Broad adaptive genetic differentiation in forage quality traits but similar phenotypic plasticity in Tunisian populations of Brachypodium hybridum in response to phosphorus deficiency

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Abstract

Facing to the harsh environmental constraints, natural populations of widespread plant species may express plastic response, evolve local adaptation or combine both mechanisms in order to maintain their genetic variation and pervasiveness across a wide ecological niche. Here, 79 lines representing height Tunisian populations of Brachypodium hybridum were cultivated in two contrasting phosphorus (P) availability levels and their genetic variation was investigated using forage quality traits to identify the main mechanisms governing their genetic variation. Our results revealed large variation among populations for most traits. PCA analysis showed the separation between coastal and inland populations in both P levels. Importantly, all traits revealed high heritability in both P treatments and the response of most of them to P deficiency was highly influenced by the population effect, which accounted 52.38% of the total variation, indicating strong population differentiation. However, although significant for most traits, only 18.13% of variation was attributed to P treatment, suggesting little plastic response to P deficiency. Importantly, as revealed by the lack of population*treatment effect for most traits, the plastic response was probably common to all populations. Moreover, we found that 56.99% of variation was attributed to environmental factors with distance to coastline, precipitation seasonality, and annual precipitation being the most important factors, suggesting that the strong population differentiation likely reflects local adaptation to specific environmental conditions. Contrary to the coastal populations, which were characterized by high levels of fiber-related traits levels, the inland ones displayed the high values of most energy-related traits, suggesting that these latter were characterized by relatively higher forage quality. Overall, our results suggested that the effect of local adaptation overrides the effect of phenotypic plasticity in explaining the variation of forage quality traits, which indicate a broad adaptive genetic differentiation among the Tunisian populations of B. hybridum in response to P deficiency.

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Abstract

Facing to the harsh environmental constraints, natural populations of widespread plant species may express plastic response, evolve local adaptation or combine both mechanisms in order to maintain their genetic variation and pervasiveness across a wide ecological niche. Here, 79 lines representing height Tunisian populations of *Brachypodium hybridum* were cultivated in two contrasting phosphorus (P) availability levels and their genetic variation was investigated using forage quality traits to identify the main mechanisms governing their genetic variation. Our results revealed large variation among populations for most traits. PCA analysis showed the separation between coastal and inland populations in both P levels. Importantly, all traits revealed high heritability in both P treatments and the response of most of them to P deficiency was highly influenced by the population effect, which accounted 52.38% of the total variation, indicating strong population differentiation. However, although significant for most traits, only 18.13% of variation was attributed to P treatment, suggesting little plastic response to P deficiency. Importantly, as revealed by the lack of population*treatment effect for most traits, the plastic response was probably common to all populations. Moreover, we found that 56.99% of variation was attributed to environmental factors with distance to coastline, precipitation seasonality, and annual precipitation being the most important factors, suggesting that the strong population differentiation likely reflects local adaptation to specific environmental conditions. Contrary to the coastal populations, which were characterized by high levels of fiber-related traits levels, the inland ones displayed the high values of most energy-related traits, suggesting that these latter were characterized by relatively higher forage quality. Overall, our results suggested that the effect of local adaptation overrides the effect of phenotypic plasticity in explaining the variation of forage quality traits, which indicate a broad adaptive genetic differentiation among the Tunisian populations of B. hybridum in response to P deficiency.

Keywords

Brachypodium hybridum; genetic variation; phosphorus deficiency; phenotypic plasticity; local adaptation

Introduction

As a result of the ongoing global anthropogenic climate change and the extensive land use change, abrupt environmental changes, including drought, nutrient deficiency, waterlogging, elevated CO^2 are expect to dramatically threatens plant biodiversity through the alteration of the geographical distribution and the genetic variation of natural plants populations. Phosphorus deficiency in agricultural and natural areas is considered one of the major environmental constraints as it gravely limits feed and food production (Pang et al., 2018). Various recent studies have demonstrated that phenotypic plasticity and local adaptation are the most important processes through which the natural populations of broadly distributed plants species overcome the threats caused environmental changes (de Villemereuil et al., 2018; Benito Garzon et al., 2019; Garate-Escamilla et al., 2019). The phenotypic plasticity is recognized as the ability of given genotype to react rapidly to environmental change via expressing various new phenotypes without genetic shifts (Tigano and Friesen, 2016). However, local adaptation is defined as the adaptive genetic changes that allow the appearance of new genotypes having greater ability to greater ability to cope with environmental variations (Tyrmi et al., 2020).

