



Comparative analysis of drought responses in *Phaseolus vulgaris* (common bean) and *P. coccineus* (runner bean) cultivars

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Abstract

Genetic improvement of crop drought tolerance has become an urgent need for increasing agricultural yields and food production, to feed a growing human population in the context of global climate change. To get insights into the most relevant mechanisms underlying drought resistance in beans, we have analysed the responses to water deficit of three *Phaseolus vulgaris* (common bean) and one *P. coccineus* (runner bean) cultivars, focusing on the accumulation of specific osmolytes, a conserved response to abiotic stress in plants. Changes in osmolyte levels were correlated with the relative tolerance to water stress of the studied cultivars. Drought tolerance in *Phaseolus* largely depends on the accumulation of *myo*-inositol; glycine betaine may also contribute to tolerance in *P. coccineus* (but not in *P. vulgaris*). Proline, another common osmolyte, is a reliable marker of the level of stress affecting bean plants, but is not directly involved in tolerance mechanisms, as its drought-dependent accumulation is lowest in the most tolerant cultivar. We suggest that, by measuring the levels of proline and *myo*-inositol in water-stressed plants, a large number of cultivars could be easily and rapidly screened to select promising candidates to be used in breeding programmes for improving drought tolerance in beans.

Introduction

For all major crops, average yields are only a fraction of record yields (20% to 50%, depending on the species). Most of these losses are due to abiotic stresses affecting the plants in the field including, amongst others, drought, soil salinity, high temperatures, cold, UV light or flooding. Drought is the single environmental stress condition most devastating for agriculture (1). Insufficient rainfall brings about a reduction of the water available for plants in the soil, decreasing their growth and productivity, and can lead to plant death and the loss of the whole crop if water deficiency is prolonged in time. Irrigation systems are required to maintain acceptable yields in arid and semiarid regions. In fact, irrigated land is much more productive than rain-fed cropland: irrigation systems currently extend over ca. 280 million hectares of arable land, which represents less than 20% of the global cultivated area but produces more than 40% of the world food (2). Losses in agricultural production are expected to increase in the near future due to the forecasted effects of climate change, which include an increase in average temperatures worldwide and the occurrence of more frequent, longer and more intense drought periods (3), and consequently a shortage of water available for irrigation; this will especially affect subsistence farming in developing countries (4).

An effective approach to increase crop productivity and food production in the next decades can be based on the improvement of abiotic stress – especially drought – tolerance of our major crops (5). Generation of genetic engineered tolerant crops will require a deep understanding of the physiological, biochemical and molecular mechanisms of plant stress tolerance, which explains why the study of the responses of plants to abiot-

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ic stress, and particularly to drought, is currently a very active area of research in plant biology – apart from the academic interest of this topic. Drought-tolerant cultivars can also be obtained through ‘classical’ breeding techniques based on sexual crosses and selection; This approach has been relatively unsuccessful in the past, if compared for example with the improvement of yields of major crops under optimal growth conditions – the basis of the so-called ‘Green Revolution’ of the 1960s and 1970s – or the generation of pathogen-resistant varieties, but now new biotechnological tools are available to the breeder to make the whole process much more efficient and quicker (5, 6). Modern agriculture is based in a very limited number of varieties of each crop, which are in general relatively sensitive to water deficit stress; therefore, it will be necessary to screen minor commercial cultivars, neglected varieties and landraces stored in seed banks, as well as wild relatives, as possible sources of genetic variability for the improvement of drought tolerance (7, 8).

The common bean (*Phaseolus vulgaris*) is the most important legume for human nutrition (9). It was independently domesticated in Central America and in the Andes (10), and is now cultivated worldwide to be consumed as dry beans or green pods. Beans are essential components of people’s diet, especially in developing countries where they are a major source of protein, minerals, vitamins and fibre (11). *Phaseolus coccineus* L., the runner bean, was domesticated more than 2,000 years ago in Mexico where it is currently the second legume in the local diet after the common bean (12); *P. coccineus* is also cultivated in many other countries, but at a small scale compared to common bean. Beans are generally grown in non-irrigated farmland despite the fact that, as all major crops, they are sensitive to drought, to the point that it can cause drastic reductions in crop yields (11), such as those reported in Romania (80%) (13), or in some areas in east Africa (ca. 75%) (14).

