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Mutation breeding for heat and drought tolerance in tepary bean (*Phaseolus acutifolius* A. Gray)

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Abstract

Tepary bean (*Phaseolus acutifolius* A. Gray) is more heat and drought tolerant than common bean (*P. vulgaris* L.). Four hundred mutant lines of two tepary accessions (G40068 and G40159) were generated by ethyl methane sulfonate (EMS) treatment. In preliminary studies of the M_5 mutant lines under abiotic stress, three mutant lines (CMT 38, CMT 109, CMT 187) were selected from six mutated lines based on morpho-physiological traits and superior yield and advanced to the M_6 generation. The M_6 mutant lines were uniform and genetically stable. These mutant lines and their original (M_0) parents were evaluated for heat and drought tolerance under greenhouse conditions. Their performance was evaluated for morpho-physiological attributes, seed yield and yield components. Under high temperature and drought conditions, the CMT 38 mutant (M_6 line) and its original tepary (M_0) accession (G40068) showed greater values of pod biomass, pod number and 100-seed biomass than the other lines tested. The CMT 109 and CMT 187 mutant lines and their G40159 original accession (M_0) also showed the highest value of seed number under high temperature and drought conditions. This suggests that the previous screening performed during the population advancement of these mutant lines, based on morphological traits like growth habit, was not detrimental to the yield variables evaluated here. Under combined heat and drought conditions, different parameters could be incorporated into tepary breeding programmes, as selection criteria to screen genotypes for tolerance to heat and drought stress. These parameters included: chlorophyll (SPAD) readings, seed biomass, 100-seed biomass and seed number because they explain the observed variance in the principal component analysis. Two additional traits (root biomass and stem diameter) were also identified as useful attributes, based on univariate analysis. The mutant lines evaluated here offer potential for further improvement of tepary bean to high temperature and drought.

Key Words: Abiotic stress; beans species; crop improvement; EMS mutagenesis; yield.
Abbreviations: EMS, ethyl methane sulfonate; M_6, Mutant line generation 6; PCA, principal component analysis; SPAD, Soil-Plant Analyses Development chlorophyll meter.

Introduction

Global warming is responsible not only for global temperature increase but also for region-specific increases or decreases in precipitation. This, in turn, has a negative impact on the production systems of crops that are vital for improving food and nutritional security of people in developing countries. Common bean, *Phaseolus vulgaris* L., is a valuable source of protein, starch and other nutrients. Drought affects 60% of the dry bean production area worldwide (Beebe et al., 2008). Temperatures > 30 °C during the day or > 20°C during the night result in significant yield reduction of common bean (Rainey and Griffiths, 2005).

Tepary bean, *Phaseolus acutifolius* A. Gray, is a traditional crop of desert and semi-arid regions of Mexico and southwestern USA (Freeman, 1912; Nabhan and Felger, 1978). Renewed interest in tepary is due to its possession of many traits that enable it to flourish in hot and dry regions: it is more heat tolerant at biological tissue levels than common bean and it produces more leaves to compensate for reduced leaf size due to heat stress (Lin and Markhardt, 1996). Tepary also has more extensive and a thinner root system, better stomatal control and more active para-heliotropism than common bean (Markhart, 1985; Bielenberg et al., 2003; Butare et al., 2012). It is part of the tertiary gene pool of common bean and considered as a potential useful donor parent of drought and heat tolerance traits for common bean improvement, through interspecific hybridization (Muñoz et al., 2004, Rao et al., 2013). Tepary could be a source of genes for the improvement of common bean through inter-specific crosses followed by backcrossing (Mejia et al., 1994). It could also serve as a valuable crop in itself, particularly for dryland environments where common bean is less adapted (Muñoz et al., 2006). An evaluation of tepary gene introgression showed that tepary DNA markers can be transferred to the interspecific progeny (Muñoz et al., 2004). However, success is limited to a lower-than-expected percentage of genome contributed by tepary (Blair et al., 2012). Introgression of heat or drought tolerance from tepary into common bean might be feasible through breeding, to generate elite lines that can tolerate up to 4°C higher temperatures. However, most of the lines obtained come from a limited number of crosses between common and tepary beans (Muñoz et al., 2004). A diversity study of the

tepary collection at the Genetic Resources Unit (GRU) of CIAT Colombia, using AFLP and microsatellite (SSR) markers showed that the genetic base of the cultivated tepary accessions is narrow (Muñoz et al., 2006; Blair et al., 2012). A similar conclusion was made after a study of variability of the seed storage proteins of wild and cultivated accessions of tepary (Schinkel and Gepts, 1988). A reason for this reduced genetic diversity might be the historic regression of tepary after the introduction of new watering technologies in Mesoamerica after 1492 (Nabhan and Felger, 1978; Nabhan, 1985; Debouck, 1992). Given this genetic extinction, future breeding rests on exploiting the significant diversity provided by wild teparies and related species (Muñoz et al., 2006), transformation (Dillen et al., 1997) or by inducing variation via mutagenesis (explored here). Traits that are particularly looked for in tepary are: uniform red seed colour, erect growth habit and grouped pod maturity (Pratt and Nabhan, 1988).

Mutagenesis has been used to broaden the genetic diversity of *Phaseolus* species (Ahloowalia et al., 2004; Blair et al., 2007; Gwata et al., 2016). The results of chemical mutagenesis of common bean with ethyl methane sulfonate (EMS) in morphological and physiological changes, as well as varietal development have been reported (Blair et al., 2007; Porch et al., 2009). With the objective of genetic improvement of tepary bean, a protocol was developed by Muñoz et al. (2013) for chemical mutation induction using EMS in two cultivated tepary accessions (G40068 and G40159).

From the research discussed above, three further questions arise: (i) Why is it important to induce mutations in tepary? (ii) Which novel traits could be achieved by mutation induction in tepary? and (iii) How would the mutant lines be used in a breeding programme for tepary and/or common beans? The main objectives of the present study were to: i) evaluate tepary bean M_6 mutant lines under conditions of high temperature and drought; and ii) identify heat and drought tolerant mutant lines that could serve as parents in breeding programmes that aim to improve heat and drought tolerance in common bean.

