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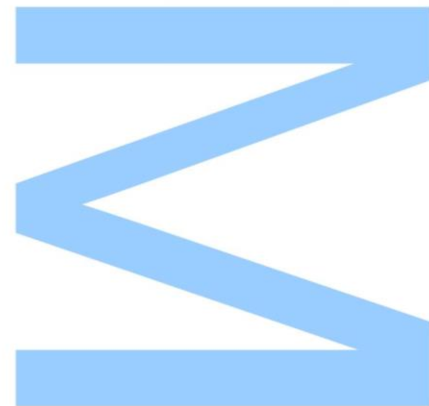
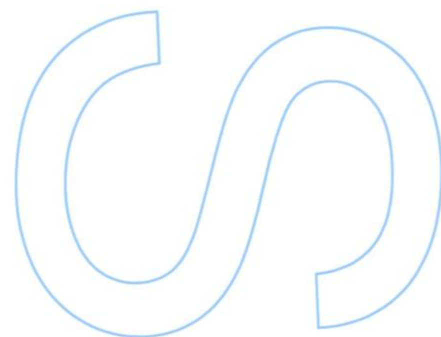
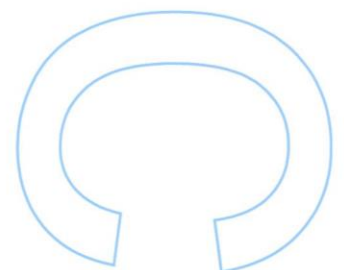
Gene expression in response to drought stress of Portuguese chickpea (*Cicer arietinum* L.) cultivars







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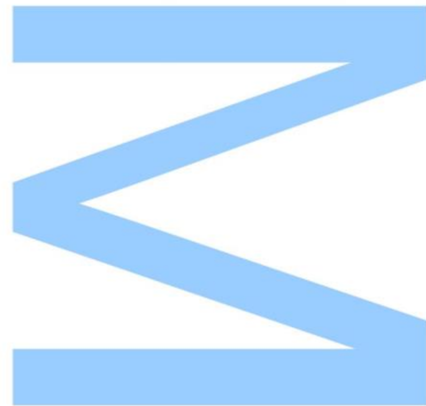

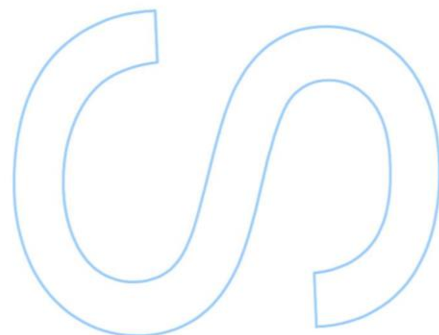

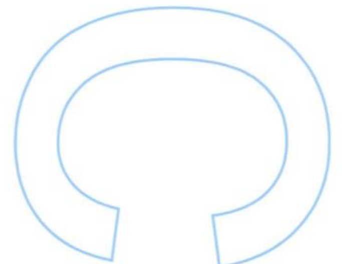

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Abstract

Drought is one of the main threats to the future of agriculture, as it is becoming more occurring, and drought tolerance is important way to overcome this imminent problem. Plant breeding is an important tool in grating abiotic stress tolerance to plants. Chickpea is third most produced pulse in the world and is consumed around the globe. With this work we intend to evaluate the response to drought stress of two Portuguese varieties via agronomic performance and gene expression. Elmo (Desi) and Elvar (Kabuli) were submitted to four treatments: hydric comfort (T1), permanent hydric stress (T2), hydric stress during vegetative stage (T3) and hydric stress during reproductive stage (T4). Development and harvest performance were evaluated and differences in gene expression between stress and control were assessed through qPCR for six genes of interest in leaf sample collected at appearance of first flower. Elmo responded to stress with a better overall production and early maturity, which are traits selected for drought tolerance. Although, Elvar had a higher biomass production, including taller plants and longer roots, which is a desirable trait to avoid drought. During stress, Phosphoenolpyruvate carboxylase kinase-1 was upregulated in Elmo, but not in Elvar. This kinase was previously upregulated in drought-tolerant plants, and overexpression conferred drought tolerance compared to wild-type. Ferredoxin-1 was upregulated in Elvar.but not in Elmo. Ferredoxin-1 is associated with cyclic electron transfer, which induces of non-photochemical quenching. It was concluded that Elmo, that showed a mechanism of drought escape, is more tolerant to drought, and more suitable for drought-tolerance breeding. It was also concluded that drought stress during the reproductive phase (T4) is the best treatment for drought tolerance selection out of the four treatments, due to higher overall variability between varieties across drought tolerance related parameters, and better representation of the stress present in the production regions

Resumo

A seca é uma das principais ameaças para o futuro da agricultura, pois está se a tornar cada vez mais ocorrente, e a tolerância ao stress hídrico é uma maneira importante de superar este problema iminente. O melhoramento vegetal é uma ferramenta importante para conceder tolerância a stress abiótico às plantas. O Grão de bico é a terceira leguminosa de grão mais produzida no mundo e é consumido por todo o mundo. Com este trabalho pretendemos avaliar a resposta ao stress hídrico de duas variedades portuguesas através do desempenho agronómico e da expressão genética. Elmo (Desi) e Elvar (kabuli) foram submetidos a quatro tratamentos: conforto hídrico (T1), stress hídrico permanente (T2), stress hídrico durante a fase vegetativa (T3) e stress hídrico durante a fase reprodutiva (T4). O desempenho de desenvolvimento e de colheita foram avaliados, e as diferenças de expressão genética entre stress e controlo foram avaliadas pela técnica de qPCR para seis genes de interesse em amostras de folhas colhidas ao aparecimento da primeira flor. Elmo respondeu ao stress com uma melhor produção global e maturidade precoce, as quais são características de seleção para tolerância à seca. Contudo, Elvar teve uma maior produção de biomassa, incluindo plantas mais altas e maiores raízes, o que é uma característica desejável para evitar a seca. Durante o stress, a proteína fosfoenolpiruvato carboxilase quinase-1 foi regulada positivamente para a variedade Elmo, mas não para a variedade Elvar. Esta quinase foi previamente regulada positivamente em plantas tolerantes à seca, e o sobre-expressão conferiu à tolerância da seca em comparação ao tipo selvagem. Ferredoxina-1 foi regulada positivamente para a variedade Elvar. mas não para a Elmo. Ferredoxinag-1 está associado com cyclic electron transfer, que induz non-photochemical quenching. Concluiu-se que Elmo, que mostrou um mecanismo de fuga de seca, é mais tolerante à seca, e mais adequado para a melhoramento de tolerância à seca. Concluiu-se também que o stress hídrico durante a fase reprodutiva (T4) é o melhor tratamento para a seleção de tolerância à seca entre os quatro tratamentos, devido à maior variabilidade global entre as variedades para os parâmetros relacionados como tolerância à seca e melhor representação do stress presente nas regiões de produção.

Keywords: drought, gene expression, yield parameters, abiotic stress, plant breeding

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Introduction

Chickpea: *Cicer arietinum* L.

Origin and Taxonomy

Chickpea (*Cicer arietinum* L.) belongs to a group of the *Fabaceae* family and its archaeological remains date back to 10000 BC and were found in south-east Turkey[4, 5] or in nearby regions (north-west Syria) [6], which corresponds to the present distribution of *Cicer reticulatum*, a chickpea ancestor.[7] However, genetic markers were used to assess patterns and levels of genetic diversity within and between accessions, and in addition to center of origin already discussed, Pakistan, Afghanistan, south-east Russia and Lebanon were suggested as centers of origin due to high values of diversity. The newly suggested centers all belong to mountainous areas, where environmental heterogeneity favors specific adaptation.[8]

Morphological description

Duke [9] described chickpea as "Annual herb, erect or spreading, much branched, 0.2-1 m tall, glandular pubescent, olive, middle, dark green or bluish green in color. Root system to 2 m deep, but major portion up to 60 cm. Leaves imparipinnate, glandular-pubescent with 3-8 pairs of leaflets and a top leaflet (rhachis ending in a leaflet); leaflets ovate to elliptic, 0.6-2.0 cm long, 0.3-1.4 cm wide; margin serrate, apex acuminate to aristate, base cuneate; stipules 2-5- toothed, stipels absent. Flowers solitary, sometimes 2-3 per inflorescence, axillary; peduncles 0.6-3 cm long, pedicels 0.5-1.3 cm long, bracts triangular or tripartite, up to 2 mm long; calyx 7-10 mm long; corolla white, pink, purplish (fading to blue), or blue, 0.8-1.2 cm long. Pod rhomboidellipsoid, 1-2 (-4}-seeded, 1.4-3.5 cm long, 0.8-2 cm wide, inflated, glandular-pubescent. Seed color cream, yellow, brown, black, or green, rounded to angular obovoid, 0.4-1.4 by 0.4-1 cm; seed coat smooth or wrinkled, or tuberculate, laterally compressed with a median groove around two-thirds of the seed, anterior beaked; germination cryptocotylar. Flowering summer (Mediterranean, Middle East), winter (India); Fruitification summer-fall (Mediterranean, Middle East), March-April (India)". Many crops from the Fabaceae family, including chickpeas, are great source of soil nitrogen, due to the symbiosis with Rhizobium bacteria, which fixates atmospheric nitrogen in the soil. [10] The entire plant surface, except the corolla, is covered with fine hair known as trichomes, many

glandular that secrete a highly acidic substance containing malic, oxalic and citric acids, which play an important role in plant protection against insects and pests.[11]

Varieties may vary in flower and seed color and size, growth duration, yield, and disease resistances. [9] Desi chickpea's seeds were shown to have a thick seed coat and various shades and combinations of brown, yellow, green and black (Fig.1) This variety presented smaller angular seeds with a rough surface. The flowers were described as pink, with some exceptions presenting white color and plants shown variable anthocyanin pigmentation, although some may not present such pigmentation on the stem. (Fig. 2 and 3) Kabuli

Figure 1 Elmo's (Desi) seeds.

chickpea's seeds were characterized as white or beige-colored, ram's head shape, thin seed coat, smooth surface, white flowers and lack of anthocyanin pigmentation on the stem. (Fig.4) These seeds have higher levels of sucrose, lower levels of fiber, larger size and higher market price when compared to Desi's.[11]

Chickpea seedlings have hypogeal emergence, and emergence occurs 7-15 days after sowing, depending on soil temperature and sowing depth. Its growth habit is indeterminate, in which the plant can keep growing after the start of flowering. Vegetative growth before flowering ranges from 40 to 80 days depending on variety, location, availability of soil moisture and weather conditions. Anther dehiscence occurs inside the bud 24 hours before the opening of the flower, characterizing chickpea's flowers as cleistogamous and self-pollinated. In favorable conditions, it takes about 6 days from fertilization to pod set. After pod set, the pod grows quickly for 10 to 15 days and then seed growth occurs. Following pod growth and seed filling, senescence of subtending leaves begins. If soil moisture levels are high, flowering and podding will continue in the upper nodes. Chickpea's harvesting occurs when 90% of the stems and pods turn light golden yellow.[11]

Figure 2 Elmo's (Desi) flowers.

Figure 3 Elmo's (Desi) stem with anthocyanin pigmentation

Climatic requirement and cultural practices

Chickpea can be grown as a winter crop in the tropics and as a summer or spring crop in the temperate environments. Temperature, day length and availability of moisture are the major abiotic factors affecting flowering. Usually, flowering is delayed under low temperatures and under short days. Chickpea is sensitive to high temperature (>35°C) as well as low temperature(<15°C) during the reproductive stage, leading to drop and reduced pod set.[11] Optimum condition include 18°C-26°C during the day and 19°C-21°C during the night. Optimum relative humidity for seed set is 21-41%.[9] The Best suited soil are deep loams or silty clay loams with pH ranging from 6.0 to 8.0. Fields should be loose tilth and good drainage, because chickpea plants are highly sensitive to poor aeration, and sowing done on conserved soil moisture.[11] Although chickpea can be grown under limited moisture conditions, adequate supply of moisture is needed for proper growth and

development, however irrigation during critical stages, such as vegetative and pod formation, is more important.[12-14]

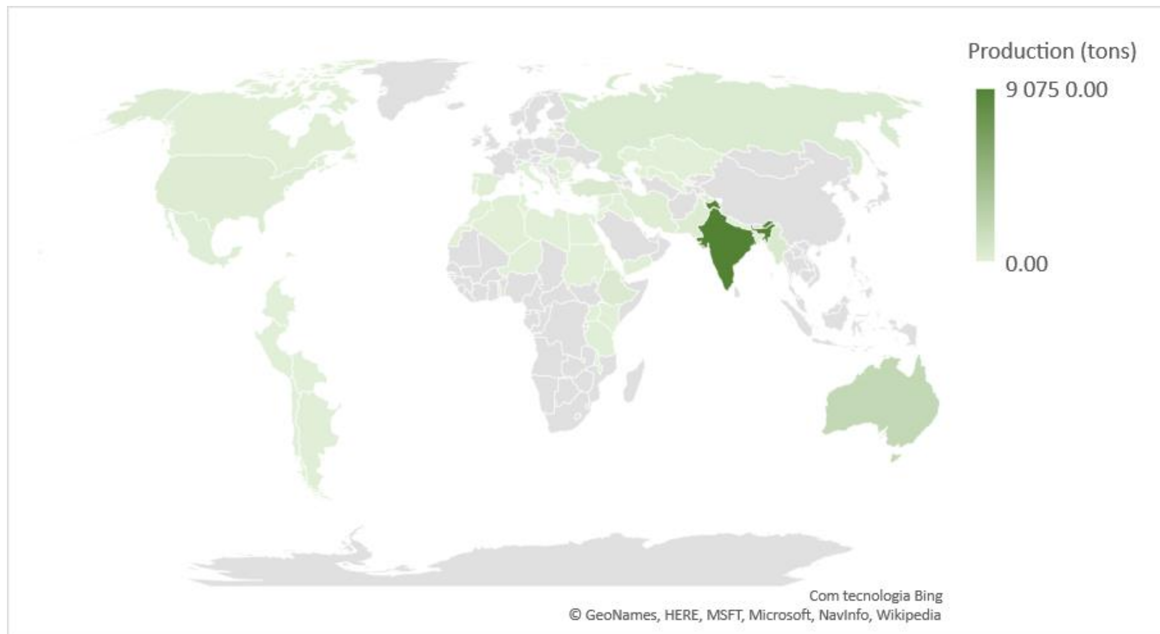
Figure 4 Elvar's (Kabuli) flower.

