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Application of anti-transpirants temporarily alleviates the inhibition of symbiotic nitrogen fixation in drought-stressed pea plants

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ABSTRACT

Stomatal closure is one of the first plant responses under a water deficit situation. This leads to a decline in transpiration but also in the plant photosynthetic activity. Legume plants grown under symbiosis with rhizobium bacteria present an inhibition of nitrogen fixation that has been shown to occur even before this of photosynthesis. One of the hypotheses to explain this rapid inhibition is the accumulation of nitrogen (N) compounds in nodules due to reduced transpiration, which would provoke the N-feedback inhibition of nitrogenase activity. The current work analyzes the effects of changes in transpiration rates in the regulation of nitrogen fixation through the application of the anti-transpirant Vapor Gard (VG) to pea (*Pisum sativum* L.) plants subjected to a progressive water deficit. VG produced a rapid inhibition of nitrogen fixation upon application. This inhibition, however, did not coincide with the accumulation of either amino acids or soluble carbohydrates observed at later drought stages in nodules. Results show that the application of VG has a beneficial, albeit temporary, effect in both maintaining the plant water status and apparent nitrogenase activity of nodulated pea plants under water-deficit conditions.

1. Introduction

Drought stress is one of the environmental factors most limiting crop productivity (Bray, 1997). Although there are several climate change scenarios, the general consensus is that the frequency of severe drought conditions is likely to increase in the future (Dai, 2012). At the physiological level, water deficit is known to reduce the plant photosynthetic activity, to activate stomatal closure by the integration of abscisic acid (ABA) and hydraulic signaling, as well as to cause metabolic impairment (Lawlor et al., 1999; Schroeder et al., 2001; Comstock, 2002; Dodd, 2013). In legumes grown under symbiotic conditions, drought leads to a rapid inhibition of symbiotic nitrogen fixation, an inhibition that has been shown to occur before this of photosynthetic activity (Djekoun and Planchon, 1991; Durand et al., 1987). Although several hypotheses have been drawn to explain the drought-induced decline in nitrogen fixation, the exact regulatory mechanisms remain unclear to date. Regulation based on a nitrogen feedback inhibition of nitrogenase activity has received much attention in the last decades. This hypothesis was built on the observation that ureides and amino acids accumulate in leaves and nodules of drought-stressed legumes (de Silva et al., 1996; King and Purcell, 2005; Ladrera et al., 2007; Serraj et al., 2001, 1998; Sulieman et al., 2010, 2014; Sulieman and Tran, 2013; Vadez et al., 2000). Nonetheless, recent works using a split-root system have shown that amino acids accumulate in nodules before any measurable decline in nitrogen fixation both in temperate and tropical climate legumes (Gil-Quintana et al., 2013a,b). One possible explanation for the observed accumulation of N compounds may involve alterations in long-distance transport of metabolites between aerial and underground plant tissues mediated by transpiration (Serraj et al., 2001; Walsh, 1990). However, the influence of transpiration in long-distance metabolite transport and its effects on the drought-induced inhibition of symbiotic nitrogen fixation have not been formally tested.

In the current work, the effects of the application of the anti-transpirant Vapor Gard (VG) have been investigated in nodulated pea plants subjected to a gradual water-deficit treatment. VG is a terpene polymer (di-1-*p*-menthene, also known as pinolene) that forms a thin film on leaves, increasing resistance to water vapor loss and, therefore, reducing plant transpiration (Bender and Lipe, 1986; Byari and Okeefe, 1982, and references therein). The application of this compound has allowed us to test whether alterations in transpiration rates were associated to reduced long-distance transport and, consequently, could explain the observed accumulation of C and N compounds in leaf and nodule tissue of drought-stressed plants. Additionally, we characterized the physiological plant responses to the application of the anti-transpirant both under well-watered and water-deficit conditions. Results

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presented here provide insights into the role of transpiration in the regulation of nitrogen fixation and discusses the potential application of these compounds to reduce the negative effects of drought in nodulated legumes.

2. Material and methods

2.1. Plant growth conditions and treatment application

Pea seeds (*Pisum sativum* L. cv Sugar snap) were surface sterilized (Labhilili et al., 1995), germinated and grown in 0.6-L pots containing a mixture of perlite:vermiculite (1:1, v/v) in a growth chamber under controlled environmental conditions (12 h photoperiod; 525 μmol m^{-2} s^{-1} photosynthetic photon flux intensity; 26 °C/22 °C day/night temperature; 60–70% relative humidity) for 4 weeks. Plants were inoculated three times with 1 ml ($OD_{600} \simeq 0.4$) of a bacterial culture of the *hup*$^-$ strain *Rhizobium leguminosarum* biovar *viciae* NLV8. The first inoculation was carried out at germination, the second one three days after planting and finally seven days after planting. Plants were watered three times a week with a nitrogen-free nutrient solution (Rigaud and Puppo, 1975).