We have selected three cultivars of *Phaseolus vulgaris* and one cultivar of its congener *P. coccineus*, to perform a comparative analysis of the responses of the plants to water deficit treatments under controlled greenhouse conditions. This work extends and complements a previous study where different responses of the same cultivars to salt stress treatments were investigated (15). Here, we have focused on a basic, conserved mechanism activated in plants subjected to different abiotic stresses, including drought; namely, the synthesis and accumulation of specific osmolytes (proline, glycine betaine or soluble carbohydrates) to maintain cellular osmotic balance, which is disrupted under stress conditions. The aims of the work were twofold. First, to get information on the molecular mechanisms of drought tolerance in *Phaseolus*, by correlating the stress-induced changes in osmolyte contents with the resistance to water deficit of the studied cultivars, estimated from their relative degree of growth inhibition under stress. Second, to confirm the possibility to use this simple and rapid analysis to identify cultivars with a higher resistance to drought at the vegetative growth stage; after confirming their tolerance regarding agronomic characteristics under stress conditions in the field,

these cultivars could be eventually used in bean breeding programmes. Therefore, this work also represents a ‘proof-of-concept’ study for future screenings including a much larger number of *Phaseolus* cultivars.

Materials and Methods

Plant material

Two commercial cultivars (‘Maxidor’ and ‘The Prince’) and one Spanish local variety (‘Judía de Franco’) of *Phaseolus vulgaris* L. and one cultivar of *Phaseolus coccineus* L. (‘Moonlight’) were used in the present study. ‘Maxidor’ and ‘The Prince’ are dwarf French bean cultivars, with a bushy growth, and considered as early cultivars: snap pods are formed 60-70 days after germination; both varieties are commonly used in Europe, especially the former. ‘Judía de Franco’ is a local landrace from the province of Teruel (Spain), which shows an indefinite growth, surpassing 3 m high and reaching maturity in 95-100 days. *P. coccineus* ‘Moonlight’ can also grow to the same size, but has a longer biological cycle (120-125 days). Seeds of ‘Judía de Franco’ were obtained from the Germplasm Bank of COMAV (Institute for Conservation and Improvement of Valencian Agrodiversity, Polytechnic University of Valencia); *P. vulgaris* seeds of ‘The Prince’ and ‘Maxidor’ cultivars were purchased from S.C. AGROSEM IMPEX S.R.L., Targu Mures (Romania), and those of *P. coccineus* cv. ‘Moonlight’ were obtained from Thompson & Morgan, AJP Garden & Crafts (UK).

Growth conditions and stress treatments

Seeds were sterilized with a diluted commercial bleach solution (sodium hypochlorite), rinsed thoroughly with water and sown on a mixture of peat, perlite, and vermiculite (2:1:1) moistened with half-strength Hoagland nutrient solution (16). Water stress treatments were initiated 16 days after sowing, when the first trifoliate leaves appeared, by completely withholding watering. Control plants grown in parallel were watered twice a week with half-strength Hoagland solution. After three weeks, when the substrate in the pots was completely dry, plants were harvested and leaf material was collected for further analysis. Treatments were carried out in a controlled environment chamber under long day photoperiod (16 h light, 8 h darkness), at 23 °C during the day and 17 °C at night, and 50-80% relative humidity.

Growth parameters

Stress-induced changes in several growth parameters (stem length, leaf number, fresh and dry weight) were determined at the end of the treatments. To better compare the degree of growth inhibition of the different cultivars, which differ in plant size, stem length and fresh weight were expressed as percentage of the absolute values measured for the non-stressed controls, which are shown in the legend of Fig. 1. To determine water content, a fraction of each sample was weighed (fresh weight, FW), dried at 65°C until constant weight (48-72 h), and then weighed again (dry weight, DW); water content of each sample (%) was calculated as: $[(FW-DW)/FW] \times 100$.

