.953	0.052	0.001*	<.001*	0.536	0.062	0.003*	<.001*
CO_2 x Antitranspirant	0.375	0.478	0.792	0.049*	0.083	<.001*	0.001*	0.020*
Watering regimes x Antitranspirant	0.309	0.365	0.051	0.491	0.582	<.001*	0.956	0.734
CO_2 x Antitranspirant x Watering regimes	0.672	0.662	0.486	0.913	0.615	0.002*	0.062	0.01*

	ETR_{max}			Leaf [ABA]	Leaf [ABA] WW	Leaf [ABA] WS	g_m
	DAT 3	DAT 7	DAT 11	DAT 7	DAT 7	DAT 7	Pooled data
ANOVA Factor	*P*-values (d.f.=71)			*P*-values	*P*-	*P*-values	*P*-values

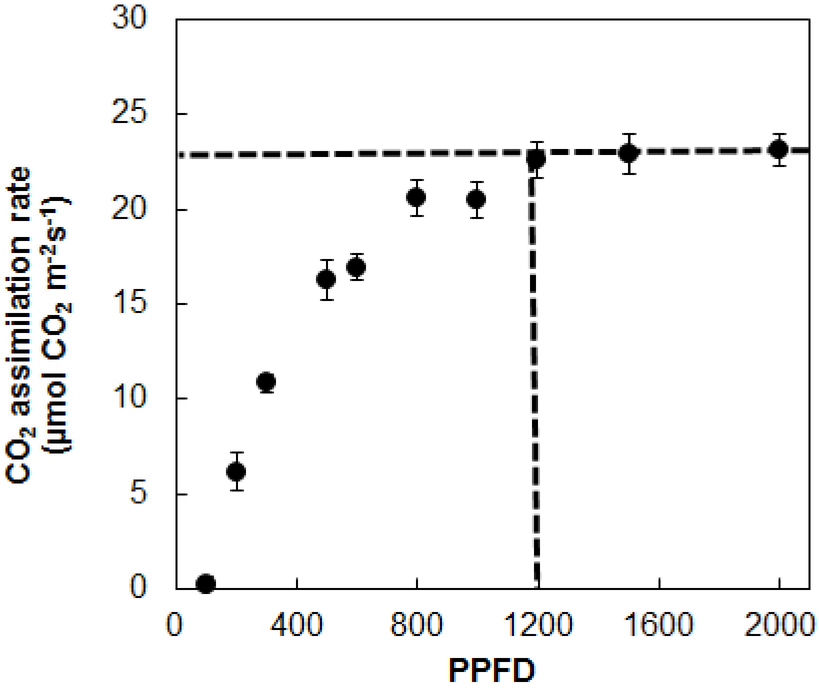
				(d.f.=71)	values (d.f.=35)	(d.f.=35)	(d.f.=35)
CO_2	<.001*	<.001*	0.667	<.001*	0.001	<.001*	<.001*
Watering regimes	0.49	<.001*	<.001*	<.001*	-	-	<.001*
Antitranspirant	0.234	0.091	0.103	0.069	0.673	0.052	0.776
CO_2 x Watering regimes	0.856	0.441	0.566	0.026*	-	-	0.627
CO_2 x Antitranspirant	0.127	0.218	0.508	0.874	0.275	0.301	0.105
Watering regimes x Antitranspirant	0.614	0.018*	0.116	0.154	-	-	0.014*
CO_2 x Antitranspirant x Watering regimes	0.726	0.459	0.978	0.129	-	-	0.928



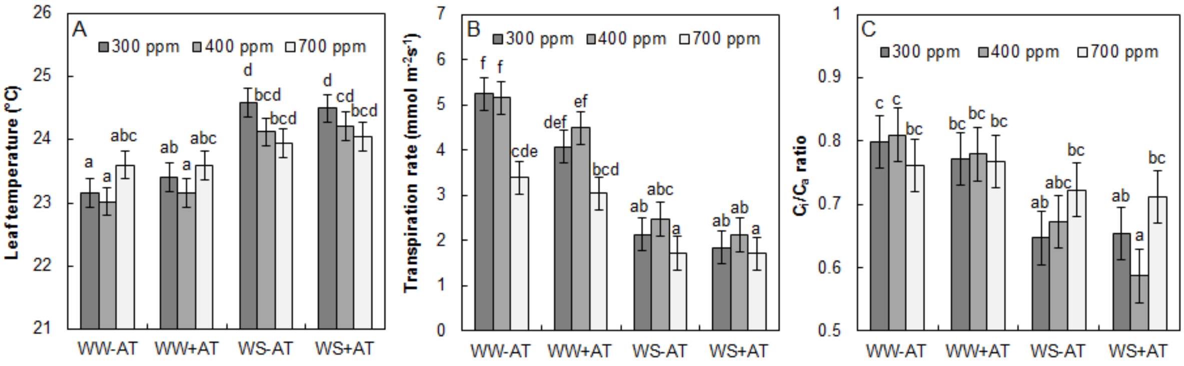
Supplementary Figure 1. Development of pot available water content (AWC, mL) over the twelve-day experimental period of well-watered (WW, solid symbols) and droughted (WS, open symbols) plants treated with (+AT) or without (-AT) 1% v/v Vapor Gard during Experiment I (A), Experiment II (B) and Experiment III (C) and development of atmospheric [CO_2] over Experiment I ($_a[CO_2]$), Experiment II ($_e[CO_2]$) and Experiment III ($_h[CO_2]$). Data points are means (n=6 for A, B and C and n=1440 for D) ±SE for A, B and C and ±SD for D.



Supplementary Figure 2. Example of experimental room with the bespoke respirator and the cardboard box used to cover plants before the light curve protocol.



Supplementary Figure 3. Light-response curves for oilseed rape seedlings. The curves were plotted on the third fully expanded leaf before Experiment I. A Li-Cor 6400 was used supplemented with a LI-6400-40 cuvette. CO_2 assimilation rates were collected at 100, 200, 300, 500, 600, 800, 1000, 1200, 1500 and 2000 PPFD. Cuvette temperature was maintained at 25°C, the flow rate was 300 µmol s^{-1}, CO_2 (C_a) was 400 ppm and the light source was maintained at 10:90 blue-red light. Data points are means ± SEM (*n*=6) with the exception of 200, 500 and 800 PPFD where *n*=3.



Supplementary Figure 4. Leaf temperature (A), transpiration rate (B) and C_i/C_a ratio (C) pooled data of winter oilseed rape plants grown at historical, ambient and elevated atmospheric CO_2 concentration under well-watered (WW) and water-stress (WS) conditions and sprayed with a 1% v/v concentration of Vapor Gard (+AT) or water (-AT). Data were collected at days after treatments application (DAT) 1, 4, 8 and 12. Data are means (n=24) ± SED. Columns with same letters are not statistically different according to Tukey's test ($P<0.05$)

Chapter 5

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In-field application of film antitranspirants shows potential yield protection from drought in *Brassica napus* L.

Pre-submitted version.

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Abstract

Crop management solutions that simulate plant water-saving strategies may help to mitigate drought damage in crops. Oilseed rape is significantly drought-sensitive from flowering to mid-pod development and drought periods lead to significant yield losses. In this work the drought-protection efficacy of different chemicals with antitranspirant activity applied just prior to key drought-sensitive phenological stages was tested on field-grown oilseed rape in two years. Drought was artificially imposed with rain-shelters. The results suggest that in-field application of 1 L ha^{-1} antitranspirant (Vapor Gard, a.i. di-1-*p* menthene, VG) at GS 6.0 (initiation of flowering) may mitigate drought-induced yield losses leading to a 0.71 t ha^{-1} seed yield increase on average and over two years of experiments when compared to the un-sprayed un-irrigated plots. No significant yield responses were found from application at GS 7.0, from increasing VG concentrations (i.e. 2 and 4 L ha^{-1}), and from an antitranspirant with short-lasting effectiveness. The data suggest that in field conditions where drought occurs during the flowering stage, application of 1 L ha^{-1} VG prior to the drought event can reduce yield loss.

Introduction

There is significant evidence that a major factor determining the yield of winter oilseed rape (OSR) is the amount of soil water available over the reproductive stages (Jensen et al. 1996; Berry and Spink, 2006; Istanbulluoglu et al. 2010). The yield components of the crop (pod number, seed number, and seed weight) are determined over a crucial period between flowering and mid-pod development (Mendham et al. 1981). This period often occurs in a seasonal time-frame (i.e. spring) of high crop water use (Vadez et al. 2014), elevated soil evaporation (Vadez et al. 2014) and low precipitation (Berry and Spink, 2006) in turn lowering the yield potential of the elite commercially-available varieties.