Results

Effect of high temperature (HT) on shoot and root morpho-physiological characteristics

Table 1 presents the results of the effect of HT on genotypic differences in yield components such as pod number, pod biomass, seed number, and 100-seed biomass and also the number and biomass of nodules (nodules variables). The genotype parameter showed an effect ($P \leq 0.01$) on seed number, pod number and nodule biomass under both conditions of temperature HT and CT (control temperature). Under HT conditions, the effect of genotype was significant for pod biomass, seed biomass and nodule number.

There was no difference between the mean values of pod numbers for the genotypes under HT and CT conditions, but the value was higher under CT (24.7 pods/plant) compared to HT (20.5 pods/plant). The mutant line CMT 38 showed a high mean value for pod number under HT and CT conditions. For pod biomass, a similar trend was observed: the mean value was higher under CT (17.2 g /plant) compared to HT (14.0 g /plant). The pod biomass of the CMT 38 mutant line was higher under both conditions of temperature and differences ($P \leq 0.05$) were observed between the CMT 38 mutant line and the other mutant lines or parental accessions G40068 and G40159 (Table 1).

The seed number/plant was also higher under CT (88.9 seeds/plant) compared to HT (69.3 seeds/plant). Under both conditions of temperature, the CMT 109 mutant line showed the highest seed number/plant. Differences ($P \leq 0.05$) were observed between this mutant line (CMT 109) and the other two mutant lines (CMT 38 and CMT 187) and differences were also observed between the mutant line CMT 109 and the tepary parental accession G40068 (Table 1).

The 100-seed biomass/plant was higher under HT (16.5) as compared to CT (15.7). Under HT, the value was higher for the tepary accession G40068 and mutant line CMT 38, and differences ($P \leq 0.05$) were observed between these genotypes and the others. Similar responses of these genotypes were observed under CT. The tepary accession G40068 and the mutant line CMT 38 showed the highest values of 100-seed biomass. The differences were also significant ($P \leq 0.05$) when compared to the other genotypes (Table 1). Nodule formation (nodules/plant) was lower under HT (3.0) as compared to CT (12.5). The tepary accession G40068, showed a high mean value under HT. The mutant lines CMT 38 and CMT 109 and the tepary accession G40068 showed the highest values of nodule formation under CT (Table 1). The nodule biomass (g/plant) was lower under HT (0.006) compared to CT (0.039). Under HT conditions, the tepary accession G40068 showed the highest nodule biomass, while the mutant line CMT 109 and the tepary accession G40159 showed the highest mean nodule biomass) under CT. This value was significantly different ($P \leq 0.05$) to the other genotypes evaluated (Table 1).

The results of the leaf biomass, stem biomass and roots biomass under HT (data not shown) showed significant differences ($P \leq 0.001$) between genotypes. The mutant line CMT 38 (1.8 g/plant) and the G40068 (1.9 g/plant) tepary accession showed the lowest mean stem biomass compared to the CMT 187 (2.5 g/plant) and CMT 109 (2.2 g/plant) mutant lines and the G40159 (2.1 g/plant) tepary accession. Significant differences ($P \leq 0.05$) were observed between the two groups of genotypes. The highest mean values for root biomass were observed in the G40068 tepary accession (0.98 g/plant) and the CMT 187 mutant line (0.95 g/plant). Significant differences ($P \leq 0.05$) were observed between the means of these genotypes and the mean values of the mutant lines: CMT 38 (0.83 g/plant), CMT 109 (0.76 g/plant) and the G40159 tepary accession (0.76 g/plant). No differences were observed between genotype means for leaf biomass under HT (data not shown). No difference between genotypes was observed for stem diameter (data not shown). Under CT, significant differences ($P \leq 0.001$) between genotypes were observed for leaf biomass and stem biomass, but not for stem diameter and root biomass. The CMT 187 mutant line showed the highest means for leaf biomass and stem biomass and significant differences ($P \leq 0.05$) were observed between this mutant line and the other genotypes (data not shown). In relation to physiological variables, under HT (results not shown), significant differences ($P \leq 0.001$) were observed between the tepary parental (M_0) accessions and the mutant lines for the efficiency of the photosystem II (QY) and the stomatal conductance. The CMT 187 (46.6) and CMT 109 (58.8) mutant lines and the G40159 (58.1) tepary accession showed the lowest mean stomatal conductance values as compared to the CMT 38 (79.8) mutant line and the G40068 (66.7) tepary accession. Significant differences ($P \leq 0.05$) in stomatal conductance were only observed between CMT 187 and CMT38 mutant lines and G40068. No differences were observed between genotypes for the SPAD and leaf temperature variables. Under CT, significant differences ($P \leq 0.001$) were observed between genotypes for the SPAD and the stomatal conductance tests. The CMT109 mutant line showed a SPAD mean higher (45.0, $P \leq 0.05$) than the CMT 38 mutant line (39.7). The G40159 tepary accession, the mutant line CMT 38 and the tepary M_0 accession G40068 showed higher means (121.9, 118.5 and 107.6) for stomatal conductance, respectively, as compared to the CMT 187 and CMT 109 mutant lines (95.9 and 75.9), respectively.

Effect of temperature under drought and irrigated soil conditions on shoot and root morpho-physiological characteristics

The results on the effect of HT and drought as compared to CT and irrigated conditions of soil on pod number, pod biomass, seed number, 100-seed biomass, nodule number and

nodule biomass are shown in Tables 2 and 3. Soil conditions (drought or irrigated) showed significant effects (P≤0.01) on pod number /plant, seed number /plant, pod biomass /plant, 100-seed biomass/plant and nodule biomass /plant under both temperature conditions. The effect of soil condition was also significant (P≤0.01) for the nodule number/plant, under HT treatment.