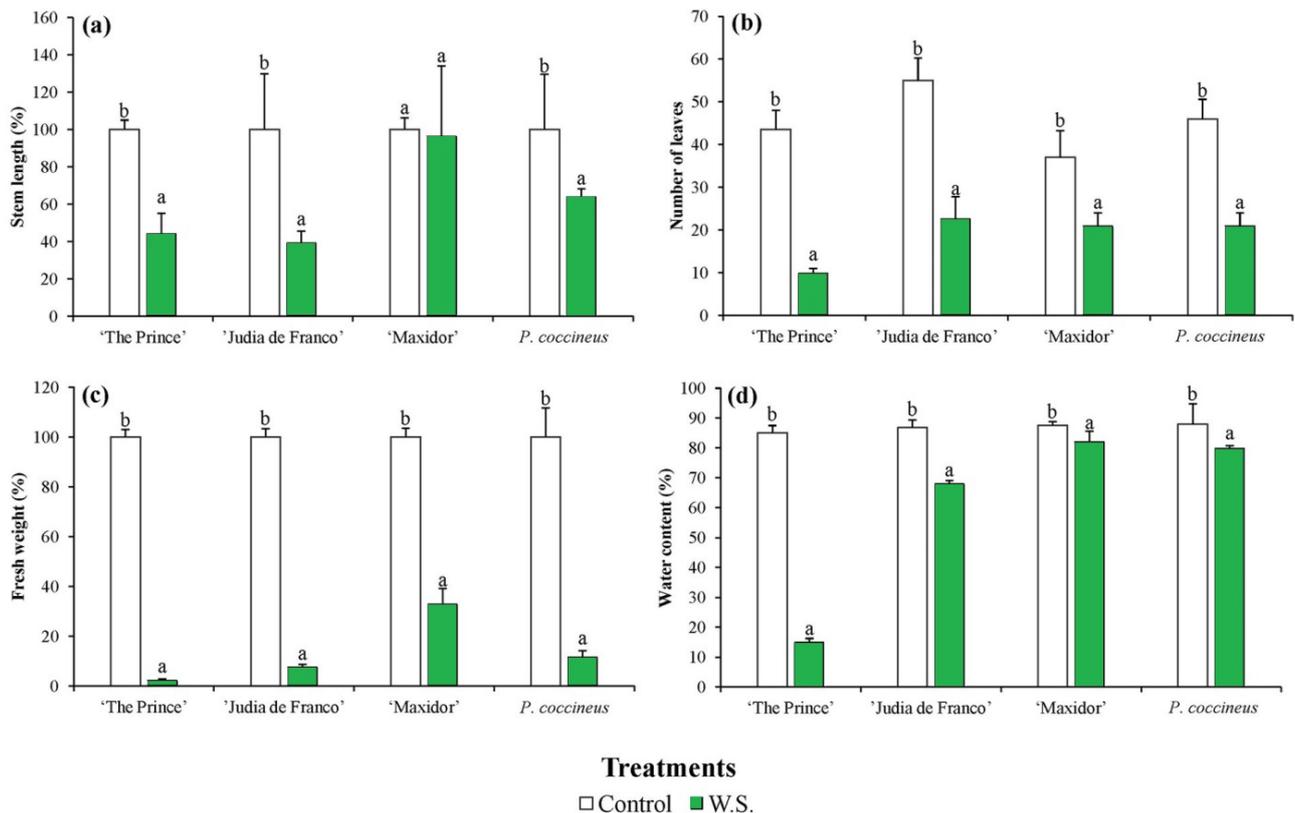


Figure 1. Water stress-induced changes in several growth parameters: (a) stem length (%), with the stem lengths of control, non-treated plants (*Phaseolus vulgaris*, cv. 'The Prince': 60.00 cm; cv. 'Judia de Franco': 174.61 cm; cv. 'Maxidor': 44.16 cm; *P. coccineus*: 219.00 cm) considered as 100% for each cultivar; (b) number of leaves; (c) Fresh weight (%), with the fresh weight of control plants (*P. vulgaris*, cv. 'The Prince': 31.54 g; cv. 'Judia de Franco': 34.87 g; cv. 'Maxidor': 17.17 g; *P. coccineus*: 30.26 g) considered as 100% for each cultivar; (d) water content (%). Measurements were performed after three weeks of treatment. The values shown are means with SE (n = 5). For each cultivar, different lower-case letters indicate significant differences between treatments according to the *t*-test ($\alpha = 0.05$).