It has been extensively hypothesized that by maintaining high soil water availability and/or plant water status over these key-periods, arable crops may exhibit a yield benefit (e.g. Salter and Goode, 1967). In Wang et al. (2009) and Wang et al. (2005), down-regulation of the Farnesyltransferase subunit, a protein involved in stomata sensitivity to ABA, gave a yield benefit in field-grown canola under drought due to a significant reduction in transpiration. Similarly, intracuticular and epicuticular wax accumulation under water-limited conditions reduces leaf transpiration leading to a sustained photosynthetic rate (Cossani and Reynolds, 2012). Thus, further exploitation of water-saving strategies or wax-simulating tools may significantly reduce the drought damage on OSR yield at key-sensitive growth stages.

Film antitranspirant (AT) capacity to reduce transpiration through stomatal occlusion is well documented (Solarova et al. 1981). Recently the mechanisms of the yield benefit of AT under drought conditions on wheat and OSR in particular in relation to the reproductive development have been explored (Weerasinghe et al. 2016; Faralli et al. 2016). The main physiological factors involved in reduced yield loss from drought following AT application are i) a higher leaf water potential (Weerasinghe et al. 2016; Faralli et al. 2016), ii) a higher pollen fertility at pollen development stage and/or a lowered ABA signaling (Weerasinghe et al. 2016; Faralli et al. 2016) and iii) a sustained photosynthetic rate (Abdullah et al. 2015; Faralli et al. 2016) leading to higher grains and/or pod production when compared to the un-treated and stressed control (Abdullah et al. 2015; Weerasinghe et al. 2016; Faralli et al. 2016)

OSR has been shown to be more drought sensitive than wheat (Hess et al. 2015) and film antitranspirant application around flowering was beneficial for the yield of pot-grown OSR subjected to water stress although a substantial difference in efficacy between two chemicals was recorded (Faralli et al. 2016). Application of AT on field-grown *Brassica campestris* gave a grain yield increase following improved plant water status and water-use efficiency under dryland conditions (Patil and De, 1978). However, there is no work investigating the effectiveness of film antitranspirants at avoiding OSR yield losses under drought conditions in the field. Thus, two field experiments under rain-shelters investigated the effectiveness of AT at sustaining the yield of droughted OSR over different phenological stages: in 2015 (Experiment I) two chemicals with antitranspirant activity were applied at three different phenological stages, whereas in 2016 (Experiment II) the chemical which showed the best yield response in four glasshouse experiments and in the field in 2015 (di-1-*p* menthene), was used in a dose-response experiment and sprayed at two phenological stages.

Materials and methods

Site, soil analysis and crop sowing

The two field experiments were carried out in Flat Nook field, a field site at Harper Adams University, Shropshire (52°46′N, 2°25′W). Soil profile, bulk density and soil texture were analysed on January the 20th 2015. A 1 m^3 soil profile pit was excavated inside the experimental area. Four bulk density samples, at 20, 30, 60, 80 cm depths, were collected inside the pit with a 300 mm^3 tin, adapted from Rowell (1994). Texture samples were collected at the same depth as bulk density samples. The soil profile was used to determine soil depth (~90 cm). Bulk density at the different depth was estimated according to Rowell (1994) and texture samples were analysed according to Toogood (1958).

Previous crops at the site were fallow (no crops) for the 2014/2015 experiment area and potatoes for the 2015/2016 experiment area. Oilseed rape seeds (cv. Excalibur, Dekalb, UK) were sown on 29 August 2014, 15 cm row spacing and 80 seeds m^{-2} (Experiment I) and on the 4 September 2015 with row spacing at 15 cm and a seed rate of 50 seeds m^{-2} (Experiment II). Soil preparation for sowing and crop management followed the standard UK agronomic practices including insecticide, fungicide, herbicide and fertilizer application.

Design and treatments in 2014/2015 (Experiment I)

The experiment was a factorial randomized block design composed of three blocks with each block in a separate rain-shelter. There were eight plots per block and the plots were ~5 meters length and ~3 meters width. The treatments consisted of two antitranspirant products each sprayed at three growth stages according to the BBCH growth scale: bud emerging (flower buds visible from the above, GS 5.1), flowering (50% of plants have the first flower open, GS 6.0), pod development (10% of pods on the main stem reached the final size, GS 7.0). There were two additional control treatments in each block: irrigated with no AT (WW) and the unirrigated with no AT (WS). Rain-shelters were moved into position on the 26th of February 2015 when plants were still at rosette stage and from this stage until harvest water was applied only on the WW plots. The two antitranspirants chosen for the experiments (Nu-Film P, a.i. poly-1-*p* menthene 96%, NFP; Vapor Gard, a.i. di-1-*p* menthene 96%, VG. Miller Chemicals and Fertilizer, Hanover, USA) were sprayed in a volume of 200 L ha^{-1} of water using a hand sprayer (0.3 MPa, 1 m s^{-1}, nozzle, Flat Fan 110/03). For each spray treatment the boom was maintained ~0.5 meters above the leaf (GS 5.0 and 6.0) and pod (GS 7.0) canopy.

Design and treatments in 2015/2016 (Experiment II)

The experiment was a factorial randomized block design composed of six blocks with eight treatments per block and the plots were ~6 meters length and ~1 meter width. Each rain-shelter contained two blocks and in each block the treatments were three VG dose rate (1, 2 and 4 L/ha) sprayed at two growth stages (GS 6.0 and GS 7.0) using the spray conditions of the 2015 experiment and two control treatments in each block: irrigated with no AT (WW) and the unirrigated with no AT (WS). Rain-shelters were moved into position the 1st of February 2016 until harvest and water was applied only to the WW plots.

Antitranspirants spray application

In 2014, a glasshouse experiment inside the Harper Adams University was carried out to investigate the effect of different dose rates, water volumes and nozzles on spray leaf-coverage and droplets number. Significant differences were found between all the variables. Collectively, the data suggested that higher dose rates and water volumes evidenced higher antitranspirant leaf coverage and smaller nozzles created very fine spray quality when compared to the bigger ones. However, farmers are generally reluctant at using high water volumes (more expensive) as well as small nozzles (that tend to occlude). The Flat Fan 110/03 at 200 L ha^{-1} water volume was chosen as a good compromise as also previously used on wheat (Weerasinghe et al. 2016). Therefore, in 2015 the two antitranspirants (NFP and VG) were applied at 1 L ha^{-1} dose rate in a volume of 200 L ha^{-1} of water using a hand sprayer (0.3 MPa, 1 m s^{-1}, nozzle, Flat Fan 110/03) at the beginning of each phenological stage as reported above. In 2016, VG was applied at 1, 2 and 4 L ha^{-1} in a water volume of 200 L ha^{-1} and using the spray conditions of 2015 Experiment.

Soil moisture measurements, irrigation and environmental conditions

In Experiment I, 80-90cm duralumin neutron probe access tubes for soil moisture data collection were placed on each plot. Soil moisture measurements were taken with a neutron probe (Institute of Hydrology Neutron Probe System, Wallingford, UK) with a probe of 80 cm length. Soil moisture readings were taken from all plots (one reading tube per plot) at 20, 30, 50 and 80 cm depth in both the experiments. Volumetric water content (VWC) was calculated for all the experiment according to the Neutron Probe handbook for sandy soil as:

$$\mathrm{VWC}\ (\%) = \left[0.79\ \mathrm{x}\ \frac{\text{counts per second}}{\text{nutron probe reading}} - 0.024\right] \mathrm{x}\ 100$$

The field capacity for the different soil depth was estimated by carrying out two overall field readings on the 15/12/2014 and the 14/01/2015, whilst the soil was at field capacity. Soil moisture data were taken on the 16/12/2014, 14/01/2015, 02/03/2015, 16/03/2015, 26/03/2015, 07/04/2015, 17/04/2015, 24/04/2015, 01/05/2015, 13/05/2015, and 04/06/2015. Irrigation was applied only to WW plots over the whole experimental period through a bespoke pipe installed in the WW plots. Water was applied from the installation of the rain-shelter until complete maturity (i.e. before harvest). Water was applied every two days to avoid soil moisture deficit to the WW plots.

In Experiment II, one duralumin tube was placed in a WW and one in a WS plot (regardless of antitranspirant application) randomly selected for each rain-shelter (n=3 for WS and WW). Soil moisture readings and calculations for VWC were done as for Experiment I and for each tube on the 19/01/2016, 21/01/2016, 26/02/2016, 24/03/2016, 26/04/2016, 23/05/2016, and 21/06/2016. Irrigation was applied to the WW-rain shelter plots by installing bespoke irrigation tapes to each WW plot. Tapes had 1 mm diameter emitters (two for each set) positioned 10 cm apart from each other and ensuring ~200 mm H_2O m^{-2} h^{-1}.