Effect of HT under drought and irrigated conditions of soil

Under HT the pod number/plant was lower under drought (14.5) as compared to the irrigated treatment (26.6) for all genotypes (Table 2). Under drought conditions the mutant line CMT 38 showed the highest value as compared to other genotypes. Significant differences (P≤0.05) were observed between this mutant line, the mutant line CMT 109 and the tepary accession G40068 (Table 2). Under irrigated conditions, two mutant lines (CMT 38 and CMT 109) showed the highest values. Significant differences (P≤0.05) were observed between these mutant lines and the mutant line CMT 187 that showed the lowest pod number (23.4) (Table 2). For pod biomass (g/plant), the value was also lower under drought (9.6) than the irrigated treatment (18.4) for all genotypes. The mutant line CMT 38 showed the highest value under drought and irrigated conditions. Under drought conditions, significant differences (P≤0.05) were observed between the mutant lines CMT 38 and CMT 109, which showed the lowest value. Under irrigated conditions, significant differences (P≤0.05) were observed between the mutant line CMT 38 and CMT 187 and the tepary accession G40159. The seed number/plant was lower under drought (45.9) compared to irrigated conditions (92.7) for all genotypes (Table 2). The tepary parental accession G40159 showed the highest value under drought conditions. Significant differences were observed between this accession, the mutant line CMT 38 and the tepary accession G40068. Under irrigated conditions, the mutant line CMT 109 showed the highest value. Significant differences (P≤0.05) were observed between this mutant line, the mutant line CMT 187 and the tepary accession G40068. There was a small difference in 100-seed biomass (g/plant), between drought (17.3) and irrigated conditions (16.1) for all genotypes. Under drought conditions, the tepary accession G40068 and the mutant line CMT 38 showed the highest value of 100-seed biomass. There were significant differences (P≤0.05) between these genotypes and the mutant lines (CMT 109, CMT 187) and the tepary accession G40159 (Table 2). Under irrigated conditions, the same genotypes also showed the highest 100-seed biomass values. Significant differences (P≤0.05) were observed between all genotypes. The nodule number/plant was very low under drought and irrigated conditions (1.01 and 5.10, respectively), for all genotypes evaluated. Under irrigated conditions, the G40068 tepary parental accession showed the highest number. There were significant differences (P≤0.05) between this tepary accession, the mutant line CMT 38 and the other genotypes (Table 2). Under drought conditions, all genotypes showed a lower level of nodule formation. With respect to nodule biomass (g/plant), only the G40068 tepary accession showed a higher mean (0.024) under irrigated conditions, and significant differences (P≤0.05) were observed between this accession and the other genotypes (Table 2).

Effect of CT under drought and irrigated conditions of soil

The results obtained with the five genotypes under normal (control) conditions of temperature (CT) in a greenhouse are shown in Table 3**.**

For each treatment (drought or irrigated), all variables were higher under CT as compared to HT. The pod number/plant was lower under drought (18.3) compared to irrigated conditions (31.2) under CT for all genotypes. The mutant line CMT 38 and the tepary accession G40068 showed the highest values under irrigated conditions, but there was no difference between the genotypes. Under drought conditions, no difference was observed between the genotypes, for this variable (Table 3). The pod biomass (g/plant) was also lower under drought (11.2) as compared to irrigated conditions (23.1) for all genotypes. Under irrigation, the mutant line CMT 38 and the tepary accession G40068 showed the highest values. There were significant differences (P≤0.05) between the mutant lines CMT 38, CMT 109 and CMT 187 and the tepary accession G40159. The mutant line CMT 38 also showed the highest value under drought conditions. There were significant differences (P≤0.05) between this mutant line and the other genotypes (Table 3). The seed number/plant was lower under drought as compared to irrigated conditions (56.8 vs 121) for all genotypes (Table 3). Under drought treatment, the mutant line CMT 109 and the tepary accession G40159 showed higher values. Significant differences were observed between these genotypes and the others (Table 3). Under irrigated conditions, the mutant line CMT 109 showed the highest value. There were significant differences (P≤0.05) between this mutant line, the mutant line CMT 38 and the tepary accession G40068. In relation to the 100-seed biomass (g/plant) variable, there was a modest difference between drought (16.2) and irrigated treatments (15.5) for all genotypes. Under drought and irrigated conditions, the mutant line CMT 38 and the G40068 showed the highest value for 100-seed biomass. Significant differences (P≤0.05) were observed between these two genotypes and the others under both conditions (Table 3). The number of nodules/plant increased considerably under CT and irrigated conditions as compared to results obtained under HT (Table 1). The value was 23.3 under irrigated conditions as compared to 1.62 under drought for all genotypes. The mutant lines CMT 38 and CMT 109 and the G40068 tepary accession showed the highest values (Table 3). The differences between all genotypes were not significant. Under irrigated conditions, the CMT 109 mutant line showed the highest nodule biomass, and there were significant differences (P≤0.05) between this mutant line and the other genotypes tested (Table 3). Under both temperature conditions and considering drought and irrigation treatments for all variables and genotypes, the values were lower under drought, except for the 100-seed biomass. In this case, the value was higher under drought (Table 2). Under HT and CT, significant differences (P≤0.05) were observed between the values obtained under drought and irrigation for all evaluated variables and genotypes. In relation to 100-seed biomass, under CT conditions, significant differences (P≤0.05) were observed between drought and irrigated conditions for the mutant lines and G40159 tepary accession. Under CT conditions, significant differences (P≤0.05) were observed only with the mutant lines (Table 3).

A significant strain effect (*Rhizobium tropici* or *Bradyrhizobium* spp.) was observed only for the number of nodules /plant (data not shown).

Multivariate analysis of shoot and root morpho-physological variables

Effect of temperature

Principal component analysis was performed to identify the major components (i.e. principal components) that could explain much of the total variation observed in the data. The PCA showed that under HT and CT, the first four components represented 73 and the 75 % of the total variance, respectively (Table 4). Under HT, the first component accounted for 46% of the variance, the second 11%, the third 9% and the fourth 7%, while under CT, the first component accounted for 53% of the variance, the second 8%, the third 7 %, and the fourth 7%. The dominance of these four components suggests that they contained the main variables that discriminate between the genotypes evaluated under HT and CT conditions (Table 4).

Table 1. Mean values of pod number, pod biomass, seed number, 100 seed biomass, nodules number and nodule biomass for the M_6 mutant lines (CMT 38, CMT 109 and CMT 187) and their original M_0 tepary accessions (G40068 and G40159), grown in greenhouses under high temperature (HT) and controlled temperature (CT) conditions.

