

Variations in proline accumulation and relative water content under water stress characterize bean mutant lines (*P. vulgaris* L.)

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Abstract

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Drought stress linked with climate change is one of the major reasons limiting the productivity of crop species. This investigation was intended to characterize 19 bean mutant (*Phaseolus vulgaris* L.) genotypes for water-deficit tolerance, by determining their relative water content and proline accumulation. The drought stress was simulated by Polyethylene Glycol (PEG₆₀₀₀). The response of the two populations with initial genotypes – variety Evros and variety Mastilen 11b were evaluated in pots arranged in a three-plot design. The pots were placed in a greenhouse with full sunlight. The plants were watered using liquid ½ Murashige and Skoog (MS) nutrient solution every 2 days (50 mL per pot). After the plants have three trifoliolate leaves (\pm 14 days after planting) the solution was combined with PEG (20% PEG equivalent to – 1,2 Mpa osmotic potential) solution treatment and the volumes were increased up to 100 mL. The control plants were watered with a ½ MS nutrient solution without PEG.

During the treatment with PEG changes of RWC % in leaves and roots were observed. Drought stress resulted in a significant reduction in fresh and dry leaf and root weights and relative water content (RWC %). Proline content of the studied mutant lines increased when plants were subjected to water deficit. The results indicated that M₃ mutant lines (variant 9, variant 16, and variant 20) exhibited the best drought tolerance and are good candidates for further evaluation to release mutant varieties or to be used as donor parents in a bean crop improvement program towards drought tolerance.

Keywords: mutant lines (*P. vulgaris* L.); water stress; relative water content; proline

Introduction

The common bean (*Phaseolus vulgaris* L.) is one of the most important and the most-consumed food legumes from the Fabaceae family (Rosales et al., 2012). Beans (*P. vulgaris* L.) are consumed all over the world and are a staple food in many countries. Beans are a good source of components of the diet, such as a source of protein, vitamins, minerals, and fiber (Bellucci et al., 2014; Broughton et al., 2003).

The beginning of 21 century is marked by global scarcity of water resources, environmental pollution, and increased salinization of soils and waters. Abiotic stress is already a major limiting factor for plant growth and will soon become even more severe as desertification covers more and more of the world's terrestrial areas (Vinocur & Altman, 2005). Significant changes in agricultural practices around the world are currently expected as population growth exceeds food supply.

There is a general consensus that water saving is very critical to plant growth and development. Various studies have shown that *P. vulgaris* L. is relatively sensitive to drought stress as compared to other grain legumes (Molina et al., 2000); nevertheless, it is cultivated under diverse environmental conditions, including relatively dry areas (Sugenith et al., 2020). In fact, globally, only a small percentage, around 7%, of the cropland planted with common bean receives adequate rainfall (Broughton et al., 2003) and in some areas, drought causes yield losses of up to 80% (Cuellar-Ortiz et al., 2008). The drought stress mainly causes low pod setting ratio, early pod abscission, and consequently low productivity (Shen & Webster, 1986; Suzuki et al., 2001), decreasing pollen stainability (Tsukaguchi et al., 2003) and flower buds (Choudhury et al., 2010).

Abiotic stresses are the major environmental challenges for crops in countries with a typical continental climate like Bulgaria. Drought, high summer and low winter, and spring temperatures restrict plant growing season and decrease productivity (Djilianov et al., 2005).