Stomatal conductance and gas-exchange

In both the experiments, leaf stomatal conductance to water vapour (*gs*) was collected over the experiment using a transient state diffusion porometer (AP4, Delta-T Devices, Cambridge, UK). The device was calibrated before every use with the calibration plate provided. Measurements of the abaxial *gs* and adaxial *gs* surface were collected from three fully expanded leaves at the top of the canopy per plant randomly selected in the three (Experiment I) and the six (Experiment II) plots and averaged. Total *gs* was then calculated as adaxial *gs* + abaxial *gs* (n=4 for Experiment I and n=6 for Experiment II). Data were collected between 9:30 and noon. Pod *gs* was analysed with the same porometer on main stem pods positioned at mid-distance between the first internode and the plant tip (n=4 for Experiment I and n=6 for Experiment II).

In Experiment I, the light-saturated CO_2 assimilation (A_{max}, µmol CO_2 m^{-2} s^{-1}) and the leaf transpiration rate (E, mmol H_2O m^{-2} s^{-1}) were measured on the first fully expanded leaf of the top canopy (n=4) using a CIRAS portable photosynthesis system (PP system, MA, USA) with a 2.5 cm^2 cuvette ensuring a saturating 1200 µmol m^{-2} s^{-1} PAR; all the data were recorded after 3–4 min at 380 ppm CO_2 level, when steady-state photosynthesis was achieved. The leaf water-use efficiency (WUE) was then calculated as A_{max}/E (n=6).

Chlorophyll fluorescence

A FluorPen 100 MAX (PSI, Czech Republic) was used to evaluate dark-adapted chlorophyll fluorescence parameters. From 9:00 to 16:00 and the same days of the stomatal conductance analysis, the tagged first fully expanded leaf of the top canopy was used for a 30 minutes dark-adaptation provided by leaf clips in Experiment I and Experiment II. OJIP test was performed and the maximum quantum yield of photosystem II in dark adapted state ($F_v/F_m = [F_m - F_o / F_m]$), the oxygen evolving complex activity ($F_v/F_o = [F_m - F_o / F_o]$) and the pool size of plastoquinone on the reducing side of the photosystem II (Area = the area above F_o and F_m).

Leaf and pod water potential

Plants were used for leaf water potential (LWP, over GS 6.0; DAS 1, DAS 4 and DAS 6) and flower/pod water potential (PWP, over GS 7.0; DAS 2 and DAS 12) analysis in Experiment I. Between 11:00 and 14:00, leaves or pods were excised with a scalpel from five plants for each treatment (n=5) and water potential was immediately analysed by a Scholander pressure chamber (SKPM 1405/50, Skye Instruments Ltd, UK). The tissues were analysed on the cut end of the petiole 1 cm from the base (leaf or flower/pod). The water potential value (MPa) was collected when water was exuding from the cut surface, seen by using a magnifying lens.

Yield assessments

At maturity (the 1st of July 2015 for Experiment I and the 19th of July for Experiment II) plots were harvested with a plot-combine harvester (Wintersteiger Nursery Master, Germany) (in total 7.5 m^2 area harvested for each plot in Experiment I and 6 m^2 in Experiment II) and the seeds for each plot were collected and stored. Seed moisture was collected daily with a moisture meter and seed were weighed by balance. Yield (t ha^{-1}) was then calculated by adjusting the area of the plot to a hectare and by adjusting the seed weight to 9% moisture. Then, 1000-seed weight (TSW) was calculated by using a 100-seed plate and a balance and seed per m^2 was calculated as the total plot seed number divided by the area of the plots.

Statistical analyses and data presentation

Temperature and rainfall for Experiment I and II are presented as monthly averages calculated from daily data collected at a weather station approximately

650 metres from the field site. The volumetric water content (VWC) of each experiment is shown as plot means. Since in Experiment I no statistically significant differences were recorded between droughted antitranspirant sprayed and un-sprayed plots, all the data from droughted plots (+ or - antitranspirant) were pooled and presented as “un-irrigated” as means. Stomatal conductance, gas-exchange and water potential data were subjected to one-way ANOVA for each day of data collection and means were separated by using a Tukey’s test ($P<0.05$). Yield data were subjected to one-way ANOVA and means were separated by using a Tukey’s test ($P<0.05$). Yield data were then subject to contrast analysis to evaluate additional statistical differences between treatment combinations. In Experiment I, plots were subjected to a significant lodging in two of the rain-shelters. Thus the lodging was scored as % of the total plots area and used as covariate in the ANOVA model. Although the covariate was significant, the residual from the ANOVA of each single plot, was plotted against the relative lodging score. The correlation analysis shows that there is a modest but significant ($P=0.024$ according to regression analysis) and negative correlation ($r= - 0.46$) between the two variables. Therefore, the covariate was used in the model for the statistical analysis. For Experiment I, data from GS 5.0 are not presented since the soil moisture deficit applied at the time of the antitranspirants application was very similar to the irrigated one (no soil moisture deficit) and therefore, the data cannot be considered solid, requiring further investigation. Yield data from Experiment I and II of un-irrigated un-sprayed, 1 L ha^{-1} VG GS 6.0 and 1 L ha^{-1} VG GS 7.0 were pooled and a *t*-test was used to test the differences over two years in seed yield. Since in Experiment II block 1 was significantly damaged by pigeons and block 6 was subjected to edge effects, only block 2, 3, 4 and 5 were used for the *t*-test

(therefore, *n*=7). All the statistical analyses were performed by using GenStat (17th edition, VSN International Ltd, UK)

Results

Weather, soil and VWC

The monthly weather data for Experiment I (2014-2015) and Experiment II (2015-2016) are shown in Table 1.

Table 1. Monthly mean temperature and total rainfall for Experiment I (2014-2015 growing season) and Experiment II (2015-2016 growing season)

	2014-2015		**2015-2016**	
Month	Mean temperature (°C)	Mean rainfall (mm day^{-1})	Mean temperature (°C)	Mean rainfall (mm day^{-1})
August	15.01	2.72	15.92	3.10
September	14.75	0.60	12.84	1.73
October	12.20	2.00	10.81	1.25
November	8.65	2.80	10.10	2.23
December	5.01	2.08	9.99	2.77
January	4.72	1.60	5.87	3.41
February	4.07	1.38	5.25	1.03
March	6.44	1.90	5.49	2.30
April	9.32	0.75	7.69	2.62
May	11.10	1.88	12.10	1.87
June	14.12	1.71	15.65	2.55
July	16.02	1.81	15.68	2.86

Analysis of the soil texture showed that Flat Nook soil is typically a sandy loam soil according to Toogood (1958). At a soil depth of 20 cm the percentage of sand was 75.8% with a 20.8% of silt and 3.4% of clay and a bulk density of 1.74 g/cm^3. At 40 cm depth the percentage of sand increased compared to the 20 cm depth to 78.9% and decreasing to 71.2% and 72.1% for 60 and 80 cm depth respectively. Silt percentage remained relatively stable at ~20% whereas clay concentration increased to 6.4 and 5.4% at 60 and 80 cm depth respectively. Bulk density steadily increased to 1.76, 1.78 and 1.84 g/cm^3 at 40, 60 and 80 cm depth respectively.

In both Experiment I and II, well-watered plots grown under rain-shelters exhibited similar VWC values that fluctuated between 40-45% for 20 and 40 cm depth and 30-35% for 60 and 80 cm depth (Figure 1). Rain-shelter and un-irrigated plots exhibited a steep decrease in VWC during both Experiments I and II. When compared to the irrigated plots, un-irrigated plots showed an average (20, 40, 60 and 80 cm depth) decrease in VWC from an initial 40% to 38%, 28% and 21% at GS 5.0, 6.0 and 7.0 respectively in Experiment I. In contrast, in Experiment II it was from an initial 43% to 30% and 24% on average at GS 6.0 and 7.0.