Brachypodium hybridum is an allotetraploid (2n = 30) grass deriving from the natural hybridization between the two diploid species *B. distachyon* (2n = 10) and *B. stacei* (2n = 20) (Catalan et al., 2012). Due to their close relationship with the major cereals in the globe (wheat, maize, rice, barley), this complex of species has been championed as model for cereal and bioenergy crops (Catalan et al., 2014; Sancho et al., 2018). Although the three species are widely distributed in the Mediterranean basin, *B. hybridum* is characterized by a broader ecological niche due to its great adaptation to harsh environmental conditions (Lopez-Alvarez et al., 2012; Manzaneda et al., 2015a), resulting in a high phenotypic and genetic variation within its natural populations (M. Neji et al., 2015a; Mohamed Neji et al., 2015b). By analyzing various morpho-physiological parameters, recent previous studies have demonstrated that, in the diploid species *B. distachyon*, the response to diverse abiotic stresses was modulated by phenotypic plasticity such as drought (Chochois et al., 2015; Manzaneda et al., 2015b), phosphorus deficiency (Baudson et al., 2019) cold (Mayer and Charron, 2020). In contrast, evidence of local adaptation to climate was detected in the same species in response to water shortage and elevated temperature stresses (Des Marais et al., 2017). Likewise, other investigations have demonstrated the relevance of phenotypic plasticity in the natural populations of the polyploid species *B. hybridum* in response to environmental stresses (Manzaneda et al., 2015; Ibrahim et al., 2020). However, although it has been widely argued that the pervasiveness of polyploids and their great adaptation to harsh environmental stresses is associated with their short-term plastic responses (Gallego-Tevar et al., 2018; Wei et al., 2019; Zigelski et al., 2019), diverse investigations have demonstrated that, as a result of polyploidization, various epigenetic mechanisms such as DNA methylation and over-accumulation of transposable elements provide polyploids with high adaptive potential that offers their natural populations a substantial local adaptation in response to environmental shifts (Gardiner et al., 2018; Baduel et al., 2019).

In Tunisia, the geographical distribution of *B. hybridum* is very large, extending from the extreme South, where the arid climate is dominant, to sub humid regions in the North. We speculate that, both phenotypic plasticity and local adaptation interact to provide the natural populations with a great ability deal with environmental stresses. Being a model for forage grasses, the analysis of the genetic variation of forage quality traits in the natural populations of *B. hybridum* will be very useful to identify populations with high nutritive value and many agronomically-useful traits that could be exploited for improving forage quality of the conventional forage grasses. In the present study, using various forage quality traits, we analyze the genetic variation of Tunisian *B. hybridum* in response to phosphorus deficiency in order to (1) assess how do the Tunisian populations of B. hybridum vary in their in their responses to phosphorus deficiency (2) explore the role of environmental heterogeneity in driving the genetic variation under phosphorus deficiency and (3) identify main mechanisms underlying such variation.

Material and methods

Plant material and growth conditions

The plant material used in the study consists of healthy seeds issued after two generations of 79 *B. hybridum* lines representing 8 Tunisian populations cultivated in controlled conditions at the laboratory of Extremophile plants, Centre of Biotechnology of Borj Cedria, Tunisia. The study populations were collected from diverse geographical sites characterized by a wide environmental heterogeneity along entire species distribution range in Tunisia, which climate varying from arid in the extreme south to sub-humid in the extreme north (Table 1 and Fig. 1).

The experiment of the present study was conducted in a glasshouse at the Faculty of Sciences of Gabes, Tunisia. First, we started by sowing four to five healthy seeds of each line in a plastic pot filled with 2.5 kg of a sterilized sandy-loam soil with a pH of 6.9. The seedlings were irrigated using distilled water for two weeks after germination and then thinned to three morphologically homogenous replicates. At this stage, the seedlings were divided into two groups and subjected to two phosphorus (P) treatments: 1mM (control) and 5 μ M (P deficiency) and irrigated using 1/10-strength Hoagland's nutritive solution as described by Neji et al. (2019). The full composition nutritive solution was used to irrigate the control seedlings, whereas the solution provided for the treated plants was alimented with only 5 μ M KH₂PO₄. The adequate amount the potassium (K) for the treated seedlings was maintained by adding KCL to the nutritive solution. The experiment was conducted for two months and the seedlings were completely randomized in a 2-factorial arrangement of the height populations and the two phosphorus levels. The temperature of the glasshouse was set at 25 °C/20 °C (day/night), the relative humidity was at 65% and the photoperiod was set at 16 h/8 h light/dark cycle.