Osmolyte quantification

Free proline (Pro) was determined as previously described (17). Dry leaf material was extracted with a 3% (w/v) aqueous sulfosalicylic acid solution; the extract was mixed with freshly prepared acid ninhydrin and incubated in a boiling water bath for 1 h. After stopping the reaction on ice, Pro was extracted with toluene and the absorbance of the organic phase was measured at 520 nm, using toluene as a blank. Pro concentration was expressed as $\mu\text{mol g}^{-1}$ DW.

To determine glycine betaine (GB) (18), dry leaf material was ground in a mortar in the presence of liquid nitrogen and suspended in Milli-Q water. The sample was mixed with potassium iodide, incubated on ice for 90 min, extracted with 1,2-dichloroethane and the absorbance of the solution was measured at 365 nm. GB concentration was expressed as $\mu\text{mol g}^{-1}$ DW.

Total soluble sugars (TSS) were quantified according to the method described in (19). Ground dry leaf material was extracted with 80% methanol by gently mixing in an orbital shaker overnight. 100 μL of the extract was mixed with 0.5 mL of 5% phenol and 2.5 mL of concentrated sulfuric acid, and the absorbance of the sample was later measured at 490 nm. TSS contents were expressed as 'mg equivalent of glucose' per gram of DW.

HPLC analysis

The soluble sugar fraction (mono- and oligosaccharides) was analysed using a Waters 1525 high performance liquid chromatography coupled to a 2424 evaporative light scattering detector (ELSD), as previously described (15). Standards of glucose, fructose, sucrose, and *myo*-inositol were used to identify peaks by co-injection. Sugars were quantified by peak integration using the Waters Empower software and comparison with the standards' calibration curves.

Statistical analysis

Data were analysed with the Statgraphics Centurion XVI software package (Statgraphics-Statpoint Technologies, Inc., Warrenton, VA, USA). Five individual plants were used as biological replicates per cultivar and per treatment. The significance of the differences between drought stress treatments and controls, for each cultivar, was evaluated with Student's *t* tests. All means throughout the text are followed by the standard error (SE).

Results

Drought-induced growth inhibition

Water deficit inhibited growth in all cultivars under study, but determination of several growth parameters revealed clear dif-