	Pods/plant		Pod biomass, g/plant		Seeds/plant		100 seeds biomass		Nodules/plant		Nodule biomass, g/plant	
	HT	CT	HT	CT	HT	CT	HT	CT	HT	CT	HT	CT
Genotype												
CMT 38	22.2 a	25.6 a	15.2 a	18.2 a	69.9bc	83.5c	18.0 a	18.0 a	3.6 a	15.3 a	0.006 ab	0.029 b
CMT 109	21.2 a	23.9 a	13.8 b	16.6 b	75.2 a	95.8 a	14.4 d	13.4 d	2.7 a	14.4 a	0.005 ab	0.054 a
CMT 187	18.9 a	24.7 a	13.4 b	16.9 b	68.3 c	91.1 b	15.9 b	15.2 b	2.8 ab	9.4 a	0.005 ab	0.029 b
G40068	20.1 a	24.9 a	14.0 b	17.3 b	60.2 d	78.0 d	18.9 a	17.3 a	5.1 a	14.1a	0.013 a	0.031 b
G40159	20.3 a	24.7 a	13.5 b	16.9 b	73.1ab	96.3 a	15.2 c	14.8 c	1.1 b	9.1a	0.003 a	0.054 a
Mean	20.5	24.8	14.0	17.2	69.3	88.9	16.5	15.7	3.0	12.5	0.006	0.0394

*Means between a yield component and treatment not followed by the same letters are significantly different at P≤ 0.05 according to Duncan's multiple rang test.

Table 2. Mean values of pod number, pod biomass, seed number, 100 seed biomass, nodules number and nodules biomass for the M_6 mutant lines (CMT 38, CMT 109 and CMT 187) and their original M_0 tepary accessions (G40068 and G40159), grown in a greenhouse under high temperature (HT) and irrigated and drought conditions.

	Pods/plant		Pod biomass, g/plant		Seeds /plant		100 seed biomass, g/plant		Nodules/plant		Nodule biomass, g/plant	
	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought
Genotype												
CMT 38	28.8 a	15.6 a	20.3 a	10.1 a	95.5 ab	44.3 b	17.5 b	19.0 a	5.9 ab	1.3 a	0.0117 b	0.0017 a
CMT 109	28.5 a	13.9 b	18.5 ab	9.1 b	103.0 a	47.3 a	13.9 e	15.5 c	4.6 bc	0.9 a	0.0083 b	0.0018 a
CMT 187	23.4 b	14.5 ab	17.4 a	9.4 ab	88.8 bc	47.7 a	15.8 c	16.4 b	4.1 bc	1.5 a	0.0078 b	0.0017 a
G40068	26.5 ab	13.8 b	18.5 ab	9.6 ab	80.2 c	40.2 c	18.7 a	19.4 a	9.4 a	0.8 a	0.0233 a	0.0009 a
G40159	25.7 ab	14. 9 ab	17.4 b	9.7 ab	96.0 ab	50.2 a	14.7 d	16.1 b	1.6 c	0.6 a	0.0033 b	0.0008 a
Mean	26.6	14.5	18.4	9.6	92.7	45.9	16.1	17.3	5.1	1.02	0.0108	0.00138

*Means between a yield component and treatment not followed by the same letters are significantly different at P≤ 0.05 according to Duncan's multiple rang test.

Table 3. Mean values of pod number, pod biomass, seed number, 100 seed biomass, nodule number and nodules biomass and number of pods for the M_6 mutant lines (CMT 38, CMT 109 and CMT 187) and their original M_0 tepary accessions (G40068 and G40159), grown in a greenhouse under controlled temperature (CT) and drought and irrigated conditions.

	Pods/plant		Pod biomass, g/plant		Seeds /plant		100 seeds biomass		Nodules/plant		Nodule biomass g/plant	
	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought
Genotype												
CMT38	32.4 a	18.7 a	24.4 a	11.9 a	113.6 b	53.4 c	17.7 a	18.5 a	28.9 a	1.6 a	0.053 b	0.007 a
CMT109	30.2 a	17.7 a	22.1 c	11.0 b	128.8 a	62.7 a	13.4 c	13.7 c	27.0 a	1.9 a	0.103 a	0.008 a
CMT187	30.9 a	18.4 a	22.6 bc	11.2 b	123.4 b	58.8 b	15.2 b	15.3 b	17.7 a	1.1 a	0.054 b	0.004 a
G40068	31.6 a	18.3 a	23.7 ab	10.9 b	108.9 b	47.1 d	17.5 a	18.7 a	25.9 a	2.3 a	0.056 b	0.005 a
G40159	31.0 a	18.4 a	22.6 bc	11.1 b	130.6 a	61.9 a	13.7 c	14.7 b	16.9 a	1.2 a	0.055 b	0.004 a
Mean	31.2	18.3	23.1	11.2	121.1	56.8	15.5	16.2	23.3	1.62	0.064	0.005

*Means between a yield component and treatment not followed by the same letters are significantly different at P≤ 0.05 according to Duncan's multiple rang test.

Table 4. Eigen values and per cent of total variation and component matrix for the principal component axes - high temperature (HT) and control temperature (CT)under greenhouse conditions.