Forage quality measurements

The shoot samples were first oven dried at 65°C for 48 h to exclude the influence of moisture and ground to 1 mm in size. In total, fourteen forage quality parameters were measured in our study, from which five were considered as fiber-related traits and nine were energy-related traits. The fiber-related traits included Neutral and acid detergent fibers (NDF and ADF), acid detergent lignin (ADL), Hemicellulose and cellulose contents. First, NDF, ADF and ADL were chemically determined using the procedure described by Van Soest et al., (1991). Then, we calculated the hemicellulose content as NDF minus ADF and the cellulose content as the difference between ADF and ADL (Sanz-Sáez et al., 2012). For the energy-related traits, we started by chemically determined the crude protein (CP), Ether extract (EE), Ash and the *in vitro* dry matter digestibility (IVDMD). CP content was determined after measuring the nitrogen content ([N]) using the Kjeldahl procedure and multiplying [N] by 6.25 (Horwitz and Chemists, 2010). Ash was determined by burning of a 0.2 g of dry matter in a muffle furnace at 550°C for 4 h. Ether extract (EE) procedure was carried out in ANKOMXT¹⁵ extraction system according to AOCS (2005). The *in vitro* dry matter digestibility (IVDMD) was measured from 0.2 g of ground shoot according to Aufrére and Demarquilly (1989). The five remaining energy-related traits were Relative Feed value (RFV), Relative Forage Quality (RFQ), Non fiber carbohydrates (NFC), Total Digestible Nutrient (TDN) and Metabolizable Energy (ME) were calculated according to Filik (2020) using the following formulas:

- RFV= RFV = [DMD * DMI]/1.29, where DMD and DMI, the dry matter intake and the digestible dry matter, respectively, were calculated where DDM (%) = 88.9 [0.779 * ADF%] and DMI (%) = 120/[NDF%]
- RFQ = (DMI * TDN)/1.23
- NFC (%DM) = 100 (CP + EE + Ash + NDF)
- TDN (% DM) = 82.38 (0.7515 x ADF %)
- ME $(MJ/kgDM) = [(TDN \times 3.6)/100 \times 4.184]$

Data analysis

The obtained data were statistically analyzed using a set of univariate and mutivariate approaches using R statistical programming environment version 3.4.4 (https://www.r-project.org/). First, the maximum and minimum values (max and min), standard deviation (SD) and coefficient of variation (CV) were computed using the function 'stat.desc' of the package 'pastecs' (Grosjean et al., 2014) to describe the variation patterns of all the analyzed traits within and among the studied populations in control and under P deficiency conditions.

We then applied the 'corrplot' package (Wei et al., 2017) to investigate the relationships between traits pairwise in both treatments using Pearson coefficient (r), with P < 0.01 set as significance level. Moreover, in both phosphorus availability conditions, we used the function lmer implemented in the package 'lme4' (Bates et al., 2014) to run a Linear Mixed Model (LMM) for each of the analyzed traits, with population as fixed effect an line within population as random effect in order to explore differences between populations and to partition the total variance ($\sigma^2_{\rm T}$) into the variance among lines ($\sigma^2_{\rm L}$), the variance among populations ($\sigma^2_{\rm P}$), and the residual variance ($\sigma^2_{\rm E}$). In our models, we used the package lmerTest (Kuznetsova et al., 2017) to test for the significance of the random affect and the function emmeans in the package emmeans (Lenth, 2017) to perform Tukey's post hocs for paiwise populations comparison. At each phosphorus level, the broad sense heritability of each trait was estimated as the ratio between the variance among lines and the variance ($H^2 = \sigma^2_{\rm L} / \sigma^2_{\rm T}$) according to (Zhang et al., 2018). Moreover, we used the functions 'prcomp' and 'autoplot' of 'ggfortify' package (Tang et al., 2016) to compute a Principal Component Analysis (PCA) on the log2-transformed data of all analyzed traits in order to explore the patterns of the population differentiation in the two phosphorus availability conditions and identify the most contributing traits.