2.2. Application of VG and drought stress

Four-week old plants were separated randomly into two sets; the first set of plants was treated with a 0.2% (v/v) solution of BioPower (Bayer, Germany) containing 6.7% (w/w) 3,6-dioxaeicosylsulphate sodium salt and 20.1% (w/w) 3,6-dioxaoctadecylsulphate sodium salt. This set of plants used as a negative control (untreated plants). The second set of plants was treated with a solution containing 2.5% (v/v) of the anti-transpirant VG (Bio-Agrichem, Spain), having 96% (v/v) di-1-*p*-menthene as active ingredient, and 0.2% (v/v) BioPower. Subsequently, each group was further divided into two subsets, one of which was maintained under well-watered conditions (hereafter referred to as control plants), while watering was withheld for the other subset of plants (drought-stressed plants). Plants were analyzed at day 0, day 1, day 2, day 3 and day 4 after the onset drought. All measurements were independent (biological replicates) and were carried out inside the growth chamber starting three hours after the onset of the photoperiod to minimize day-to-day variations. Root, leaf and nodule samples were collected, snap-frozen in liquid nitrogen and kept at −80 °C for further determinations.

2.3. Water relations

Leaf water potential (Ψ_w) was measured in the first fully expanded leaf 2 h after the beginning of the photoperiod using a pressure chamber (Soil Moisture Equipment, USA) as previously described (Scholander et al., 1965). Nodule Ψ_w was analyzed using C52 sample chambers coupled to a Wescor HR-33T Dew Point Microvoltmeter (Wescor, USA). Stomatal conductance was measured in the youngest fully-expanded leaf using an AP4 porometer (Delta-T Devices, UK). Plant transpiration rates were gravimetrically determined daily on a whole-plant basis. The pots used for plant growth have a lid that covers most of the surface of the pot so evaporation from the substrate is negligible. To calculate water content, shoots and roots were excised, weighed (fresh weight, FW) and placed in paper bags for dry weight (DW) determinations. Aliquots of nodules were also weighed and kept for DW. To estimate DW, plant tissue was desiccated in an oven for 72 h at 80 °C. The percentage of water content of the different tissues was calculated using the following formula: $[(FW - DW)/FW] \times 100$.

2.4. Symbiotic nitrogen fixation measurements

Apparent nitrogenase activity (ANA) was estimated as H_2-evolution of intact plants in an open flow-through system under N_2:O_2 (79%:21%, v/v) using an electrochemical H_2 sensor (Qubit System Inc., Canada) as previously described (Witty and Minchin, 1998). The H_2 sensor was calibrated with high purity gases (Praxair, Spain) using a gas mixer (Air Liquide, Spain) flowing at the same rate as the sampling system (500 ml min^{-1}).

2.5. Analytical determinations

The content of sucrose, fructose and glucose, and free amino acids were analyzed as earlier described (González et al., 2001; Larrainzar et al., 2014).

2.6. Statistical analyses

Data are reported as mean ± standard deviation of $n = 3$–6 biological replicates. A two-way analysis of variance (ANOVA) was carried out to analyze whether there was an interaction between the factor water regimen and the factor VG application. Results of the ANOVA are included as Supplementary material (Appendix Table A.1). Following the ANOVA, mean comparison was performed using a Bonferroni-adjusted *t*-test ($p < 0.0125$). Data were analyzed using Microsoft Excel and SPSS software.

3. Results

3.1. Physiological effects of the application of Vapor Gard in plants subjected to drought stress

To characterize the physiological effects of the VG treatment, we monitored the levels of transpiration, Ψ_w, stomatal conductance and water content of both pea plants under well-watered and drought stress conditions.

Application of the anti-transpirant produced a rapid reduction of transpiration rates in treated plants starting at day 1 (Fig. 1A). This reduction was maintained in VG-treated drought-stressed plants, while in well-watered plants the effect of the anti-transpirant was partially lost at day 4. In the untreated set, drought stress caused a significant drop in transpiration rates by the end of the experiment (Fig. 1A). In terms of stomatal aperture, the film formed by the application of VG provoked a rapid decline in stomatal conductance that was maintained during the whole experiment in treated plants (Fig. 1B). Untreated drought-stressed plants showed a progressive reduction in stomatal conductance, reaching values close to those of VG-treated plants at day 4 (Fig. 1B). In parallel to the observed decline in stomatal conductance, plants experiencing water deficit showed a gradual reduction in both leaf and nodule Ψ_w values (Fig. 1C and D, respectively). In contrast, drought-stressed plants treated with VG did not show a reduction in the levels of leaf Ψ_w values until the last day of the drought period (Fig. 1C), while maintaining nodule Ψ_w values close or above those of plants without water restriction (Fig. 1D).