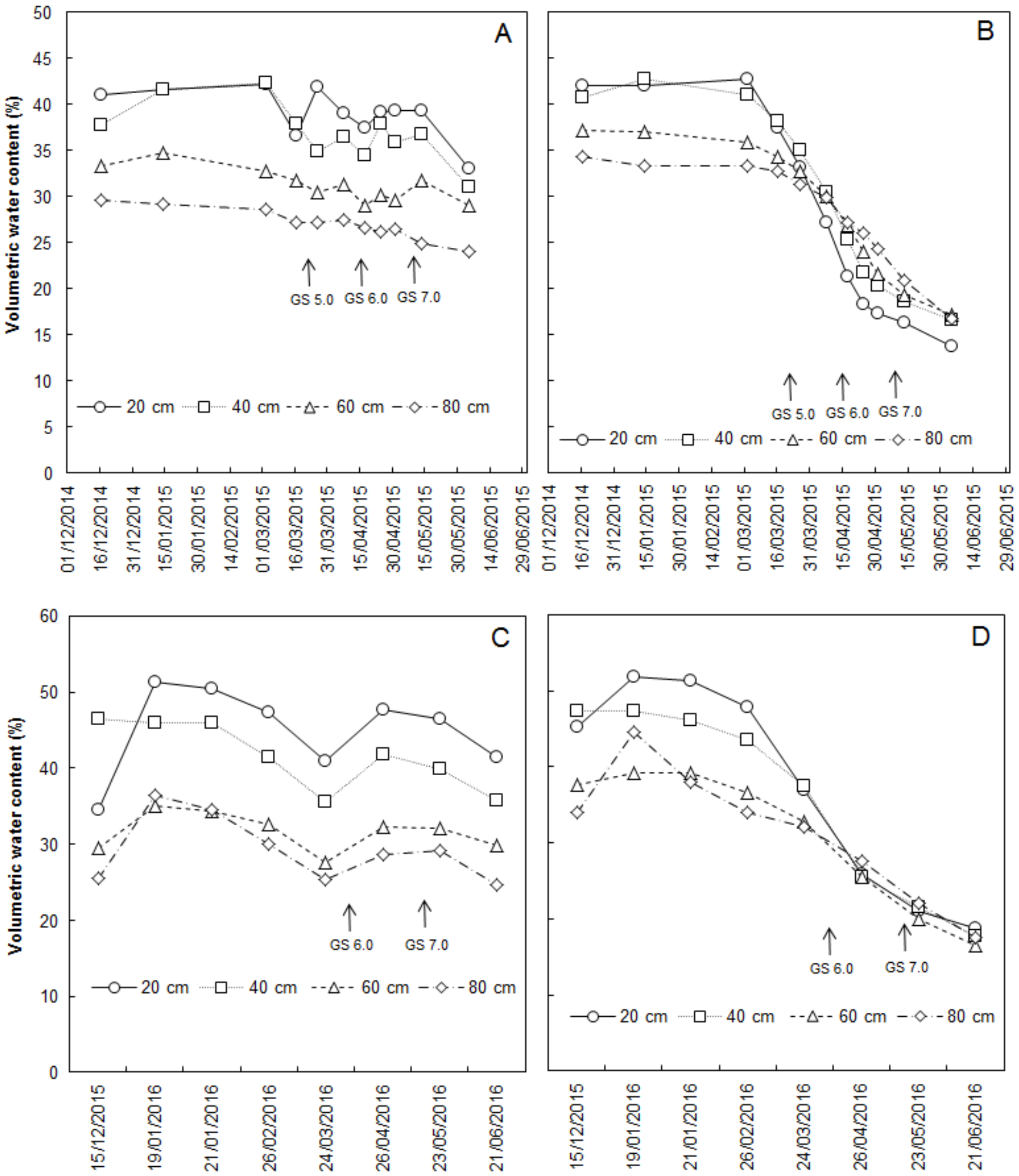

Figure1. Volumetric water content (VWC, %) for Experiment I (A, irrigated plots, B un-irrigated plots) and Experiment II (C, irrigated plots, D un-irrigated plots) collected with the neutron probe at 20, 40, 60 and 80 cm depth. Arrows represent the growth stages at which chemicals were applied. Data are means (n=3 for A and B and n=21 for B; in C, all the means are n=3 except for 80 cm depth where n=2)

Stomatal conductance, gas-exchange and chlorophyll fluorescence over GS 6.0

In both the experiments, Total *gs* of WW plots over GS 6.0 fluctuated from ~1200 to 500 mmol m^{-2} s^{-1}. Over GS 6.0 WS plots exhibited a decrease in Total *gs* at all the DAS when compare to the WW plots (Figure 2). Compared to the WW un-sprayed plots, the WS un-sprayed exhibited a lower Total *gs* by ~50% in Experiment I and by ~25% in Experiment II ($P<0.001$ for both and for all the DAS). Indeed at all the DAS WS significantly decreased abaxial and adaxial *gs* with the latter showing a smaller reduction. At the same time, gas-exchange analysis in Experiment I showed that WS plots exhibited a lower capacity at assimilating CO_2 compared to the WW plots ($P<0.001$ for all the DAS) leading to higher leaf WUE values when compared to the WW plots.

In Experiment I, application of NFP significantly reduced adaxial *gs* on DAS 3 and DAS 6 without affecting abaxial *gs* compared to the WS un-sprayed. However, no significant differences were found in Total *gs* and CO_2 assimilation rate when compared to the WS plots. Application of NFP decreased the transpiration rate compared to the droughted un-sprayed plots by 13% leading to slightly higher leaf WUE values.

In both the Experiment, VG (1 L ha^{-1} dose rate) significantly reduced adaxial *gs* throughout GS 6.0 compared to the WS un-sprayed plots ($P<0.001$). However a small increase, despite not being significant, was found in the abaxial surface values compared to the WS un-sprayed on DAS 6 and DAS 16. Total *gs* was significantly reduced by VG treatment on most of the DAS. When the experiments showed low conductance values (i.e. DAS 10 and 12 of Experiment I and DAS 6 of Experiment II) the effect was not significant. Steady lower total *gs* values compared to the WS un-sprayed were recorded even at DAS 18 and DAS 20. In Experiment II, higher VG dose rate (2 and 4 L ha^{-1}) did not show any additional *gs* reduction when compared to the 1L/ha. VG application in Experiment I did not affect CO_2 assimilation showing similar trends to the WS un-sprayed plots but it was accompanied by an overall 15% reduction in transpiration rate leading to significantly higher WUE values (Figure 2H) when compared to the WS plots. For all the Experiments and treatments, no differences were found between chlorophyll fluorescence traits.