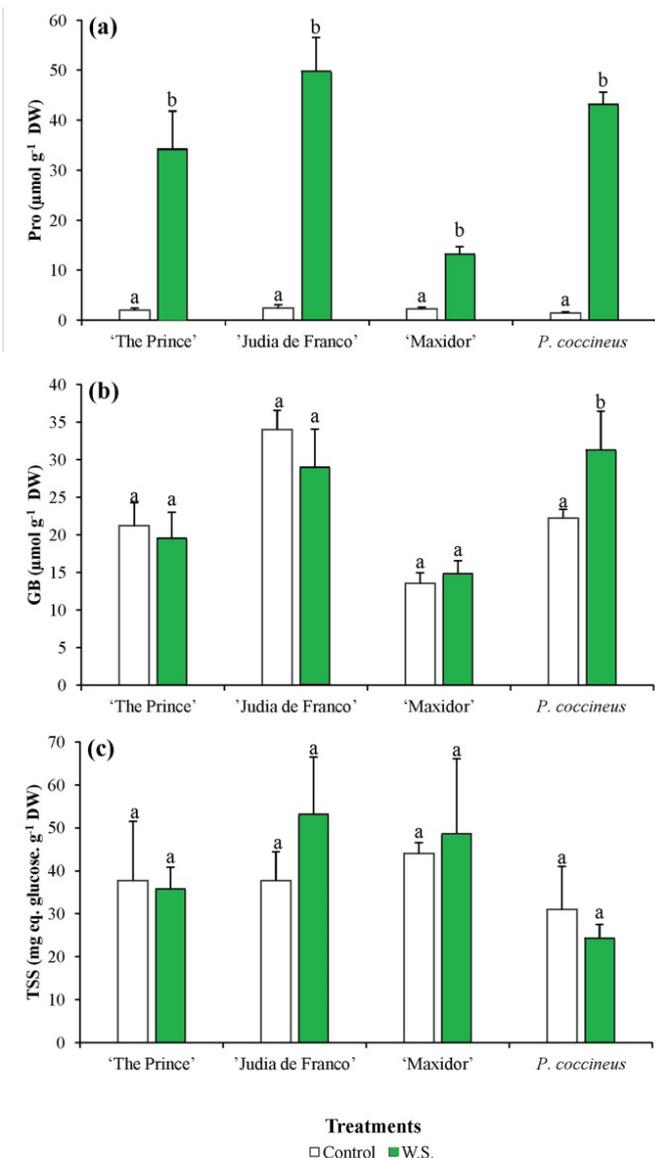


Figure 2. Water stress-induced changes in osmolyte levels: (a) proline (Pro), (b) glycine betaine (GB), and (c) total soluble sugars (TSS) in leaves of *Phaseolus* plants of the studied cultivars. Measurements were performed after three weeks of treatment. The values shown are means with SE (n = 5). For each cultivar, different lower-case letters indicate significant differences between treatments according to the t-test ($\alpha = 0.05$).

ferences in their relative resistance to water stress. Considering the relative reduction of stem length, compared with the non-stressed plants, the most affected cultivars were 'The Prince' and 'Judía de Franco', with a 60% reduction, followed by *P. coccineus* (40%), while cv. 'Maxidor' plants did not show any significant change in stem length under water stress conditions (Fig. 1a). The number of trifoliolate leaves was reduced in all cultivars after the stress treatment, mostly in 'The Prince' (ca. 4.5-fold), followed by 'Judía de Franco' (2.7-fold) and *P. coccineus* (2.2-fold), with the smallest decrease, about 1.7-fold, observed again in cv. 'Maxidor' (Fig. 1b). The fresh weight of the plants of the four cultivars was strongly affected by drought; a reduction of 97% of the corresponding control was registered in 'The

