



Mechanisms for water-use efficiency between bean cultivars tolerant to drought are different

Víctor Montero Tavera¹, César Leobardo Aguirre Mancilla², Jorge Alberto Acosta Gallegos¹, Juan Gabriel Ramírez Pimentel², Ana Isabel Mireles Arriaga³ and Jorge Eric Ruiz Nieto³

¹Campo Experimental Bajío, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Celaya 38110, Guanajuato, México.

²División de Estudios de Posgrado e Investigación, Instituto Tecnológico de Roque, Tecnológico Nacional de México, Celaya 38110, Guanajuato, México.

³Departamento de Agronomía, División de Ciencias de la Vida, Universidad de Guanajuato, Campus Irapuato-Salamanca, Irapuato 36500, Guanajuato, México. *Author for correspondence. E-mail: jorge.ruiz@ugto.mx

ABSTRACT. The water-use efficiency (WUE) has been proposed as an alternative to mitigate the effects of climate change in agriculture and to reduce pressure on water resources, mainly in species with high water requirements which are grown under rainfed conditions. The aim of this study was to characterize the instantaneous, integral and molecular WUE of four bean cultivars contrasting in their response under limiting water conditions to compare the component mechanisms of this trait between drought tolerant and susceptible cultivars. Results indicated that tolerant cultivars increased their instantaneous WUE in comparison to susceptible ones; however, there was a difference between cultivars since Pinto Villa had a higher stomatal conductance and transpiration rate, leading to a higher water cost to produce seed than Pinto Saltillo. Furthermore, *yef2*, *rrn16*, *rpoC2*, *hardy*, *ndhK*, *erecta* and *yef1* WUE genes were only overexpressed in Pinto Saltillo under limited water conditions, which turned out to be the most WUE efficient cultivar. Therefore, the component mechanisms of WUE are different even between drought tolerant cultivars and the mechanisms by which the tolerant cultivars increased their instantaneous and integral WUE were different.

Keywords: *Phaseolus vulgaris*; physiological characterization; expression profiles; water regimens; production water cost.

Mecanismos de eficiência do uso da água entre cultivares feijão tolerante à seca são diferentes

RESUMO. A eficiência do uso da água (UEA) foi proposta como uma alternativa para mitigar os efeitos das mudanças climáticas na agricultura e reduzir a pressão sobre os recursos hídricos, principalmente em espécies com altos requerimentos de água que são cultivadas sob condições de sequeiro. O objetivo deste estudo foi caracterizar o UEA instantâneo, integral e molecular de quatro cultivares de feijão, contrastando sua resposta em condições de água limitadas para comparar os mecanismos componentes desta característica entre cultivares tolerantes à seca. Os resultados indicaram que os cultivares tolerantes aumentaram seu UEA instantâneo em comparação aos suscetíveis; entretanto, houve diferença entre as cultivares, uma vez que Pinto Villa apresentou maior condutância estomática e taxa de transpiração, levando a um custo maior de água para produção de sementes do que Pinto Saltillo. Além disso, os genes UEA de *yef2*, *rrn16*, *rpoC2*, *hardy*, *ndhK*, *erecta* e *yef1* foram apenas sobreexpressos em Pinto Saltillo sob condições de água limitadas, o que se revelou ser a cultivar mais eficiente do UEA. Portanto, os mecanismos componentes da UEA são diferentes mesmo entre as cultivares tolerantes à seca e os mecanismos pelos quais as cultivares tolerantes aumentaram sua UEA instantânea e integral foram diferentes.

Palavras-chaves: *Phaseolus vulgaris*; caracterização fisiológica; perfis de expressão; regimes hídricos; custo da água de produção.

Introduction

World population will increase around 9 billion by 2050 and its water requirements will be of 12,400 km³ instead of the 6,800 km³ (Godfray et al., 2010). Even after improving irrigation efficiency in agriculture, water resources management and modernized rainfed production, 3,300 km³ of water will still be required (Molden et al., 2010), whereby the water demand will increase to satisfy the needs of urban and industrial use,

generating competition within and among sectors, reducing the availability of water for agricultural production (Hanjra & Qureshi, 2010). Furthermore, climate change is inflicting a high impact on agriculture by altering the spatial and temporal distribution of rainfall, which limits water availability (Crimmins, Dobrowski, Greenberg, Abatzoglou, & Mynsberge, 2011). Water stress is one of the main factors that restrict plant growth, development, survival and production (Ahuja, de Vos, Bones, & Hal, 2010). Only

20% of the agricultural area is irrigated and in this area 40% of food world production is harvested, therefore 60% of global agricultural production takes place under rainfall conditions (Sauer et al., 2010). One way to mitigate the effects of climate change and reduce agricultural water consumption is by generating cultivars with higher water-use efficiency (WUE) (Ruiz-Nieto et al., 2015), mainly in species with high water requirements grown under rainfed conditions. According to Jensen et al. (2012), legumes can reduce the emissions of greenhouse gases and have high rates of soil carbon incorporation, for these reasons they represent an option within the actual agricultural production systems to decrease the effects of climate change. Common bean is one of the main worldwide legume crops with 26.5 million tons produced in 2014 (FAOSTAT, 2017). However, 60% of the bean production is obtained in rainfed areas prone to experience water deficits, where typical drought periods might reduce up to 80% its production (Rosales et al., 2012). The characterization of plant mechanisms and their genetic interactions with the environment will provide the opportunity to establish water-efficient crop systems to cope climate change (Hatfield et al., 2011). WUE describes the relation between carbon fixation and water loss in plants, because this last evaporates from interstitial tissues of the leaves each time stomata open to assimilate CO₂ (Bramley, Turner, & Siddique, 2013). The identification of intraspecific genetic variation for WUE has allowed breeders to begin selection programs for this trait to improve drought tolerance (Devi et al., 2011). As for dry bean, Rosales et al. (2012) indicated that in the cultivar Pinto Saltillo, WUE is one of its mechanisms in response to limited water availability, which was confirmed by our researching group (Ruiz-Nieto et al., 2015). To complement previous research, the aim of this study was to characterize instantaneous, integral and molecular WUE in four common bean cultivars contrasting in their response to limited water conditions, to compare the component mechanisms of this trait focusing on the drought tolerant cultivars Pinto Villa and Pinto Saltillo.