Production distribution and economic values

Chickpea (*Cicer arietinum* L.) is an important pulse cultivated globally, ranked third in production after beans (*Phaseolus vulgaris* L.) and peas (*Pisum sativum* L.), with 14.77 million tons in 2017 and harvested area of 14.56 million hectares. For 2017, Asia accounted for 73.5% of the world production. India, as the world's largest producer, recorded 9.07 million tons in 9.53 million hectares harvested, followed by Australia, with 2.00 million tons in 1.06 million hectares harvested, and Myanmar, closing the top 3 producers of 2017 with 0.52 million tons in 0.37 million hectares harvested. In Europe, the largest producer is by far the Russian Federation with 0.41 million tons and a harvested area of 0.45 million hectares, second place goes to Spain with 0.05 million tons and 0.05 million hectares harvested and Italy is third with 0.03 million tons and 0.02 million hectares.[1] In Portugal,

production reached 1665 and 1506 tons with 1987 and 1833 hectares of harvested area for 2016 and 2017, respectively.[15]

In 2016, the following production made up a gross production value worldwide of 7740.5 million US dollars, 1.26 US million from Portugal. Still in the year 2016, Portugal imported 10998 tons of chickpea, valued at 11.229 million US dollars, and exported 4916 [1]



Source: [1]

Figure 5 Chickpea worldwide production in 2017

Nutritional value

According to United States Department of Agriculture's National Nutrient Database for Standard reference legacy release, chickpea has a high protein content, around 20% per dry weight¸ and dietary fiber, around 12% per dry weight. Chickpea's amino acid profile is high percentage of branch-chain amino acids, presenting 71.5 mg of leucine per g of protein, 43.1 mg of isoleucine per g of protein and 42.3 mg of valine per g of protein, covering the requirements of 59 mg of leucine per g of protein, 30 mg of isoleucine per g of protein and 39 mg of valine per g of protein, for a consumption of 0.66 g of protein per kg per day, disclosed by World Health Organization. The only limiting essential amino acid in chickpea is methionine, 13.2 mg per g of protein, which the requirement is 16 mg per g of protein, although methionine and cysteine combine requirement of 22 mg per g of protein is achieved.[16, 17] The lipid content is about 6%, for dry weight, mostly composed of

unsaturated fatty acids like linoleic (18:2) and oleic (18:1) acids. [17, 18] Carbohydrate in chickpea represent 10%, for dry weight. Starch content varies from 41% to 50% of total carbohydrates, 65% being available starch. [17, 18] Chickpea is also a good source for minerals such as iron (Fe), magnesium (Mg), manganese (Mn) and zinc (Zn), and vitamins such as thiamin (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), folic acid (B_9), γ- and α-tocopherols (E), β-Carotene, which is converted to vitamin A, plus other carotenoids.[18] Nutritional values are different between Kabuli and Desi varieties. Carbohydrate percentage is valued at 55% and 47.7%, crude fiber 3.9% and 11.2%, fat 5.1% and 3.7%, for Kabuli and Desi respectively.[19]

Health benefits

Many interesting compounds have been identified in chickpea with reported health benefits.[18, 20, 21] Phenolic compounds, anthocyanins and antioxidant peptides are the main compounds with antioxidant properties found in chickpea. This range of compounds includes flavonols, flavone glycosides, oligomeric and polymeric proanthocyanidins, cinnamic acid, salicylic acid, hydroxycinnamic acid, p-coumaric acid, gallic acid, caffeic acid, vanillic acid, ferulic acid, chlorogenic acid and more phenolic compounds as well as non-phenolic organic acids.[22] Isoflavones were also found in chickpea.[23] Though domestic processing reduces some phenolic compounds as well as bioaccessibility. [20]

Compared to wheat- based meal, chickpea led to a smaller glucose and insulin concentration in plasma, proving to have a lower glycemic index then wheat.[24] This property may result from antinutritive compounds and phenolic compounds, that impair starch digestion and glucose absorption, and also chickpea's starch resistance to intestinal lectins, making them a suitable source of energy for diabetic patients.[18, 20] Chickpea-based diet may be used for obesity management, due to its high fiber content, as it was associated with weight loss during a hypocaloric diet, reduction of systolic blood pressure measurements and total cholesterol in humans, with reduction of serum triglycerides and LDL in rats. [18, 20, 25, 26] Chickpea contain various bioactive compounds that have shown anticancerous potential.[18, 20] C-25, antifungal proteins proved to be an antiproliferative agent against human oral carcinoma cells.[27] A Sterol, β-sitosterol, reduced chemical-induced colon tumor counts in rat. [28] Chickpea albumin hydrolysate shown potent antitumor activity in mice inoculated with hepatic carcinoma cells.[29] Furthermore, chickpea flour prevented azoxymethane-induced aberrant crypt foci in CF-1 mice's colon.[30] Chickpea's ability to lower plasma cholesterol level makes it a great food for

cardiovascular disease prevention, since hypercholesteremia is a characteristic of CVD.[18, 20, 31] Angiotensin I-converting enzyme inhibitor peptides have anti-hypertensive and some peptides found in chickpea have inhibitory potential for Angiotensin I-converting enzyme, thus potential for anti-hypertensive, though in vivo and clinical studies are needed to assess bioaccessibility.[32-34] Phytoestrogen found in chickpea like formononetin, biochanin A, genistein, and daidzein shown potential as therapeutic agents in diabetes, androgen related cancer and estrogen deficiency diseases.[18, 20]

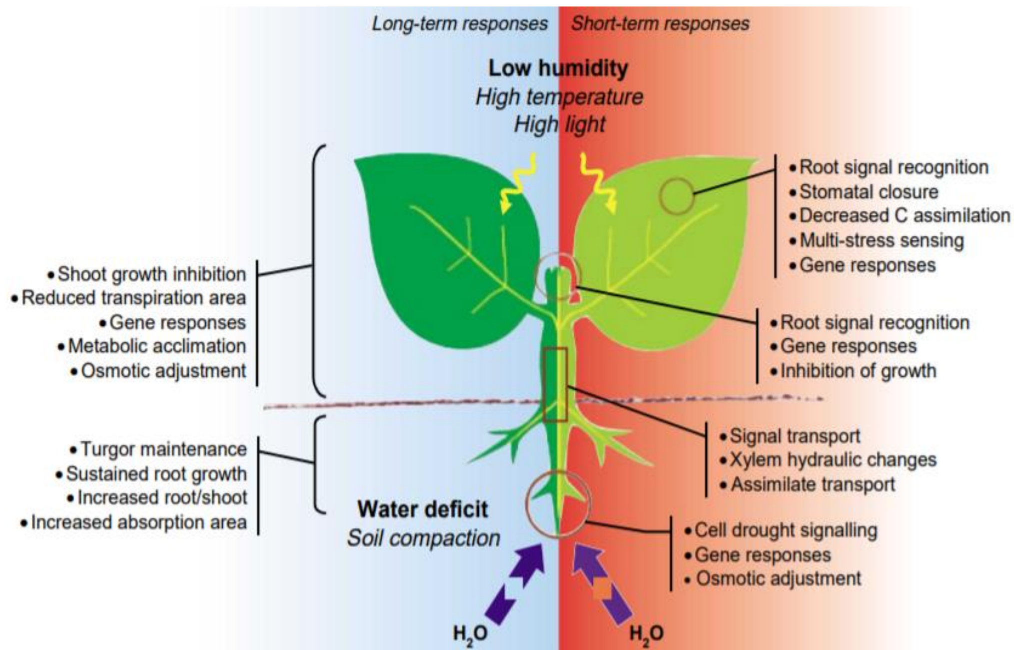
Drought importance

Drought is projected to become more prevalent in the future due to climate change. It affects the carbon cycle, reducing terrestrial ecosystem productivity, increases tree mortality. These effects of drought result in more exacerbating climate changes and higher drought stress.[35]

Whole plant response

Drought stress triggers biochemical and physiological changes in plants, such as stomatal closure [36, 37], cellular growth inhibition[38], diminishing of photosynthesis [reviewed in 39] and increase of photorespiration (Fig. 6) [40].

To overcome the water deficit, plants use several mechanisms: drought escape (earliness and finishing the life cycle before water reduction), drought avoidance (water loss reduction via stomata and water uptake augmentation via root), drought tolerance (osmotic adjustment via phyto-hormones and osmoregulation), drought resistance (changing metabolic pathway like antioxidant metabolism), drought abandon (loss of plant part) and drought adaptation (adaptation via genetic mutation and modification for long stress periods). [41, 42] Plants accumulate organic osmolytes and inorganic ions to maintain cell turgor and water uptake. Organic osmolytes include sugars (e.g. sucrose) [Reviewed in 43], polyols (e.g. mannitol) [44], amino acids and derivatives (e.g. proline)[45], quaternary methylated ammonium compounds (e.g. glycine betaine) [46], tertiary sulfonium compounds (e.g. Dimethylsulfoniopropionate (DMSP)) [reviewed in 43]. Potassium uptake has been detected during drought tolerance, which may be related to initial osmoregulation.[47]. In addition, increase in proline content, decrease in chlorophyll content, stomatal closure and diminishing of photosynthesis rate [Reviewed in 37, 48], were detected in chickpea.



Source: [2]

Figure 6 "Whole-plant responses to drought stress. Left, long-term or acclimation responses; right, short-term responses."

Functional molecules

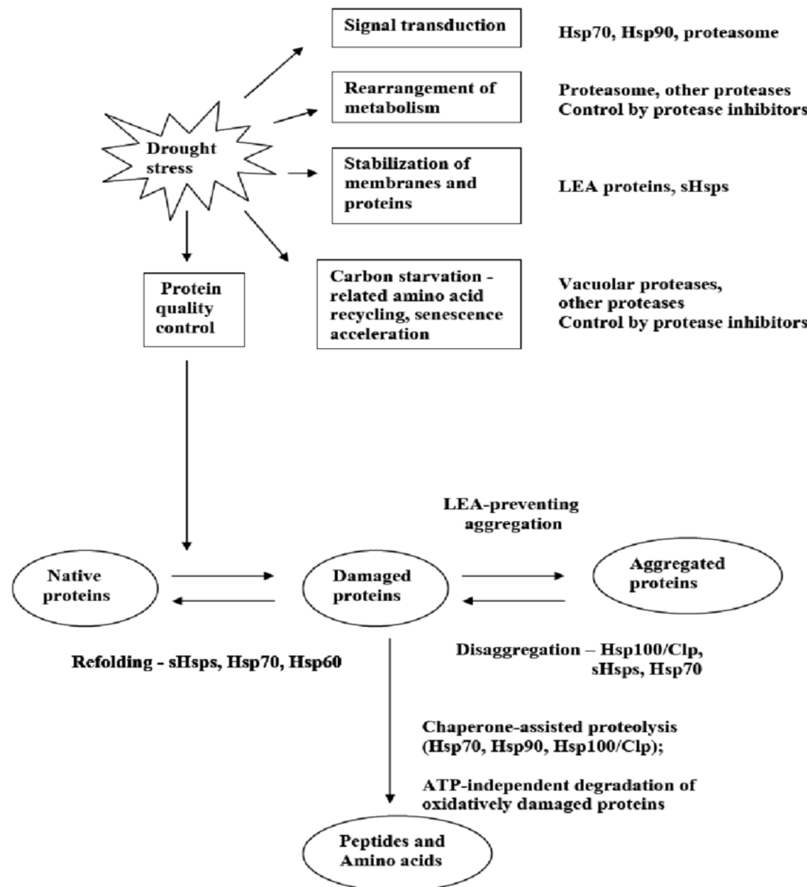
During dehydration, protective proteins of macromolecules are produced, such as late embryogenesis abundant proteins (LEA) and heat shock proteins (HSP)[49].

LEA proteins are mostly hydrophilic proteins expressed in the seeds of many plants, where they were first found, but also in other plant organs, as well as in many other organisms [50]. According to Hundertmark and Hincha [51], they can be divided into 9 groups, found in Arabidopsis thaliana. LEA proteins play roles mainly in dehydration tolerance and storage of seeds, and in whole-plant stress resistance to abiotic stress, such as drought, salt, and cold. Their peptide sequence is mainly composed of hydrophilic amino acids. As for the structure, it changes according to the family, some present amphipathic α-helix involved in ion fixation, others have specific structures for chaperone like functions or are randomly coiled in aqueous solution [Reviewed in 37, 52, 53]. For instance in chickpea, LEA4 has been reported to be strongly induced by drought, salt, heat, cold, abscisic acid (ABA), indole-3-acetic acid (IAA), Gibberellin (GA3) and methyl jasmonate (MeJA).[54], whereas LEA1 and LEA2 have upregulation in response to salinity stress. [55]

HSP are organized into five major families based on their approximate molecular weight. They can be found in the cytosol and many organelles, such as endoplasmic reticulum, chloroplasts, mitochondria and nucleus.[Reviewed in 56, 57]. HSPs can be found both in plants and animal cells, in which they undertake a chaperone function during abiotic and biotic stress. HSPs are responsible for protein folding, assembly, translocation and degradation, assistance of protein refolding under stress conditions and stabilization of proteins. They also may play a regulatory role in the innate immune response in plant cells. HSP 70 and HSP 91 have been found in large-scale transcriptome analysis in chickpea [58].

Proteases and protease inhibitors also play their role in organ senescence for protein remobilization during drought stress. Based on the amino acid residue at the active site that is directly involved in peptide bond hydrolysis, proteases can be classified into aspartic, cysteine, serine and threonine peptidases [Reviewed in 3]. Cysteine endopeptidases are the most reported proteases influenced by drought, usually induced [59, 60]; furthermore, the involvement of aspartic proteases, serine endopeptidases and aminopeptidases in response to drought has been reported. Proteases act mainly inside the vacuoles during macromolecules degradation and defense against pathogens. In the cytosol and nucleus proteolysis occurs to eliminate misfolded, damaged and/or regulatory proteins. These enzymes act via rearrangement of metabolism through selective degradation of key enzymes and/or degradation of short-lived proteins involved in cell signaling, removal of oxidatively damaged, improperly folded or irreversibly denatured proteins, recycling of carbon-starvation-related amino acids and hastening of senescence under source-sink regulation, protection against potential biotic stress [Reviewed in 3]. Protease inhibitors may act in the inhibition of proteases activated on water deficit, osmoprotection, resulting from their highly hydrophilic nature, and defense against biotic stress caused by viral, bacterial or fungal pathogens, nematodes or herbivorous arthropods during the period of reduced growth under drought conditions [Reviewed in 3].

Cross-talk between LEA proteins, HSPs, proteases and proteases inhibitors during the response to drought stress has been reported. These functional proteins act in unison, in addition, gene expression studies pointed to simultaneous up-regulation under drought stress. (Fig. 7)



Source: [3]

Figure 7 “Drought stress response and cross-talk between dehydrins, chaperones (HSPs), proteases and protease inhibitors in maintaining cell protein conformation and function.”

Besides these functional proteins, ABA transporters, aquaporins, ion channels and pumps, amino acids and quaternary ammonium compounds transporters, sugar and sugar alcohols transporters, play an important role as transmembrane channels and transporters in drought resistance mechanisms [Reviewed in 61].

Reactive oxygen species (ROS) are produced under normal condition, however during prolonged drought stress, a certain level of phytotoxicity can be reached causing oxidative damage to cellular components, such as cellular membranes.