Regarding the water content of plants, although there was variability at the aerial part level (Fig. 2A), application of VG prevented the drought-induced reduction in water content in the underground organs of plants treated with the anti-transpirant (Fig. 2B and C).

3.2. Changes in transpiration rates affect symbiotic nitrogen fixation independently of the accumulation of carbon and nitrogen compounds in different tissues

In untreated plants, drought stress caused a progressive decline in the levels of apparent nitrogenase activity (ANA), while application of the anti-transpirant VG led to early declines in ANA rates both in control and drought-stressed treated plants (Fig. 3). At day 3 and 4, however, this VG-induced reduction in ANA was reversed and there were no significant differences between the ANA values recorded for VG-treated plants under water deficit and well-watered plants (Fig. 3).

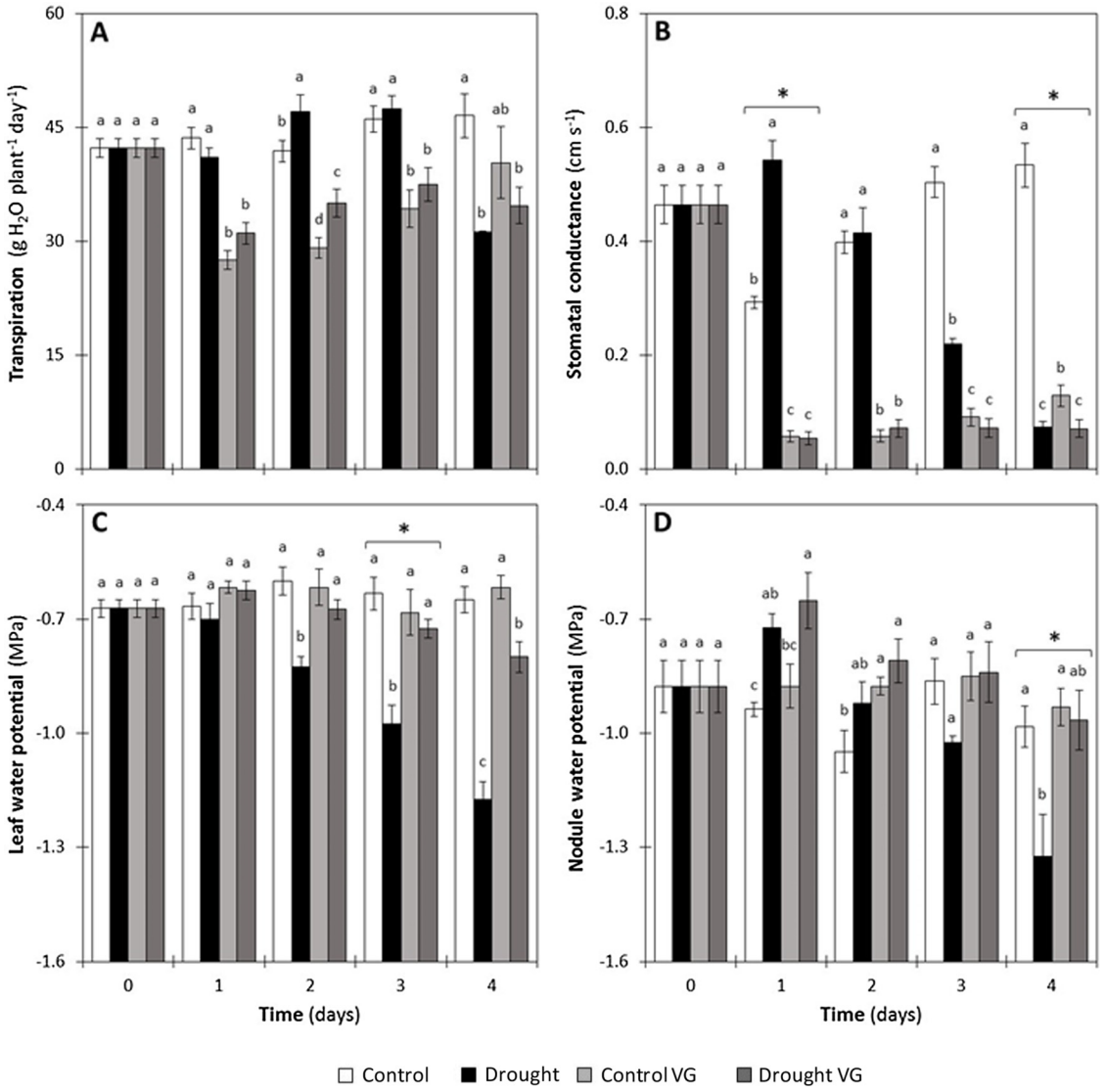

Fig. 1. Effects of the application of Vapor Gard (VG) on the rates of transpiration (A), stomatal conductance (B), leaf water potential (C) and nodule water potential (D) of pea plants subjected to a progressive drought. Values represent means ± standard error (SE) ($3 \leq n \leq 6$ biological replicates). Different letters refer to statistically significant differences between treatments [two-way ANOVA ($p < 0.05$) followed by a Bonferroni-adjusted *t*-test ($p < 0.0125$)] for each day. Interactions between factors are indicated with an asterisk (*).