Material and method

Plant material

Four cultivars of contrasting response to drought stress were included in this study, Pinto Villa (PV) considered as tolerant (Cuellar-Ortiz, Arrieta-Montiel, Acosta-Gallegos, & Covarrubias, 2008), Canario 60 (C60) as susceptible, while Rosales et al. (2012) consider Pinto Saltillo (PS) and Bayo Madero (BM) as tolerant and susceptible, respectively. Three cultivars are of indeterminate

prostrate Type III growth habit, of short photoperiod response and belong to the Durango race, whereas C60 is of determinate type I growth habit from the Nueva Granada race.

Experimental conditions

Seeds were germinated in a humid chamber and five days later the seedlings were transplanted into pots of 16 cm height and 18 cm greater diameter with type peat substrate (Sunshine® mix No. 3). Based on the Field Capacity (FC) of 1.89 g of water g of substrate⁻¹ two water regimes were established at 20 and 60% of the FC as limiting and optimal respectively (Ruiz-Nieto et al., 2015). Treatments were kept gravimetrically through the daily weighting of each pot and maintenance irrigation. To avoid evaporation and percolation losses, the pots were covered with foil and the drain holes were sealed, thus ensuring that the plants were always kept in the corresponding water regimens. The experiment was conducted during 75 days in greenhouse conditions at the installations of Campo Experimental Bajío (20°31' north latitude, 100°45' west longitude and 1,765 masl). The average maximum and minimum temperatures were 31.1°C and 11.9°C, with averages maximum and minimum relative humidities of 88.3% and 55.9%, the natural photoperiod was around 12h during the growing cycle.

Variables measured

For plant development and using five replications, leaf area (LA, cm²) was determined following Jesus, Vale, Coelho, and Costa (2001), plant height (PH, cm) was also measured; total biomass (TB, g) was recorded after drying the plants at 80°C overnight, at the same time plants were separated into above ground biomass (AB, g) and roots (RB, g). The foliage relative water content (RWC, %) (Ruiz-Nieto et al., 2015). Negative impressions were obtained on the underside of representative leaves with clear acrylic lacquer, thus stomata density (SD) was determined from those impressions by counting the number of stomata per 1.323 mm² corresponding to the optical field used. Using the same images, stomata aperture area (SA, μm²) was evaluated with the software Image Measurements KLONG 13.1.3.8; stomata conductance (g_s, mmol H₂O m⁻² s⁻¹), photosynthesis (A, μmol CO₂ m⁻² s⁻¹) and transpiration (E, mol H₂O m⁻² s⁻¹) rates were measured with the aid of an infrared gas analyzer CI-340 (CID Bio-Science, Inc.) with a chamber of 6.25 cm² and a constant air flow of 0.3 L min⁻¹. Measurements were performed between 12:00 and 13:00 hours of the day with 70% relative humidity, considering representative leaves at each plant growth stage under each treatment. All variables were evaluated in the vegetative (V3), reproductive (R6) and

physiological maturity (R8) stages, which were considered when plants presented three fully developed leaves, the onset of flowering and physiological maturity, respectively. Other variables related to reproductive habits and production were evaluated including days to flowering (DF), pods per plant (PP) and seeds per pod (SP), seed yield per plant (SYP), and the weight of 100 seeds (W100S). Water consumed per plant (WCP, mL) during the whole cycle was measured throughout its daily record. Instantaneous and intrinsic WUE were characterized by the relationship between photosynthesis and transpiration rates (A/E , $\mu\text{mol CO}_2 \text{ mol H}_2\text{O}^{-1}$), as well as photosynthesis and stomata conductance (A/g_s , $\mu\text{mol CO}_2 \text{ mmol H}_2\text{O}^{-1}$). Integral WUE was evaluated by the relationship between total biomass and water consumed (TB/WCP, g L^{-1}), also by seed production and water consumed (SYP/WCP, g L^{-1}). Using this last ratio water footprint (WF, L kg^{-1}) was estimated as the number of liters of water needed to produce one kg of seeds. Data were analyzed using a completely randomized design with five replications; Tukey ($p < 0.05$) tests were carried out using the SAS System for Windows 9.1.

Amplification of genes by PCR

The presence-absence of 16 fragments previously identified as related to WUE by Ruiz-Nieto et al. (2015) was determined in the genome of the four bean cultivars by PCR (Table 1).

Genomic DNA was extracted from leaf tissue and five replications per treatment using the procedure described by Devi, Punyarani, Singh, and Devi (2013). DNA purity was estimated by spectrophotometry and its integrity evaluated by submitting it to electrophoresis in 1 % agarose with 1X TBE buffer (1 mM pH 8 EDTA, 40 mM boric acid, 40 mM Tris). Gels were stained with ethidium bromide ($0.5 \mu\text{g mL}^{-1}$) and results were exposed with UV light in a photodocumenter BIO-RAD Universal Model Universal Hood II. Fragment genes were amplified in a total volume of 20 μL containing 12 μL of sterile distilled water, 2 μL of 10 mM dNTP's, 2 μL of 10X PCR buffer, 0.8 μL of 50 mM MgCl_2 , 2 μL of 10 mM corresponding primers pair, 0.2 μL of 5 U μL^{-1} Taq and 1 μL of 30 ng μL^{-1} genomic DNA. The amplification program was: 1 cycle at 95°C for 5 min., 25 cycles at 95°C for 1 min., corresponding Tm to each primer pair for 2 min. and 72°C for 2 min., 1 final cycle at 72°C during 2 min. Reaction products were separated by electrophoresis in 1% agarose with 1X TBE buffer.