Principal components	CP1	CP2	CP3	CP4
HT				
Eigen value	7.30	1.79	1.41	1.05
Variance proportion	0.46	0.11	0.09	0.07
Cumulative proportion variance	0.46	0.57	0.66	0.73
Shoot biomass (g/plant)	0.365	-0.395	0.027	0.019
Pods biomass (g/plant)	0.358	-0.160	0.079	-0.032
Stem biomass (g/plant)	0.296	-0.154	-0.179	0.131
Stem diameter (mm)	0.150	-0.024	0.025	0.368
Leaf biomass (g/plant)	0.322	-0.037	-0.038	0.126
Pod number	0.336	-0.067	0.003	-0.132
Seed total biomass (g/plant)	0.354	-0.008	0.086	-0.054
100-seed biomass	-0.103	0.361	0.323	-0.234
Seed number	0.352	-0.138	-0.029	0.032
Root biomass (g/plant)	0.221	0.224	-0.187	0.128
Nodule biomass	0.173	0.553	0.086	0.007
Nodule number	0.174	0.508	0.172	0.105
SPAD chlorophyll content	-0.176	-0.009	0.068	0.652
Leaf stomatal conductance	0.009	0.035	0.577	-0.101
Efficiency of photosystem II	0.108	-0.399	0.369	-0.317
Leaf temperature (°C)	0.002	0.204	-0.549	-0.435
CT				
Eigen value	8.48	1.26	1.19	1.08
Variance proportion	0.53	0.08	0.07	0.07
Cumulative proportion variance	0.53	0.61	0.68	0.75
Shoot biomass	0.337	-0.078	-0.061	-0.019
Pod biomass	0.329	-0.127	-0.031	-0.036
Stem biomass (g/plant)	0.299	0.060	-0.223	0.049
Stem diameter (mm)	0.188	0.034	-0.200	0.302
Leaf biomass (g/plant)	0.311	0.037	-0.049	0.003
Pods number	0.308	-0.103	-0.086	-0.065
Seed total biomass	0.326	0.183	0.001	-0.045
100-seed biomass	-0.056	-0.660	0.431	0.132
Seeds number	0.328	0.069	-0.164	-0.078
Roots biomass	0.253	-0.040	-0.120	-0.101
Nodules biomass	0.223	0.271	0.306	0.389
Nodules number	0.168	0.297	0.513	0.454
SPAD chlorophyll content	-0.259	0.217	-0.145	0.022
Leaf stomatal conductance	0.11	-0.152	0.392	0.357
Efficiency of photosystem II	0.046	0.493	0.255	-0.405
Leaf temperature (°C)	-0.135	-0.098	-0.267	0.463

Values in bold indicate the traits that were decisive in genotype differentiation.

The traits that separated genotypes in the first component included shoot biomass, pod biomass, total seed biomass and seed number under HT and CT. Under HT, only pod number/plant differed between genotypes. The traits that contributed most to the discrimination in the second component were: nodule biomass, nodule number under HT and 100-seed biomass and the efficiency of photosystem II under CT. In the third component, the separation of genotypes was mainly due to leaf stomatal conductance under HT and CT, leaf temperature under HT and nodule number under CT. In the fourth component, the main traits were: SPAD readings under HT and nodule number and leaf temperature under CT (Table 4).

Effect of HT and CT under drought and irrigated conditions

Effect of HT under drought and irrigated conditions

The PCA showed that, under drought and irrigation, the first five components represented 79% and 80 % of the total variance, respectively (Table 5). Under drought conditions, the first component accounted for 39% of the variance, the second 14 %, the third 10%, the fourth 9% and the fifth 7%; while under irrigated conditions, the first component accounted for 30% of the variance, the second 21%, the third 14 %, the fourth 9% and the fifth 6%. The dominance of these five components suggests that they contained the main variables that discriminate the genotypes evaluated under drought and irrigation (Table 5).

The traits that discriminated genotypes in the first component included shoot biomass, pod biomass, pod number and seed total biomass under drought and irrigated treatments. The traits that contributed most to the discrimination in the second component are 100-seed biomass under drought conditions and leaf biomass, and seed number and nodule biomass under irrigated conditions. In the third component, the differences between genotypes were mainly due to the root biomass under drought and irrigated conditions; and the nodule biomass and nodules number under drought conditions. Under irrigated conditions, differences were due to the efficiency of photosystem II and leaf temperature.

Table 6. Eigen values and per cent of total variation and component matrix for the principal component axes - control temperature (CT) under drought and irrigated conditions in a greenhouse.

Principal components	CP1	CP2	CP3	CP4	CP5
CT -Drought condition					
Eigen value	6.67	2.10	1.75	1.41	1.18
Variance proportion	0.42	0.13	0.11	0.09	0.07
Cumulative proportion variance	0.42	0.55	0.66	0.75	0.82
Shoot biomass (g/plant)	0.368	0.179	-0.020	0.079	-0.059
Pods biomass (g/plant)	0.348	0.238	-0.070	0.143	-0.038
Stem biomass (g/plant)	0.312	-0.077	0.227	0.057	-0.093
Stem diameter (mm)	0.162	-0.062	-0.061	-0.506	-0.040
Leaves biomass (g/plant)	0.302	0.033	0.012	-0.223	-0.091
Pods number	0.343	0.120	-0.150	0.154	-0.040
Seed total biomass (g/plant)	0.332	0.288	-0.103	0.130	-0.023
100-seed biomass	-0.045	0.573	-0.314	0.168	0.057
Seeds number	0.319	-0.234	0.209	-0.021	-0.059
Roots biomass (g/plant)	0.136	0.170	0.210	-0.334	0.532
Nodules biomass	-0.189	0.334	0.459	0.181	0.195
Nodules number	-0.149	0.270	0.549	0.104	-0.190
SPAD chlorophyll content	0.172	-0.293	0.371	0.317	0.228
Leaf stomatal conductance	-0.033	0.316	0.228	-0.340	0.439
Efficiency of photosystem II	0.292	-0.042	0.229	-0.080	-0.175
Leaf temperature (°C)	0.053	-0.129	-0.021	0.465	0.579
CT-irrigated conditions					
Eigen value	4.85	3.43	1.76	1.64	1.01
Variance proportion	0.30	0.22	0.11	0.10	0.06
Cumulative proportion variance	0.30	0.52	0.63	0.73	0.79
Shoot biomass	0.439	-0.091	0.021	0.019	0.100
Pods biomass	0.365	-0.279	0.083	0.061	0.008
Stem biomass (g/plant)	0.280	0.346	-0.139	-0.063	0.029
Stem diameter (mm)	0.250	0.100	-0.107	-0.099	-0.517
Leaves biomass (g/plant)	0.330	0.166	-0.036	-0.050	0.349
Pods number	0.401	-0.182	0.045	0.011	0.049
Seed total biomass	0.307	-0.353	0.011	0.052	-0.113
100-seed biomass	-0.029	-0.492	0.027	-0.067	-0.179
Seeds number	0.319	0.284	-0.306	0.146	0.113
Roots biomass	-0.108	0.272	-0.114	0.413	-0.353
Nodules biomass	0.092	0.244	0.506	0.229	-0.014
Nodules number	0.021	0.022	0.701	0.028	-0.090
SPAD chlorophyll content	0.128	0.291	-0.196	-0.195	0.081
Leaf stomatal conductance	-0.135	-0.188	-0.115	0.387	0.331
Efficiency of photosystem II	-0.103	0.144	0.363	-0.421	0.508
Leaf temperature (°C)	0.005	-0.007	0.110	0.625	0.117

Values in bold indicate the traits that were decisive in genotype differentiation.