To increase legume productivity under water stress, it is imperative first to understand tolerance mechanisms. Plants have evolved several adaptations including escape and avoidance, compatible solute accumulation, antioxidant regulation, and hormonal regulation. Compatible solute accumulation is a fundamental strategy for applying osmoprotectant and osmotic adjustment under water stress (Nadeem et al., 2019). These compatible solutes accumulate primarily in drought-stressed cells without interfering with the macromolecules and are either hydroxyl compounds like oligosaccharides, polyhydric alcohols, and sucrose or nitrogen-containing compounds such as amino acids and proline, polyamines and ammonium compounds (Majumdar et al., 2019). The mechanism of osmoprotection is based on the close association of non-toxic elements with numerous components of the cell, whereas osmotic regulation assists in maintaining turgor through maintaining the water contents of cells (Slama et al., 2015). During drought stress, proline plays an important role and acts as a signaling compound to regulate mitochondria function and affects cell proliferation by means of activating particular genes, which are essential for recovery from stress (Solanki et al., 2015). Proline accumulation aids in retaining membrane integrity by decreasing the oxidation of lipids through guarding cellular redox potential and scavenging free radicals (Shinde et al., 2016).

Among the several methods used to characterize internal plant water status under drought conditions, RWC is an integrative indicator (Choudhury et al., 2010; Parsons & Howe, 1984) used to identify drought tolerance of varieties (Liu et al., 2003; Matin et al., 1989). Slow development

of water deficit may induce osmotic adjustment resulting in the maintenance of appropriate water content of leaf during the period of water deficiency. As a result, plants of French beans can survive longer under a water deficit. Varieties showing a smaller drop in leaf water content set more pods than the varieties showing a large drop in leaf water content (Choudhury et al., 2010). Most bean breeding programs for tolerance to drought used conventional breeding methods exploiting only the available natural variability. The selection within available germplasm for a long time exhausted the genetic variability (Amri-Tilioune et al., 2018).

The development of new varieties tolerant to water stress with high yield is therefore important to anticipate bean yield losses. Induced mutation can be efficiently used as an alternative to induce genetic variability (Moussa, 2011). Mutation breeding is an alternative solution to producing various mutant forms. For quite a long time now, the use of mutagens has been proven to be beneficial in cowpea (Gnankambary et al., 2019b) and in groundnut (Gunasekaran & Pavadai, 2015).

Consequently, the aim of this study was to determine the effects of drought stress on proline accumulation and relative water content in nineteen bean mutant lines (*Phaseolus vulgaris* L.).

Material and Methods

Nineteen mutant lines in M₃ generation obtained by ethyl-methane sulfonate (EMS) treatment from varieties Mastilen 11b and Evros (the latest originating from Line 564) were selected on a single plant descent selection method. Seeds were treated by EMS in the Molecular Biology Laboratory at the Maritsa Vegetable Crops Research Institute, Plovdiv (Sofkova-Bobcheva et al., 2021).

The mutant lines were evaluated under a greenhouse - photoperiod of 16 h natural light and ambient temperature of 23 – 25°C.

All seeds were sown directly into a moistened mixture of peat (50%), perlite (50%) in pots (Ø = 10 cm). The substrate was kept moderately moist, using Murashige and Skoog solution. The plants were watered using liquid ½ MS nutrient (Murashige and Skoog) solution every 2 days (50 mL per pot). After the plants had three trifoliate leaves (14 days after planting) the solutions were combined with PEG₆₀₀₀ and the volumes were increased to 100 mL. The plants were treated with a liquid of ½ MS nutrient solution without PEG₆₀₀₀ as control, and the same liquid containing 20% PEG₆₀₀₀ (equivalent to – 1.2 MPa) for drought tolerance selection. In all periods plants were controlled from pests and diseases. The treatment with PEG₆₀₀₀ was terminated after the plants were 28 days.

Relative Water Content

The relative water content was estimated according to the method of Gulen & Eris (2003) using the equation:

$$\text{RWC \%} = [(\text{FW}-\text{DW}) / (\text{TW}-\text{DW})] \times 100$$

Leaf discs and root were weighed to determine the fresh weight (FW), soaked in distilled water at 25°C for 4 h to determine the turgid weight (TW), then oven-dried at 70°C for 24 h (leaves) and 80°C for 72 h (root) to determine the dry weight (DW).