Table 1. Genetic elements considered to characterize WUE.

Gen	Primers Forward and Reverse	Product (pb)	Tm (°C)
¹ <i>ndhK</i>	F: CAGGCTCCCATAGCAATAACA R: AGGCGGACCTCATTTTAACC	112	60
² <i>rpoC2</i>	F: ACACCAATTAGTAGGTATGAGAGGA R: GCAGTATCTACAACCCCTTTACG	150	58
³ <i>rps19</i>	F: TTGGTCCAGAGCATCTACCA R: CGCATGTCCTCGGAAGTTTA	154	60
⁴ <i>rm16</i>	F: CCCAGGCGGGATACTTAACGC R: GGAATTTCCGGTGGAGCGGT	218	59
⁵ <i>rm23</i>	F: GGACGGAGGAGGCTAGGTTA R: GGCTCGAGGCATTTTCTCTA	103	60
⁶ <i>yf1</i>	F: TCCAGAACATGAACAAGTAAAAATG R: GTGTTATTTTCGATTTCTTTTGT	126	59
⁷ <i>yf2</i>	F: TTTTGTGAAACAGCCCTTCC R: GCCTTAAACATGAGGCCCTA	173	60
⁸ <i>cp2</i>	F: CCAGACGTTTCGTCAGTGTGA R: CAGCTTCGCACACAAACGAA	133	60
⁹ <i>g564</i>	F: GCGGTTTTCCACTCATCACC R: GGCGCATGTGTTGTGATTGT	178	60
¹⁰ T-2-3	F: TTAAACCCATTTTCGCTCCAC R: GGAGGAGGTTGGTGATTCAA	114	60
¹¹ T2-12	F: ACGAAATGTCGGATTTCAGC R: TCAGAGGAAATTTGGCAAGG	247	60
¹² <i>apx2</i>	F: CATACTGGCACAAGGTGCTG R: CGCATTATGACGTTGCTGTC	289	60
¹³ <i>erecta</i>	F: CCACTCCAAACTCCAGTGGT R: CTGAATGGCAAGTCAGTGGA	139	60
¹⁴ <i>hardy</i>	F: CCCAGTAAGCCATTTTCCAG R: GGTGTGGAGATGGAAGACA	123	60
¹⁵ <i>bzip</i>	F: GACGACGACAAGATGGTTCCTAGTGAG R: GGAACAAGACCCGTTTCATTCATGAAT	581	60
¹⁴ LR0064F	F: TGGAGGAGACCCTTCATGTGGG R: GGCTTGTGCTCCCCTTTCTGTTCCAC	145	60

GenBank accessions: ¹JZ715498, ²JZ715502, ³EU196765.1 (80705-80486), ⁴EU196765.1 (97773-98003), ⁵EU196765.1 (102505-102636), ⁶JZ715503, ⁷JZ715504, ⁸JZ715505, ⁹JZ715507, ¹⁰JZ715508, ¹¹JZ715510, ¹²NM_001354113, ¹³AF350505, ¹⁴CK901535. References: ¹(Wilson et al., 2009), ²(Karaba et al., 2007).

Evaluation of expression levels by RT-PCR

Expression profiles were generated with the 16 genetic elements for the four cultivars from leaf tissue and using five replications under the two water regimens (20 and 60%) and at two growth stages (V3 and R6). Sampling at physiological maturity was not considered since there was a general decline in physiological activity related to plant age instead of treatments. Total RNA was extracted from leaf tissue representative of growth stage using the procedure of Logemann, Schell, and Willmitzer (1987) and RNA purity was determined by spectrophotometry, subsequently RNA integrity was tested by denaturing electrophoresis in 1.5% agarose with 12.3 M formaldehyde and 10X MOPS. Results were documented in the way above described. Later, single strand cDNA was synthesized by retrotranscription with the aid of the kit Super Smart™ kit. Basal expression was normalized based on the expression level of the 26S reference gene (Montero-Tavera, Escobedo-Landín, Acosta-Gallegos, Anaya-Lopez, & Ruiz-Nieto, 2017). WUE related genes were amplified in PCR reactions with 300 ng μL^{-1} of cDNA as template and with corresponding number cycles

according with the normalization of the 26S gene. Products were analyzed by electrophoresis in 1% agarose with 1X TBE buffer. Densitometric analyzes using the software TotalLabQuant TL120 1D v2009 were performed over the images visualized.

Validation of expression profiles by qPCR

To confirm the observed differences in the expression profiles, the expression levels of *rpoC2*, *ndhK*, *ycf1*, *apx2* and *erecta* genes were determined in the experimental conditions at which these genes expressed differentially (RT-PCR). The *rpoC2* gene was evaluated in PV and C60 at the V3 stage and in PS at the R6 stage; the differential expression of gene *apx2* in PV and BM at the V3 stage; the *erecta* gene in PV in the V3 stage; genes *ndhK* and *ycf1* were evaluated in PS at the R6 stage. The fluorophore used was SYBR[®], by using 25 μL reactions containing 12.5 μL of 2X Maxima SYBR Green/ROX qPCR Master Mix, 1.5 μL of 300 mM (previously evaluated) corresponding oligonucleotide pair, 1 μL of 50 ng μL^{-1} cDNA and 10 μL of nuclease free water, with three replicates. The reaction conditions were: 1 cycle at 95°C for 10 min., 30 cycles at 95°C for 15 s, 60°C for 30 s and 72°C during 30 s. In the same reaction, the absence of nonspecific products was verified by generating melt curves with the following conditions: 95°C for 15 s, 50°C during 1 min. and 95°C for 15 s. The reaction efficiencies for the genes were determined by calibration curves using five cDNA (1:10) serial dilutions and three replicates. CT (threshold cycle) values were obtained with the software StepOne V2.3 and results were analyzed with the software REST 2009 V2.0.13 with 2,000 bootstrap iterations.