The chloroplast is the organelle with higher production of such species due to the water photolysis, in which electrons are extracted from the water molecule and transferred across the electron transport chain, where electron “leakage” can occur, resulting in ROS. The major ROS scavenging enzymes are superoxide dismutase (that dis-mutates superoxide into molecular oxygen or hydrogen peroxide), catalase (that catalyzes hydrogen peroxide to molecular oxygen and water), and enzymes and metabolites from the ascorbate-glutathione cycle (same as catalase). ROS may also play a role in signaling during drought [Reviewed in 37, 62]. The scavenging enzymes described previously have been identified in chickpea submitted to drought [63, 64].

Regulatory proteins

In addition to the functional mechanisms involved in drought stress response, another group of molecules, the regulatory proteins, is also involved in the drought-responsive network. This group includes all the protein factors involved in the regulation of signal transduction and gene expression that act during the stress response. The regulatory proteins include: transcription factors (TFs), protein kinases, phosphatases, enzymes involved in phospholipid metabolism, ABA biosynthesis and other signaling molecules [65].

TFs act by binding to cis-regulatory elements in the promoter region of different stress-related genes. The major cis-acting regulatory elements are ABA-responsive element (ABRE) and dehydration-responsive/C repeat (DRE/CRT) that are a binding site of TFs in ABA-dependent and ABA-independent gene expression, respectively [65-68].

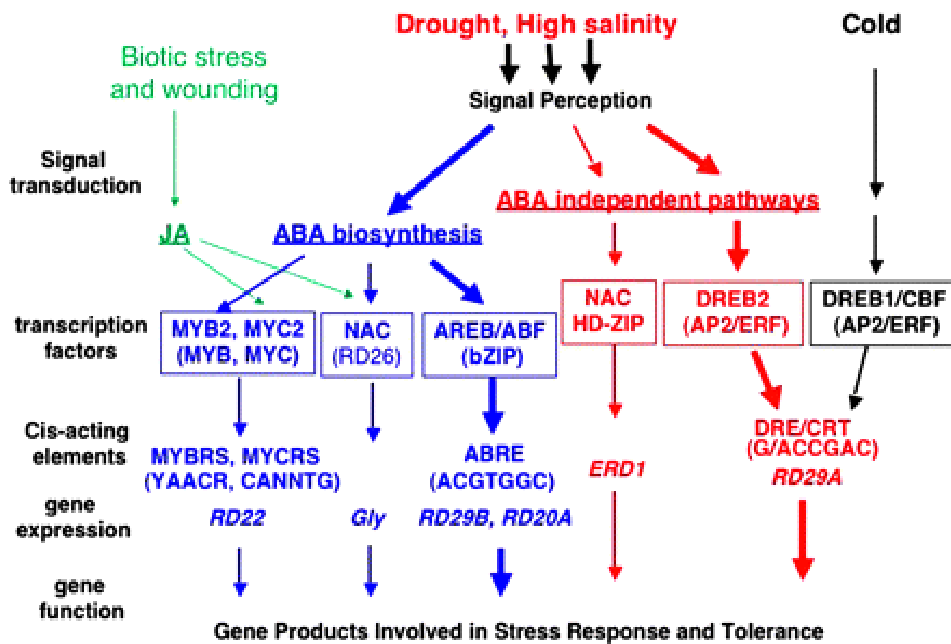
Besides these many other cis-acting regulatory elements are involved in stress-responsive gene expression. Table 1 summarizes both the cis-acting elements and their respective transcription factors. A single copy of ABRE is not sufficient for ABA-responsive transcription, it may require coupling elements CE1 and CE3 or two ABRE sequences to induce ABA-responsive expression of genes [66]. Basic-domain leucine zipper (bZIP) transcription factors or ABRE-binding proteins (AREB) or ABRE-binding factors (ABFs) bind to ABRE and activate ABA-dependent gene expression. Activation of AREB/ABF proteins requires an ABA-mediated signal, like ABA-dependent phosphorylation via type-2 SNF1-related protein kinases (SnRK2-type) [58, 69].

Other cis-acting regulatory elements are MYCR and MYBR, to which MYC-like basic helix-loop-helix (bHLH) transcription factor and MYB transcription factor bind, respectively [66, 68, 70].

NAC transcription factor expression is induced by cold, high salinity, drought, ABA, and MeJA, and they bind to NAC recognition site (NACR) [66, 68, 71].

DRE/CRT are cis-acting regulatory elements involved in water deficit and cold. DRE binding proteins (DREB)/C-repeat-binding factor1 (CF1) belong to APETALA2/Ethylene Response Element Binding Factors (AP2/ERF) family bind to DRE/CRT elements. DREB2s are involved in drought-responsive gene expression and DREB1s are involved in cold-responsive gene expression [68, 72, 73].

Cross-talk between DRE and ABRE has been identified, showing that DRE may act as a coupling element of ABRE [66]. Figure 8 (Shinozaki and Yamaguchi-Shinozaki, 2007) sums up all the transcription factors involved in drought stress response. These TFs were also found in drought-responsive gene expression in chickpea [58, 68, 74]



Source: (Shinozaki and Yamaguchi-Shinozaki, 2007)

Figure 8 Transcriptional regulatory networks of abiotic stress signals and gene expression

Table 1 Cis-Acting regulatory elements in osmotic- and cold-stress-responsive gene expression.[1]

Cis element	Sequence	Type of transcription factors that bind to *cis* elements	Gene	Stress condition	References
ABRE	PyACGTGGC	bZIP	Em, RAB16	Water deficit, ABA	[75]
CE1	TGCCACCGG	ERF/AP2	HVA1	ABA	[76, 77]
CE3	ACGCGTGCCTC	Not known	HVA22	ABA	[78, 79]
ABRE	ACGTGTC	bZIP	Osem	ABA	[78, 79]
ABRE	ACGTGGC, ACGTGTC	bZIP	RD29B	Water deficit, ABA	[80]
MYBR	TGGTTAG	MYB	RD22	Water deficit, ABA	[81, 82]
MYCR	CACATG	bHLH	RD22	Water deficit, ABA	[81, 82]
DRE	TACCGACAT	ERF/AP2	RD29A	Water deficit, cold	[83-85]
CRT	GGCCGACAT	ERF/AP2	Cor15A	Cold	[85, 86]
LTRE	GGCCGACGT	ERF/AP2	BN115	Cold	[87]
NACR	ACACGCATGT	NAC	ERD1	Water deficit	[88]
ZFHDR	Not yet reported	ZFHD	ERD1	Water deficit	[88]
ICRr1	GGACACATGTCAGA	Not known	CBF2/DREB 1C	Cold	[89]
ICEr2	ACTCCG	Not known	CBF2/DREB 1C	Cold	[89]

Other regulatory proteins involved signal translation [37] are: mitogen activated protein kinase(MAPK), that are serine/threonine kinases able to phosphorylate a wide range of

[1] Table obtained from 66. Yamaguchi-Shinozaki, K. and K. Shinozaki, *Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters.* Trends in Plant Science, 2005. **10**(2): p. 88-94.

substrates, including other kinases and/or transcription factors [90]; SNF-1-Like Kinases (SnRKs), that belong to the SNF1/AMPK family and are involved in ABA-dependent stress response by activating TFs [69]; protein phosphatase 2C (PP2C) known as a negative regulator of ABA signaling pathway [91]; phospholipases cleave phospholipid that act as second messengers, like phospholipase C (PLC) that produces inositol triphosphate and diacylglycerol, resulting in a increase of cytosolic calcium in guard cells [37, 92].

Aims

This work focused on analyzing drought effects in the transcriptome of two Portuguese chickpea cultivars, Elmo (Desi) and Elvar (Kabuli), submitted to four drought stress conditions. Both agronomic performance and analysis of transcription activity for genes involved in response to drought stress and affected by dehydration provide new information about the cultivars being studied, specifically on their ability to overcome such adversity through activation of stress inducible genes. Summed up, this study aimed to obtain valuable information about the cultivars, mainly their characteristics regarding drought stress tolerance.

Material and method

Plant material and stress treatment

The main proposal of this investigation was the characterization of the chickpea local cultivars' transcriptomes, through qPCR. This study produced essential information to be further used in chickpea breeding for drought tolerance.

The two Portuguese chickpea cultivars used in this study, were bred at the Estação Nacional de Melhoramento de Plantas, in Elvas, Portugal (INIAV): Elmo (Desi type) and Elvar (Kabuli type), characterized as intermediate to late maturity and early to intermediate maturity, respectively. This study was conducted in a greenhouse, at *Campus* de Vairão of

the University of Porto. Plants were grown in individual 9 L pots containing 5 kg of soil mix consisting of 2 dirt:1 turf and 5 g of phosphate.

Figure 9 Elmo (Desi) plant during development.

A complete randomized design was used to evaluate 2 cultivars to 4 treatments (T_1, T_2, T_3 and T_4) in 4 replicates, totalizing 32 plants. The four drought treatments are control (T_1), permanent hydric stress (T_2), hydric stress induction during the vegetative stage (T_3), hydric stress induction during the reproductive stage (T_4). The water volume for each stress treatment condition was calculated based on the volume of water in each pot with soil mix at field capacity (V_{fc}) using the following equation:

$$V_{fc} = \frac{m_{total} - m_{dry}}{\rho_{water}} \qquad \text{(Eq. 1)}$$

V_{fc} – water volume in the pot at field capacity;

m_{total} – total weight of the pot filled with soil mix at field capacity;

m_{dry} – total weight of the pot filled with dry soil mix;

ρ_{water} – water density (1 kg/dm^3).

The average dry weight (m_{dry}) of 2 pots filled with 5 kgs of soil mix, was recorded after soil preparation. Then for soil mix field capacity determination, the same two pots with dry soil

mix were over irrigated with 1.5 L of water and drained overnight. Afterwards the weight was recorded (m_{total}). Average water volume present in soil mix at field capacity (V_{CC}) was calculated by subtracting m_{dry} from m_{total}.

$$m_{total} = \text{pot weight} + \text{soil weight at field capacity} \qquad \text{(Eq. 2)}$$

$$m_{seco} = \text{pot weight} + \text{dry soil} \qquad \text{(Eq. 3)}$$

$$V_{CC} = m_{total} - m_{seco} \qquad \text{(Eq. 4)}$$

Based on this information, the amount of water applied to each water stress condition was:

Hydric stress: 30% field capacity

Control: 65% field capacity

Each pot was covered with aluminum foil to prevent water loss by evaporation.

Twelve leaves were collected at 4 sampling times (early flowering, one week after stress treatment change, pod set and dry seed) for each cultivar and stress condition and stored at -80ºC until DNA and RNA extraction. The sampling early flowering was used in this study.

Figure 10 Elvar (Kabuli) plants during development.

Agronomic and morphological parameters

Average greenhouse temperature

For the duration of this experiment, temperature was recorded in Celsius, at 9:00am and 5:00pm. Monthly average greenhouse temperature was calculated.

Growth and development

The following growth and development parameters were recorded weekly:

- Plant height: length from stem base to the tip of main stem
- Phenological Stage: classification accordingly to Legume ipmPIPE (Table 2)
- Days to first flower: DAS to first flower appearance

Harvest

The following morphological and yield parameters were recorded at harvest:

- Plant height: length from stem base to the tip of main stem
- Biomass weight: dried plant weight after 48 hours in a drying chamber at 65 °C
- Root length: length of stretched root post cleaning
- Root weight: weight of root including *Rhizobium* clusters
- Number of main stem's ramifications
- Number of pods per plant
- Pod weight per plant
- Individual pod weight: quotient of pod weight per plant over the number of pods per plant
- Number of *Rhizobium* clusters per plant
- Number of seeds per plant
- Seed weight per plant
- Harvest Index: quotient of seed weight over full plant weight

Table 2 Chickpea's growth stages.[2]

Growth phase	Phenological stage	Description
Vegetative	VE	Seedling emergence
	V1	First multifoliate leaf has unfolded from the stem
	V2	Second multifoliate leaf has unfolded from the stem
	V3	Third multifoliate leaf has unfolded from the stem
	V4	Fourth multifoliate leaf has unfolded from the stem
	Vn	Nth multifoliate leaf has unfolded from the stem
Reproductive	R1	Early bloom, one open flower on the plant
	R2	Full bloom, most flowers are open
	R3	Early pod visible
	R4	Flat pod, pod has reached its full size and is largely flat.
	R5	Early seed, seed in any single pod fill the pod cavity
	R6	Full seed, all seeds fill the pod cavity which is rounded
Physiological Maturity	R7	Leaves start yellowing and 50% of the pods have turned yellow
	R8	90% of pods on the plant are golden-brown

Gene Expression

For this analysis, leaf samples were collected of two varieties from two treatments, at appearance of first flower. Six genes of interest and two housekeeping genes were used for relative gene expression evaluation via qPCR.

[2] Adapted from 93. Schwartz, H.F., and Langham, M. A. C. Ann. Rept. Bean Improv. Coop. 51:4-5. *PIPE – Pest Information Platform for Extension and Education.* 2008, 93. Ibid.

RNA with PureZOL^{TM} RNA isolation reagent

RNA was extracted, following the instruction of PureZOL^{TM} RNA isolation reagent's instruction manual, on three biological replicates of chickpea leaf samples collected at early flowering.

The following reagents were used for RNA extraction: liquid nitrogen, PureZOL^{TM} RNA Isolation Reagent from Bio-Rad®, 97% ethanol ACS grade from AppliChem®, Centrifuge 5415 D from Eppendorf®, Chloroform ACS grade from Sigma Aldrich®, Isopropyl alcohol from Sigma Aldrich® , RNase-free water (DEPC-treated water) from Sigma Aldrich®, 75% ethanol (prepared in DEPC-treated water), 0.1M NaOH 1mM EDTA plastic ware cleaning solution, and RNase ZAP.

All material used was either rinsed in 0.1 M NaOH 1 mM solution, followed by several rinses in DEPC-treated water or autoclaved. Working surface and micropipettes were cleaned and wiped with RNase ZAP.

About 100 mg of frozen tissue was weighed in a 2 mL polypropylene tube, previously dipped in liquid nitrogen, followed homogenization with periodical addition of liquid nitrogen to avoid thawing. Tissue was processed into a powder and the tubes were kept in ice until addition of PureZOL^{TM} reagent. Then, 1 mL of PureZOL^{TM} was added to each tube, mixed with the homogenized tissue and incubated 5 minutes at room temperature. Afterwards, the lysate was centrifuged at 12,000 g for 10 minutes to remove insoluble debris from the disruption step and the supernatant was transferred to new 2 mL tube. Per 1 mL of PureZOL^{TM} used in the previous step, 0.2 mL of chloroform was added to the tube, followed by 15 seconds of vigorous shaking and a 5 min incubation at room temperature, occasionally mixing the samples. Next, a centrifugation at 12,000 g for 15 minutes separated the mixture into 3 phases, an upper, colorless aqueous phase, a white interphase and a lower, red organic phase. RNA was exclusively in the aqueous phase, while DNA and proteins remained in the interphase and organic phase. The aqueous phase was transferred to a new 2 mL tube without disturbing the interphase, 0.5 mL of isopropyl alcohol per 1 mL of PureZOL^{TM} was added and the mixture was incubated 5 minutes at room temperature. After that, the mixture was centrifuged at 12,000 g for 10 minutes and the supernatant was discarded, leaving a white pellet of RNA in the tube. Then 1 mL of 75% ethanol was added for each mL of PureZOL^{TM} to wash the pellet, the sample was vortexed, followed by a centrifugation at 7,500 g for 5 minutes. After discarding the supernatant, the pellet was air-dried for 5 minutes and re-suspended in 100µL of DEPC-treated water. The RNA sample was separated in 4

7aliquots of 23µL, stored at - 80°C until RT-qPCR was performed, and 1 aliquot of 8µL stored at -20°C until the northern blot and nanodrop analysis were executed.