Proline Concentration

The proline concentration was determined spectrophotometrically using the ninhydrin method of Bates (1973). First, fresh leaf samples were homogenized in 3% sulfosalicylic acid, followed by the addition of 2 mL each of ninhydrin and glacial acetic acid, after which the samples were heated to 100°C. The mixture was then extracted with toluene, and the free toluene was quantified at 520 nm.

$$\text{proline } \mu\text{mol} / \text{g FW} = [(\mu\text{g proline/ml} \times \text{mL toluene}) / 115.5 \mu\text{g}/\mu\text{mole}] / [(\text{g sample}) / 5]$$

Statistical Analysis

Data were expressed as mean \pm standard error and were analyzed statistically. The means were compared statistically using Duncan's multiple-range test at the level of $p < 0.05$. Principal component analysis (PCA) was used to check the similarity between the responses of stress within each genotype, and the similarity between accessions. The average mean and the significance interval of the studied characteristics were calculated by descriptive statistics (IBM SPSS Statistics19, 2010).

Results

Drought stress adversely affected all bean genotypes studied. The highest values of root and leaf fresh weights were observed in the control untreated plants. Growth parameters (leaves and root fresh weight) of the genotypes were reduced under water deficit stress compared to the control, which may have been due to the loss of turgor and reduction of relative water content. The data of leaves RWC % are presented in Figure 1.

Drought challenge may decrease cell division and elongation, which can lead to leaf weight reduction. Similar results have been reported in several crop species, such as common bean (Emam et al., 2010), faba bean (Siddiqui et al., 2015), corn (Lorens et al., 1987). Among the studied variants, drought significantly decreased leaf fresh weight in variants 4, 5, 6, and 8. In these lines, RWC % reduction

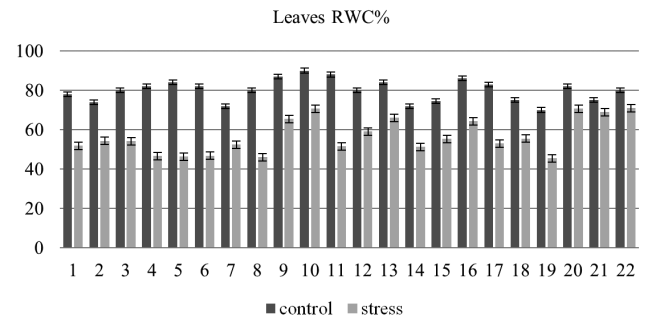


Fig. 1. Relative water content in leaves of mutant lines subjected to water stress by PEG₆₀₀₀

*control – untreated plants; stress – plants treated by PEG₆₀₀₀

was from 84% (control plant) to 46% for treated plants. This trend may be indicative of sensitivity to drought stress. The bean mutant genotypes tested in this study showed variability in phenology and physiological responses to water deficit. Water stress decreased leaf relative water content and biomass in all the tested genotypes. Among the studied variants 10, 20, and 22, the variety Lody exhibited the highest RWC % after stress while variant 19 had the lowest RWC % - 45% under water deficit stress. Leaf RWC % of all accessions remained similar under well-watered conditions. This genotypic variation in RWC may be attributed to differences in the ability of the varieties to absorb more water from the soil and / or the ability to control water loss through the stomata. These findings are in agreement with those reported in cowpeas and soybeans (Cortes & Sinclair, 1986), and in sugar bean (McCree & Richardson, 1987). In the present experiment, leaf RWC of all genotypes decreased significantly depending on water deficit level, which suggested differences in leaf hydration, leaf water deficit and physiological water status in the different studied variants. This result strongly supports the findings of Siddiqui et al. (2015) in faba bean genotypes. The authors suggest that the differences in RWC in genotypes could be associated with their capacity for water absorption from the soil.