Additionally, we used LMM to depict the relative contribution of genetically fixed variation and phenotypic plasticity in the traits variation in response to phosphorus deficiency. The model was fitted using the line, population and treatment as fixed effects, whereas the interactions line * treatment and population * treatment were considered as random effects. The significant treatment indicates a plastic response to phosphorus deficiency, the significant effect of line and population indicates implies genetically based differentiation, whereas the significant effects of line * treatment, population * treatment indicate differential plasticity among lines and populations. Hence, for variance partitioning, the variance associated with treatment was defined the component of phenotypic plasticity, whereas the sum of variance associated with the remaining factors was defined as the component of genetically fixed variation.

We further investigated the relative effect of environmental heterogeneity on the genetic variation of forage quality traits of the Tunisian natural populations of B. hybridum under phosphorus deficiency using full and partial redundancy analyses (RDA) with variance partitioning. RDA analyses were performed using of 'vegan' package (Oksanen et al., 2013) on a matrix of response variables formed by the data of the forage quality traits a set of 23 explanatory variables. These latter were divided into two independent matrices of climatic and geographical variables. The first matrix included 19 climatic variables extracted from WorldClim version 2.1 database (http:// worldclim.org/) (Fick and Hijmans, 2017) for the period 1970–2000 at the highest resolution (1 km2), whereas the second one was formed by the four geography related features; longitude, latitude, altitude and the distance to coastline. To keep only the most relevant and significant explanatory variables, we applied a stepwise forward model selection process using 'rda'. 'ordistep' functions on the response variables matrix and each of the two explanatory variables matrices (Borcard et al., 2018). Then, in order to disentangle the effects of climate and geography on the variation forage quality under phosphorus deficiency, we conducted three different RDAs: (1) a full model including all the environmental variables identified in the forward selection procedure (climate + geography), (2) a partial model of climate controlling for geography effect (climate | geography), and (3) a partial model of geography controlling for climate effect (geography | climate). We then used the used the function 'varpart ' to estimate the pure contribution of climate and geography on explaining the forage quality variation. The results of from the RDA and pRDA models were further used to estimate the independent contributions of climate, geography and their joint effect in explaining the variation. The pure contribution of climate was calculated as the fraction of explained variance in both the full RDA and the partial (climate | spatial) model. The pure contribution of geography was estimated as the proportion of explained variance in the full RDA and also explained by the partial (geography | climate) model (Peres-Neto et al., 2006). Finally, the join contribution of both climate and geography (spatial [?] climate) was considered as the as the remaining explained variance in the full RDA not associated to the pure effects of both climate and geography.

Results

Variability in forage quality traits and population differentiation in control conditions

In control conditions, moderate to high variation among populations was detected for the analyzed traits, with CV values ranging from 8.65% to 29.37%, with a mean CV value of 15.98% for all analyzed traits. The most variable traits were Hemicellulose, ADL, RFV, ME and IVDMD with CV values of 29.37%, 24.65%. 23.35% and 22.98%, respectively, whereas the least amounts of variation were observed for NFC, ADF, NDF and TDN (CV < 10%) (Supplementary file 1). Remarkably, the fiber-related traits were found more variable among the studied populations than the energy-related traits, with CV averaging on 17.07% and 15.38%for both traits sets, respectively. At the population level, narrow and comparable amplitude of variation in all the studied populations with coefficients of variation above 10%, except for Hemicellulose content which displayed CV values ranging from 10.21% in Enfidha population and 21.95% in Fayedh population. Pairwise populations comparison revealed a significant population differentiation for all analyzed traits, except for the two energy-related traits IVOMD and Ash. Obviously, despite the contrasted climate conditions characterizing the studied populations, the observed the population differentiation followed a coastal-inland gradient, with striking differences observed between coastal and inland populations. The inland populations originated from Douar El Haj Whiss, Fayedh, El Kef and Jbal Zaghouan displayed the highest mean values of most energy-related traits, suggesting that these populations were characterized by relatively higher forage quality compared to the coastal populations. In contrast, these latter which are originated from Haouaria. Raouad, Sejnen and Enfidha exhibited the highest values of the fiber-related traits contents (NDF, ADF and ADL), which suggests their relatively lower nutritive value.