RNA separation by electrophoresis

Electrophoresis was performed to evaluate integrity of the isolated RNA in a 1% agarose gel. RNA was first denatured, placing 4µL of RNA solution at 90°C in a thermocycler for 1 min and then on ice. 1 µL of stock ethidium bromide (10 mg/mL) was had to each sample as loading dye, to a final concentration of 0.2µg/µL. The samples and the Ladder were loaded into the gel. Electrophoresis was run until RNA migrated 2-3cm into the gel. The molecules were read with UV light and photographed. (Fig.33)

RNA extractions were repeated when 28S rRNA/18S rRNA ratio was lower than 1.8. [94-96]

The material and equipment used for the RNA separation procedure was the following: TAE 1x mixture, ethidium bromide, agarose, agarose gel rig, electrophoresis tray, power supply, microwave, 1kb DNA Ladder, Gel Doc XR+ from Bio-Rad® and thermocycler Bio-rad, CFX96.

RNA Concentration

Acid nucleic concentration, A260/280 ratio was obtained by Nanodrop™, using 1µL of RNA per sample. RNA extracts with a A260/280 ratio equal or higher than 1.7 were selected for cDNA synthesis.[97]

Bio-Rad® iScript™ cDNA Synthesis

For cDNA synthesis was perfomed using iScript™ cDNA Synthesis Kit from Bio-Rad®. For the reaction was used, 1µg of RNA, 4µL of 5x iScript Advanced Reaction Mix, 1µL of iScript Reverse Transcriptase and Nuclease-free water to 20µL were mixed together. The thermal cycling protocol was 20 min at 46°C for reverse transcription and 1 min at 95°C. The cDNA synthesized was stored -20°C.

Quantitative Real-Time PCR

Quantitative Real-Time PCR (qPCR) was used to quantify gene expression using chickpea reference and target genes in three biological replicates and three technical replicates of the different treatment combinations, using the delta-delta Ct method ($2^{-\Delta\Delta Ct}$ method).[98].

Two housekeeping genes were used for qPCR normalization: glucose-6-phosphate dehydrogenase (G6PD), and TIP41-like protein (TIP41), which were previously validated [74]. Six genes of interest involved in photosynthesis reactions, plant development, basic metabolic reactions and response to oxidative stress were select from transcription analysis. (Table 3) The genes and respective primers were selected from previous studies. [74, 99] (Annex I)

Table 3 List, designation and function of six qPCR genes of interest.

Gene of Interest Primer	Designation	Function	References
Ca_03790	*Cicer arietinum* phosphoenolpyruvate carboxylase kinase 1 [PPCK1]-like	Light-dependent phosphoenolpyruvate carboxylase phosphorylation	[100]
Ca_08236	*Cicer arietinum* arabinogalactan [AGP] protein 14	Development, root hair length and density, seed set, and senescence	[101]
Ca_15236	*Cicer arietinum* NAC domain-containing protein (NAC20)	Regulation of the transcriptional reprogramming associated with plant stress responses	[102]
Ca_05907	*Cicer arietinum* zinc finger CCCH domain-containing protein 20	Stimulation of plant oxidative stress signal pathway	[103]
Ca_20991	*Cicer arietinum* chlorophyll a-b binding protein 7, chloroplastic	Light-harvesting complex and photoprotective non-photochemical quenching	[104]
Ca_00047	*Cicer arietinum* ferredoxin	transfer electrons in a wide variety of metabolic reactions, including photosynthesis	[105]

For each variety, 3 biological replicates for two treatments (control and stress) at flowering, were selected based on A260/280 ratio and nucleic acid concentration.

Sso Advanced™ SYBR® Green Supermix from Bio-Rad® was used during qPCR procedures with a concentration of 1x. Forward and reverse primers were used at 0.25µM. A calibration assay was made from consecutive cDNA dilution of 1:5, starting at 50ng/µL. Assays cDNA concentration was 2 ng/µL with dilutions using RNA nanodrop reads as reference. (Fig. 34)

The reaction setup per well was 5 µL of Sso Advanced™ SYBR® Green Supermix, 250 nM of forward and reverse primers, 2 ng of cDNA and Nuclease-free water to 10 µL. Mastermix for each gene enough for all reactions was prepared by adding Sso Advanced™ SYBR® Green Supermix, forward and reverse primers and Nuclease-free water. Then 9 µL of each mastermix was added to the respective wells and then 1 µL of cDNA was added to the mastermix.

The thermal cycling protocol was the following: Polymerase activation, 30 seconds at 98°C, then denaturation, 15 seconds at 95ºC, annealing/extension and plate read, 30 seconds at 60°C, for 40 cycles. Melting curve analysis was performed from 65°C to 95°C at 0.5°C increment at 5 seconds.

Statistical Analysis

Statistical analysis was performed with SAS University Edition software. Duncan's new multiple range test was used to determine significant differences between means of the analyzed variables, with a maximum significance level of 5% (different letters represent statistical differences; * - represents statistically significance).

The tested model for days to flowering was the following:

$$y_{ijk} = P_i + T_j + V_k + P_i \times T_j + P_i \times V_k + T_j \times V_k + \varepsilon_{ijkl} \quad \text{(Eq.6)}$$

y_{ijk} = Days to flowering;

P_i = Plant (i=1,2,3,4);

T_j = Treatment (j=1,2,3,4; T1=1, T2=2, T3=3, T4=4);

V_k = Variety (k=1,2; Elmo=1, Elvar=2);

ε = Error.

The tested model for all dependent variables relative to harvest was the following:

$$y_{ijk} = P_i + T_j + V_k + P_i \times T_j + P_i \times V_k + T_j \times V_k + \varepsilon_{ijkl} \quad \text{(Eq.7)}$$

y_{ijk} = Dependent variable being studied;

P_i = Plant (i=1,2,3,4);

T_j = Treatment (j=1,2,3,4; T1=1, T2=2, T3=3, T4=4);

V_k = Variety (k=1,2; Elmo=1, Elvar=2);

ε = Error.

The tested model for ΔCt for each housekeeping gene by primer was the following:

$$y_{ijk} = R_i + P_j + T_k + V_l + R_i \times P_j + R_i \times T_k + R_i \times V_l + P_j \times T_k + P_j \times V_l + T_k \times V_l + \varepsilon_{ijkl} \quad \text{(Eq.8)}$$

y_{ijk} = ΔCt for each housekeeping ;

R_i = Technical replicate (i=1,2,3)

P_j = Biological replicate (j=1,2,3);

T_k = Treatment (k=1,2; Control=1, Stress=2);

V_l = Variety (l=1,2; Elmo=1, Elvar=2);

ε = Error.

The tested model for ΔCt for each housekeeping gene by primer and variety was the following:

$$y_{ijk} = R_i + P_j + T_k + T_k \times P_j + \varepsilon_{ijkl} \quad \text{(Eq.9)}$$

y_{ijk} = ΔCt for each housekeeping ;

R_i = Techinal replication (i=1, 2, 3)

P_j = Plant (j=1,2,3);

T_k = Treatment (k=1,2; Control=1, Stress=2);

ε = Error.

The results will be presented in relative transcript abundance ($-\Delta\Delta Ct = -(\Delta Ct_{target} - \Delta Ct_{calibrator})$).

Results

Agronomic and morphological parameters

Monthly average temperature

The monthly average temperature in the greenhouse varied (Fig. 11), during chickpea's development from: 22.5°C in November 2018, 19.7°C in December 2018, 17.4°C in January 2019, 25.5°C in February 2019, 26.8°C in March 2019, 23.6°C in April 2019, and 27.2°C in May 2019.

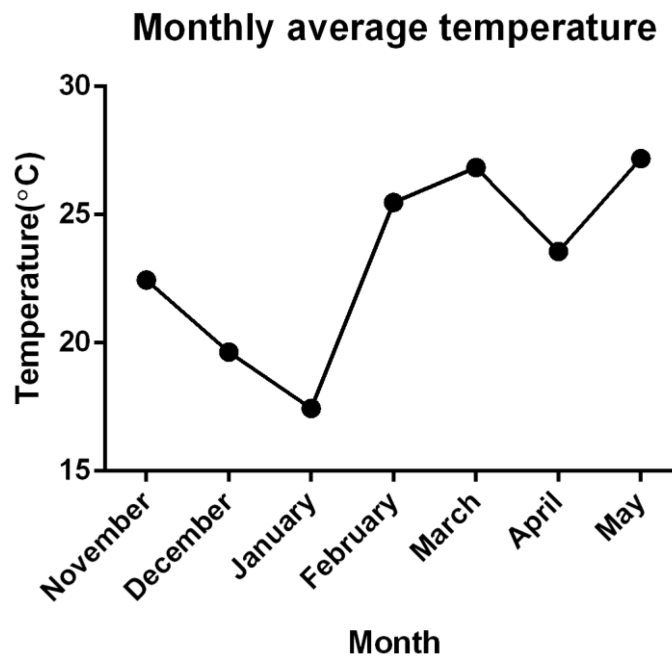


Figure 11 Monthly average temperature during chickpea's development.

These temperatures were within the optimum recommended temperature during daytime, for chickpea development, which varies between 18°C and 26°C. [9]

Growth and development

Average Height

Figures 12 and 13 present sigmodal curves for plant height of two cultivars of chickpea, Elmo and Elvar, respectively, submitted to four treatments, T1 (hydric comfort), T2 (hydric stress), T3 (hydric stress before flowering) and T4 (hydric stress after flowering).

In Elmo, no evident variation in plant height can be detected for the vegetative stage, but T1 (hydric comfort) stands out, after flowering, as the tallest treatment.

In Elvar, T3 (hydric stress until flowering) stands out with a subtle lower plant height compared with the other treatments, and T4 (hydric stress after flowering) appear to be slightly taller than the other treatments, after flowering.

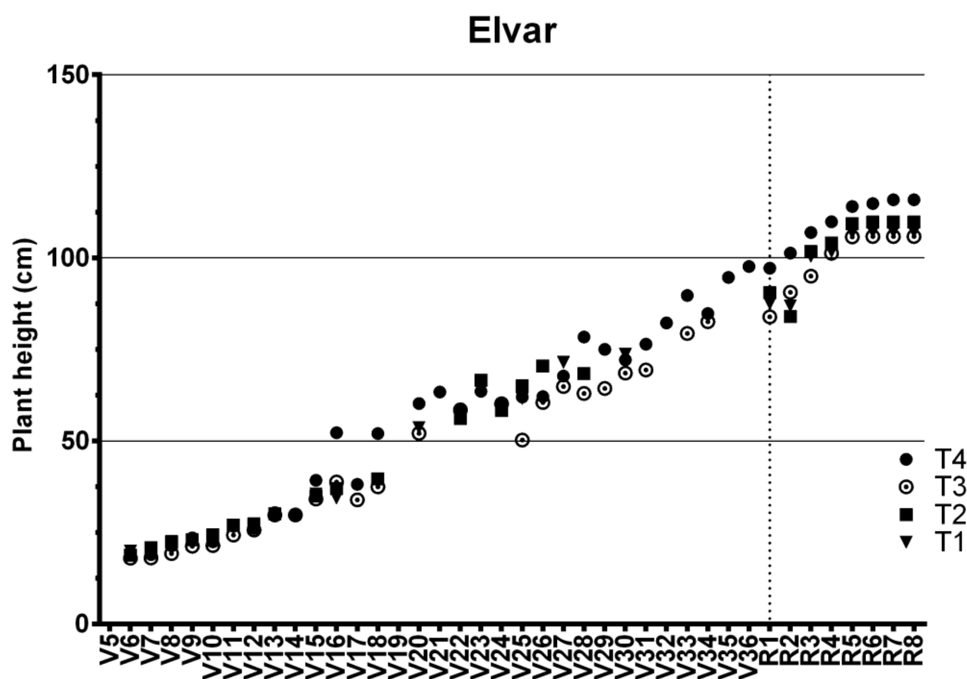


Figure 13 Elvar's average plant height vs phenological stage..

- dotted line represents flowering

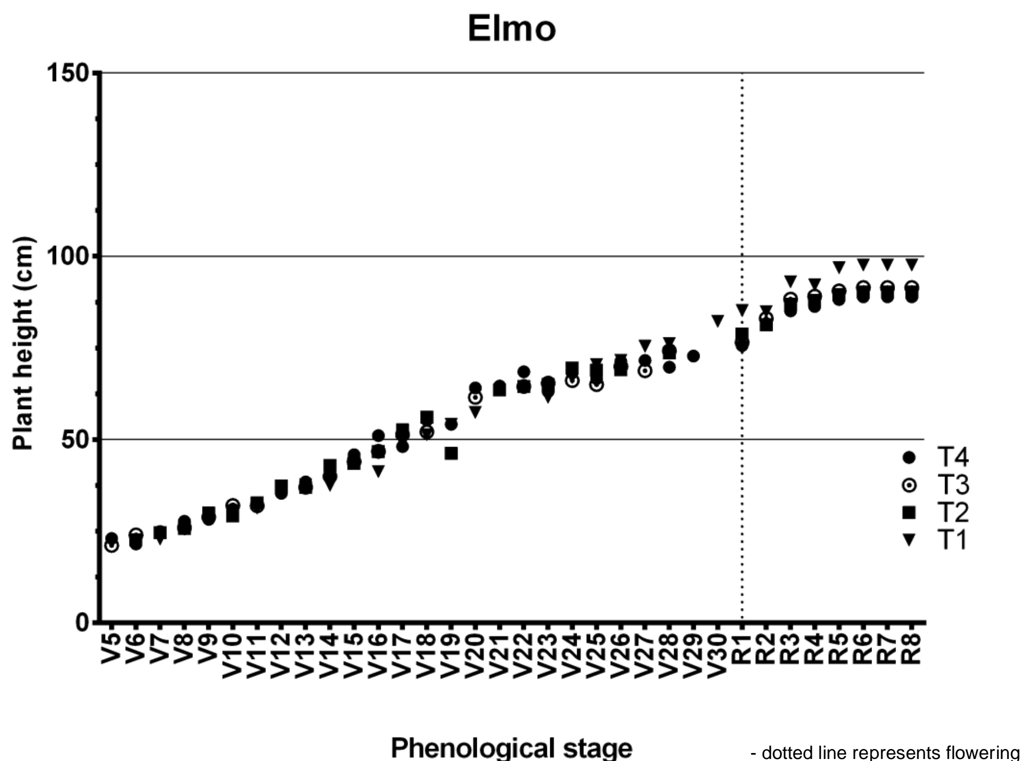


Figure 12 Elmo's average plant height vs phenological stage

Days to Flowering

The model presented for days to first flower showed a coefficient of determination (R^2) of 0.85 and coefficient of variation of 6.9%. The only significant explanatory variable was Variety. ($p<0.01$).