At drought stress, the root RWC % decreased less than the leaves (Figure 2). The differences in RWC in all genotypes could be associated with their ability of water absorption. Thus, we concluded that genotypes 5, 6, and 9, followed by pairs 20, and 19 showed 68 - 74% RWC after treatment and could have a better ability to resist drought stress. According to Devarshi & Khanna-Chopra (2010) under stress, the drought tolerant wheat plants exhibited better root water relations in terms of turgor potential and RWC as compared to sensitive genotypes.

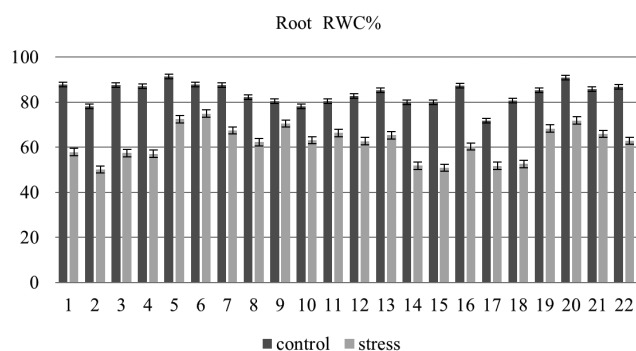


Fig. 2. Relative water content in root of mutant lines subjected to water stress by PEG₆₀₀₀

Data are represented as the means \pm SD and are derived from 3 replicates

*control – untreated plants; stress – plants treated by PEG₆₀₀₀

Differences within mutants in RWC may also be a result of maximizing soil water reserves by fully extracting water in the existing rooting zone and/ or extending rooting depth to increase water reserve for the crop. Other researchers have investigated the role of increased root length and density in the maintenance of guard cells. Martin et al. (1989) proposed the mechanism for drought tolerant barley varieties that maintained high RWC under water stress. They referred that similarly, stomatal conductance may be a useful indicator in discriminating drought tolerant and non-tolerant varieties in the glasshouse, such as water stress, developing quickly in the glasshouse due to limited soil volume in the pots.

The physical features such as length of stem, root and leaves exhibited reduction with increasing time of drought stress. The growth arrest seen in roots and leaves can be considered as a possible mechanism to preserve carbohydrates for sustained metabolism, prolonged energy supply and for better recovery after stress relief. It is believed that retardation of root growth and the decrease in its activity combined with stomatal closure caused by the accumulation of abscisic acid produced in roots and then accumulated in guard cells in response to water deficit are the causes of decrease in RWC reported by Khan et al. (2007).

Drought stress induces changes in metabolic parameters that are associated with the tolerance of plants to water shortage. Accumulation of free amino acids (especially proline) and soluble carbohydrates by plant tissue under water deficit conditions is an adaptive response (Sheela Devi & Sujatha, 2014).

In this study, proline accumulation was significant in the stressed mutant lines (9, 16, 19, 20) compared to the parental varieties and control variety Lody (Figure 3).

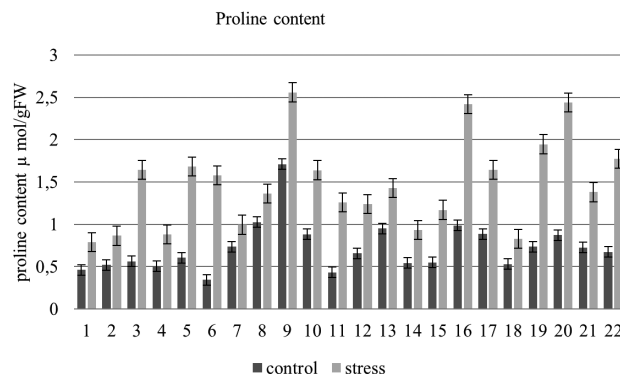


Fig. 3. Proline content in leaves of mutant lines subjected to water stress by PEG₆₀₀₀

The data are represented as the means \pm SD and are derived from 3 replicates

*control – untreated plants; stress – plants treated by PEG₆₀₀₀

Leaf proline concentration, on the contrary of RWC, significantly increased in response to water stress, about 1.5 fold (variant 18) to 4.5 fold (variant 6), as average, for the plants treated.