Transpiration and transport of solutes across the plant are two closely related physiological processes. Therefore, we investigated the changes in the content of soluble carbohydrate in leaves, roots and nodules of pea plants treated with VG under drought conditions (Table 1). Both the variations in the content of fructose and glucose presented similar trends in the aerial part, showing a progressive accumulation under drought conditions in untreated plants. This accumulation of soluble carbohydrates was observed in plants treated with the anti-transpirant only at day 4 of drought. Sucrose levels, in contrast, did not show a clear trend during the experiment, although drought-stressed plants presented higher levels of this compound in all the tissues tested. In nodules, sucrose was found the main soluble carbohydrate, with average values of around 50 μmol g^{-1} DW in well-watered plants, while fructose and glucose were detected at very low levels. Drought stress caused a moderate accumulation of sucrose in nodules, an accumulation that was less pronounced in nodules of plants treated with the anti-transpirant VG.

To understand whether these variations in the rates of ANA could be related to the levels of N metabolites in the plant, the content of the total soluble pool of amino acids was measured in different tissues (Table 2). Similarly to the trends observed for carbohydrates, water deficit led to the gradual accumulation of amino acids in all the tissue tested. In leaves, this accumulation was significant from day 3 onwards, while in roots amino acids started to accumulate at day 2. Nodules, in contrast, presented significant differences only at the last day of the experiment. In samples of plants treated with VG, however, this accumulation was attenuated, being significant only during the last days of the experiment.

In a parallel series of experiments, foliar application of a 15 μM solution of the fungal toxin fusicoccin, a well-known activator of stomatal opening, led to the exacerbation of the severity of drought in most of the parameters tested (data not shown). Application of fusicoccin to plants with limited water availability caused a rapid decline in ANA rates, as early as day 1 after the onset of drought, which occurred even prior to any significant decline in the plant water status parameters checked. This early inhibition in nitrogen fixation in fusicoccin-treated drought plants, however, did not correlate to changes either in the concentration of sucrose or soluble amino acids in the different plant tissues tested.

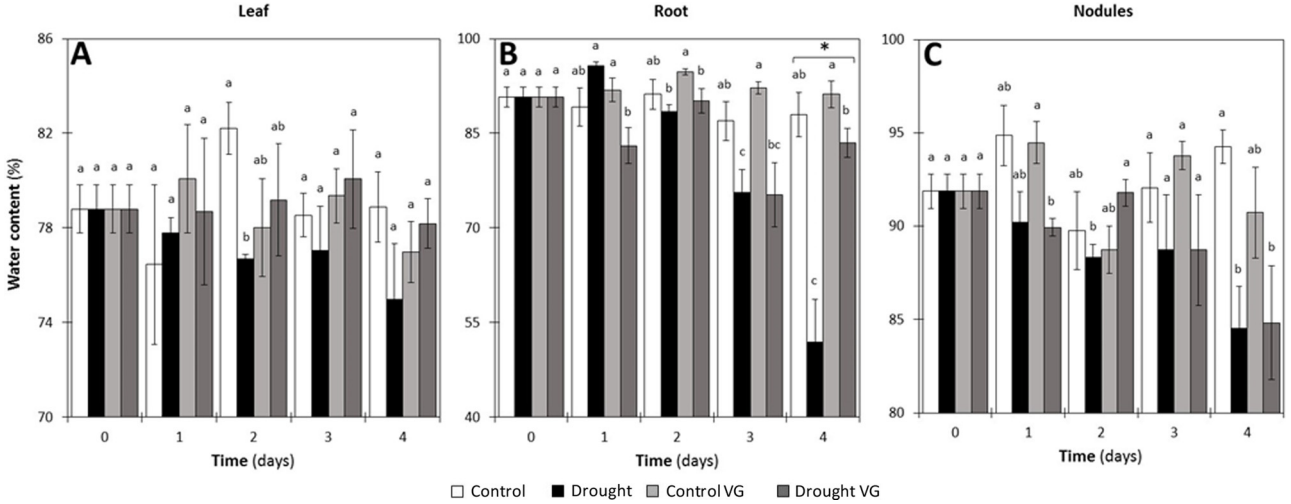

Fig. 2. Effects of the application of VG on leaf water content (A), root water content (B), and nodule water content (C) of pea plants subjected to progressive drought stress. Values represent means ± SE ($3 \leq n \leq 6$ biological replicates). Different letters refer to statistically significant differences between treatments [two-way ANOVA ($p < 0.05$) followed by a Bonferroni-adjusted *t*-test ($p < 0.0125$)] for each day. Interactions between factors are indicated with an asterisk (*).