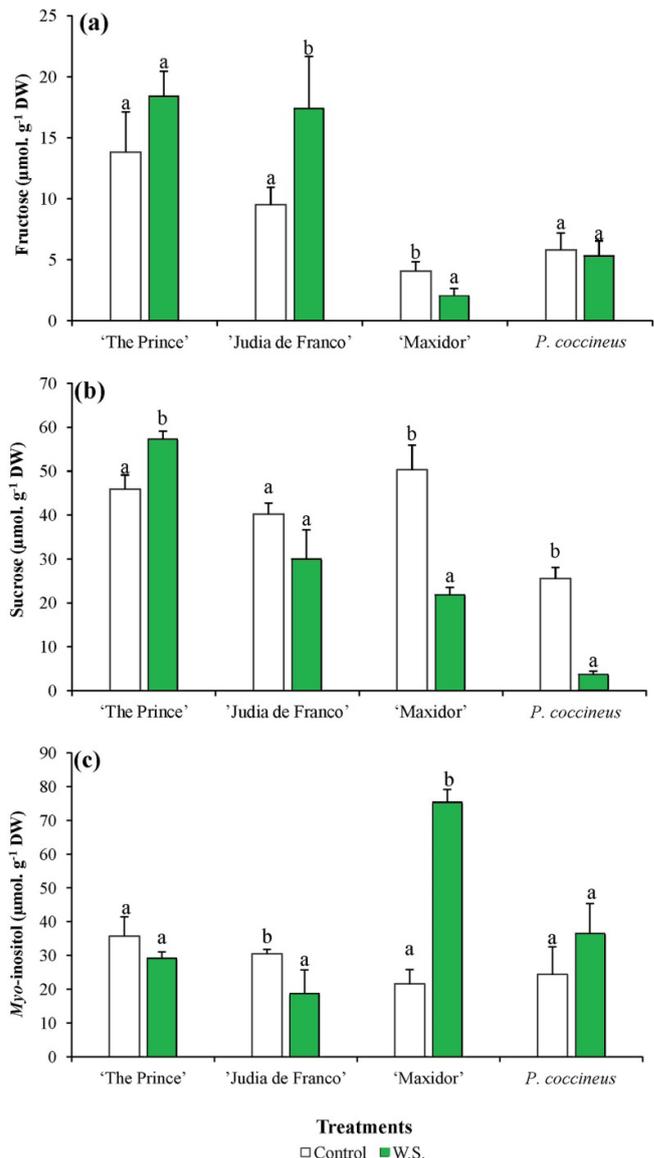


Figure 3. Water stress-induced changes in the levels of: (a) fructose, (b) sucrose, and (c) myo-inositol, separated by HPLC. Measurements were performed after three weeks of treatment. The values shown are means with SE (n = 5). For each cultivar, different lower-case letters indicate significant differences between treatments according to the t-test ($\alpha = 0.05$).

Prince', around 90% in 'Judía de Franco' and *P. coccineus*, and 67% in 'Maxidor' (Fig. 1c). The decrease of fresh weight was partly due to loss of water, which was significant, albeit quantitatively different, for all cultivars. Again, the strongest reduction of water content, ca. 85% was measured in cv. 'The Prince', in comparison with 32% in 'Judía de Franco', 20% in *P. coccineus* and 18% in 'Maxidor' (Fig. 1d). Taken together, these results allowed establishing the relative degree of drought tolerance of the studied cultivars: *P. vulgaris* cv. 'Maxidor' is clearly the most tolerant, followed by *P. coccineus* cv. 'Moonlight', whilst *P. vulgaris* cv. 'The Prince' is the most sensitive, probably because the mechanisms to avoid cellular dehydration under water stress are much less efficient than in the other cultivars.

Osmolyte contents

Leaf Pro levels increased significantly under drought stress in all cultivars, by 12 to 15-fold in plants of *P. vulgaris* cv. 'The Prince' and cv. 'Judía de Franco', and in *P. coccineus* cv. 'Moonlight' plants, but by only 4-fold in those of cv. 'Maxidor' (Fig. 2a). Leaf GB contents showed no significant variation under water deficit conditions in the three *P. vulgaris* cultivars, but a 1.5-fold increase, approximately, in *P. coccineus* (Fig. 2b). Mean amounts of total soluble sugars (TSS) did not vary significantly, as compared with the controls, in stressed plants of any of the investigated *Phaseolus* varieties (Fig. 2c), but this kind of measurements may be masking significant changes in the levels of individual carbohydrates, which could be relevant for drought tolerance.

To detect possible drought-induced changes in the levels of individual sugars, the soluble fraction was fractionated by HPLC. Three major peaks were observed in the chromatograms, which were identified as corresponding to fructose, sucrose and *myo*-inositol by co-elution with commercial purified standards. When analysing these individual carbohydrates, some differences were found between control and stressed plants. Fructose levels increased significantly only in *P. vulgaris* cv. 'Judía de Franco', differences were not significant in cv. 'The Prince' or in *P. coccineus*, and a small (but significant) decrease was observed in cv. 'Maxidor' (Fig. 3a). A decrease in mean sucrose contents was measured in all cultivars except 'The Prince' (Fig. 3b). Yet, the most interesting finding was the large increases in mean *myo*-inositol contents detected in plants of cv. 'Maxidor', and to a lesser extent of *P. coccineus*, subjected to water stress (Fig. 3c), although in the latter cultivar differences were not statistically significant.

Discussion

As for other stresses, the most general effect of drought on plants, and the easiest to quantify, is inhibition of growth, which allows plants to survive under adverse conditions by re-directing their resources (metabolic precursors and energy) from normal metabolism and biomass accumulation to the activation of specific stress defence mechanisms (2, 20). By measuring several growth parameters in water-stressed plants we could establish the relative degree of resistance of the investigated bean cultivars. *Phaseolus vulgaris* cv. 'Maxidor' was the most tolerant – even more than cv. 'Moonlight', the tested *P. coccineus* cultivar, although this species has been previously reported as being more tolerant than *P. vulgaris* (21, 22). On the other hand, common bean 'The Prince', followed by 'Judía de Franco', were the most drought-sensitive cultivars.