Result and discussion

During the conduction of the greenhouse experiment none plant showed the typical drought symptoms as wilting and/or bending in the 20% water regimen. Under the 20%, drought tolerant cultivars increased their root biomass with respect to the susceptible ones, although this response that was more evident at the V3 stage. Results were like those described by Shao, Chu, Jaleel, and Zhao (2008), who mention that the radicle of tolerant cultivars developed faster under stress conditions. On average PV and PS had 1.2 and 0.8 g more root biomass respectively than susceptible cultivars. Same trends were observed for foliage dry weight and LA. PV and PS had 0.75 and 1.05 g more of foliage dry weight respectively, than susceptible cultivars, while for LA, PV and PS had on average 33.5 and 39.5 cm^2 more than susceptible cultivars, respectively. This suggests that the root biomass increased in the tolerant cultivars in response

to limited water availability, which enabled them to satisfy the water requirements of a larger leaf area. Both LA and foliage biomass were similar in PV and PS, but root biomass was higher in PV suggesting that foliage maintenance in this drought tolerant cultivar had higher water requirements; consequently, its water consumption was higher. The four cultivars decreased their g_s and E under 20% water regimen, comparable results were observed by Rosales et al. (2012) with PS and BM. Regarding g_s , the reduction of its magnitude in the four cultivars was higher mainly at the V3 stage where high physiological activity was displayed. In this growth stage PV and PS decreased their g_s 50 and 61 $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$, respectively. Remarkably, cultivar C60 in the same stage had a greater reduction in its g_s (34 $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$) with respect to BM. As expected, there was a highly significant positive correlation ($R^2 = 0.98$) between g_s and SA, which indicates that the effect of limited water regimen in the stomata response can be evaluated by any of these two variables. Tolerant cultivars had a lower E rate due to greater ability to modulate their stomata and between tolerant cultivars; PV had a higher E rate than PS due to greater g_s , therefore PV will have higher water consumption at the end of its biological cycle. Under limited conditions, A increased in the four cultivars however the response of tolerant cultivars was higher and reciprocal to instant and intrinsic WUE. In PV and PS at the V3 stage, A was 12.1 and 11.8 $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$, respectively, while in BM and C60 at the same stage was 7.5 and 6.5 $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$, respectively. A highly significant positive correlation ($R^2 = 0.89$) was observed between RWC and A . The value of both variables increased in the four cultivars under 20% water regimen and mainly at the V3 stage; although A and RWC were higher in the tolerant cultivars than in the susceptible ones. The four cultivars tended to reduce their stomata density under the 20% water regime, although tolerant cultivars had higher stomata density, according to Reynolds-Henne et al. (2010) a reduction in stomata density is related to adaptation under stress conditions. The four cultivars increased their instantaneous and intrinsic WUE under 20% water regimen, however the tolerant cultivars were more efficient mainly due to their ability to assimilate CO_2 under limited water conditions throughout a high photosynthetic rate. Ruiz-Nieto et al. (2015) mentioned that this trait is important for the identification of drought tolerant bean genotypes (Zlatev & Lidon, 2012). Meanwhile, in the instantaneous assessments between tolerant cultivars, PS was more efficient despite that both cultivars had high A . Differences between them were since PV had higher water requirements that were negatively correlated with WUE (Table 2).

Table 2. Variables evaluated during the plant development.

Variable	Stage	PV20%	PV60%	PS20%	PS60%	BM20%	BM60%	C6020%	C6060%
LA	V3**	183.6 bc	411.3 a	189.5 bc	423.2 a	152.2 c	344.1 a	149.4 c	248.2 b
	R6**	428.7 c	718.7 ab	400.0 c	682.7 ab	367.4 c	762.8 a	375.1 c	598.4 b
	R8**	385.8 c	574.9 ab	340.1 c	512.1 b	356.1 c	648.4 a	356.3 c	508.6 b
PH	V3**	22.8 de	34.2 c	25.3 d	55.8 b	14.9 ef	70.4 a	9.8 f	13.8 f
	R6**	27.9 d	64.2 b	34.0 cd	73.6 b	42.2 c	145.8 a	33.0 cd	37.0 cd
	R8**	25.6 c	54.2 b	30.0 c	63.0 b	36.2 c	121.2 a	28.2 c	32.8 c
TB	V3**	5.6 a	5.6 a	5.5 a	5.4 a	4.0 b	4.3 b	3.3 c	3.8 bc
	R6**	10.5 c	15.3 a	10.0 cd	15.4 a	9.5 d	14.7 a	9.7 cd	13.8 b
	R8**	7.5 b	10.0 a	6.9 b	9.9 a	6.9 b	9.8 a	7.1 b	9.0 a
AB	V3**	2.7 cd	3.9 a	3.0 bc	3.4 b	2.1 ef	2.6 cd	1.8 f	2.3 de
	R6**	6.8 c	8.8 ab	6.3 c	8.9 ab	6.0 c	9.4 a	6.2 c	8.0 b
	R8**	5.1 b	6.3 ab	4.9 b	5.9 ab	5.4 b	7.0 a	5.3 b	6.0 ab
RB	V3**	2.9 a	1.7 cd	2.5 b	2.0 c	1.9 c	1.7 cd	1.5 d	1.5 d
	R6**	3.7 d	6.5 a	3.7 d	6.5 a	3.5 d	5.3 c	3.5 d	5.8 b
	R8**	2.4 cd	3.6 a	2.0 de	4.0 a	1.5 f	2.8 bc	1.8 ef	3.0 b
RWC	V3**	82.6 a	78.8 ab	84.8 a	80.8 ab	77.3 ab	72.1 ab	71.8 ab	66.3 b
	R6*	70.6 ab	62.5 ab	69.1 ab	64.2 ab	69.6 ab	75.1 a	67.1 ab	61.0 b
	R8**	64.8 a	53.7 bc	61.8 ab	50.8 c	65.2 a	70.2 a	54.2 bc	49.2 c
SD	V3**	35 bc	39 ab	37 ab	41 a	35 bc	34 bc	31 c	32 c
	R6**	35 bc	40 ab	38 ab	42 a	30 c	32 c	31 c	35 bc
	R8**	37 ab	41 a	36 ab	39 a	29 c	33 bc	32 bc	33 bc
SA	V3**	102.0 abc	118.1 a	78.0 d	94.1 bcd	90.1 bcd	98.2 bc	86.76 cd	105.5 ab
	R6**	48.2 c	68.6 a	29.4 d	51.2 bc	58.2 abc	70.0 a	52.9 bc	63.3 ab
	R8**	26.4 bc	24.2 bc	16.6 c	24.3 bc	33.3 ab	50.0 a	36.8 ab	46.4 a
A	V3**	12.1 a	10.3 ab	11.8 a	8.1 bc	7.5 bc	5.3 c	6.3 c	6.5 c
	R6**	6.5 ab	5.5 bc	7.4 a	5.8 abc	4.3 c	5.8 abc	5.3 bc	4.7 c
	R8**	3.1 bc	2.4 cd	2.1 de	1.4 c	2.5 cd	4.5 a	3.8 ab	2.7 cd
E	V3**	4.2 bc	5.2 bc	4.0 c	4.9 abc	5.4 a	5.3 a	5.0 bc	5.2 a
	R6**	3.1 bc	3.4 bc	2.8 c	3.4 bc	3.8 ab	4.6 a	3.0 c	3.1 bc
	R8**	1.4 cd	1.5 cd	1.1 d	1.5 cd	1.7 bc	2.6 a	2.0 b	2.1 b
g	V3**	274 ab	324 a	181 c	242 bc	245 bc	255 b	232 bc	266 ab
	R6**	121 abc	140 ab	91 c	107 c	123 abc	151 a	102 c	116 bc
	R8**	54d	49 d	30 e	51 d	60 cd	108 a	72 bc	85 b