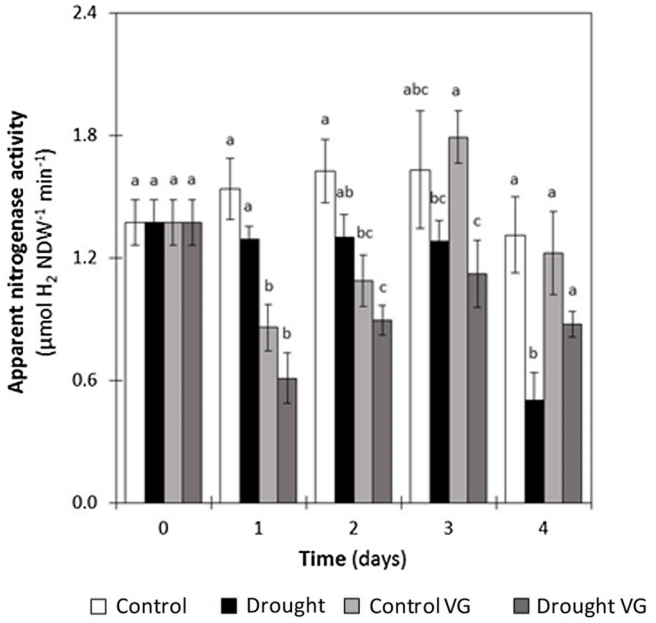

Fig. 3. Effects of the application of VG on the apparent nitrogenase activity (ANA) of pea plants subjected to progressive drought stress. Values represent means ± SE ($3 \leq n \leq 6$ biological replicates). Different letters refer to statistically significant differences between treatments [two-way ANOVA ($p < 0.05$) followed by a Bonferroni-adjusted *t*-test ($p < 0.0125$)] for each day. Interactions between factors are indicated with an asterisk (*). NDW, nodule dry weight.

4. Discussion

In the present study, we employed the anti-transpirant VG as a tool to analyze the effects of reduced transpiration in the regulation of symbiotic nitrogen fixation and long-distance metabolite transport in drought-stressed pea plants. Anti-transpirant compounds used in agriculture are polymers that form a thin film on the leaf surface to temporarily reduce plant transpiration. The application of these compounds received a considerable amount of attention in the 60–70 s due to their potential to save significant amounts of water and reduce plant damage cause by water deficits (Plaut et al., 2004). However, the fact that several studies suggested that film-forming polymers would inhibit photosynthesis (and, therefore, crop productivity) to a greater extent than transpiration partially discouraged investigations in the field (Kramer and Boyer, 1995). Nonetheless, subsequent works have shown increased crop yields and improved plant tolerance to drought upon anti-transpirant application under water-limiting conditions (Bender and Lipe, 1986; Brillante et al., 2016; Byari and Okeefe, 1982; del Amor et al., 2010; Faralli et al., 2017; Iriti et al., 2009, and references thereafter). Anti-transpirants have been also used to decrease stress associated with other abiotic stresses such as ozone damage (Francini et al., 2011), as well as to enhance plant resistance to physical damage and fungal infections (Percival and Boyle, 2009; Sutherland and Walters, 2002). In the current work we observed that drought-stressed plants treated with VG were able to maintain water status parameters similar to those of well-watered plants in the short term. Application of the anti-transpirant VG provoked a rapid reduction in the levels of transpiration (Fig. 1A) and stomatal conductance (Fig. 1B) both in well-watered and drought-stressed plants. Two-way ANOVA analyses indicate that, in general, only at day 4 there is an effect of the water regimen applied to treated plants, a stage at which the anti-transpirant effectiveness starts diminishing in terms of reduced transpiration and stomatal conductance. Thus, an improved drought response in VG-treated plants experiencing water deficit was observed during the first three days of the experiment, with plants that presented Ψ_w and water content values comparable to those of plants under full water availability conditions (Fig. 1C and D; Fig. 2).