In order to depict with more details the patterns of differentiation among the studied populations in control

conditions, the dataset derived from the fourteen forage quality parameters were analyzed by Principal component analysis. PCA results revealed that the differentiation among populations was mainly explained by the first two axes (PC1 and PC2) which accounted for 71.2% of the total variance. PC1 explained 52.8% of the total variance and displayed high positive correlations (r > 0.71) with ADF, ADL, RFV, Hemicellulose and Cellulose and relevant negative associations with CP, EE, NFC, TDN, RFQ and ME (|r| > 0.76). The second PC, which explained only 18.4% of the total variance, exhibited significant correlations with the three parameters NFC (r = 0.89), Hemicellulose (r = -0.84) and NDF (r = -0.67). Thus, the first PC and it's strongly associated parameters were the most important contributing to separation among the studied populations. As shown in figure 2A, PC1 allowed to intermix the studied populations in two major groups. The inland populations formed a second group along the positive side of PC1 and seem to exhibit considerable forage quality by expressing relatively high levels of CP, RFV, CP, EE and ME. Whereas, the coastal populations formed a second group along the positive side of PC1. This group of populations exhibited relatively high values of fiber contents (ADL, ADF and NDF), indicating relatively low forage quality compared the inland ones.

Heritability and traits correlations

Overall, broad-sense heritability (H^2) was remarkably high and differed slightly within the two phosphorus availability conditions among analyzed traits. For the fiber related traits, Hemicellulose displayed the lowest heritability level $(H^2 = 0.57)$, whereas the most heritable trait was ADL $(H^2 = 0.95)$ with an average H^2 values of 0.78 and 0.77 in control and under P deficiency, respectively. For the energy-related traits, CP, RFV, RFQ and ME showed the highest heritability values in both phosphorus levels $(H^2 = 0.85)$, whereas NFC was found the least heritable trait with H^2 values of 0.6 and 0.62 in control and under P deficiency, respectively (Table 2). Moreover, as shown in figure 3, no significant change was found in traits correlations patterns between control and under phosphorus deficiency conditions. Fiber related traits exhibited strong positive correlations between each other, with coefficient of correlation (r) ranging from 0.57 between ADL and NDF to 0.98 between ADF and Cellulose content. Strong positive correlations were also detected between pairwise of energy-related traits, with r values ranging from 0.61 between CP and RFQ to 0.99 between RFV and RFQ. In contrast, in both phosphorus supplies, strong negative correlations were detected between fiber and energy related traits. The lowest correlations were observed between NDF and the two traits RFV and CP (r = -0.36 and r = -0.49, respectively), whereas the strongest ones were detected between ADF and the two traits ME and TDN (r = -1).

Patterns of response to phosphorus deficiency and population differentiation

Linear mixed-effects model (LMM) revealed significant differences in all forage quality traits across phosphorus availability treatments, except for of CP, EE and NFC, pointing towards signs of phenotypic plasticity in response to the phosphorus deficiency (Table 3). Broad differences were also observed among populations and among lines within populations (Table 3). Overall, patterns of plasticity did not differ among populations nor among lines. Significant genetic variation in plasticity among populations and among lines was detected for only the three parameters RFV, IVDMD and EE indicating that the response of these traits was not in the same direction for all the populations and lines. Furthermore, variance partitioning inferred from LMM revealed that the population and line effects were the main sources of variance for all analyzed traits. except for IVDMD and Ash, indicating the forage quality parameters in *B. hybridum* were under a strong genetic control. Noticeably, as shown in figure 4, the population effect was highly significant and contributed with more 60% for most analyzed traits, which suggest strong genetic population differentiation in response to phosphorus deficiency. In addition, except for the non-significant part attributed to ADL (1.29%), considerable variance proportions were attributed to the line effect for most of analyzed traits, ranging from 8.09% in ADF to 56.53% in NFC. On the other hand, the effect of P treatment on the different parameters was variable. The highest fractions of variance sourced from the P treatment were observed in IVOMD and Ash (50.11% and 46.31%, respectively), followed by ADL, RFV, NDF and ADF (23.71%, 22.21%, 21.2% and 19.34%), indicating that these traits exhibited the highest plastic response to phosphorus deficiency. For the remaining traits showing significant P treatment effect, the magnitude of their plastic response was low to moderate, with fraction of variance due to P treatment ranging from 6.74% in RFQ and 11.63 in ME. Overall, for all analyzed traits, 52.38% of variation to phosphorus deficiency was attributed to the population effect followed by the line and P treatment effects (24.67% and 18.13%, respectively). The variance attributed to the population*treatment and line*treatment effects were 4.07 and 0.76%, respectively, supporting the lack of genetic variation in plasticity among both populations and lines (figure 4). Taken together, these results suggest a strong genetic differentiation among populations under phosphorus deficiency. Such differentiation was further supported by PCA results. with roughly the same traits association revealed in control conditions, the first two PCs summarizing 54.6% and 18.1% of the total variation, respectively, separated the inland and the coastal populations in two major groups along PC1 (figure 2B).