Pinto Villa (PV), Pinto Saltillo (PS), Bayo Madero (BM), Canario 60 (C60), limited water regimen (20%) and optimal (60%), vegetative stage (V3), reproductive stage (R6), maturity (R8), leaf area (LA, cm²), plant height (PH, cm), total biomass (TB, g), foliage biomass (AB, g), root biomass (RB, g), relative water content (RWC, %), stomatal density (SD, stomata per 1.323 mm²), stomatal aperture area (SA, μm²), photosynthesis (A, μmol CO₂ m⁻² s⁻¹), transpiration (E, mol H₂O m⁻² s⁻¹), stomatal conductance (g, mmol H₂O m⁻² s⁻¹). Values with the same letter within averages rows are statically equal Tukey (p < 0.05), (*) significant differences p < 0.05, (**) highly significant differences p < 0.01.

The SYP of the four cultivars was reduced under the 20% regimen, although tolerant cultivars had a higher number of pods and seeds. Cultivar PV showed higher SYP than PS under both water regimens; despite that the number of pods was similar, PV had more SP. Also, under the water limited regimen the seed weight of all cultivars was reduced; although PV produced more seeds, its size was reduced by 26.5%, while in PS, BM and C60 the reduction was of 4.8, 10.6 and 11.2%, respectively; this suggest the action of the yield component compensation (Kahlon, Board, & Kang, 2011). In the integral measurement of WCP the four cultivars limited their consumption under the 20% water regimen, particularly PS whose SYP/WCP relationship indicated an efficient water use under limited conditions, with an increase in its WUE of 28%, while there were no differences between PV and BM. Although PV was the highest yielder cultivar, when its WUE was measured in integral terms, it was like the one

under the optimal water regimen. Cultivar C60, despite of being considered as susceptible, it increased its integral WUE (SYP/WCP) under the restrictive water regimen 20%, however with respect to PS the difference was 0.34 g L⁻¹. Results on WUE indicated that seed production of PS under limited conditions had a lesser water cost than that of PV and the susceptible cultivars. Among tolerant and susceptible cultivars, the difference in integral WUE was due to the ability to produce seed with minimum water consumption under limited water conditions. While between tolerant cultivars, PV and PS, the difference in the same evaluation was due to the water cost of their seed production. For the TB/WCP relationship, the four cultivars were more efficient in the 20% water regimen and the greatest difference was observed between susceptible cultivars, where C60 was more efficient than BM in the early stage of its biological cycle, stage in which it had less water requirements (Table 3).

Table 3. Variables evaluated for reproduction, production and WUE.

Variable	Stage	PV20%	PV60%	PS20%	PS60%	BM20%	BM60%	C6020%	C6060%
DF	I**	39 cd	37 cde	34 e	35 de	41 bc	40 cd	46 ab	47 a
PP	I**	3.2 bc	4.6 a	3.2 bc	5.0 a	2.2 c	3.4 b	2.8 bc	3.2 bc
SYP	I**	2.4 cd	4.2 a	2.0 cde	3.3 b	1.3 e	2.5 c	1.5 de	2.7 bc
SP	I*	3.1 a	3.3 a	2.4 ab	2.5 ab	2.1 ab	2.3 ab	2.3 ab	3.0 a
W100S	I**	24.1 b	32.8 a	25.8 ab	27.1 ab	29.5 ab	33.0 a	25.3 ab	28.5 ab
WCP	I**	2,407 bc	4,612 a	1,710 c	3,770 ab	2,057 c	3,983 a	2,125 c	4,080 a
	V3**	2.8 a	2.0 b	2.9 a	1.7 bc	1.4 cd	1.0 d	1.3 cd	1.3 cd
A/E	R6**	2.1 b	1.7 bc	2.6 a	1.5 cd	1.1 d	1.3 d	1.8 bc	1.4 cd
	R8*	2.0 a	1.5 bcd	1.8 abc	0.9 e	1.4 d	1.7 abcd	1.9 ab	1.5 cd
	V3**	0.044 b	0.032 c	0.064 a	0.034 bc	0.031 cd	0.021 d	0.030 cd	0.025 cd
A/g _s	R6**	0.054 b	0.039 cd	0.081 a	0.054 b	0.035 d	0.039 cd	0.048 bc	0.038 d
	R8**	0.057 a	0.048 bc	0.070 ab	0.027 e	0.042 cd	0.042 cd	0.053 bc	0.033 de
	V3**	13.3 a	4.8 c	12.2 a	4.6 c	8.8 b	4.3 c	11.0 ab	4.6 c
TB/WCP	R6**	8.7 abc	5.8 e	8.8 ab	6.1 de	7.9 bcd	6.6 cde	10.5 a	6.0 de
	R8**	3.5 bc	2.3 d	4.3 ab	2.7 cd	3.5 bc	2.9 cd	4.7 a	2.7 cd
SYP/WCP	I**	0.98 ab	0.94 ab	1.14 a	0.89 bc	0.66 c	0.64 c	0.80 bc	0.66 c
WF	I**	1,033 cd	1,082 cd	877 d	1,136 bc	1,564 a	1,492 ab	1,305 ab	1,506 a