Figures 14 and 15 present the development of two cultivars of chickpea, Elmo and Elvar, respectively, submitted to four treatments (T1, T2, T3, and T4).

Within cultivars, days to the first flower did not show significant differences among treatments ($p<0.05$), however there were significantly differences between varieties($p<0.05$): flowering occurred 16 days earlier (Fig. 16) in Elmo (108 days after sowing) than in Elvar (124 days after sowing).

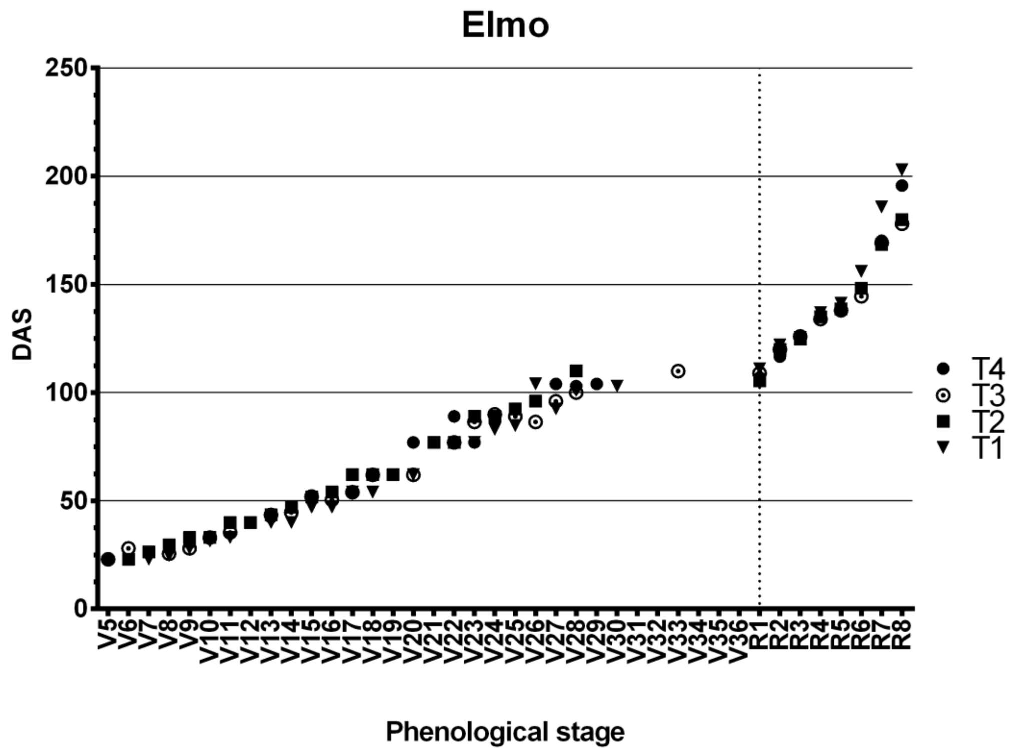


Figure 14 Elmo's phenological stage vs days after sowing.

- dotted line represents flowering; DAS=Days after sowing

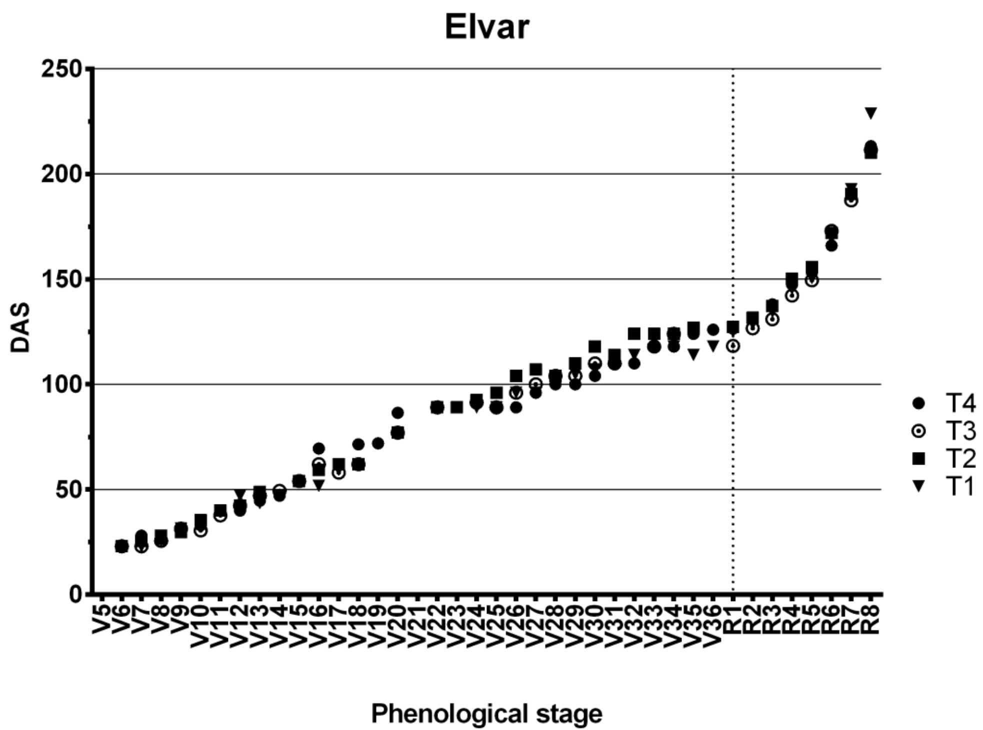


Figure 15 Elvar's phenological stage vs days after sowing

- dotted line represents flowering; DAS=Days after sowing

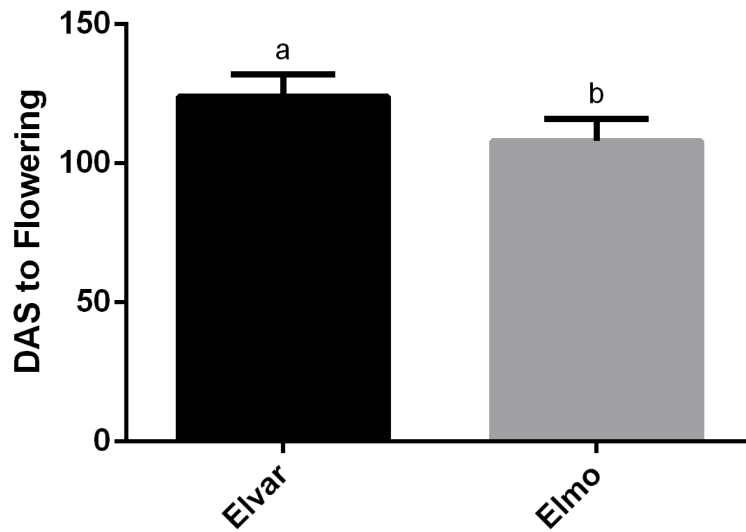


Figure 16 Effect of variety in average DAS to flowering.

Harvest

Plant height

The model presented for plant height showed a coefficient of determination (R^2) of 0.85 and Coefficient of Variation of 10.0%. The only significant explanatory variable was Variety ($p<0.01$).

At harvest (Fig. 17), Elvar was taller (106.3cm) than Elmo (88.0cm).

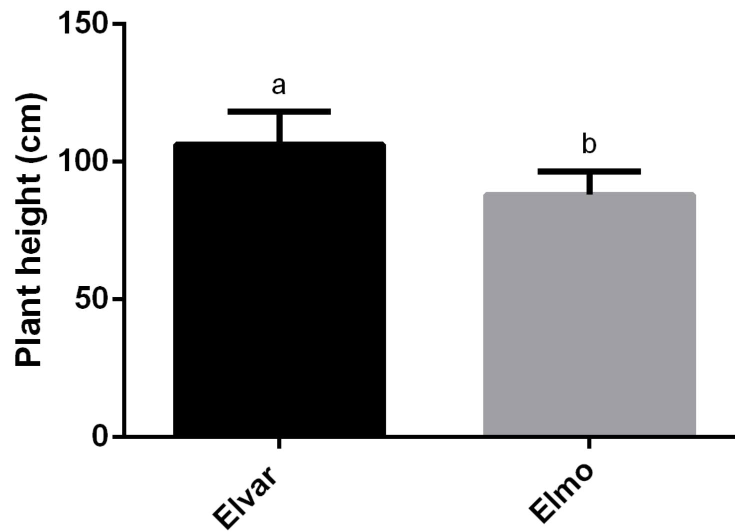


Figure 17 Effect of variety in average plant height at harvest.

Biomass weight

The model presented for total biomass weight showed a coefficient of determination (R^2) of 0.83 and Coefficient of Variation of 15.7% The only significant explanatory variable was Plant. ($p<0.01$).

Root Length

The model presented for root length showed a coefficient of determination (R^2) of 0.86 and Coefficient of Variation of 12.1%. The significant explanatory variables were Treatment and Treatment x Variety ($p<0.05$).

Treatment

Figure 18 shows the effect of the treatments in root length. In general, chickpea plants submitted to T4 treatment (hydric stress after flowering) had a longer ($p<0.05$) root length (48.7 cm) compared to the other three treatments that ranged from 42.8 cm in chickpea plants submitted to T3 (hydric stress before flowering) to 37.4 cm in chickpea plants submitted to T1 treatment (hydric comfort).

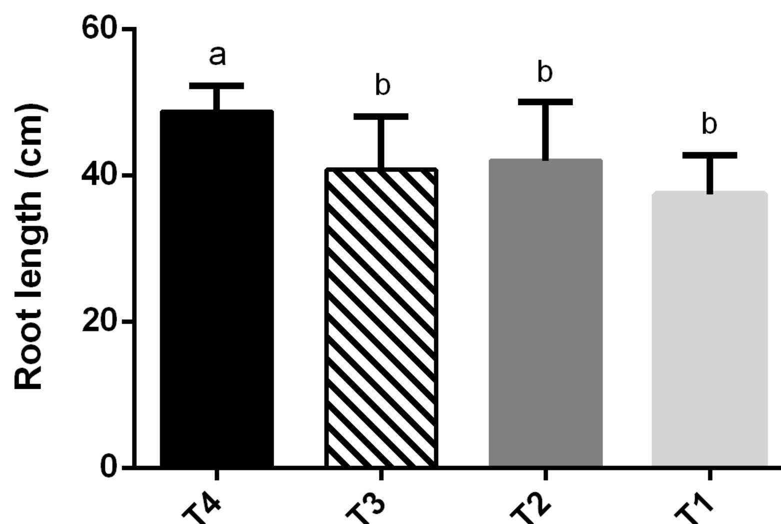


Figure 18 Effect of treatment in root length.

Interaction between treatment and variety

Figure 19 represents variety x treatment interaction for average root length. There were no significant differences on root length between Elmo and Elvar plants submitted to treatments T1 (hydric comfort; 36.6 and 38.8 cm, respectively), T3 (hydric stress before flowering; 44.5 and 37.2 cm, respectively), or T4 (hydric stress after flowering; 47.7 and 50.0 cm, respectively). However, chickpea plants submitted to T4 needed to lengthen its roots 10 cm to reach for water than those submitted to treatment T1.

In contrast, chickpea plants of both varieties respond differently when submitted to treatments T2 (permanent hydric stress): Elvar roots (48.4 cm) grew about 13 cm longer than Elmo' (35.7 cm).

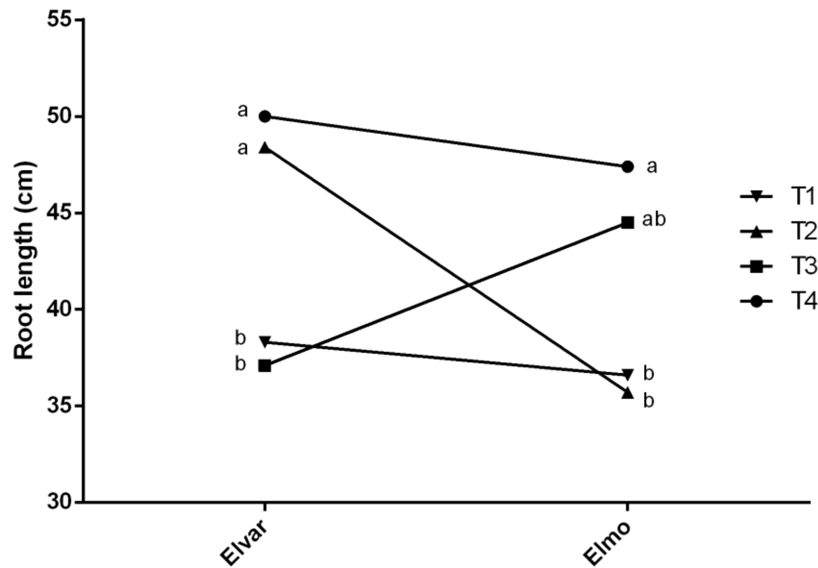


Figure 19 Interaction between variety and treatment in root length.

Root Weight

The model presented for root weight showed a coefficient of determination (R^2) of 0.77 and Coefficient of Variation of 38.5%. No explanatory variables were statistically significant.

Number of stem's ramifications

The model presented for number of main stem's ramifications showed a coefficient of determination (R^2) of 0.63 and Coefficient of Variation of 18.5%. No explanatory variables were statistically significant.

Number of pods per plant

The model presented for number of pods per plant showed a coefficient of determination (R^2) of 0.98 and Coefficient of Variation of 9.7%. The significant explanatory variables were plant, treatment, variety, plant x variety and treatment x variety ($p<0.01$).

Treatment

Figure 20 shows the effect of the treatments in the average number of pods per plant. Chickpea plants submitted to treatments T4 (hydric stress after flowering), T3 (hydric stress before flowering) and T1 (hydric comfort), produced higher number of pods per plant (48,

52 and 49 pods, respectively) compared to 41 pods produced by chickpea plants submitted to T2 (permanent hydric stress).

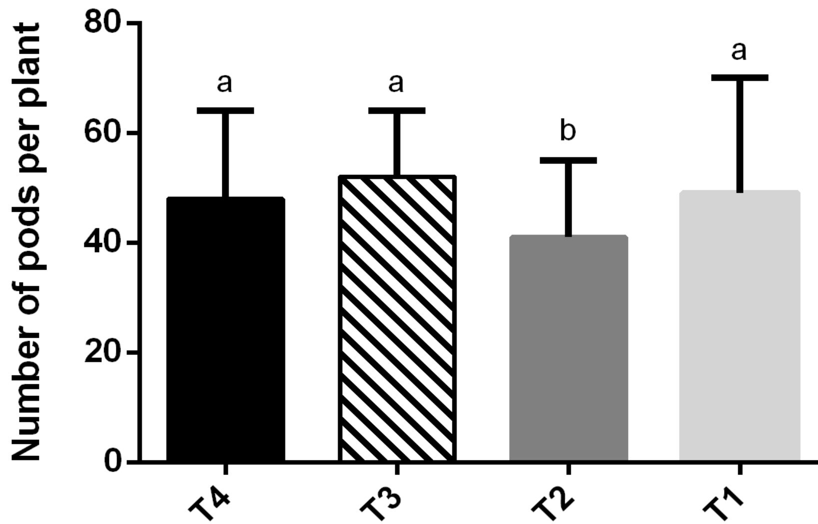


Figure 20 Effect of treatment in average number of pods per plant.