To better understand how genetic and environmental effects interact to mediate the phenotypic plasticity of forage quality traits in the Tunisian populations of *B. hybridum*, we investigated norms of reaction for the height studied populations under control and phosphorus deficiency conditions. Consistent with ANOVA results, among all the studied forage quality traits, only RFV, IVOMD and EE displayed crossed reaction norms, confirming the variation in phenotypic plasticity among studied populations. This result suggests that the Tunisian *B. hybridum* populations exhibited contrasted plasticity to adapt to phosphorus deficiency and there was a genetic basis for their plastic response. Generally, in contrast to coastal populations, the inland populations showed a considerable increase in their RFV and EE levels under phosphorus deficiency (figure 5 3-C and figure 5 D-1). For IVDMD (figure 5 C-2), negative responses were shown in all populations, with less pronounced decrease in the three coastal populations Enfidha, Haouaria and Raouad. All the remaining traits showing significant treatment effect displayed non-flat and parallel reaction norms (figure 5), indicating similar plastic response patterns among populations. Noticeably, except for the fiber-related traits (ADF, NDF and ADL) and cellulose content which are negatively sloped, the other treats increased significantly under P deficiency, suggesting significant beneficial effect of phenotypic plasticity in improving the forage quality.

Association between forage quality and environmental factors under phosphorus deficiency

Forward selection of the analyzed environmental factors separately identified only three factors as sufficiently predictive of variation in forage quality among the studied populations, from which Precipitation Seasonality (BIO15) and Annual Precipitation (BIO12) were related to climate, and the distance to coastline as geography-related factor. The full RDA model combining the three retained variables revealed a significant environment-forage quality association, with 56.99 % of forage quality variation explained by the environmental factors. The first two canonical axes from the full RDA model cumulated 56.16% of the total variation, with 41.36% of the variation explained by the first axis. Noticeably, the distance to coastline contributed alone with 32.73% of the explained variation, whereas the two climate related variables (BIO15 and BIO12) explained together 24.26% of the total variation (16.23% and 7.94%, respectively) (figure 6). On the other hand, the partial RDA models indicated that the that the pure contribution of geography exceeded the pure effect of climate factors in explaining (46.42% vs 33.92%) the variation of forage quality under phosphorus deficiency in Tunisian populations of *B. hybridum*(26% and 19% of the explained variance, respectively). However, 19.64% of the explained variation was found indiscernible among the different factors (Climate [?] Geography) (Table 3).

Discussion

Polyploidy has played a major role in crop improvement and plant breeding. In plant kingdom, the process is generally associated with enhanced adaptation to environmental pressures and ecological niche expansion in diverse plant species (Scarrow et al., 2020). Thus, the most recent breeding programs have focused on polyploid candidates to be harnessed in improving agricultural staples. The allotetraploid species *Brachypodium hybridum* and its two diploid progenitors are considered as a model system for cereal crops and forage grasses (Brutnell et al., 2015; Scholthof et al., 2018). Due to its great tolerance to environmental stress, previous studies have demonstrated that *B. hybridum* is characterized by a wider ecological niche compared to its diploid progenitors (López-Alvarez et al., 2012; Manzaneda et al., 2015b) and it natural populations of *B. hybridum* harbor an extensive phenotypic and genetic variation in its native circum-Mediterranean range (Neji et al., 2015a; Neji et al., 2015b; Shiposha et al., 2020). Importantly, Rancour et al., (2012) have demonstrated that the composition of cell walls in the diploid species B. distachyon is very similar to agriculturally important forage grasses. However, until today the variation of forage quality-related traits in *Brachypodium* species is poorly uninvestigated. The unique study undertaken using some morphological traits and cell wall composition related-traits revealed an extensive variation in a core collection of the diploid species B. distachyon (Tyler et al., 2014). Hence, more detailed studies could be very useful for identifying traits and/or populations that could exploited for forage crops improvement. In the present study, we have investigated the genetic variation of 79 diverse lines representing 8 Tunisian populations of B. hybridum issued after two generations of a spontaneous selfing using a large set of forage quality traits. Congruently with our results obtained using various morpho-physiological traits in the same B. hybridum , the present study revealed a substantial variation among populations coupled with a relatively low within-population variation under low and high phosphorus availability (Neji et al., 2019). Similar variation patterns were also observed in F1 generation of ten Tunisian populations of B. hybridum exposed to drought stress (Ibrahim et al., 2020).