Pinto Villa (PV), Pinto Saltillo (PS), Bayo Madero (BM), Canario 60 (C60), limited water regimen (20%) and optimal (60%), integral evaluation (I), vegetative stage (V3), reproductive stage (R6), maturity (R8), days to flowering (DF, days), number of pods per plant (PP, No.), seed yield per plant (SYP, g), seeds per pod (SP, No.), weight of one hundred seeds (W100S, g), water consumed per plant (WCP, ml), instant UEA (A/E, $\mu\text{mol CO}_2 \text{ mol H}_2\text{O}^{-1}$), intrinsic UEA (A/g_s, $\mu\text{mol CO}_2 \text{ mmol H}_2\text{O}^{-1}$), integral UEA (TB/WCP, g L^{-1}) and (SYP/WCP, g L^{-1}), water footprint (WF, L kg). Values with the same letter within averages rows are statically equal Tukey ($p < 0.05$), (*) significant differences $p < 0.05$, (**) highly significant differences $p < 0.01$.

Among tested cultivars PS was the most efficient to use water under limited conditions. None difference in the genome of the four cultivars was observed, this indicates that those differences in water-use under limited conditions are not due to presence and/or absence of genomic factors, but rather to their transcription and respective regulation as suggested by Ruiz-Nieto et al. (2015). With the expression profiles generated by RT-PCR and densitometry, the differences in transcription

related to WUE were evaluated and such differential character was confirmed by qPCR (Figure 1).

In the V3 stage the *apx2* gene increased its expression level in the four cultivars, which indicates that it has an important biological role in response to induced oxidative stress in all genotypes, including the response to drought stress (Wilson et al., 2009). *erecta*, *cp2*, and *rpoC2* genes were expressed in response to limited water conditions in PV, the last gene was also differentially expressed in C60.

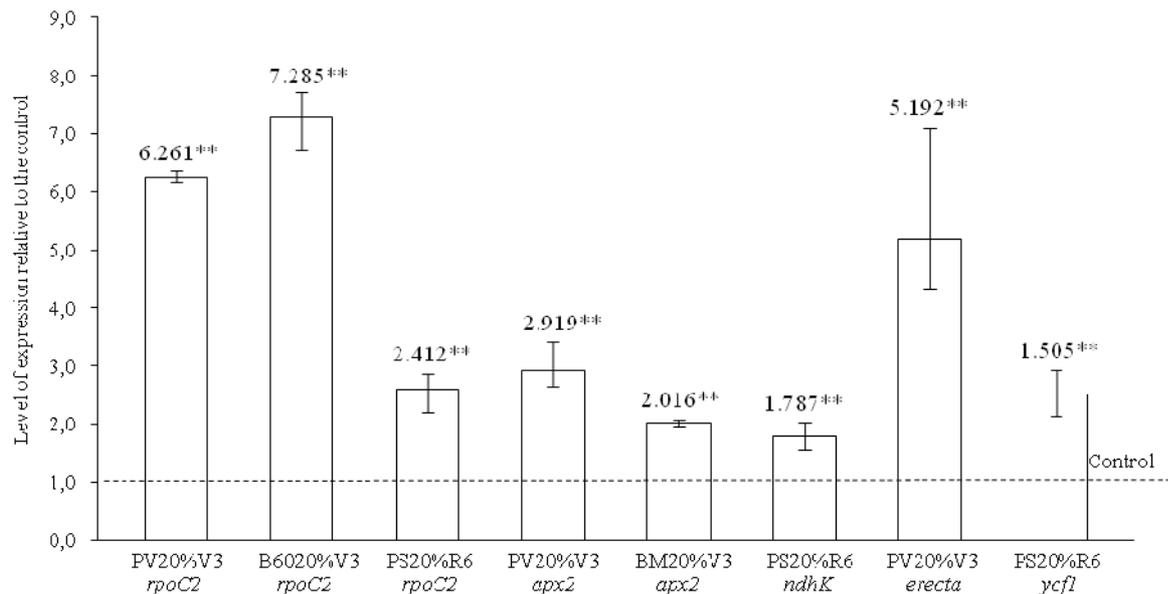


Figure 1. Evaluation of gene expression levels by qPCR. Pinto Villa (PV), Pinto Saltillo (PS), Bayo Madero (BM), Canario 60 (C60), limited water regimen (20%), vegetative stage (V3), reproductive stage (R6), WUE related genes (*rpoC2*, *apx2*, *ndhK*, *erecta*, *ycf1*), expression level in the 60% water regimen for each corresponding condition (control). significant differences $p < 0.05$ (*), highly significant differences $p < 0.01$ (**).