In the fourth component, the main traits were: stem diameter under drought and irrigated conditions and leaf temperature under drought, stem diameter and nodule number under irrigated conditions. In the fifth component, the main traits were the SPAD readings and stem diameter under drought and irrigated conditions, respectively (Table 5).

Effect of CT under drought and irrigated conditions

The PCA showed that under drought and irrigated conditions, the first five components represented 82% and the 79% of the total variance, respectively (Table 6). Under drought conditions, the first component accounted for 42% of the variance, the second 13 %, the third 11%, the fourth 9% and the fifth 7%, while under irrigated conditions, the first component accounted for 30% of the variance, the second 22%, the third 11%, the fourth 10% and the fifth 6%. The dominance of these five components suggests that they contain the main variables that discriminate the genotypes evaluated under drought and irrigated conditions (Table 6).

The traits that separate genotypes in the first component included shoot biomass, pod biomass, pod number and seed number under drought and irrigated conditions, and seed total biomass under drought conditions. The traits that contributed most to the discrimination in the second component were: 100-seed biomass under both drought and irrigation, stem biomass; and total seed biomass under irrigated conditions. In the third component, the separation of genotypes was mainly due to the nodule biomass and nodule number under drought and irrigated conditions. In the fourth component, the main traits were: leaf temperature under drought and irrigated conditions and the stem diameter under drought conditions. Under irrigated conditions, the main traits were root biomass, leaf stomatal conductance and the efficiency of photosystem II. In the fifth component the main traits were: root biomass and stem diameter under drought and irrigated conditions, respectively (Table 6).

Discussion

In all experiments the stress treatments (high temperature and drought) were effective as all genotypes performed less well under these stresses compared to control conditions. The treatments were also effective in discriminating between good performing and poor performing lines in stress treatments, with the mutant line CMT 38 showing superior characteristics in pods/plant and pod biomass/plant compared to its parental line G40068 and other mutant lines.

There are three main points for discussion: (i) Why is it important to induce mutations in tepary? (ii) Which novel traits are sought from mutagenesis in tepary bean? and (iii) How would the mutant lines be used in a breeding programme for tepary and / or common bean improvement? On the first point, although genetic variability among tepary wild accessions is high (Muñoz et al., 2006; Blair et al., 2012), these are in general more heat and drought tolerant, they also show agronomic disadvantages such as
indeterminate growth habit and very small seeds. The chemical mutagen (EMS) was used in this study to obtain variability in the cultivated accessions (Muñoz et al., 2013). The introduction of characteristics, such as an indeterminate erect growth habit, is necessary in the case of large-scale production, to facilitate mechanical harvesting and mechanical weed removal. This growth habit was also obtained in common beans, using breeding and screening, because it does not exist in traditional varieties. In terms of seed colour, it would probably be necessary to introduce a uniform red seed colour, the colour preferred by the consumers of Central America. On the second point, in the first generations of the mutant populations, lines with deleterious phenotypic variations were observed: dwarf plants, plants with apparent virosis, yellowing, or sterile plants. The selection of mutant lines, presenting desirable characteristics: plants with a determinate growth habit and/or a larger seed size, was carried out, through the generational development of the mutant lines (data not shown). In the present study, the CMT 38 and CMT 187 tepary mutant lines had larger seed size, as reflected by their higher values of 100-seed biomass, compared to the original accessions (G40068 and G40159) under CT conditions (Table 1). Gwata et al., 2016 showed that genotype does not affect seed size of three mutant tepary bean genotypes. The seed size was smaller, as compared to that reported for tepary in other studies. The analysis of a common bean variety and its 34 NaN_3-induced mutants (M_6 generation) showed that the seed yield and yield components differed among the 34 common bean mutants (Wang et al., 2010).

Heat and drought reduce yield and quality and restrict the geographic adaptation of common beans (Rainey and Griffiths, 2005; Beebe et al., 2008). The HT treatment applied to common bean genotypes reduced the yield components: seed number, pod number, mean seed weight and seeds/pod (Rainey and Griffiths, 2005b). In contrast, tepary accessions that produce substantial numbers of pods and seeds under very HT conditions or drought were reported (Rainey and Griffiths, 2005; Rao et al., 2013; Polania et al., 2016). The mutant lines evaluated here under HT and drought conditions, showed a yield higher or comparable to the original accessions G40068 and G40159. This indicates that screening based on morphological traits is useful and not detrimental to seed yield and yield components. G40068 and G40159 were outstanding in their adaptation to terminal drought stress. The superior performance of these accessions was associated with their ability to mobilize photosynthates from leaves and stems to developing grains. Tepary was superior to common bean in combining several desirable traits that contribute to adaptation to terminal drought stress (Rao et al., 2013). Under rainfed conditions, these two accessions yielded more than any elite line or accession of *P. vulgaris* under terminal drought, thus demonstrating the advantages that this species has over *P. vulgaris* under terminal drought stress (Rao et al., 2013, 2017).

The PCA analysis under heat and drought conditions of all traits evaluated showed that the four first components (CP_1, CP_2 CP_3 CP_4) represented 73% of the total variance under HT conditions (Table 4). The SPAD readings appeared to explain the variance in the PCA under HT but not under CT. Three variables: root biomass, stem diameter and biomass do not explain the variance in the analysis performed under HT or CT. But these variables appear to explain the variance in the PCA analysis, when the drought or irrigated conditions of the experiment were considered under HT and CT conditions (Tables 5 and 6). This indicates that these traits can be used to select better adapted genotypes under drought conditions. A greater capacity to develop roots that go deep into the soil can provide a better adaptation to conditions of water stress (White and Castillo, 1992; Polania et al., 2009, 2016). There is a direct correlation between drought and heat stresses, since during heat stress water availability can be at a deficit caused by the high temperature (Omae et al., 2012). It is necessary to identify specific morpho-physiological traits that contribute to improved resistance to combined stresses of heat and drought in beans, and that could be useful as selection criteria in breeding.