Variety

Figure 21 shows the effect of the variety in the average number of pods per plant. Elmo produced higher number of pods per plant (59 pods) than Elvar (35 pods).

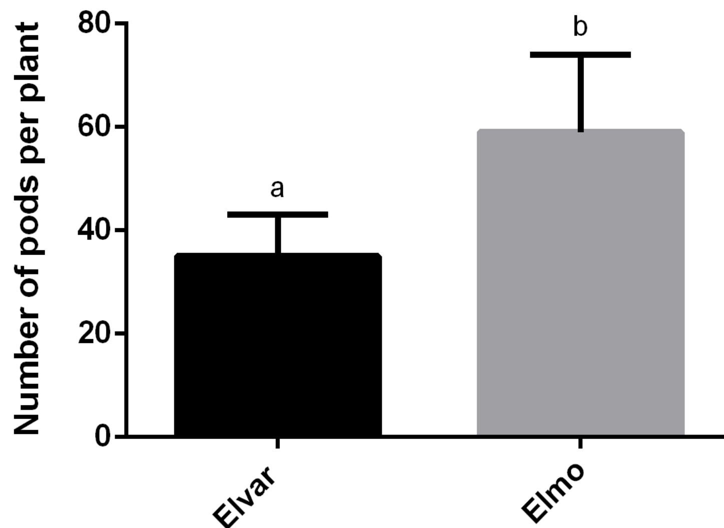


Figure 21 Effect of variety in average number of pods per plant.

Interaction between treatment and variety

Figure 22 represents variety x treatment interaction for the average number of pods per plant. There were no significant differences on the number of pods per plant submitted to either treatments T2 (permanent hydric stress) or T4 (hydric stress after flowering), however, each plant of Elmo produced about 20 more pods than Elvar'.

In contrast, chickpea plants of both varieties respond differently when submitted to treatments T1 (hydric comfort) and T3 (hydric stress before flowering): Elvar plants produced (30 and 45 pods, respectively) about 20 less pods than Elmo' (68 and 59 pods, respectively).

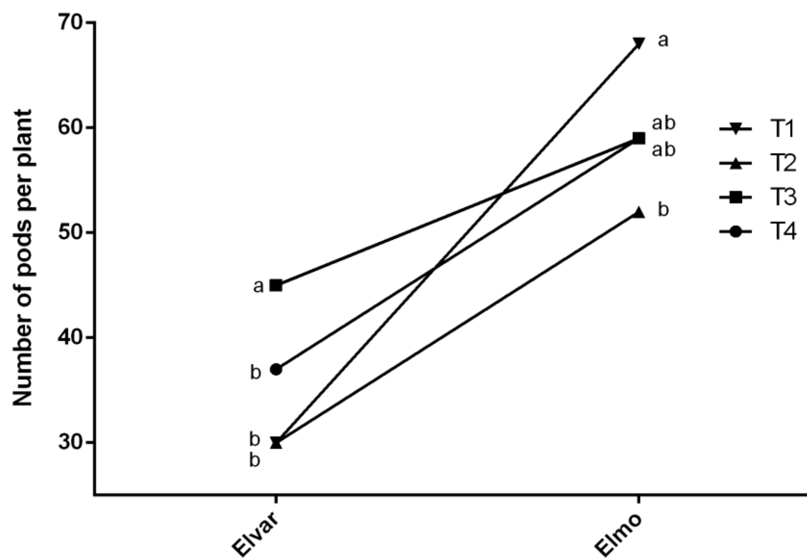


Figure 22 Interaction between variety and treatment in average number of pods per plant.

Pod weight per plant

The model presented for pods weight per plant showed a coefficient of determination (R^2) of 0.92 and Coefficient of Variation of 14.2%. The significant explanatory variables were plant and variety ($p<0.01$).

Variety

Figure 23 shows the effect of the variety on the average pods weight per plant. Chickpea plants of the variety Elmo produced higher pod weight per plant (39.3 g) than those of variety Elvar (28.6 g).

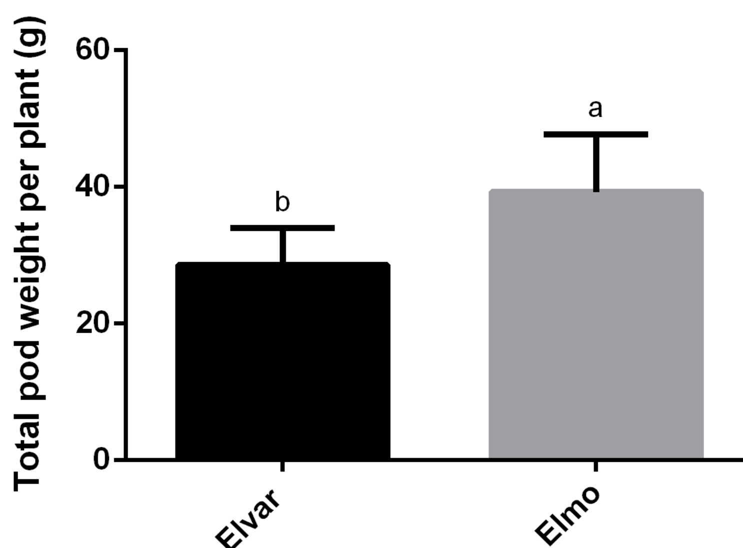


Figure 23 Effect of variety in average total pod weight per plant.

Number of *Rhizobium* clusters per plant

The model presented for number *Rhizobium* clusters per plant showed a coefficient of determination (R^2) of 0.78 and Coefficient of Variation of 57.4%, which indicates a high variability in the data. The significant explanatory variables were plant, treatment x variety ($p<0.05$). Due to the high coefficient of variation of this parameter no analysis was performed.

Number of seeds per plant

The model presented for number of seeds per plant showed a coefficient of determination (R^2) of 0.98 and Coefficient of Variation of 11.8%. The significant explanatory variables were plant ($p<0.01$), treatment ($p<0.01$), variety ($p<0.01$), plant x treatment ($p<0.05$), plant x variety ($p<0.01$), treatment x variety ($p<0.05$).

Treatment

Figure 24 shows the effect of the treatments in the average number of seeds per plant. Chickpea plants submitted to treatments T4 (hydric stress after flowering), T3 (hydric stress

before flowering) and T1 (hydric comfort) produced higher number of seeds (55, 62, and 55 seeds, respectively) than chickpea plants submitted to treatment T2 (permanent hydric

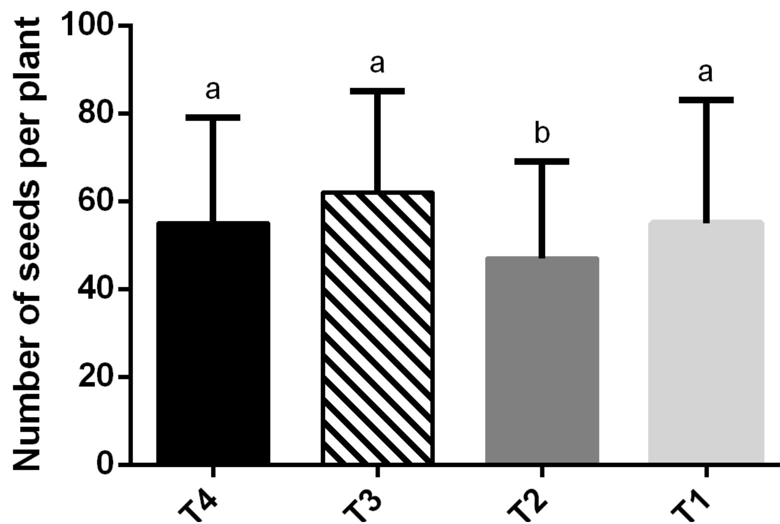


Figure 24 Effect of treatment in average number of seeds per plant.

stress), with 47 seeds.

Variety

Figure 25 shows the effect of the variety on the average number of seeds per plant. Elmo produced higher number of seeds (74 seeds) than Elvar, with 35 seeds.

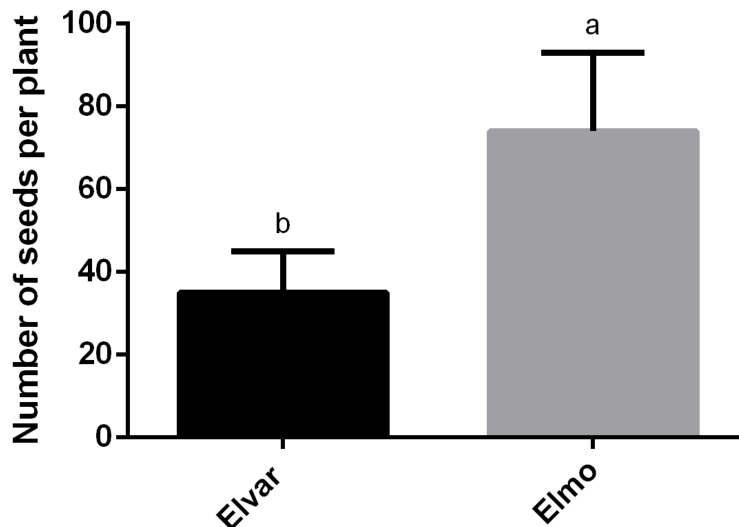


Figure 25 Effect of variety in average number of seeds per plant.

Interaction between treatment and variety

Figure 26 represents variety x treatment interaction for the average number of seeds per plant. There were no significant differences on the number of seeds per plant submitted to either treatments T2 (permanent hydric stress), T3 (hydric stress before flowering) or T4 (hydric stress after flowering), however, each plant of Elmo produced about 40 more seeds than Elvar'.

In contrast, chickpea plants of both varieties respond differently when submitted to treatment T1 (hydric comfort): Elvar plants produced (28 seeds) about 50 less pods than Elmo' (81 pods).

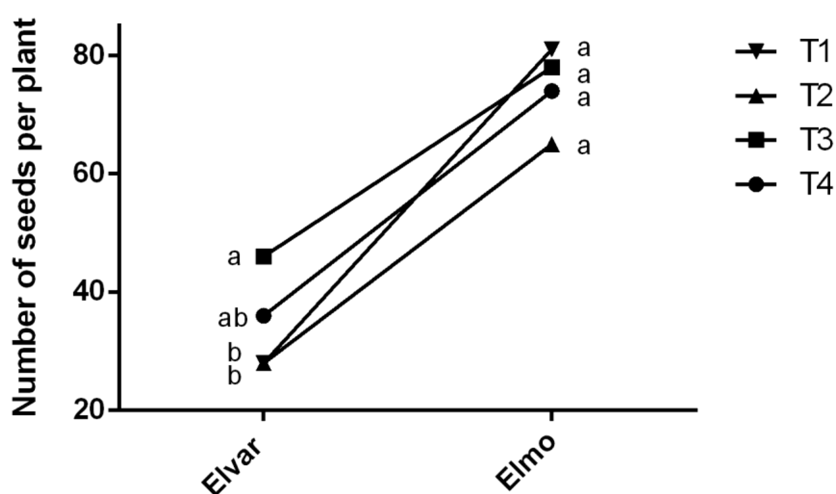


Figure 26 Interaction between variety and treatment in average number of seeds per plant.

Seed weight per plant

The model presented for seeds weight per plant showed a coefficient of determination (R^2) of 0.96 and coefficient of variation of 15.4%. The significant explanatory variables were plant, variety and plant x variety ($p<0.01$).

Variety

Figure 27 shows the effect of the variety in the average seeds weight per plant. Elmo yielded a heavier total seed weight per plant (21.0 g) than Elvar, with 12.6 g.

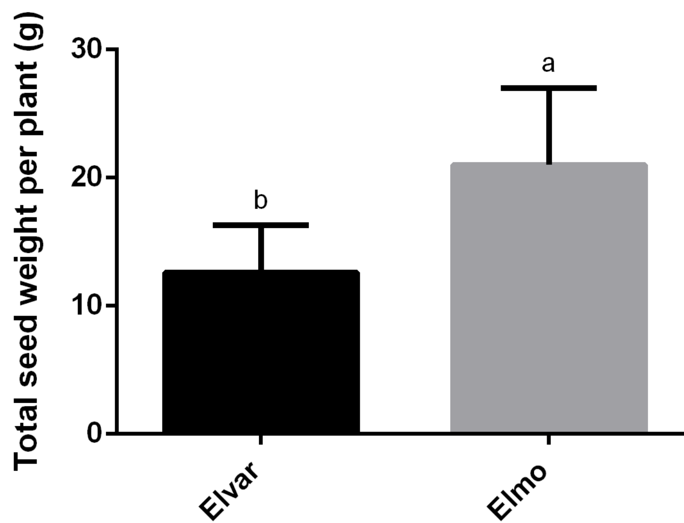


Figure 27 Effect of variety in average total seed weight per plant.

Harvest index

The model presented for harvest index showed a coefficient of determination (R^2) of 0.88 and Coefficient of Variation of 25.3%. The significant explanatory variable was variety ($p<0.01$).

Variety

Figure 28 shows the effect of the variety on the average harvest index. Variety Elmo reached a higher harvest index (19%) than Elvar (11%).

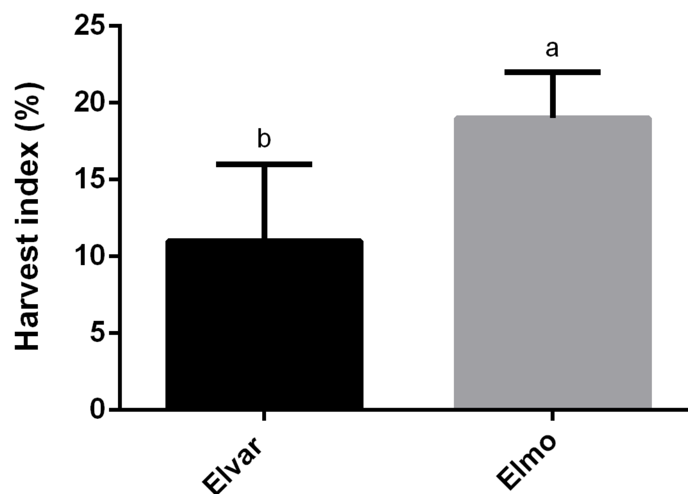


Figure 28 Effect of variety in average yield index.

Average pod individual weight

The model presented for Average pod weight showed a coefficient of determination (R^2) of 0.76 and Coefficient of Variation of 15.7%. The significant explanatory variable was variety ($p<0.05$).

Variety

Figure 29 shows the effect of the variety in the average pod weight. Elvar produced heavier pods (1.30 g per pod), than Elmo (0.74 g per pod).