Furthermore, our results revealed relatively high heritability level of all forage quality traits in both P availability conditions. Similar high heritability levels were also revealed in forage quality in diverse important grasses, including Sorghum bicolor (Shiringani and Friedt, 2011), Zea mays L. (Wang et al., 2016) and Thinopyrum intermedium (Mortenson et al., 2019). These results suggest that the genetic variation of the forage quality in *B. hybridum* are under strong genetic control, which may helpful selecting progenies and enhancing forage quality traits in future breeding programs. Additionally, similar to the findings of (Lin et al., 2020) in the autotetraploid alfalfa (*Medicago sativa L.*), strong negative correlations were obtained between fiber-related traits and energy-related traits. According to (Jung et al., 2012), high levels of ADF, ADF noticeably reduce the digestibility of cellulose and Hemicellulose, which greatly lessen the overall forage quality. In both P availability conditions, PCA results revealed that the studied populations were divided in a group of coastal populations particularly characterized by high level of fibers contents and a group of continental populations characterized by high values of energy-related traits, indicating that these latter exhibited relatively higher forage quality. In our previous study, we have demonstrated that the coastal populations are more tolerant to P deficiency than the continental ones (Neji et al., 2019). (Nanamori et al., 2004) have demonstrated that Brachiaria hybrid cv. Mulato, a tropical forage grass highly adapted to lowfertility acid soils deficient in phosphorus (P), tolerates low P supply by enhancing the sugar catabolism in leaves which may increase polysaccharides (ADF and NDF) in cell walls. Moreover, it has been demonstrated that a high lignin level confers cells walls rigidity, which may enhance the tolerance to environmental stresses (Liu et al., 2018). Hence, we speculate that, the tolerance of the coastal populations of B. hybridum to P deficiency is strongly associated a considerable lignin and polysaccharides accumulation in leaves and tillers cell walls, which may explain their relatively lower forage quality compared to the continental populations. In agreement with our results, Lin et al., (2020) revealed that the drought/salt tolerant alfalfa cultivars were characterized by low forage quality, whereas the susceptible ones displayed relatively higher quality. These results suggest that, generally, the susceptibility to environmental stresses confers to crops species better forage quality.

Various previous investigations have demonstrated that the genetic variation of natural plant species in responses to environmental stresses could be shaped by both heritable genetic variation and phenotypic plasticity (Matesanz et al., 2017; Brunet and Van Etten, 2019). In Tunisian *B. hybridum* populations, our previous investigations have demonstrated that both processes controlled the genetic variation of morphophysiological traits under phosphorus deficiency and drought stress, with more plastic response to low phosphorus availability (44.09% vs 32. 56\% of genetically fixed response) (Neji et al., 2019), whereas greater genetically fixed response under drought stress (40.05% vs 33.54%) (Ibrahim et al., 2020). Likewise, the results of the present study revealed that the genetic variation of forage quality traits in response to phosphorus deficiency, in both morpho-physiological and forage quality traits was mainly due to the population effect, whereas it was dominated by the line effect in response to drought. These patterns was in line the population grouping patterns revealed in the present

study and strongly supports the high divergence among populations in response to phosphorus deficiency. Importantly, most analyzed traits displayed parallel reaction norms and an obvious lack of population by phosphorus deficiency effect, indicating that the plastic response of the forage quality traits in response to low P availability was broadly similar among the Tunisian *B. hybridum* populations. Similar patterns of common plastic response was observed in many plants species to diverse environmental stresses, such as the response of *Calamagrostis epigejos* (Poaceae) to nutrient deficiency (Lehmann and Rebele, 2005), *Spartina densiflora* (Poaceae) to salt stress (Grewell et al., 2016), *Zea mays* L. (Poaceae) to temperature variations (Sun et al., 2016) and *Lepidium subulatum* L. (Brassicaceae) to drought (Matesanz et al., 2017).