On the last point, how would the mutant lines be used in a breeding programme for tepary and/or common beans? These genetically stable mutant lines, which were selected for their phenotypic characters, and/or for their tolerance to HT and drought, could have two possible uses in a bean breeding programme. First, these mutant lines could be included in interspecific crosses, between *P. vulgaris* and *P. acutifolius,* to try to introgress these physiological characteristics to common bean. Second, they could be used for the improvement per se of the species *P. acutifolius*.

Materials and methods

Plant materials

We evaluated two accessions of tepary bean, *P. acutifolius* A. Gray (G40068 from Arizona, USA and G40159 from Sonora, Mexico, and three mutant M_6 lines (CMT 38, CMT 109 and CMT 187), which were uniform and genetically stable. The mutant line CMT 38 was obtained from the G40068 accession, while CMT 109 and CMT 187 mutant lines were obtained from the G40159 accession. . These mutant lines were selected based on two key traits: large seed size and/or a determinate growth habit and superior yield from previous experiments, where M_5 mutant lines were evaluated under drought and high temperature conditions in greenhouse tests (data not shown). The evaluated tepary mutant lines were obtained from a protocol established by Muñoz et al. (2013), using ethyl methane sulfonate (EMS).

Experimental conditions

The experiments were conducted at the International Center for Tropical Agriculture (CIAT) in Palmira, Colombia, located at latitude 3° 29' N, longitude 76° 21' W and 965m above sea level.

Two experiments were conducted simultaneously in two separate greenhouses, to evaluate the M_6 mutant lines and the two tepary parental (M_0) accessions (G40068 and G40159) to high temperature and drought stress conditions. Experiment 1 was carried out with a high temperature treatment (HT) in a greenhouse. Experiment 2 was carried out at normal (control temperature) conditions (CT) in another greenhouse. Both experiments included three replicates and were conducted using pots with a Mollisol soil from Palmira. The seeds were germinated in wet paper towels and uniform seedlings were selected for transplanting into pots. The plants of each accession and of the mutant tepary lines from the two experiments were inoculated at 10 days after sowing with

Rhizobium tropici (strain CIAT 899) or *Bradyrhizobim* spp. (strain CIAT461) as is normal practice. To obtain high temperature conditions and simulate the changes in temperature between day and night, conditions in the greenhouse were modified using heaters, ventilation and thermostats. The HT treatment was set at 29±5 ºC during the day and >24 ºC during the night, with an average relative humidity of 65%. The maximum day/night temperatures of the greenhouse for normal conditions (CT) were set at day/night of 30°C /20°C. Data on relative humidity and temperature were monitored with thermo-hygrometers that registered the parameters every 15 minutes. The mean and minimal/maximal temperatures were calculated per day.

Plants were grown in optimal conditions of soil moisture (80% field capacity) for 10 days and were then submitted to their respective treatment with soil moisture, either at 80% field capacity (irrigated) or 40% (drought). In both cases, the pots were weighed twice a week and water was added to bring back the required moisture level.

Measurement of shoot and root morpho-physiological characteristics

Plants were harvested between 80 to 86 days under drought and at 100 days under irrigated conditions. At the mid-pod filling growth stage, the following non-destructive measurements were made: leaf chlorophyll content of fully expanded leaves was measured using a non-destructive, hand-held chlorophyll meter (SPAD-502 chlorophyll metre, Minolta Camera Co., Ltd., Japan). The principle is based on the difference in light attenuation at wavelengths 430 and 750 mm. From the difference in light attenuation, a numerical SPAD (Soil-Plant Analysis Development) unit, ranging from 0 to 80, is calculated by the microprocessor in the SPAD-502 chlorophyll metre. The efficiency of photosystem II (QY) in leaves adapted to light (Fv'/Fm') was also determined. The stomatal conductance (mmol m^{-2} s^{-1}) was measured with a portable leaf porometer (Deacagon SC-1) on a fully expanded young leaf of one plant within each replication. Measurements were made late in the morning (10 am -12 noon) on clear and sunny days. The leaf temperature was measured with an infra-red thermometer (Telatemp AG-42D, Telatemp Co, US). At the time of harvest, plants were cut at soil level and dry weights of different shoot biomass components (stem, leaves, pod biomass and pod number, seed number, seed biomass per plant) were recorded. The roots of each pot were washed free of soil. Root weight per plant was determined after the roots were dried in an oven at 60 °C for 48h.

Statistical analysis

A separate analysis was conducted for each experiment. The sources of variation within each experiment were: replications and genotypes. All data were analyzed using SAS software (v. 9.2). Values marked with * or ** are statistically significant at probability levels of 5% and 1%, respectively. The mean values were compared with the Duncan test. A Principal Component Analysis (PCA) was performed on the measured variables and was based on Pearson correlation matrix and Euclidean distances. Eigen values for all principal components (PC) were shown. Eigen vectors generated by the PCA were used to identify parameters that best differentiated the genotypes in each experiment.

Conclusion

Mutation induction in G40068 and G40159 cultivated tepary accessions, increased the genetic variability in morpho-physiological characteristics of the species. In addition, the CMT 38, CMT 109 and CMT 187 mutant lines showed seed yield values per plant comparable to or higher than that of the original accessions, under heat and drought conditions. The identification of four key plant traits in the PCA analysis (SPAD readings, seed biomass, 100-seed biomass and seeds number) explained a major part of the variance under heat and drought conditions, and suggests that these traits and two others (root biomass and stem diameter, identified from the univariate analysis) could be incorporated into tepary breeding programmes as selection criteria to screen the tepary accessions and their mutant lines, for combined tolerance to heat and drought stresses.

Mutation breeding has potential to generate phenotypic and genotypic variations in tepary bean that can be exploited by plant breeders in the development of new cultivars with improved adaptation to heat and drought stress.

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References

Ahloowalia BS, Maluszynski K, Nichterlein K (2004) Global impact of mutation derived varieties. Euphytica 135:187-204.

Beebe S, Rao IM, Cajiao C, Grajales M (2008) Selection for drought resistances in common bean also improves yield in phosphorus limited and favorable environments. Crop Sci. 48:582-592.