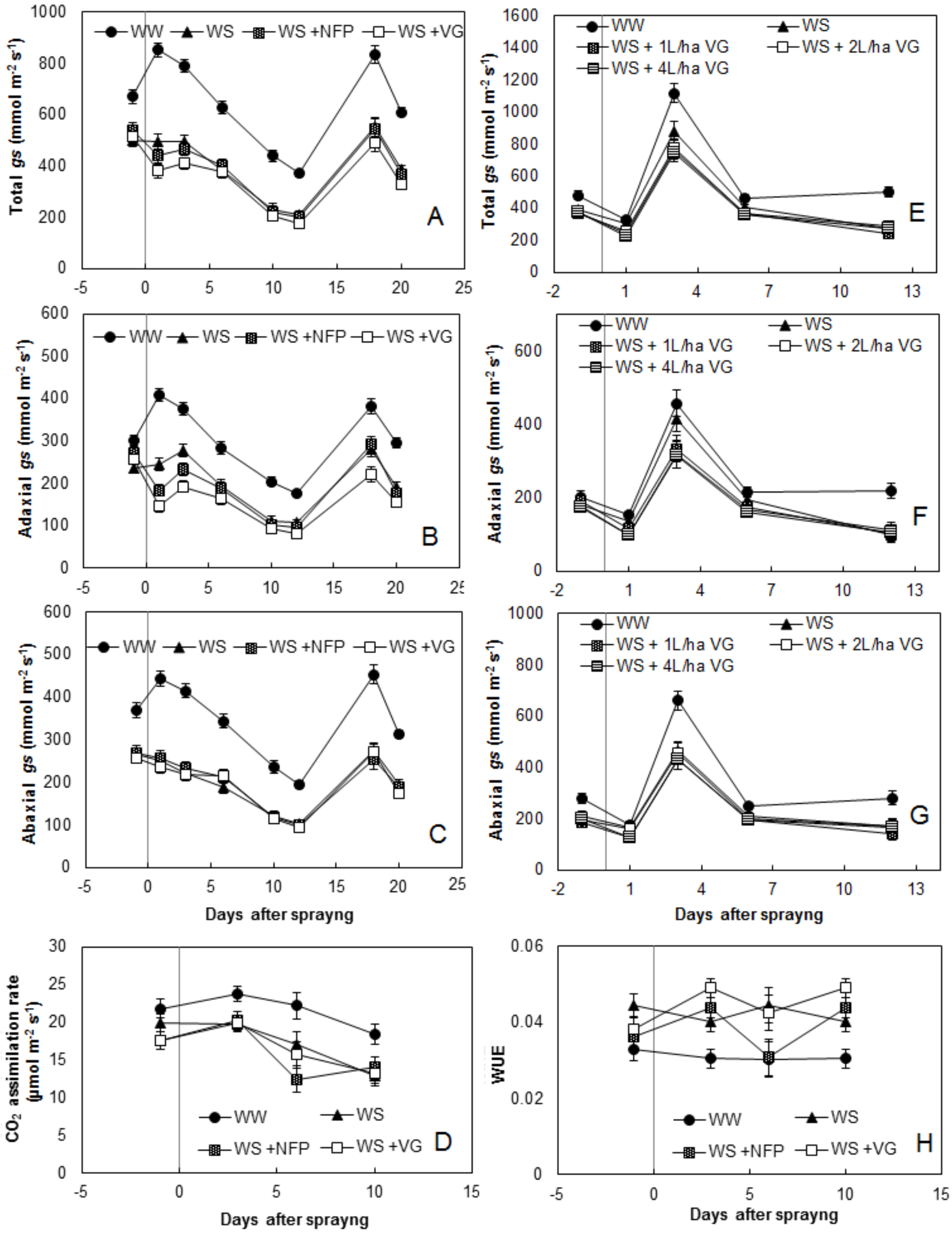

Figure 2. Total, adaxial and abaxial leaf stomatal conductance (*gs*) for oilseed rape plots over GS 6.0 of Experiment I (A, B and C) and Experiment II (E, F and G). For Experiment I data are means (n=4) ± SE collected in irrigated (WW), un-irrigated (WS), un-irrigated treated with 1 L ha^{-1} Nu Film P (WS+NFP) and un-irrigated treated with 1 L ha^{-1} Vapor Gard (WS+VG). In Experiment II data are means (n=6) ± SE collected in irrigated (WW), un-irrigated (WS), un-irrigated treated with 1 L ha^{-1} Vapor Gard (WS+ 1L/ha VG), un-irrigated treated with 2 L ha^{-1} Vapor Gard (WS+ 2L/ha VG) and un-irrigated treated with 4 L ha^{-1} Vapor Gard (WS+ 4L/ha VG). CO_2 assimilation rate (D) and leaf water-use efficiency (H, WUE, µmol mol^{-1}) calculated as the ratio between CO_2 assimilation rate and transpiration for oilseed rape plots over GS 6.0. Data are means (n=4) ± SE and collected in Experiment I.

Stomatal conductance over GS 7.0

In WW plots and over the two experiments, the pod *gs* was between ~120 and ~150 mmol m^{-2} s^{-1} on average whereas adaxial *gs* fluctuated between ~150 mmol m^{-2} s^{-1} and ~200 mmol m^{-2} s^{-1} in Experiment I and II respectively (Figure 3). In WS plots, the average pod *gs* was ~70 and 100 mmol m^{-2} s^{-1} in Experiment I and II respectively, that was ~40% less of the WW plots. Similarly, the adaxial *gs* of the WS plots was ~45% lower than that of the WW plots.

In Experiment I, NFP application did not have a significant effect on pod *gs*. In contrast a slight reduction of adaxial *gs* was recorded on DAS 1 that however was not statistically significant.

Application of VG at 1 L ha^{-1} had a strong and significant effect at reducing pod *gs* in Experiment I, whereas no significant differences were recorded in Experiment II. Similarly, 1 L ha^{-1} VG decreased adaxial *gs* on DAS 1, 4 and 6 in Experiment I whereas in Experiment II no statistical significant differences were recorded. Increasing dose rate (i.e. 2 and 4 L ha^{-1}) had a negligible effect at reducing both adaxial and pod *gs* in Experiment II, despite pod *gs* being significantly lower than that of the WS un-sprayed plots on DAS 1, 4 and 6.

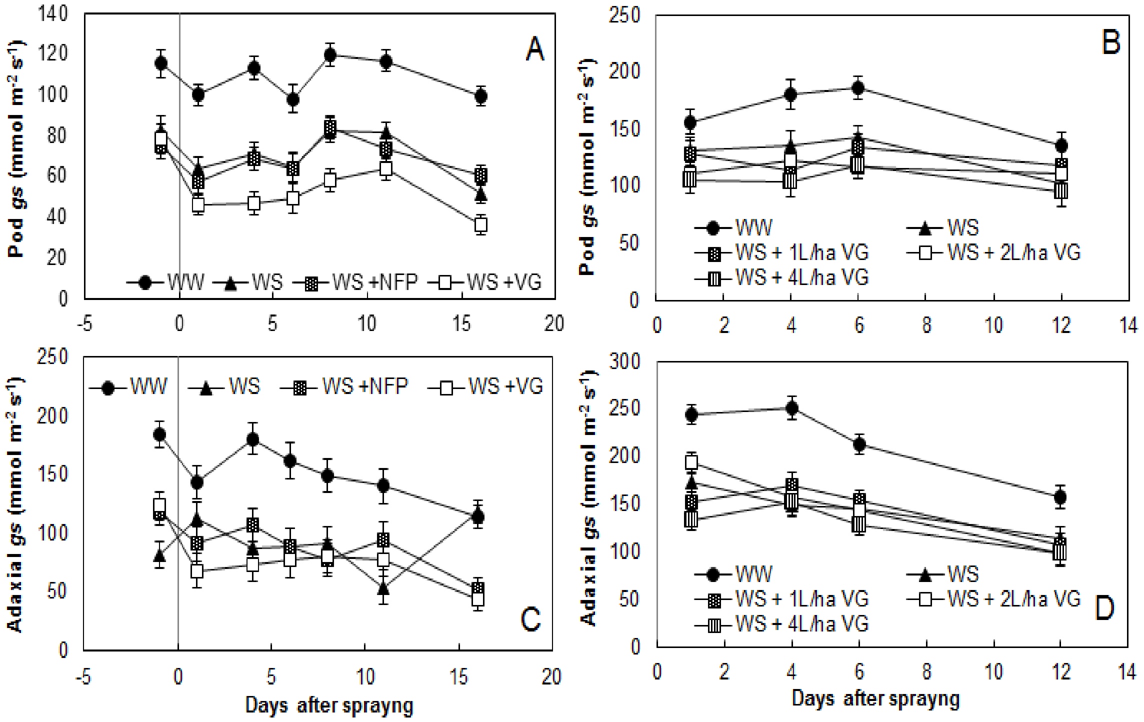

Figure 3. Pod and leaf adaxial stomatal conductance (*gs*) for oilseed rape plots over GS 7.0 of Experiment I (A and C) and Experiment II (B and D). For Experiment I data are means (n=4) ± SE collected in irrigated (WW), un-irrigated (WS), un-irrigated treated with 1 L ha^{-1} Nu Film P (WS+NFP) and un-irrigated treated with 1 L ha^{-1} Vapor Gard (WS+VG). In Experiment II data are means (n=6) ± SE collected in irrigated (WW), un-irrigated (WS), un-irrigated treated with 1 L ha^{-1} Vapor Gard (WS+ 1L/ha VG), un-irrigated treated with 2 L ha^{-1} Vapor Gard (WS+ 2L/ha VG) and un-irrigated treated with 4 L ha^{-1} Vapor Gard (WS+ 4L/ha VG).

Leaf and pod water potential

Leaf water potential of WW plots was between -1 and -1.2 MPa whereas the PWP in WW plants was slightly less negative (~ -0.9 on average) (Figure 4). Drought had an effect on both LWP and PWP leading to lower values by ca. 2-fold on average respectively. While no differences in LWP and PWP were found between NFP sprayed and un-sprayed plots, statistically significant less negative values were found in VG-sprayed plots by 33% and 25% respectively averaged over all the dates when compared to WS plots.

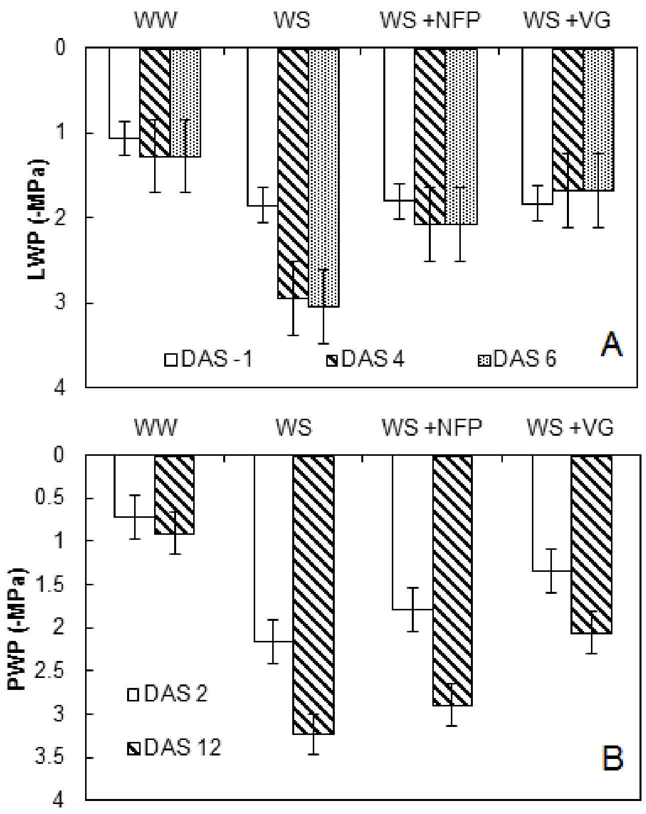

Figure 4. Leaf water potential (LWP, A) and pod water potential (PWP, B) for oilseed rape plots over GS 6.0 and GS 7.0 respectively. Data are means (n=5) ± SE collected in irrigated (WW), un-irrigated (WS), un-irrigated treated with 1 L ha^{-1} Nu Film P (WS+NFP) and un-irrigated treated with 1 L ha^{-1} Vapor Gard (WS+VG). DAS represents days after spray application. Data from Experiment I.