We further analyzed whether changes in transpiration were associated to an inhibition of symbiotic nitrogen fixation and/or to transport of C and N compounds through the plant. Application of the anti-transpirant VG induced an early inhibition of ANA at day 1 (Fig. 3). In VG-treated plants nitrogen fixation rates were, however, restored to control values at day 3 both in the set of plants kept under well-watered conditions and in those under water deficit (Fig. 3). Therefore, we observed a beneficial effect of the application of the anti-transpirant both in maintaining the plant water status, as well as active symbiotic nitrogen fixation rates in drought-stressed pea plants in the short term. It is worth noting that the observed changes in ANA were independent of the plant transpiration rates or the levels of amino acids in the different tissues (Fig. 1A and Table 2, respectively). These observations are in contrast to earlier works in which reduced transpiration was suggested to provoke an accumulation of N compounds in nodules under drought stress as a consequence of decreased xylem transport (Serraj et al., 2001, and references thereafter). Therefore, the rapid reduction in ANA levels upon the application of the anti-transpirant VG in the present work could not be explained by the hypothesis of a N-feedback

Table 1
Effects of the application of VG on the levels of fructose, glucose and sucrose in leaves, roots and nodules of pea plants subjected to a progressive drought. Values represent means ± SE ($3 \leq n \leq 6$ biological replicates). Different letters refer to statistically significant differences between treatments [two-way ANOVA ($p < 0.05$) followed by a Bonferroni-adjusted *t*-test ($p < 0.0125$)] for each day. Interactions between factors are indicated with an asterisk (*). DW, dry weight.