Bielenberg DG, Miller JD, Berg VS (2003) Paraheliotropism in two *Phaseolus* species: combined effects of photon flux density and pulvinus temperature, and consequences for leaf gas exchange. Environ Exp Bot. 49: 95-105.

Blair MW, Porch T, Cichy K, Galeano CH, Laringuet P, Pankhurst CE, Broughton W (2007) Induced mutants in common beans (*Phaseolus vulgaris*) and their potential use in nutrition quality breeding and gene discovery. Isr J Plant Sci. 55:191-200.

Blair MW, Pantoja W, Muñoz, LC (2012) First use of microsatellite markers in a large collection of cultivated and wild accessions of tepary bean (*Phaseolus acutifolius* A. Gray). Theor Appl Genet. 125:1137–1147.

Butare L, Rao I, Lepoivre P, Polania J, Cajiao C, Cuasquer J, Beebe S (2012) Phenotypic evaluation of interspecific recombinant inbred lines (Rils) of *Phaseolus* species for aluminium resistance and shoot and root growth response to aluminium- toxic acid soil. Euphytica 186 (3) 715-730.

Debouck DG (1992) Frijoles (*Phaseolus* spp.). *In*: Hernández Bermejo E, León J (ed) Cultivos marginados: otra perspectiva de 1492. Food and Agriculture Organization of the United Nations, Rome, Italy. 45-60.

Dillen W, De Clercq J, Goossens A, van Montagu M, Angenon G (1997) *Agrobacterium*-mediated transformation of *Phaseolus acutifolius* A. Gray. Theor Appl Genet. 94: 151-158.

Freeman GF (1912) Southwestern beans and teparies. Univ Ariz Agric Exp Station Bull. 68: 573-619.

Gwata ET, Shimelis H, Matova M (2016) Potential of improving agronomical attributes in tropical legumes using two mutation breeding techniques in southern Africa. In: Petr K (ed) Alternative crops and cropping systems, IntechOpen Ltd, London, UK. 71-85.

Lin TY, Markhart AH (1996) *Phaseolus acutifolius* A. Gray is more heat tolerant than *P. vulgaris* in the absence of water stress. Crop Sci. 36 (1) 110-114.

Markhart III AH (1985) Comparative water relations of *Phaseolus vulgaris* L. and *Phaseolus acutifolius* A. Gray. Plant Physiol. 77: 113-117.

Mejía JA, Muñoz C, Jacobsen HJ, Roca WM, Singh SP (1994) Interspecific hybridization between common and tepary beans: Increased embryo growth, and efficiency of hybridization

through recurrent and congruity backcrossing. Theor Appl Genet. 88: 324–331.

Muñoz LC, Blair WM, Duque MC, Tohme J, Roca W (2004) Introgression in common bean x tepary bean interspecific congruity-backcross lines as measured by AFLP markers. Crop Sci. 44: 637-645.

Muñoz LC, Duque MC, Debouck DG, Blair WM (2006) Taxonomy of tepary bean and wild relatives as determined by amplified length polymorphism (AFLP) markers. Crop Sci. 46: 1744-1754.

Muñoz LC, Toro O, Muñoz JE, Debouck DG (2013) Inducción de mutantes por vía química para el mejoramiento genético de *Phaseolus acutifolius* A. Gray. Presented at the VII congreso Colombiano de Botánica, Universidad del Tolima, Ibague, Colombia 6 - 10 Agosto 2013.

Nabhan GP (1985) Gathering the desert. The University of Arizona Press, Tucson, Arizona, USA. 209p.Nabhan GP, Felger RS (1978) Teparies in southwestern North America - A biogeographical and ethnohistorical study of *Phaseolus acutifolius*. Econ Bot. 32: 3-19.

Omae H, Kumar A, Shono M (2012) Adaptation to high temperature and water deficit in the common bean (*Phaseolus vulgaris* L.) during the reproductive period. J. Bot. doi:10.1155/2012/803413.

Polanía JA, Rao IM, Beebe S, García R (2009) Desarrollo y distribución de raíces bajo estrés por sequía en frijol común (*Phaseolus vulgaris* L.) en un sistema de tubos con suelo. Agron colomb. 27: 25-32.

Polania JA, Poschenrieder C, Beebe S, Rao IM (2016) Effective use of water and increased dry matter partitioned to grain contribute to yield of common bean improved for drought resistance. Front Plant Sci. 7: 660.

Porch T, Blair WM, Laringuet P, Galeano CH, Pankhurst CE, Broughton W (2009) Generation of a mutant population for tilling common bean genotype BAT 93. J Am Soc Hort Sci. 134 (3) 348-355.

Pratt RC, Nabhan GP (1988) Evolution and diversity of *Phaseolus acutifolius* genetic resources. In: Gepts P (ed) "Genetic resources of Phaseolus beans: their maintenance, domestication, evolution and utilization", Kluwer Academic Publ., Dordrecht, Holland. 409-440.

Rainey KM, Griffiths PD (2005) Evaluation of *Phaseolus acutifolius* A. Gray plant introductions under HTs in a control environment. Genet Resour Crop Evol. 52: 117-120.

Rainey KM, Griffiths PD (2005b) Differential response of common bean genotypes to high temperature. J Am Soc Hort Sci. 130: 18-23.

Rao IM, Beebe SE, Polania J, Ricaute J, Cajiao C, Garcia G, Rivera M (2013) Can tepary bean be a model for improvement of drought resistance in common bean? Afr J Crop Sci. 21: 265-281.

Rao IM, Beebe SE, Polania J, Grajales M, Cajiao C, Ricaurte J, Garcia R, Rivera (2017) Ability to remobilize photosynthate to increase yield under drought. J Agric Sci. 155:857-875.

Schinkel C, Gepts P (1988) *Phaseolin* diversity in the tepary bean, *Phaseolus acutifolius* A. Gray. Plant Breed. 101: 292-301.

Wang HH, Jeng TL, Chen ML, Chuang, Sung JM (2010) Molecular and morpho-agronomic characterization of NaN_3-induced common bean mutants. Crop Environ Bioinf. 7 (12) 221-231.

White JW, Castillo JA (1992) Evaluation of diverse shoot genotypes on selected root genotypes of common bean under soil water deficits. Crop Sci. 32: 762-765.