It is well established that all plants share a series of basic, conserved mechanisms of response to abiotic stress, including the accumulation of different osmolytes in the cytoplasm to help maintain cellular osmotic balance (2, 20). Yet, it is not so clear the relative importance and contribution of different osmolytes to the stress tolerance of a given species. Comparative analyses of related genotypes, such as those reported here

for four *Phaseolus* cultivars, may help to answer this question, by correlating their relative degree of resistance to stress with changes in the levels of specific osmolytes.

Proline is one of the commonest osmolytes in plants, and seems to be a reliable marker of stress in *Phaseolus*, as in many other genera. Many reports have shown significant increases in Pro contents in *Phaseolus* plants submitted to water stress (23, 24), but the correlation between Pro accumulation and stress tolerance remains unclear since apparently contradictory results have been reported using different bean varieties (25, 26). The present study revealed that Pro accumulation is a common response to water stress in the four analysed bean cultivars, in agreement with the aforementioned published results. Yet, Pro cannot contribute significantly to their stress tolerance, since the levels reached in the most tolerant variety, cv. 'Maxidor', were by far lower than in the other cultivars. In this case, Pro should be considered as a marker of the level of stress affecting the plants, and these results simply reflect the fact that 'Maxidor' plants were less stressed than those of the other cultivars after three weeks without watering.

Glycine betaine is another osmolyte synthesised in response to water stress in many different plant groups (27), but there are only a handful of references describing the presence of this osmolyte in *Phaseolus* (23, 28), although at concentrations lower than those reported here. However, the applied water stress treatments did not lead to significant increases of GB contents in the analysed *Phaseolus* varieties, with the exception of water-stressed *P. coccineus* plants, suggesting that GB may contribute to drought tolerance in runner beans, but not in common beans.

Assessing the role of soluble sugars (e.g. sucrose, glucose or fructose) in the mechanisms of stress tolerance has the added difficulty that their role as compatible solutes may be masked by their multiple biological functions in plants, as direct photosynthesis products, components of primary metabolism and regulatory molecules (29). We did not detect significant changes, correlated with the water stress treatment, in the levels of total soluble sugars in the analysed *Phaseolus* cultivars. However, after separation of the carbohydrate fraction by HPLC, strong increases in *myo*-inositol contents were observed in cv. 'Maxidor', the most tolerant cultivar of *P. vulgaris*, and to a lesser extent in *P. coccineus* cv. 'Moonlight', the second most resistant of the tested cultivars. Therefore, this polyalcohol appears to play a significant role in the stress tolerance mechanisms in *Phaseolus* taxa. Although variation of soluble carbohydrate contents in beans under water deficit conditions has been previously observed (30, 31), publications on the presence of *myo*-inositol in this genus are scarce and, to our knowledge, there are no data on its accumulation in response to water stress.

There are numerous papers reporting a wide range of drought tolerance within the genus *Phaseolus* (32-34), supporting the possibility of selecting the most resistant genotypes to be used in breeding programmes to improve this important trait in beans. Other studies have focused on the physiological mechanisms of response to water deficit in beans, genetic re-

sources and breeding strategies for drought tolerance (35, and references therein), but there are not many data on biochemical markers which could facilitate the selection of resistant varieties. Our results suggest that a large number of bean cultivars can be simply and rapidly screened for drought tolerance by determining the accumulation of specific osmolytes in leaves under water deficit conditions. The relatively more resistant varieties should have higher *myo*-inositol contents but lower Pro levels (as those plants would be less stressed). In this way, promising candidates could be easily selected at the stage of vegetative growth. The pre-selected cultivars could then be subjected to a more extensive analysis in terms of agronomic performance (e.g. yield) under water stress conditions in the field, and those with confirmed higher drought resistance could be eventually used in bean breeding programmes.

Conflict of interest statement

The authors declare no commercial or financial conflict of interest.

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