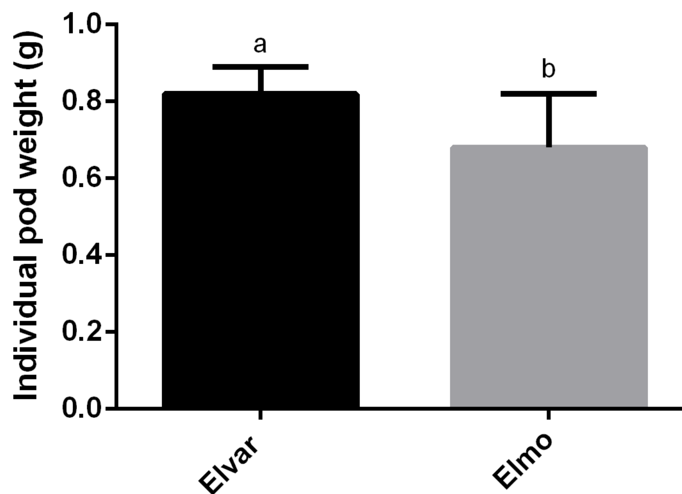


Figure 29 Effect of variety in average pod weight.

Quantitative Real-Time PCR

Overall analysis of Variance

Ca_03790 (*Cicer arietinum* phosphoenolpyruvate carboxylase kinase 1 [PPCK1]-like)

The model presented for expression of Ca_03790 showed a coefficient of determination (R^2) of 0.59 and 0.52, for TIF41 and G6PD housekeeping genes, respectively. No explanatory variables were statistically significant for the two housekeeping genes.

Ca_08236 (Cicer arietinum arabinogalactan [AGPS] protein 14)

The model presented for expression of Ca_03790 showed a coefficient of determination (R^2) of 0.88 and 0.82, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for the two housekeeping genes were biological replicate ($p<0.01$), treatment ($p<0.01$), variety ($p<0.01$), biological replicate x treatment ($p<0.01$), biological replicate x variety ($p<0.01$) and treatment x variety ($p<0.01$).

Ca_15236 (*Cicer arietinum* NAC domain-containing protein (NAC20))

The model presented for expression of Ca_15236 showed a coefficient of determination (R^2) of 0.91 and 0.89, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were treatment ($p<0.01$), biological replicate ($p<0.01$), variety ($p<0.01$) biological replicate x treatment ($p<0.05$) and technical replicate x treatment ($p<0.05$).

Ca_05907 (*Cicer arietinum* zinc finger CCCH domain-containing protein 20)

The model presented for expression of Ca_05907 showed a coefficient of determination (R^2) of 0.76 and 0.53, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.01$), treatment ($p<0.01$), variety ($p<0.05$), variety x treatment ($p<0.01$), biological replicate x treatment ($p<0.05$).

Ca_20991 (Cicer arietinum chlorophyll a-b binding protein 7, chloroplastic)

The model presented for expression of Ca_20991 showed a coefficient of determination (R^2) of 0.96 and 0.98, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.01$), treatment ($p<0.01$), variety ($p<0.01$), biological replicate x treatment ($p<0.01$), biological replicate x variety ($p<0.01$) and treatment x variety ($p<0.01$).

Ca_00047 (*Cicer arietinum* ferredoxin)

The model presented for expression of Ca_20991 showed a coefficient of determination (R^2) of 0.84 and 0.70, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.01$), treatment ($p<0.05$), variety ($p<0.01$), treatment x variety ($p<0.01$) and biological replicate x variety ($p<0.05$).

Treatment

Figure 30 shows the effect of the treatments in the expression for genes of interest during stress. Ca_03790 (*Cicer arietinum* phosphoenolpyruvate carboxylase kinase 1 [PPCK1]-like) appears to be upregulated, with a fold change of 1.62, during stress although the swift

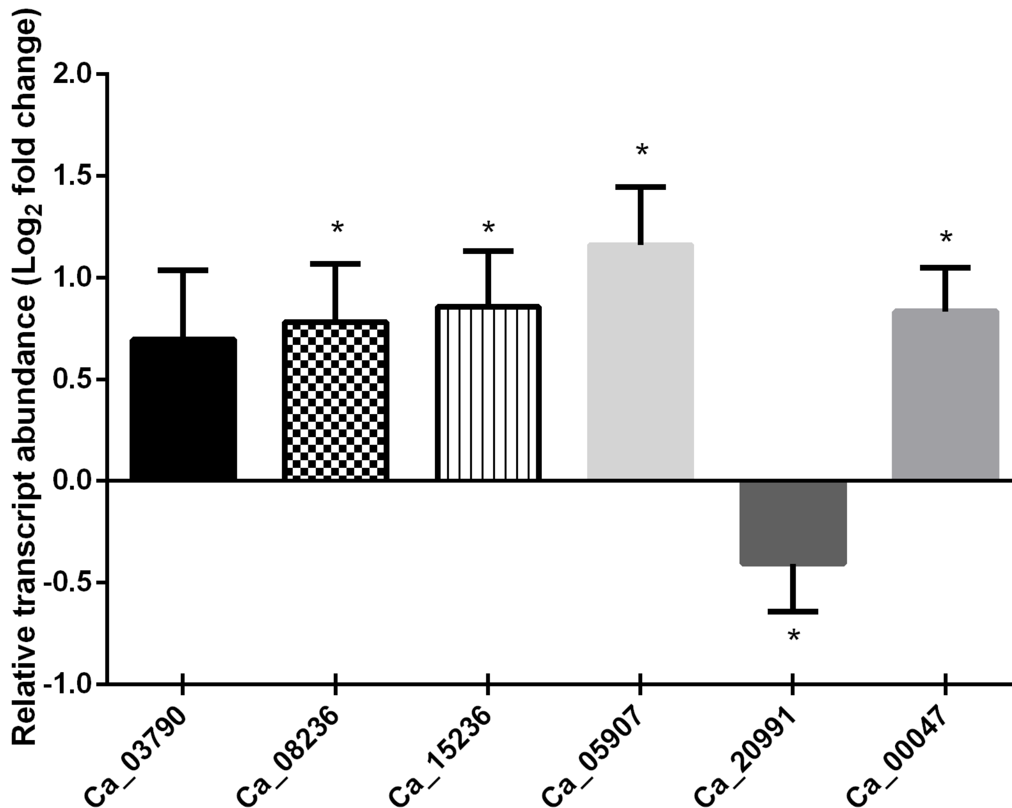


Figure 30 Effect of treatment in relative transcript abundance.

was not statistically significant.

Ca_08236 (*Cicer arietinum* arabinogalactan [AGPS] protein 14), Ca_15236 (*Cicer arietinum* NAC domain-containing protein (NAC20)), Ca_05907 (*Cicer arietinum* zinc finger CCCH domain-containing protein 20), and Ca_0047 (*Cicer arietinum* ferredoxin) were significantly upregulated, with a fold change of 1.72, 1.81, 2.23 and 1.78, respectively.

Ca_20991 (*Cicer arietinum* chlorophyll a-b binding protein 7, chloroplastic) was significantly downregulated, with a fold change of 0.75.

Variety

Figure 31 shows the effect of variety in the expression for genes of interest between varieties. In this graph, Ca_03790 (*Cicer arietinum* phosphoenolpyruvate carboxylase

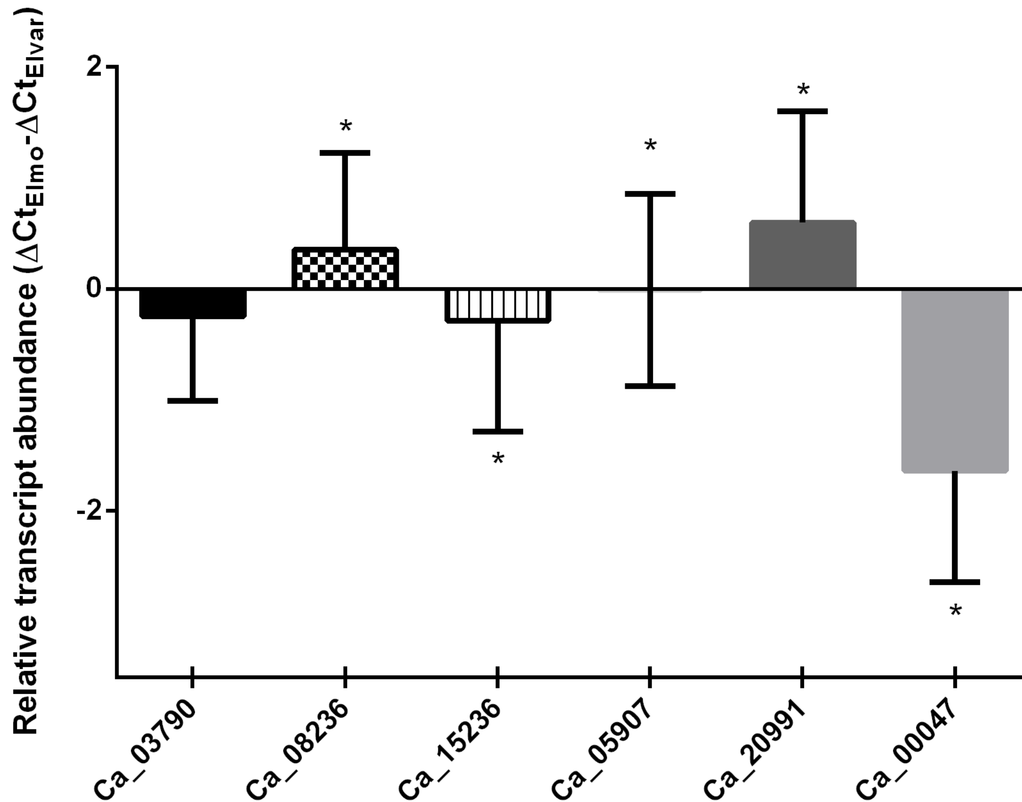


Figure 31 Effect of variety in relative transcript abundance;

kinase 1 [PPCK1]-like), Ca_15236 (*Cicer arietinum* NAC domain-,containing protein (NAC20)) and Ca_00047 (*Cicer arietinum* ferredoxin) showed an overall higher expression for the Elvar variety, with differences in relative expression of 0.84, 0.82 and 0.32, respectively. However Ca_03790 (*Cicer arietinum* phosphoenolpyruvate carboxylase kinase 1 [PPCK1]-like) was not significantly different between varieties.

Ca_08236 (*Cicer arietinum* arabinogalactan [AGP] protein 14) and Ca_20991 (*Cicer arietinum* chlorophyll a-b binding protein 7, chloroplastic) showed an overall higher expression for the Elmo variety, with differences in relative expression of 1.28 and 1.51, respectively.

Ca_05907 (*Cicer arietinum* zinc finger CCCH domain-containing protein 20) was significantly differentially expressed, but the results are inconclusive to consider the highest expressing variety.

Analysis of Variance: Elmo

Ca_03790 (*Cicer arietinum* phosphoenolpyruvate carboxylase kinase 1 [PPCK1]-like)

The model presented for expression of Ca_03790 in the variety Elmo showed a coefficient of determination (R^2) of 0.92 and 0.97, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.01$), treatment ($p<0.01$) and biological replicate x treatment ($p<0.01$).

Ca_08236 (Cicer arietinum arabinogalactan [AGP] protein 14)

The model presented for expression of Ca_037-90 showed a coefficient of determination (R^2) of 0.85 and 0.94, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.01$), treatment ($p<0.01$) and biological replicate x treatment ($p<0.01$).

Ca_15236 (*Cicer arietinum* NAC domain-containing protein (NAC20))

The model presented for expression of Ca_15236 showed a coefficient of determination (R^2) of 0.78 and 0.76, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.05$), technical replicate ($p<0.01$) and treatment ($p<0.05$).

Ca_05907 (*Cicer arietinum* zinc finger CCCH domain-containing protein 20)

The model presented for expression of Ca_05907 showed a coefficient of determination (R^2) of 0.97 and 0.97, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.01$), treatment ($p<0.01$) and treatment x biological replicate ($p<0.01$).

Ca_20991 (*Cicer arietinum* chlorophyll a-b binding protein 7, chloroplastic)

The model presented for expression of Ca_20991 showed a coefficient of determination (R^2) of 0.96 and 0.96, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.01$), technical replicate ($p<0.05$), treatment ($p<0.01$) and treatment x biological replicate ($p<0.01$).

Ca_00047 (*Cicer arietinum* ferredoxin)

The model presented for expression of Ca_20991 showed a coefficient of determination (R^2) of 0.94 and 0.88, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes was biological replicate ($p<0.01$), treatment ($p<0.01$) and biological replicate x treatment ($p<0.01$).

Analysis of Variance: Elvar

Ca_03790 (*Cicer arietinum* phosphoenolpyruvate carboxylase kinase 1 [PPCK1]-like)

The model presented for expression of Ca_03790 in the variety Elvar showed a coefficient of determination (R^2) of 0.92 and 0.91, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.01$) and biological replicate x treatment ($p<0.01$).

Ca_08236 (Cicer arietinum arabinogalactan [AGP] protein 14)

The model presented for expression of Ca_03790 showed a coefficient of determination (R^2) of 0.996 and 0.990, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.01$), treatment ($p<0.01$) and treatment x biological replicate ($p<0.01$).

Ca_15236 (*Cicer arietinum* NAC domain-containing protein (NAC20))

The model presented for expression of Ca_15236 showed a coefficient of determination (R^2) of 0.56 and 0.49, for TIF41 and G6PD housekeeping genes, respectively. No explanatory variables were statistically significant.

Ca_05907 (*Cicer arietinum* zinc finger CCCH domain-containing protein 20)

The model presented for expression of Ca_05907 showed a coefficient of determination (R^2) of 0.99 and 0.97, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.01$), treatment ($p<0.01$) and treatment x biological replicate ($p<0.01$).

Ca_20991 (Cicer arietinum chlorophyll a-b binding protein 7, chloroplastic)

The model presented for expression of Ca_20991 showed a coefficient of determination (R^2) of 0.99 and 0.98, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.01$), treatment ($p<0.01$) and treatment x biological replicate ($p<0.01$).

Ca_00047 (*Cicer arietinum* ferredoxin)

The model presented for expression of Ca_20991 showed a coefficient of determination (R^2) of 0.69 and 0.52, for TIF41 and G6PD housekeeping genes, respectively. The significant explanatory variables for two housekeeping genes were biological replicate ($p<0.01$) and treatment ($p<0.01$).