			Day 0	Day 1	Day 2	Day 3	Day 4
Fructose (μmol g^{-1} DW)	**Leaf**	Control	2.1 ± 0.7	1.8 ± 0.7 **b**	1.1 ± 0.5 **b**	1.8 ± 0.8 **ab**	2.4 ± 1.1 **b**
		Drought		8.2 ± 1.4 **a**	5.3 ± 0.9 **a**	30.7 ± 12.5 **ab**	90.0 ± 18.4 **a**
		Control + VG		4.1 ± 1.8 **ab**	1.1 ± 0.8 **b**	0.9 ± 0.6 **b**	6.1 ± 1.7 **b**
		Drought + VG		6.2 ± 1.6 **ab**	6.0 ± 0.8 **a**	5.9 ± 1.3 **a**	39.3 ± 12.8 **a**
		Interaction (Drought * VG)		ns	ns	ns	*
	Root	Control	2.7 ± 1.3	0.0 ± 0.0 **b**	0.0 ± 0.0 **c**	0.0 ± 0.0 **b**	0.0 ± 0.0 **b**
		Drought		7.6 ± 3.1 **ab**	9.8 ± 1.7 **a**	6.6 ± 0.6 **a**	9.3 ± 3.1 **a**
		Control + VG		1.1 ± 1.1 **b**	1.4 ± 1.4 **bc**	0.0 ± 0.0 **b**	2.9 ± 2.9 **ab**
		Drought + VG		10.7 ± 2.7 **a**	4.2 ± 1.2 **ab**	5.6 ± 1.2 **a**	6.3 ± 3.5 **ab**
		Interaction (Drought * VG)		ns	*	ns	ns
	Nodule	Control	0.6 ± 0.5	0.0 ± 0.0 **a**	0.0 ± 0.0 **a**	0.0 ± 0.0 **a**	0.0 ± 0.0 **a**
		Drought		0.4 ± 0.4 **a**	0.7 ± 0.7 **a**	1.5 ± 1.5 **a**	3.2 ± 2.0 **a**
		Control + VG		0.0 ± 0.0 **a**	0.0 ± 0.0 **a**	0.0 ± 0.0 **a**	0.0 ± 0.0 **a**
		Drought + VG		0.0 ± 0.0 **a**	0.1 ± 0.1 **a**	0.0 ± 0.0 **a**	2.5 ± 1.2 **a**
		Interaction (Drought * VG)		ns	ns	ns	ns
Glucose (μmol g^{-1} DW)	**Leaf**	Control	72.3 ± 9.9	51.9 ± 5.5 **a**	43.3 ± 3.4 **a**	45.7 ± 6.0 **a**	51.0 ± 6.4 **b**
		Drought		74.0 ± 9.1 **a**	64.1 ± 6.8 **a**	146.7 ± 41.5 **a**	294.1 ± 39.9 **a**
		Control + VG		49.7 ± 9.3 **a**	51.9 ± 11.4 **a**	57.7 ± 7.4 **a**	51.1 ± 5.7 **b**
		Drought + VG		54.6 ± 8.6 **a**	72.1 ± 19.3 **a**	69.2 ± 7.2 **a**	145.0 ± 31.8 **a**
		Interaction (Drought * VG)		ns	ns	*	*
	Root	Control	56.7 ± 15.9	18.9 ± 8.1 **ab**	24.2 ± 9.5 **b**	3.7 ± 1.9 **c**	28.9 ± 7.8 **b**
		Drought		75.4 ± 30.2 **ab**	169.4 ± 51.6 **a**	120.9 ± 22.7 **a**	82.3 ± 12.1 **a**
		Control + VG		10.8 ± 4.8 **b**	14.4 ± 6.4 **b**	6.0 ± 4.0 **bc**	17.9 ± 7.6 **b**
		Drought + VG		54.8 ± 9.8 **a**	66.7 ± 25.3 **ab**	51.9 ± 10.0 **a**	59.0 ± 20.5 **ab**
		Interaction (Drought * VG)		ns	ns	*	ns
	Nodule	Control	1.7 ± 0.7	1.7 ± 1.3 **a**	1.7 ± 0.8 **a**	1.2 ± 0.5 **a**	0.4 ± 0.3 **a**
		Drought		4.6 ± 1.4 **a**	2.5 ± 2.3 **a**	1.5 ± 1.5 **a**	6.6 ± 3.8 **a**
		Control + VG		0.8 ± 0.5 **a**	1.6 ± 0.5 **a**	1.4 ± 0.6 **a**	0.7 ± 0.5 **a**
		Drought + VG		0.7 ± 0.7 **a**	5.7 ± 3.9 **a**	0.0 ± 0.0 **a**	3.9 ± 2.6 **a**
		Interaction (Drought * VG)		ns	ns	ns	ns
Sucrose (μmol g^{-1} DW)	**Leaf**	Control	490.9 ± 45.1	327.3 ± 23.8 **b**	330.4 ± 26.9 **a**	438.1 ± 25.2 **a**	325.2 ± 32.4 **ab**
		Drought		547.8 ± 34.7 **a**	444.0 ± 50.6 **a**	532.4 ± 37.4 **a**	337.5 ± 75.3 **ab**
		Control + VG		196.5 ± 31.1 **c**	218.0 ± 9.3 **b**	321.0 ± 11.6 **b**	270.5 ± 28.4 **b**
		Drought + VG		259.5 ± 24.5 **bc**	279.0 ± 44.2 **ab**	445.9 ± 50.0 **ab**	428.3 ± 29.8 **a**
		Interaction (Drought * VG)		*	ns	ns	ns
	Root	Control	58.5 ± 6.6	97.5 ± 11.2 **a**	66.12 ± 9.6 **a**	65.1 ± 6.9 **bc**	58.5 ± 5.7 **a**
		Drought		75.5 ± 3.7 **ab**	121.1 ± 17.1 **a**	117.7 ± 12.2 **a**	162.5 ± 49.8 **a**
		Control + VG		33.4 ± 2.1 **c**	66.15 ± 11.6 **a**	46.1 ± 4.2 **c**	67.4 ± 13.9 **a**
		Drought + VG		47.8 ± 7.0 **bc**	76.1 ± 9.4 **a**	82.0 ± 6.6 **ab**	126.2 ± 54.2 **a**
		Interaction (Drought * VG)		ns	ns	ns	ns
	Nodule	Control	61.4 ± 6.8	65.1 ± 7.9 **ab**	50.2 ± 8.4 **a**	53.6 ± 13.5 **ab**	54.3 ± 4.8 **b**
		Drought		88.8 ± 4.5 **a**	90.9 ± 11.2 **a**	80.5 ± 4.7 **a**	96.1 ± 11.9 **a**
		Control + VG		32.9 ± 2.4 **c**	45.1 ± 9.3 **a**	51.7 ± 4.5 **b**	36.5 ± 7.2 **b**
		Drought + VG		37.2 ± 1.7 **bc**	64.8 ± 5.7 **a**	55.8 ± 6.0 **ab**	65.5 ± 10.2 **ab**
		Interaction (Drought * VG)		ns	ns	ns	ns

Table 2
Effects of the application of VG on the total amino acid content in leaves, roots and nodules of pea plants subjected to a progressive drought. Values represent means ± SE ($3 \leq n \leq 6$ biological replicates). Different letters refer to statistically significant differences between treatments [two-way ANOVA ($p < 0.05$) followed by a Bonferroni-adjusted *t*-test ($p < 0.0125$)] for each day. Interactions between factors are indicated with an asterisk (*). DW, dry weight.