Yield and yield components analysis

In Experiment I watered un-sprayed plots showed an average seed yield of 3.56 t ha^{-1} (Figure 5). Water deprivation decreased the seed yield and seed m^{2} yield component by 43% compared to the watered plots leading to an average seed yield of 2.01 t ha^{-1}. NFP sprayed at GS 6.0 and GS 7.0 onto droughted oilseed rape increased the seed yield compared to the droughted un-sprayed plots leading to 2.87 and 2.42 t ha^{-1} seed yield respectively. In particular, NFP application at GS 6.0 increased seed m^{2} yield component by 27% when compared to the droughted un-sprayed plots. With respect to the droughted un-sprayed plots, VG-treated plots at GS 6.0 and GS 7.0, showed an increase in seed yield leading to 2.49 and 2.26 t

ha^{-1} accompanied in GS 6.0 by a 25% seed m^2 yield component increase. When lodging is added in the ANOVA model as covariate, it has a significant effect in seed yield ($P=0.016$) and seed m^2 ($P=0.015$) but not in TSW ($P=0.681$). Collectively, the covariate has a big effect on the yield values that fluctuated between -20% and +25% when compared to the ANOVA model. When compared to the ANOVA, the ANCOVA model increased seed yield by 16% and 23% in irrigated un-sprayed and droughted +VG 6.0 plots respectively whereas decreased seed yield by 23%, 9%, 3% and 14% in droughted un-sprayed, +NFP 6.0, +NFP 7.0, +VG 7.0 plots respectively. In contrast seed m^2 was increased by 15% and 19% in irrigated un-sprayed and droughted +VG 6.0 plots respectively and decreased by 22%, 6%, 3% and 16% in droughted un-sprayed, + NFP 6.0, +NFP 7.0, +VG 7.0 plots respectively.

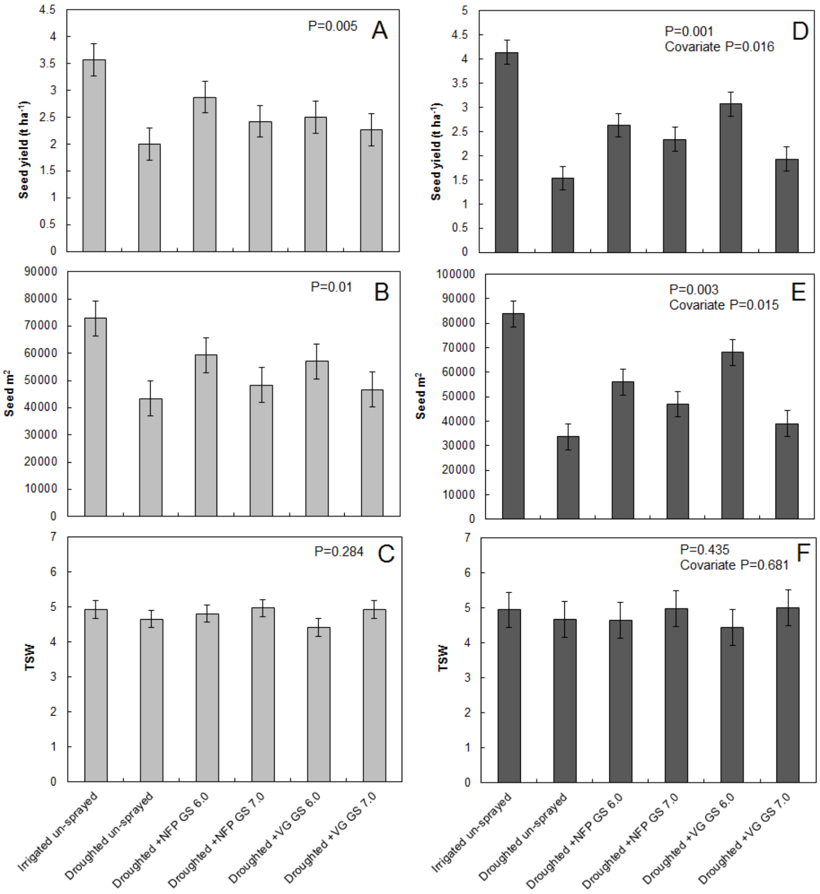

Figure 5. Seed yield (t ha^{-1}, A and D), seed per m^2 (B and E) and thousand-seed weight (TSW, C and F) of oilseed rape plots grown under irrigated and un-irrigated (droughted) conditions and sprayed at flowering (GS 6.0) or pod development (GS 7.0) stages with 1 L ha^{-1} of Nu-Film P (NFP) or Vapor Gard (VG). In A, B and C data were analysed with ANOVA while in D, E and F data were analysed with ANCOVA (lodging score as covariate). Data are means (n=3) ± standard error of the differences of the means (SED). Data from Experiment I

In Experiment II watered un-sprayed plots showed an average seed yield of 4.22 t ha^{-1} and a seed m^{-2} of 85,000 (Figure 5). Water deprivation decreased the seed yield and seed m^2 yield component by 33% compared to the watered plots leading to an average seed yield of 2.85 t ha^{-1}. TSW was not affected by water deprivation leading to similar values (~4.92 g). VG applied over GS 6.0, despite not being significant, increase seed yield by 14%, 14% and 23% at 1, 2 and 4 L ha^{-1} respectively when compared to the un-irrigated un-sprayed plots. In contrast and when compared to the un-irrigated un-sprayed plots, the VG application over GS 7.0, although not significant, increased seed yield by 12% and 14% when sprayed at 1 and 2 L ha^{-1} whereas a 7% decrease was recorded at 4 L ha^{-1} application. Since TSW was never affected by both watering regimes and VG, the seed yield variation was governed only by a similar reduction/increase in seed m^{-2}.

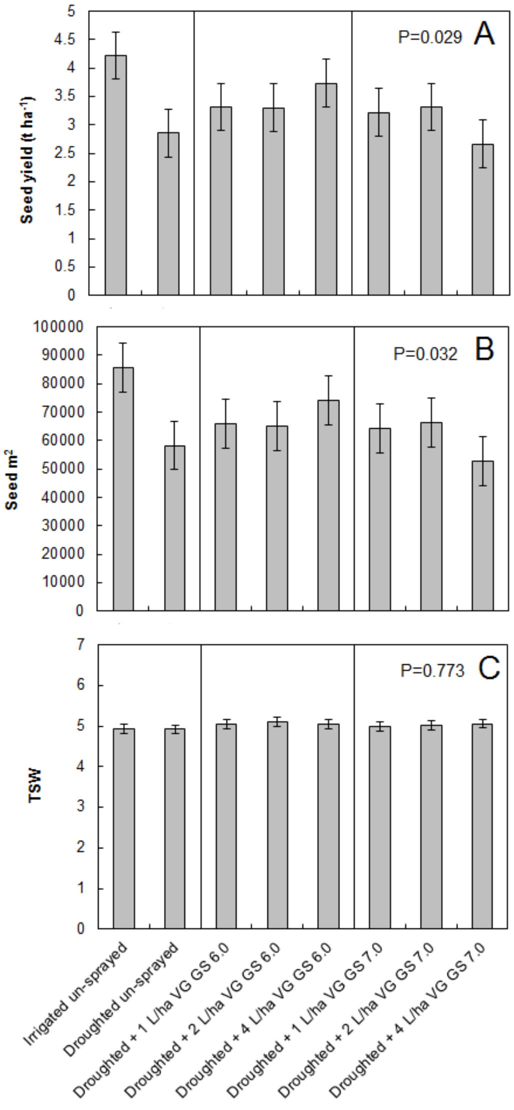

Figure 6. Seed yield (t ha^{-1}, A), seed per m^{2} (B) and thousand-seed weight (TSW, C) of oilseed rape plots grown under irrigated and un-irrigated (droughted) conditions and sprayed at flowering (GS 6.0) or pod development (GS 7.0) stages with 1 L ha^{-1}, 2 L ha^{-1} and 4 L ha^{-1} of Vapor Gard (VG). Data were analysed with ANOVA. Data are means (n=5) ± standard error of the differences of the means (SED). Data from Experiment II

On average the two field experiments showed that un-irrigated plots have an average decrease in seed yield by 40% (Fig. 7). Application of 1 L ha^{-1} VG just prior to GS 6.0 did have a significant effect at sustaining the yield of un-irrigated OSR plots by 0.71 t ha^{-1} on average when compared to un-sprayed plots. On the contrary, the effect of 1 L ha^{-1} VG application just prior to GS 7.0 was not significant.