Hayat et al. (2012) reported that overproduction of proline in plants exposed to various environmental stress imparts stress tolerance by maintaining cell turgor, stabilizing membranes, and bringing concentrations of reactive oxygen species (ROS) to normal ranges.

The accumulation of proline in plants reduces the toxic effects of ions on enzymes activity and also lowers the generation of free radicals formed by drought stress. Also, proline is associated with recovery resistance by serving as a source of respiratory energy to the plants under stress. Underwater stress genotypes 20, 9 and 16 (from 2.439 $\mu\text{mol}/\text{FW}$ to 2.559 $\mu\text{mol}/\text{FW}$) gave the maximum value for proline content, and genotype 1 and 2 being at par with genotype 18 exhibited lower value for the content of proline.

The results obtained with mutant line 16 (more decrease of RWC) is contradictory to results obtained for the proline accumulation, where it was found that this variant showed a high degree of proline accumulation and may have a large degree of drought tolerance. A possible explanation for this contradiction may be the growth strategy of the mutant line during drought stress. This line might suspend growth in order to reduce the detrimental effect of drought stress. Turgor-related processes (cell elongation, etc.) therefore no longer played a vital role during this period of stress in our study. The large-scale proline accumulation may be involved in a number of protective functions during stress. This may explain the lack of ability of this variant to maintain RWC values. It is evident that when the RWC patterns of different

varieties are studied during drought stress, careful consideration should be given to differences in growth strategies (van Heerden & de Villiers, 1995).

In our experiments, although accumulated to high concentrations, free proline (variant 19 – 1.946 $\mu\text{mol/g FW}$ and variant 3 – 1.644 $\mu\text{mol/g FW}$) did not seem to contribute to the maintenance of foliar RWC. This is in accordance with the suggestion that increased proline levels are associated not only with osmoregulation. Proline also plays essential roles in the absence of stress, being involved in many developmental processes - proline concentration increases during pollen and seed maturation. There is some confusion, often found in the literature, between the concepts of ‘stress responses’ and ‘stress tolerance’. Even though stress tolerance mechanisms are based on specific stress responses, not all responses are relevant for tolerance. On this line, proline accumulation can be considered as a general ‘response’ to abiotic stress in many plant species, but proline may be involved in stress tolerance mechanisms, depending on the species (Sugenith et al., 2020).

The principal component analysis (PCA) was used to check the similarity between the responses to the stress within each variety and the similarity between accessions. A PCA was performed for all mutant lines and control and including the mean values of all measured parameters and the two applied conditions (control and water stress).

Based on the PCA scatter plot (Figure 4), we identified some accessions (2, 14, 15 and 18) with low (0.827 – 1.170 $\mu\text{mol/g FW}$) concentrations of proline and RWC (51 - 55%) to Reference line from Equator. On the other hand, three mutant lines (9, 16, and 20) showed high proline contents (2.420 – 2.550 $\mu\text{mol/g FW}$) and limited growth inhibition, indicating that these lines can be considered as the most tolerant. Similarly, the relative position of other variants (13, 22) along the Rx-axis should allow a ranking of their tolerance to water deficit.

Considering the multiple functions of proline (protein synthesis, antioxidative reactions, immune responses, influences flowering time), it is logical to assume that proline accumulation would be associated with higher stress tolerance, and this has indeed been demonstrated for many plants and wild species.

In the present study, responses to drought have been analyzed in all the studied 22 *P. vulgaris* L. genotypes. However, the statistical analyses performed with all experimental data provided a clear overall picture of the responses to stress of the *P. vulgaris* L. variants. Both ‘variant’ and ‘treatment’, as well as their interaction, had a highly-significant effect on all growth traits analyzed, and on proline contents of water deficit. In all cases, growth inhibition was mostly reflected in the stress-induced reduction of fresh weight and water contents of leaves and increased of proline levels.