Interaction between treatment and variety

Figure 32 shows the interaction between treatment and variety in the expression for genes of interest during stress. There were no significant differences in relative transcript abundance for Ca_08236 (*Cicer arietinum* arabinogalactan [AGP] protein 14), Ca_05907

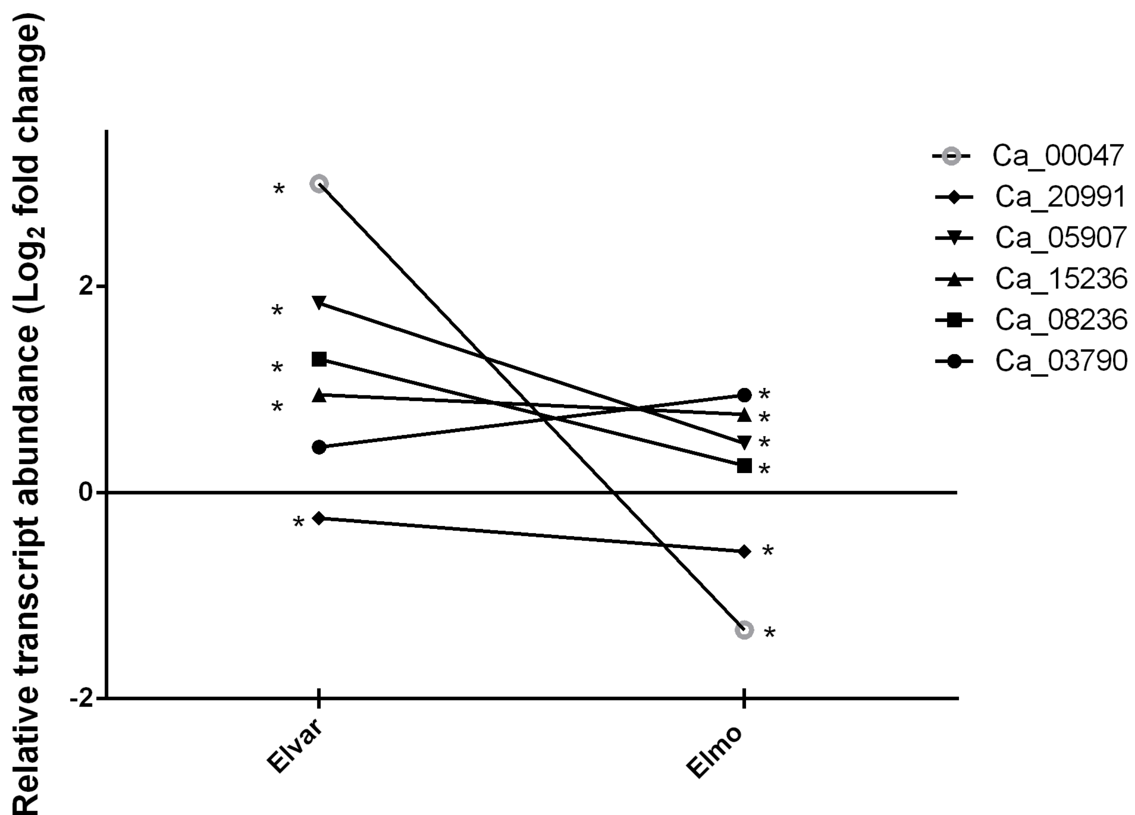


Figure 32 Interaction between treatment and variety in relative transcript abundance.

(*Cicer arietinum* zinc finger CCCH domain-containing protein 20), Ca_15236 (Cicer arietinum NAC domain-containing protein (NAC20)) or Ca_20991 (*Cicer arietinum* chlorophyll a-b binding protein 7, chloroplastic), however, Elvar's leaves expressed about 1 fold change higher than Elmo'.

In contrast, chickpea leaves of both varieties expressed differently Ca_03790 (*Cicer arietinum* phosphoenolpyruvate carboxylase kinase 1 [PPCK1]-like) and Ca_00047 (*Cicer arietinum* ferredoxin): Elvar's leaves had 0.5 and 7.5 difference in fold change, respectively, compared to Elmo'.

Discussion

When evaluating drought stress tolerance in chickpea, the most important selection criteria are earliness (highest priority), initial growth vigor, seed weight, harvest index and pods per plant. [41, 106] Drought at the end of the reproductive stage (terminal drought) is the most common form of drought stress, which affects the top chickpea production regions, resulting in a significant yield loss. [107, 108].

DAS to flowering is a good indicator of earliness, but in this study no significant differences were detected among treatments. Significant difference was detected between varieties. Elmo took 108 days to flower and Elvar took 124 days. Based on this data we conclude that Elmo variety flowers earlier than Elvar.

Elmo also develop faster than Elvar, reaching an average of 50 cm of plant height in less DAS (Fig. 12 and 13), indicating a higher early growth rate.

Harvest index was also generally higher for Elmo (19%) compared to Elvar (10%) (fig.22). Indicating a better ratio of total biomass to total seed production for Elmo. However, no statistical difference was found between treatments.

Average pod weight was heavier for Elvar(1.30 g per pod) compared to Elmo (0.74 g per pod) (Fig.29), which agrees with previous studies that state that Kabuli varieties tend to have bigger seeds than desi.[11] This characteristic might explain the overall heavier average individual pod weight. However, no statistical difference was found among treatments. Average pod weight, which correlated to seed weight was not overall affected across treatment, and seed weight has high heritability, make it a good criteria for early-breeding generations.[106]

Number of pods per plant was higher for Elmo (59 pods) compared to Elvar (35 pods) (Fig.25). In Elmo, the highest number of pods per plant was obtained during T1 (hydric comfort) (68 pods per plant), followed by T3 (hydric stress after flowering) with 59 pods. In Elvar, the highest number of pods per plant was obtained during T3 (hydric stress after flowering). This pattern for higher yield during T3 (hydric stress after flowering) compared to T2 (permanent hydric stress) and T4 (hydric stress after flowering) support that terminal drought results in higher production loss.[107]

The low production for Elvar during T1 (hydric comfort) may be due to its lateness. High greenhouse temperatures (>35°C) during late reproductive stage may have caused heat stress. [109]

Root length were longer for Elvar during T2 (permanent hydric stress), which is a desirable trait for improvement of drought tolerance.[41, 110] This finding might suggest a drought avoidance mechanism via root development, but due to pot confinement the radicular system didn’t develop.[41]

Gene expression can give an insight on the plant’s mechanism of drought tolerance. [41, 74, 99]

Phosphoenolpyruvate carboxylase kinase overexpression was previously correlated with enhancement of heat and/or drought stress in wheat, Arabidopsis and C4-PEPC transgenic rice, via Ca^{2+} and H_2O_2 signaling, and PEPC upregulation which is involved in many development process and nitrogen fixation in C_3 plants.[111-114] In chickpea, this gene was upregulated in drought-tolerant variety.[99] Phosphoenolpyruvate carboxylase kinase-1 was upregulated in Elmo during stress, whereas in Elvar no significant regulation occurred. Indicating an involvement of this protein in drought responses mechanisms of Elmo.

Arabinogalactan proteins are involved in plant development such as cell proliferation, cell viability, cell division, reproductive development, regeneration of somatic embryos, abiotic stress, and cellular signaling[101] Arabinogalactan protein 14 regulates root hair elongation in response to environmental stimuli.[115] This protein was found to be downregulated in both drought sensitive and tolerant varieties.[99] AGP14 was upregulated in both varieties during stress, but Elvar had an higher relative transcript abundance. Differences between varieties where found among treatments.

Overexpression of CCCH-type zinc finger proteins was correlated to drought tolerance ABA and JA mediated.[116] Moreover, OZF1, a CCCH-type zinc finger family protein, when

overexpressed enhanced tolerance to oxidative stress by upregulating antioxidant enzymes such as ascorbate peroxidase(APX) and glutathione S-transferase(GST).[103] CCCH-type zinc finger protein 20 was upregulated in both varieties during stress, showing no difference between varieties among treatments.

NAC transcription factor is involved in abiotic stress response, including in chickpea.[71, 117] In soybean, ÑAC20 expression was induced by salt and drought stress, and overexpression promoted lateral root formation and auxin signaling, increased freezing tolerance.[102] NAC20 was more expressed in drought-tolerant variety roots compared to drought-sensitive variety.[74] NAC20 was upregulated during stress but there significant differences among varieties.

Chlorophyll a-b binding proteins are apoproteins of light-harvesting complex of photosystem II that work like antenna and captures light and deliver excitation energy to the photosystem.[118] These proteins also have been involved in stomatal response to abscisic acid.[119] Arabidopsis chlorophyll a/b-binding protein 7 (AtLHCB7) mutant was not affected in light capture efficiency of photosystem II, but showed lower rates of light-saturated photosynthesis and a diminished irradiance threshold for induction of photoprotective non-photochemical quenching, suggesting a non-photochemical quenching function dissipate energy under excess light.[104] Chlorophyll a/b-binding protein 7 was downregulated for both varieties during hydric stress. This response may be correlated to diminishing of photosynthesis and chlorophyll content during hydric stress.[Reviewed in 37, 48]

Ferredoxin are involved in electrons transferring between photosystem I and $NADP^+$ reductase during photosynthesis, and for ferredoxin-dependent enzymes involved in nitrogen and sulfur assimilation, and in nitrogen fixation.[105] Arabidopsis ferredoxin-1, which is involved with cyclic electron transfer, were upregulated during drought stress, although overall ferredoxin downregulated due to ferredoxin-2 downregulation.[120] Cyclic electron transfer occurs when electron are not used to produce NAPDH, and it was been related with induction of non-photochemical quenching.[105] In chickpea, ferredoxin-1 were more expressed in drought-tolerant variety compared to drought-sensitive.[74] In this study, chickpea ferredoxin-1 was upregulated for Elvar and downregulated for Elmo. The higher expression under stress by Elvar may be correlated with a smaller downregulation of Chlorophyll a/b-binding protein 7, as it’s also involved in non-photochemical quenching.

Significant differences between plant of the same treatment and variety for the evaluated parameters may be correlated to environmental or genetic variability. A study with less environmental may be indicated to assess this variability.

Conclusion

Clustering all the information, it can be interpreted that Elmo variety is more tolerant to drought stress, since it presents higher yield than Elvar. Desi chickpea has been described as been more tolerant than Kabuli to drought stress. Elmo' main mechanism for drought-tolerance were drought escape, since it appears to have early maturity. Elvar' main mechanism for drought-tolerance was drought avoidance, since it presented more biomass production and root development than Elmo. The best treatment for drought tolerance's selection is T4 (hydric stress after flowering), as it has a higher variability between varieties for drought tolerance selection criteria. Terminal drought is the most occurring type of drought and the most severe, making T4 the most representative for chickpea production regions.

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Annex

Annex I: Primers table

Table 4 Primers list

Gene ID	Sequence	Length (bp)	Tm (°C)	GC%	**Self-complementarity**	**Self 3' complementarity**	Product length (bp)
Ca_03790	GCTGAGATTTTTGAGGCGGTTA	22	59.25	45.45	3	2	63
	AGATCGGAAGATTCTGGATGGA	22	58.42	45.45	5	0	
Ca_08236	CTGATGCACCAGCTCCAAGTC	21	61.28	57.14	8	1	69
	GAGAAGCAAAAGCAGTTGGAACA	23	60.18	43.48	4	2	
Ca_05907	TCGCACAAATCCAACCATACA	21	58.22	42.86	2	0	58
	CGCCGTTGAATCGCTCAT	18	58.6	55.56	3	2	
Ca_15236	AAGGCACCAAAACTGATTGG	20	56.79	45	4	3	229
	GGCTTGTTGCTGTTGTCAGA	20	58.98	50	3	3	
Ca_20991	TGCTCTCCAGCAGTGTACCA	20	60.83	55	4	2	176
	TTTGGGTTTGATCCTCTTGG	20	55.52	45	4	0	
Ca_00047	GCAAGCACACCAGCTTTGTA	20	59.33	50	4	2	313
	CAACTTTGCCAGCACAAGAA	20	57.42	45	6	2	

TIF41	GTTGTACTTCGGGAGAGTTGCT	22	60	50			115
	GGAGCTTCTGGCTTATGATGCT	22	60	50			
G6PD	ACAACGATACCAGGGTGTTACC	22	60	50			116
	TCTCCCATGATGCCTTTAACTC	22	58	45			

Annex II: Electrophoresis gel

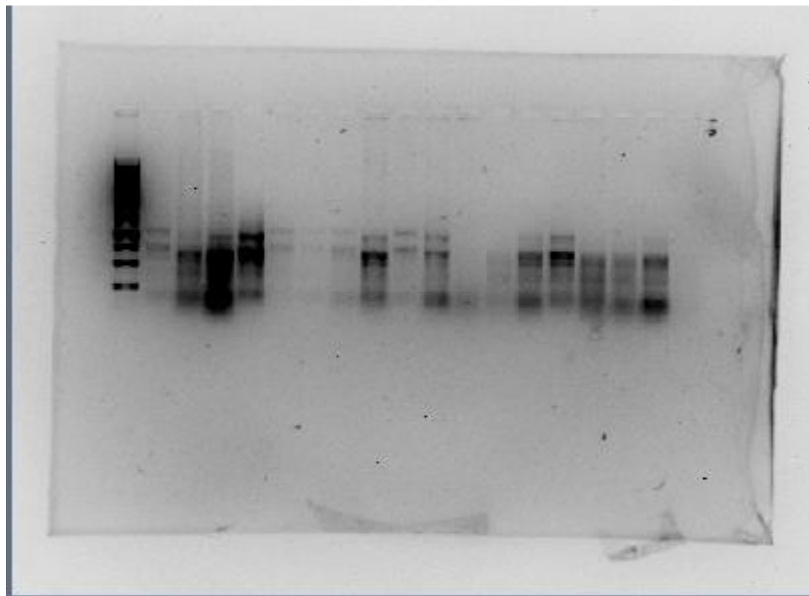

Figure 33 Example of RNA electrophoresis gel.

Annex III: qPCR Calibration curve

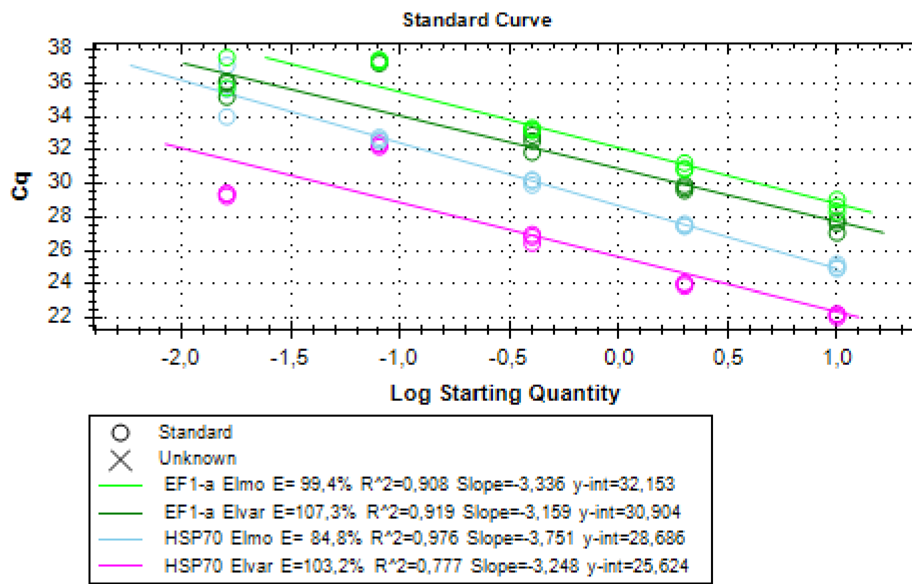

Figure 34 cDNA concentration calibration.