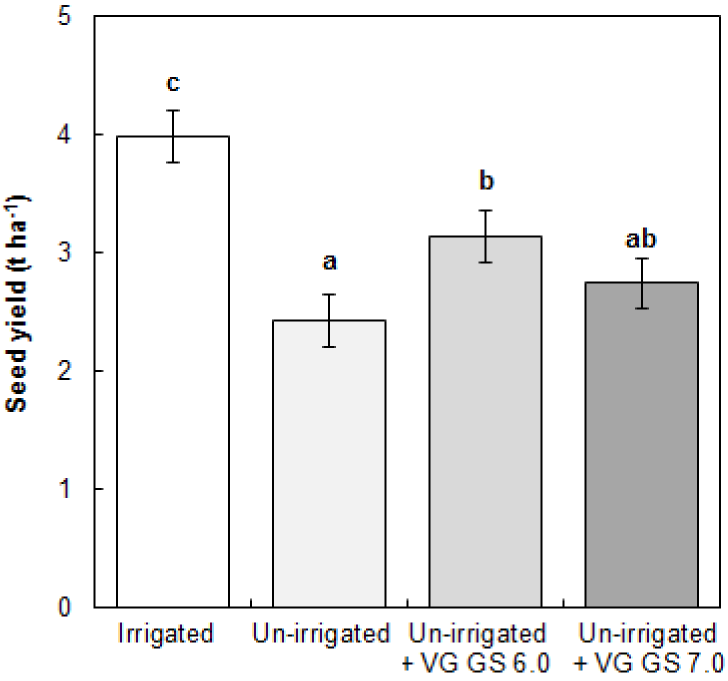

Figure 7. Pooled seed yield (t ha^{-1}) data for Experiment I and Experiment II of oilseed rape plots subjected to irrigation, reduced water availability through rain-shelters and treated with 1 L ha^{-1} Vapor Gard (+VG) or not (-VG) just prior to flowering (GS 6.0) or pod development (GS 7.0). Data are means (*n*=7) and error bars represent standard error of the differences of the means according to the ANOVA (P<0.001). Different letters represent significant differences according to the Tukey's test (P<0.05).

Discussion

The effect of water deficit on field-grown oilseed rape at GS 6.0 and GS 7.0

In our experiments the crop was grown under rain-shelters (built at the end of the winter) to decrease the soil moisture and therefore artificially induce water stress to the crop. As in Weerasinghe et al. (2016), an average of 2-3°C differences in temperature between the inside and the outside of the rain-shelter were recorded in days with high temperatures and elevated light irradiance. However, since in this work only plots grown under rain-shelters are compared, the temperature differences are irrelevant.

Data of *gs* from Experiment I and II and water potential analysis from Experiment I showed that, at the dates of AT application, the un-irrigated plots were significantly stressed. In addition, soil moisture data showed significant decreases in VWC in both top and sub-soil that match with the *gs* reduction of un-irrigated plots. Since the rain-shelters were built at the end of winter for both the years, the VWC reduction was much larger at GS 7.0 antitranspirant application than GS 6.0. In Experiment II, the VWC of the un-irrigated plots was higher than that of Experiment I at GS 6.0 and GS 7.0. This was due to the significantly lower temperatures of March and April 2016 that led to lower evaporative demand and thus a possible lower total evapotranspiration. At the same time and in both the experiments, the irrigated plots showed constant VWC at all the soil depths that were very similar to the winter values. This suggests that on irrigated plots, plants had access to high water availability throughout the experimental period. On the other hand, the VWC recorded in this work is high for a sandy loam soil. Indeed the VWC for a sandy loam top-soil would be expected to be in the range of 31% ± 8.6 (SD) (Hall et al. 1977). However, bulk density and organic matter variations would explain some of this variation as they are both known to influence VWC (Hall et al. 1977).

In both the experiments, total *gs* of un-irrigated plots was significantly lower than that of the irrigated one. Despite that, in Experiment II the reduction was less evident throughout the GS 6.0 stage. Our data showed that stomatal closure occurred at field scale when water availability decreased, but the reduction was much lower than for an artificial drought stress imposed in pots (Faralli et al. 2016). Similarly, lower CO_2 assimilation capacity was found in un-irrigated plots when compared to the irrigated one and this may be accompanied by lower assimilate production over the flowering stage. However, the non-significant differences in chlorophyll fluorescence traits between un-irrigated and irrigated plots suggests that photosynthetic down-regulation is only stomatal-driven (at least at the soil moisture deficit applied in this work) and drought does not directly affect photochemistry possibly due to the resilience of the PSII to water deprivation (as already reported by Muller et al. 2010). To confirm this, leaf WUE was increased in un-irrigated plots with respect to the irrigated one (Figure 2H) therefore showing a water-stress induced water-saving strategy triggered by stomatal closure. Similarly, in Jensen et al. (1996), oilseed rape plots grown in a sandy soil and stressed over reproductive stages showed gas-exchange and water potential reductions that are matching with our data. Indeed, in our experiments drought affected water potential data, leading to more negative values in un-irrigated plots. Altogether, the data showed overall significant detrimental effects on field-grown OSR at a physiological level, that were clearly less prominent when compared to glasshouse work (e.g. Faralli et al. 2016; Champoliver and Merrien, 1996) but consistent with other field reports (e.g Jensen et al. 1996; Morgensen et al. 1997; Istanbulloglu et al. 2010).

In both the Experiments, un-irrigated plots showed a significant decrease in seed yield when compared to the irrigated one. Mainly, the reduction was due to a significant decrease in seed m^2, in accordance with many other reports (Berry and Spink, 1996; Berry and Spink, 2009) where seed m^2 is a main target to increase OSR yield. On the contrary no significant differences were found in TSW plots in contradiction with other reports showing significant TSW compensation under drought (e.g. Champolivier and Merrien, 1996). However, since in our experiments un-irrigated plots did not received supplementary watering until harvest, is possible that the TSW compensation was significantly reduced due to the prolonged stress conditions. In this work, we confirm that soil moisture deficit during the OSR reproductive period is a key factor for seed number determination and therefore further efforts should focus at improving OSR resilience to drought focusing on reproductive physiology, a field that has not been particularly studied in OSR.

The effect of film antitranspirant on oilseed rape at GS 6.0 and GS 7.0

Our data on OSR physiology show that AT application at 1 L ha^{-1} decreased *gs* and did not affect CO_2 assimilation. One major problem related to the use of AT was that often the reduction in water loss was accompanied by a reduction in CO_2 assimilation (Solarova et al. 1981). However it has been shown that the increasing atmospheric CO_2 may counteract the reduction in CO_2 uptake (del Amor et al. 2010). Moreover, the recent literature shows an increasing amount of successful work using biotechnological approaches that focus on triggering water-saving strategies in crops leading to ameliorative physiological responses under drought (especially OSR and Arabidopsis; e.g. Wang et al. 2005 and 2009 and Yang et al. 2016) thus confirming the importance of water-saving strategies and their success to improve crops resilience to water deficit especially in conditions (e.g. the present atmospheric CO_2 concentration ~404 ppm) where Rubisco is less limited when compared to the past (e.g. 1960 with an atmospheric CO_2 concentration of ~300 ppm).

Collectively, the data over GS 6.0 suggest that VG had a major effect on seed m^{-2} and therefore it is possible to hypothesize that the higher plant water status during GS 6.0 following AT application significantly sustained seed set (as already reported on wheat by Weerasinghe et al. 2016). At the same time, linear regression showed no significant effect of the dose rate (P=0.12), suggesting that no yield benefit can be achieved by increasing VG rate at the magnitude of stress applied on this work. On the contrary, no statistically significant effects were recorded when antitranspirants were applied at GS 7.0. One reason of this could be the fact that at GS 7.0 the artificial soil moisture deficit applied with rain shelter was much stronger than that applied at GS 6.0 and therefore is possible to speculate that VG is not efficient when a strong drought-induced stomatal closure is triggered. In addition the dose response experiment, showed slight (not significant) decreases in seed yield at 4 L ha^{-1} when compared to the un-treated un-irrigated plots. To confirm that, linear regression showed non-significant effects of the dose response (P=0.85) and similarly, non-linear models (e.g. double exponential, exponential, quadratic and Gaussian models, data not shown) were not significant. Since previous work showed that application of VG on both stressed and un-stressed plants significantly reduced ABA concentrations in both leaf and reproductive organs (Iriti et al. 2009; Faralli et al. 2016), it is possible that the different yield response to VG over GS 6.0 and GS 7.0 could be due to the sensitivity to ABA of the two phenological stages. Indeed, while ABA has been reported to be involved in early reproductive failure on wheat (Westgate et al. 1996) and soybean (Liu et al. 2004) the accumulation of ABA in wheat spikelets during the grain filling stage is considered a desirable trait (Foulkes et al. 2011). This is because ABA counteracted the detrimental effect of ACC (thus ethylene) on grain filling thus leading to higher seed weight and lower seed abortion under stress. Despite no work has been done on OSR regarding the effect of ABA/ACC ratio during pod development/seed filling stage, we can hypothesize that VG application over GS 7.0 decreased ABA concentration in pods and seeds and therefore reduced the beneficial effects of ABA during the seed filling stage. Indeed, in de Bouille et al. (1986), ABA accumulated in OSR seeds during the late stage of pod development/ initiation of seed filling, suggesting that, as for other crops, ABA may possibly modulate assimilate flux to seeds and thus induce seed maturation.