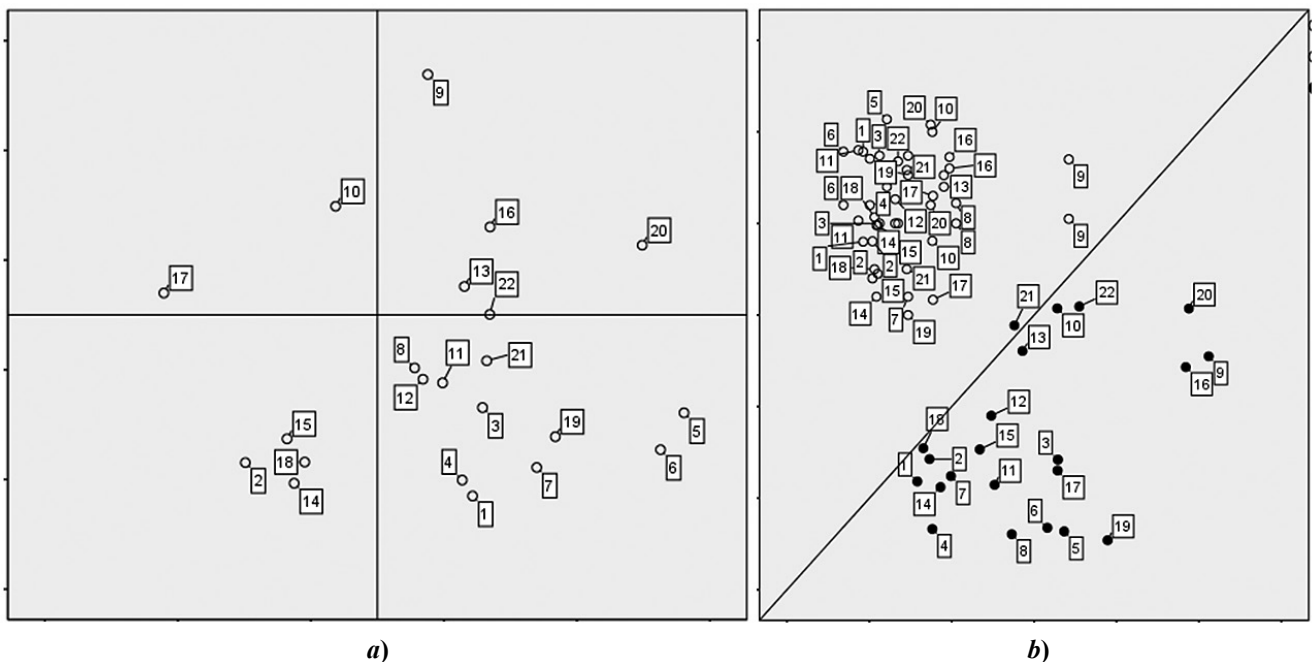


Fig. 4. Scatter plot of the principal component analysis (PCA) score conducted with the analyzed traits in mutant lines: a) control; b) proline accumulation in stress

Conclusion

The current study deduced that the material evaluated contained useful genetic diversity for drought tolerance. Proline allows identifying differences in stress response even in closely related genotypes. All morphological and physiological characteristics of 22 genotypes of bean reduced under drought stress. We observed all genotypes of bean behaved differently under water stress.

In the present study, we found some genotypes with high proline contents and limited inhibition of RWC in stress conditions (mutant lines 9, 16 and 20) and others with low proline accumulation during the stress (mutant lines 2, 14, 15 and 18). For further study, these genotypes can be used to uncover molecular mechanism(s) involved in building the tolerance of bean plants to drought stress. The mutant lines, with the best drought tolerance, are good candidates for further evaluation in field condition to their further release as variety or their use as donor parents in bean improvement programs for drought tolerance.

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