The *cp2* gene was expressed under conditions related to WUE in PS as well as *ycf2* and *rrn16* genes. At the R6 stage none of the studied elements were expressed in all four cultivars; *g564* differentially expressed in C60 and BM, while LR0064 only did in BM. *rpoC2*, *hardy*, *erecta*, *ycf1*, *ndhK*, and *bzip* transcription factor had a higher expression level in PS. The last element was also differentially expressed in PV. According to Recchia, Caldas, Beraldo, Silva, and Tsai (2013) the *bzip* transcription factor is related to flowering and response to abiotic stress, therefore it was expected that *bzip* was expressed differentially in both cultivars at the R6 stage. The T2-3 fragment of unknown function was expressed in response to limited water in C60, BM and PS at the R6 stage; this suggests that its expression is important in the onset of flowering in these conditions (Table 4).

Table 4. Differential gene expressions in the 20% water regimen.

Gen	PVV3	PVR6	PSV3	PSR6	BMV3	BMR6	C60V3	C60R6
<i>ndhK</i>	-	-	-	+	-	-	-	-
<i>rpoC2</i>	+	-	-	+	-	-	+	-
<i>rps19</i>	-	-	-	-	-	-	-	-
<i>rrn16</i>	-	-	+	-	-	-	-	-
<i>rrn23</i>	-	-	-	-	-	-	-	-
<i>ycf1</i>	-	-	-	+	-	-	-	-
<i>ycf2</i>	-	-	+	-	-	-	-	-
<i>cp2</i>	+	-	+	-	-	-	-	-
<i>pol3</i>	-	-	-	-	-	+	-	+
T2-3	-	-	-	+	-	+	-	+
T2-12	-	-	-	-	-	-	-	-
<i>apx2</i>	+	-	+	-	+	-	+	-
<i>erecta</i>	+	-	-	+	-	-	-	-
<i>hardy</i>	-	-	-	+	-	-	-	-
<i>bzip</i>	-	+	-	+	-	-	-	-
LR0064	-	-	-	-	-	+	-	-

Pinto Villa (PV), Pinto Saltillo (PS), Bayo Madero (BM), Canario 60 (C60), vegetative stage (V3), reproductive stage (R6), differential expression related to WUE (+), expression not related to WUE (-).

Although most of the genes that differentially expressed did in the drought tolerant cultivars, the differential expression of *apx2* and *rpoC2* in the V3 stage, of T2-3, LR0064, and *g564* fragments in the R6 stage in susceptible cultivars, indicates that these cultivars also have responsive elements to limited water regimen, such as observed in the instantaneous and integral WUE characterization. However, the susceptible cultivars were not as efficient as the tolerant under that stressful condition. In the characterization of WUE both drought tolerant cultivars were more efficient to use water under limited conditions with respect to susceptible; nevertheless, tolerant cultivars increased their WUE throughout instant and integral mechanisms that were essentially different. Therefore, it was expected that the genetic elements herein characterized had different expression patterns. Results suggests that it is possible to improve the WUE trait by means of assisted

selection with *ycf2*, *rrn16*, *rpoC2*, *hardy*, *erecta*, *ndhK*, and *ycf1* genes, using them as expression markers. Although some elements did not differentially express as it was reported (Ruiz-Nieto et al., 2015), likely since we used a different seed lot with respect to the seed used originally to identify WUE related genes, therefore it would be expected that as the selection of inbreed line proceeds, the number of differential expression elements would increase.

Conclusion

Drought tolerant cultivars displayed different WUE, since under limited water conditions PS was able to modulate its stomatal and keep a high photosynthetic rate with a low transpiration, while PV despite of also having a high CO₂ assimilation, it had a higher conductance and transpiration rate than PS. Consequently, the seed production had a lower water cost in PS. The genes *ycf2*, *rrn16*, *rpoC2*, *hardy*, *ndhK*, *erecta*, and *ycf1* were differentially expressed under limited conditions relative to physiological WUE only in cultivar PS, confirming that the mechanisms for the efficient use of water are essentially different between drought tolerant cultivars.

Acknowledgements

This research was supported by INIFAP-SAGARPA 1225619346 project.

References

- Ahuja, I., de Vos, R. C., Bones, A. M., & Hall, R. R. (2010). Plant molecular stress responses face climate change. *Trends in Plant Science*, 15(12), 664-674. doi: 10.1016/j.tplants.2010.08.002
- Bramley, H., Turner, N. C., & Siddique, K. H. (2013). Water use efficiency. In C. Kole (Ed.), *Genomics and breeding for climate-resilient crops* (p. 225-268). Berlin, Heidelberg, GE: Springer. doi: 10.1007/978-3-642-37048-9_6
- Crimmins, S. M., Dobrowski, S. Z., Greenberg, J. A., Abatzoglou, J. T., & Mynsberge, A. R. (2011). Changes in climatic water balance drive downhill shifts in plant species' optimum elevations. *Science*, 331(6015), 324-327. doi: 10.1126/science.1199040
- Cuellar-Ortiz, S. M., Arrieta-Montiel, M. P., Acosta-Gallegos, J., & Covarrubias, A. A. (2008). Relationship between carbohydrate partitioning and drought resistance in common bean. *Plant Cell & Environment*, 31(10), 1399-1409. doi: 10.1111/j.1365-3040.2008.01853.x
- Devi, K. D., Punyarani, K., Singh, N. S., & Devi, H. S. (2013). An efficient protocol for total DNA extraction from the members of order Zingiberales-suitable for