			Day 0	Day 1	Day 2	Day 3	Day 4
Total amino acid content (μmol g^{-1} DW)	**Leaf**	Control	99.68 ± 10.8	104.3 ± 12.0 **ab**	108.2 ± 10.0 **a**	102.6 ± 7.5 **b**	103.8 ± 14.1 **b**
		Drought		145.2 ± 17.2 **a**	146.3 ± 18.8 **a**	222.1 ± 25.3 **a**	192.3 ± 26.7 **a**
		Control + VG		73.1 ± 6.9 **b**	88.7 ± 9.8 **a**	53.3 ± 7.5 **c**	54.9 ± 5.1 **c**
		Drought + VG		119.9 ± 2.1 **a**	125.4 ± 38 **a**	113.3 ± 16.7 **b**	117.1 ± 10.9 **ab**
		Interaction (Drought * VG)		ns	ns	*	ns
	Root	Control	14.1 ± 3.3	17.0 ± 3.5 **a**	14.7 ± 1.7 **a**	14.4 ± 4.3 **bc**	22.5 ± 5.5 **c**
		Drought		24.8 ± 5.3 **a**	51.6 ± 13.9 **a**	100.5 ± 17.8 **a**	618.3 ± 103.6 **a**
		Control + VG		15.7 ± 5.8 **a**	16.2 ± 5.4 **a**	6.6 ± 3.5 **c**	9.7 ± 2.9 **c**
		Drought + VG		32.6 ± 8.7 **a**	18.3 ± 2.9 **a**	106.3 ± 47.9 **ab**	108.4 ± 21.4 **b**
		Interaction (Drought * VG)		ns	*	ns	*
	Nodule	Control	82.5 ± 16.5	129.2 ± 21.7 **a**	121.9 ± 14.1 **a**	112.1 ± 16.8 **ab**	82.2 ± 10.3 **b**
		Drought		106.9 ± 15.0 **a**	115.1 ± 16.1 **a**	139.3 ± 13.9 **a**	218.4 ± 8.2 **a**
		Control + VG		87.0 ± 4.9 **a**	70.7 ± 19.1 **a**	57.2 ± 11.0 **b**	71.7 ± 17. 5 **b**
		Drought + VG		125.4 ± 30.8 **a**	110.1 ± 7.8 **a**	82.8 ± 14.5 **ab**	178.6 ± 23.5 **a**
		Interaction (Drought * VG)		ns	ns	ns	ns

regulation of symbiotic nitrogen fixation.

Carbon limitation through inhibition of sucrose synthase activity, protein or expression levels has been also discussed as a factor contributing to the observed drought-induced inhibition of ANA in different legume species (González et al., 1995; Marino et al., 2006; Ladrera et al., 2007; Larrainzar et al., 2009). As a consequence, the inhibition of sucrose synthase has been shown to lead to an accumulation of sucrose in nodules of drought-stressed plants. However, in the present work application of the anti-transpirant actually induced a transient decline in the content of sucrose at day 1, discarding a limitation at the level of sucrose synthase in this case. Furthermore, the application of the stomatal-opening inducer fusicoccin led to an early inhibition of nitrogen fixation that did not correlate to changes in the levels of sucrose or soluble amino acids in the various plant tissues analyzed (data not shown). Thus, using two contrasting pharmacological approaches to manipulate plant transpiration, our results suggest that the inhibition of ANA is not mediated by C and N transport-based regulatory mechanisms.

Previous studies have suggested that VG application at concentrations of 6% (v/v) was effective for a period of 30–40 days as a strategy to extend the cultivation of sweet corn during the dry season in the tropics (Plaut et al., 2004). More recent work, however, estimates that application of 1% (v/v) VG may be effective for a period of 20–25 days to reduce water stress in oilseed rape under field conditions (Faralli et al., 2016). In order to test for how long we could observe the protective effect of VG under our experimental conditions, we also carried out a second experiment extending the drought period for three additional days (data not shown). This extended experiment showed that the beneficial effects of the anti-transpirant in maintaining the plant water status and nitrogen fixation were only temporary. Parameters such as transpiration and water content of different tissues were significantly reduced from day 5 onwards, while ANA values of VG-treated drought-stressed plants were comparable to those of untreated plants by day 6. Therefore, although treatment with VG can be considered beneficial to temporarily maintain the plant water status and active nitrogen fixation rates in pea plants under water deficit conditions, when the drought period is extended, the protection of the anti-transpirant appears to be lost.

5. Conclusions

In conclusion, our work shows that artificial alterations of plant transpiration produce a rapid, temporal decline in the rates of nitrogen fixation in pea plants and that this decline could not be explained by earlier proposed N-feedback regulation mechanisms. In agreement with previous studies (Gil-Quintana et al., 2013a,b), the observed accumulation of C and N compounds at later drought stages appears to be a general drought stress response not necessarily related to the regulation of nitrogen fixation or associated with reduced transpiration rates. Finally, application of film-forming polymers such as VG partially attenuates the negative effects of drought stress of nodulated pea plants, although the protective effect of the anti-transpirant is only temporary and it is largely dependent on the experimental conditions in which it is applied.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.agwat.2018.10.014.

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