Application of film antitranspirant has been previously used in a broad range of crops to mitigate drought induced yield losses (e.g on sorghum in Fuehring, 1975) and recently on field-grown wheat (Weerasinghe et al. 2016) and pot-grown oilseed rape (Faralli et al. 2016). There is only one work available testing the efficiency of different AT to avoid yield losses on a crop belonging to the same OSR family (*Brassica campestris*) (Patil and De, 1978). Mobileaf (the film forming chemical), increased seed yield irrespective of the N supply in both years with an average of 0.41 t ha^{-1} following an increase in plant water status. In these experiments the control un-irrigated and un-treated showed a lower seed yield than in our work on average (1.60 t ha^{-1}). The lower seed yield found by Patil and De (1978) when compared to our work may be for two reasons. First, the crop was a spring variety, and it is well known that spring varieties generally exhibit lower yield than the winter crop. Second, the crop was grown under dry-land conditions with high temperature (~25°C whilst in the present work the average spring temperature was ~12°C) in both years and low precipitation. Patil and De’s (1978) results suggest that the film-forming water-saving properties may be beneficial even in situations where heat and water stress occur in combination. Therefore, further investigations on the efficiency of AT should be done under different environmental conditions and on a broader range of crops to better define their use and potentials.

Conclusions

This work suggests a potential useful application of the antitranspirant VG when applied at 1 L ha^{-1} just prior to GS 6.0 on OSR subjected to reduced water availability to sustain grain yield. Consistent with previous glasshouse work, physiological data gave evidence collectively of an improvement in plant water status. This followed a reduction in stomatal conductance that did not significantly affect the carbon assimilation. Further investigations in different disadvantageous environments are required to exploit the potential applications of AT.

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Chapter 6

General Conclusions

Oilseed rape's response to transient drought periods over reproduction.

Although there is an urgent need to develop high-yielding and abiotic stress tolerant oilseed rape varieties (as pointed out at the International Rapeseed Congress, held in Saskatoon (Canada) on July 2015) the mechanisms underlying the physiological basis of OSR to water deprivation are still not well elucidated. Indeed, when the literature focusing on OSR and drought stress is compared to other major food crop such as maize, wheat, rice and soybean the information is sparse.

This study results in a substantially large amount of data related to the physiological performances of OSR under water deprivation, elucidating a few mechanisms that may be a starting point for further investigation. Collectively, the data of the present study show that:

- Consistent with a few other reports (e.g. Jensen et al. 1996) OSR shows isohydric or pessimistic response to drought with a fast stomatal closure (Chapter 2, 3 and 4).
- Photosynthetic downregulation following stomatal closure under stress occurs even at mild water deficit, and it may disrupt carbohydrate production and allocation leading to yield losses. Despite not being measured in the work presented in this thesis, the fact that OSR has a low osmotic adjustment potential (as evidenced by Jensen et al. 1996) is an indication of the low capability of the crop at maintaining turgor in water limited conditions and thus low capacity at avoiding photosynthetic inhibition under drought (Chapter 2, 3 and 4).

- Both leaf and reproductive organs are sensitive to water deprivation and similar responses (water status, ABA accumulation) occur during stress (Chapter 2 and 3)
- Bud temperature, as reported previously in *Brassica rapa* (Guo et al. 103 and 2015), may be an efficient and not time-consuming technique to detect stress in OSR over reproduction and screen potential genotypes for reproductive stage drought tolerance (Chapter 3)
- ABA accumulated in all the tissues analysed following drought treatments. To our knowledge, this work is the first showing ABA accumulation in different reproductive organs and different developmental periods (Chapter 2 and 3). Further elucidation of the role of ABA with reproductive efficiency under drought and potential cross-talk with other hormones (e.g. ACC) are required for different phenological stages and organs.
- Although the data were recorded at vegetative stages, CO_2 may have had an impact at increasing OSR yield potential in recent years. It is not clear whether increasing photosynthetic capacity can increase sink strength, thus further investigations should i) elucidate the role of the elevated atmospheric CO_2 on OSR yield ii) evaluate the capacity of elevated atmospheric CO_2 at increasing both source and sink activity and iii) better explore the relations between elevated atmospheric CO_2 and drought tolerance on OSR (Chapter 4)
- Over the key period of early flowering until pod developmental stage, seed number is the main yield component affected by drought leading to substantial decreases in seed dry matter production. Similarly, pod number decreases at increasing drought intensities (Chapter 2, 3 and 5)

In this context, unlike wheat (e.g. Ji et al. 2010), in OSR there is no data available regarding reproductive stage drought tolerance germplasm variability. Indeed further investigations are required in this direction to screen drought tolerant genotypes and understand the mechanisms underlying the reproductive stage resilience to water deprivation. Our data can be therefore considered a starting point for selection of tolerant genotypes following the above evidence.

The role of film antitranspirants at minimising drought damage: strength and weakness.

Film antitranspirants have been considered one of the most promising tools to preserve water in plants and thus improve crops' drought tolerance. This is supported by the enormous amount of literature available focusing on their potential use from ~1950 to ~1970.

In the present work the efficiency of a commercial available antitranspirant (Vapor Gard) at counteracting the detrimental effects of drought on OSR was demonstrated. In Chapter 2, 3 and 5 the central hypothesis "*the yield of droughted oilseed rape can benefit from a film-forming antitranspirant application over the most drought-sensitive phenological stages due to improvements at plant water status level*" has been supported through four glasshouse and two field experiment. In addition, the hypothesis "*the increasing atmospheric* CO_2 *concentration may counteract the source-limiting effect of the film-forming treatment leading to a substantial higher efficiency of the compounds under stress conditions*" has been positively tested in Chapter 4.

There are however additional key points that deserve in-depth investigation:

- The glasshouse work was done by standardizing the spray conditions to a situation where the adaxial leaf canopy application of the chemical was uniform. This may not occur in the field. As a matter of fact, visual observations of the antitranspirant deposition in the field confirm that the spray uniformity observed in the glasshouse experiment did not reflect spray uniformity in the field. Therefore, greater exploitation for agronomical purpose can be potentially achieved in terms of spray deposition and thus further work should focus on the application of the chemical at field level.
- Antitranspirants are expensive. Despite the fact that 1 L ha^{-1} dose rate may be a sustainable cost (from ~20 to 25 £ ha^{-1}), farmers may be reluctant to use a chemical in a situation where the yield response may not reflect a significant economic gain. Thus, while the biotechnological water-saving approaches can remove this problem by producing drought-tolerant varieties, possible low-cost antitranspirant with similar efficiency to VG should be developed and tested.
- The approach used by Wang et al. (2005 and 2009) is in line with our findings. By triggering stomatal guard cells sensitivity to ABA, canola lines were capable at minimising yield losses under water-limited environments following decreases in leaf transpiration. Our results suggest that while this can be useful at the present atmospheric CO_2 concentration (~403 ppm), the predicted higher [CO_2] in the next few years will allow a different photosynthesis-transpiration trade-off, possibly avoiding the photosynthetic down-regulation if plant water is maintained. Therefore, greater exploitations of water-saving techniques under elevated CO_2 are possible and additional efforts should be addressed in this direction.

- Our experiments were only evaluating the interactions between drought and antitranspirant factors (with the exception of Chapter 4 where CO_2 was included). It is likely that drought periods in the field are often accompanied by elevated temperatures. It is evident that “pessimistic” plants are generally not well adapted to drought x heat environments. On a general basis, the behaviour expected under drought from a “pessimistic” crop that responds to the water shortage with a pronounced stomatal sensitivity and a high intrinsic WUE aimed at soil water conservation. However, it leads to higher susceptibility to thermal stress (i.e. chlorophyll bleaching and PSII down-regulation). In this context, a lot of work should be done to evidence possible detrimental or beneficial effects of an extra-induced stomatal conductance reduction following antitranspirant application.
- In this work, only film antitranspirants were tested. However, there are three main group of chemicals with antitranspirant activity that, considering their different mechanism of action (ABA-dependent such as the metabolic, ABA-independent such as the film-forming and reflectant such as kaolin), can be potentially exploited for different purposes on a large number of crops. Therefore, further projects should be developed for their agronomic exploitation in different environments.

To conclude, although much work still needs to be done, this study could be considered as a preliminary but important step in the field of OSR yield stability under stress conditions, thus setting the basis for further key research prospects on OSR drought tolerance, crop management and crop improvement for stress tolerance.

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