- diverse PCR based downstream applications. *SpringerPlus*, 2(1), 669. doi: 10.1186/2193-1801-2-669
- Devi, M. J., Bhatnagar-Mathur, P., Sharma, K. K., Serraj, R., Anwar, S. Y., & Vadez, V. (2011). Relationships between transpiration efficiency and its surrogate traits in the rd29A: DREB1A transgenic lines of groundnut. *Journal of Agronomy and Crop Science*, 197(4), 272-283. doi: 10.1111/j.1439-037X.2011.00464.x
- FAOSTAT. (2017). Retrieved on June 9, 2017 from <http://fao.org/faostat>.
- Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Pretty, J., Thomas, S. M., & Toulmin, C. (2010). Food security: the challenge of feeding 9 billion people. *Science*, 327(5967), 812-818. doi: 10.1126/science.1185383
- Hanjra, M. A., & Qureshi, M. E. (2010). Global water crisis and future food security in an era of climate change. *Food Policy*, 35(5), 365-377. doi: 10.1016/j.foodpol.2010.05.006
- Hatfield, J. L., Boote, K. J., Kimball, B. A., Ziska, L. H., Izaurralde, R. C., Ort, D., Thomson, A. M., & Wolfe, D. (2011). Climate impacts on agriculture: implications for crop production. *Agronomy Journal*, 103(2), 351-370. doi: 10.2134/agnonj2010.0303
- Jensen, E. S., Peoples, M. B., Boddey, R. M., Gresshoff, P. M., Hauggaard-Nielsen, H., Alves, B. J., & Morrison, M. J. (2012). Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. *Agronomy for Sustainable Development*, 32(2), 329-364. doi: 10.1007/s13593-011-0056-7
- Jesus, W. C., Vale, F. X. R., Coelho, R. R., & Costa, L. C. (2001). Comparison of two methods for estimating leaf area index on common bean. *Agronomy Journal*, 93(5), 989-991. doi: 10.2134/agnonj2001.935989x
- Kahlon, C. S., Board, J. E., & Kang, M. S. (2011). An analysis of yield component changes for new vs old soybean cultivars. *Agronomy Journal*, 103(1), 13-22. doi: 10.2134/agnonj2010.0300
- Karaba, A., Dixit, S., Greco, R., Aharoni, A., Trijatmiko, K., Marsch, N., Krishnan, A., Nataraja, K., Udayakumar, M., & Pereira, A. (2007). Improvement of water use efficiency in rice by expression of HARDY, an Arabidopsis drought and salt tolerance gene. *Proceeding of the National Academy of Science*, 104(39), 15270-15275. doi: 10.1073/pnas.0707294104
- Logemann, J., Schell, J., & Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. *Analytical Biochemistry*, 163(1), 16-20. doi: 10.1016/0003-2697(87)90086-8
- Molden, D., Oweis, T., Steduto, P., Bindraban, P., Hanjra, M. A., & Kijne, J. (2010). Improving agricultural water productivity: between optimism and caution. *Agricultural Water Management, Comprehensive Assessment of Water Management in Agriculture*, 97(4), 528-535. doi: 10.1016/j.agwat.2009.03.023
- Montero-Tavera, V., Escobedo-Landín, M. A., Acosta-Gallegos, J. A., Anaya-Lopez, J. L., & Ruiz-Nieto, J. E. (2017). 26S: Novel reference gene from leaves and roots of common bean for biotic stress expression studies based on PCR. *Legume Research: An International Journal*, 40(3), 439-433. doi: 10.18805/lr.v0iOF.7859
- Recchia, G. H., Caldas, D. G. G., Beraldo, A. L. A., Silva, M. J., & Tsai, S. M. (2013). Transcriptional analysis of drought-induced genes in the roots of a tolerant genotype of the common bean (*Phaseolus vulgaris* L.). *International Journal of Molecular Science*, 14(4), 7155-7179. doi: 10.3390/ijms14047155
- Reynolds-Henne, C. E., Langenegger, A., Mani, J., Schenk, N., Zumsteg, A., & Feller, U. (2010). Interactions between temperature, drought and stomatal opening in legumes. *Environmental and Experimental Botany*, 68(1), 37-43. doi: 10.1016/j.envexpbot.2009.11.002
- Rosales, M. A., Ocampo, E., Rodríguez-Valentín, R., Olvera-Carrillo, Y., Acosta-Gallegos, J., & Covarrubias, A. A. (2012). Physiological analysis of common bean (*Phaseolus vulgaris* L.) cultivars uncovers characteristics related to terminal drought resistance. *Plant Physiology and Biochemistry*, 56(1), 24-34. doi: 10.1016/j.plaphy.2012.04.007
- Ruiz-Nieto, J. E., Aguirre-Mancilla, C. L., Acosta-Gallegos, J. A., Raya-Pérez, J. C., Piedra-Ibarra, E., Vázquez-Medrano, J., & Montero-Tavera, V. (2015). Photosynthesis and chloroplast genes are involved in water-use efficiency in common bean. *Plant Physiology and Biochemistry*, 86(1), 166-173. doi: 10.1016/j.plaphy.2014.11.020
- Sauer, T., Havlík, P., Schneider, U. A., Schmid, E., Kindermann, G., & Obersteiner, M. (2010). Agriculture and resource availability in a changing world: The role of irrigation. *Water Resources Research*, 46(6), 1-12. doi: 10.1029/2009WR007729
- Shao, H. B., Chu, L. Y., Jaleel, C. A., & Zhao, C. X. (2008). Water-deficit stress-induced anatomical changes in higher plants. *Comptes Rendus Biologies*, 331(3), 215-225. doi: 10.1016/j.crvi.2008.01.002
- Wilson, P. B., Estavillo, G. M., Field, K. J., Pornsiriwong, W., Carroll, A. J., Howell, K. A., ..., Pogson, B. J. (2009). The nucleotidase/phosphatase SAL1 is a negative regulator of drought tolerance in Arabidopsis. *The Plant Journal*, 58(2), 299-317. doi: 10.1111/j.1365-313X.2008.03780.x
- Zlatev, Z., & Lidon, F. C. (2012). An overview on drought induced changes in plant growth, water relations and photosynthesis. *Emirates Journal of Food and Agriculture*, 24(1), 57. doi: 10.9755/ejfa.v24i1.10599

Received on September 8, 2017.

Accepted on December 15, 2017.

License information: This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.