Moreover, as aforementioned, the results of the present study revealed a striking differentiation among coastal and inland populations. Importantly, our previous studies have demonstrated that, although spread over heterogeneous environments, the Tunisian B. hybridum populations are highly interconnected by a high gene flow via long-distance seed dispersal and their genetic variation was mainly shaped by natural selection (Neji et al., 2015b). According to (Abebe et al., (2015), natural selection is a key evolutionary mechanism through which plants improve their adaptation to environmental constraints. Hence, taken together, the findings of our present study suggest that that environmental factors may be more important than geographic distances in shaping the genetic variation of forage quality traits. Similar patterns have been previously highlighted in natural populations of diverse plant species (Bischoff et al., 2006; Raabová et al., 2007; Bischoff and Hurault, 2013). On the other hand, it has been demonstrated that environmental heterogeneity can triggers the genetic variation in functional traits, leading to the divergence among locally adapted populations along environmental gradients (Lázaro-Nogal et al., 2015; Akman et al., 2020; Radersma et al., 2020). Hence, similar to various previous investigations (Lowry et al., 2009; Busoms et al., 2015; Popovic and Lowry, 2020), our results strongly suggest local adaptation among coastal and inland populations. Additionally, according to (Honjo and Kudoh, (2019), the local adaptation occurs generally when the traits variation is genetically fixed, which is in agreement with the strong genetic basis revealed for most of analyzed traits in our study. Recently, (Capblancq et al., (2018), have demonstrated the reliability of the redundancy analysis in detecting signatures of adaptive differentiation. In our study, we found significant associations between environmental factors and the variation of forage quality traits under phosphorus deficiency. As we found a clear separation between the coastal and the inland populations, we expected to detect greater contribution of the geographical factors, the distance to coastline in particular, in comparison with the climate and soil factors. However, our RDA analysis that the climate variables were the key drivers of the genetic variation of the forage quality traits under phosphorus deficiency, a pattern highly consistent with local adaptation. In line with our results, previous studies suggesting the major role of local adaptation in underlying the genetic variation of diverse plant species such as Arabidopsis thaliana (Lasky et al., 2012), Hordeum vulgare L. (Abebe et al., 2015), Solanum pimpinellifolium (Gibson and Moyle, 2020) have demonstrated the major contribution of climate factors, in comparison with the geographical in inducing the adaptive genetic variation. However, the similarity between the Tunisian B. hybridum populations the plastic response for most forage quality traits under P deficiency indicates that the their adaptation to markedly contrasted environmental conditions did not have an important impact on the magnitude of plasticity.

In conclusion, the present revealed that the Tunisian populations *B. hybridum* displayed a considerable genetic variation of forage quality traits in response to phosphorus deficiency. Our results that revealed that a strong genetic basis of this variation with a similar and relatively low plastic response to phosphorus deficiency. We have detected an obvious differentiation between the coastal and the inland populations, with the coastal ones seem to be with higher forage quality under both high and low phosphorus availability. Additionally, we have observed a significant association between environmental factors and the genetic variation of forage quality traits in response to phosphorus deficiency, with the climate factors contributing more than the geography and soil factors, indicating a broad adaptive genetic variation in response to phosphorus deficiency. Considering the importance of *B. hybridum* as a model for forage crops, future works investigating the genetic basis of forage quality traits under diverse environmental stresses will be useful for screening relevant-quality traits for future breeding programs.

Authors contribution

M.N contributed in the experiments design, analyzed the data and wrote the manuscript. S.T. contributed in the forage quality analyses and C.A supervised the work.

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To my new son Nebrass, the source of my motivation, and to my wife for her patience and support!

Conflict of interest

All the authors agree that there was no conflict of interest to declare.

Data accessibility statement

Upon acceptance, data will be archived in Dryad

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Figure 1: Geographical locations of Tunisian populations of Brachypodium hybridum



Figure 2: Two-dimensional PCA plot showing the relationships among 79 lines representing the 8 populations of *Brachypodium hybridum*. A: Control conditions; B: Under phosphorus deficiency. D: Douar El Haj Wniss, E: Enfidha, F: Fayedh, H: Haouaria, K: El Kef, R: Raouad, S: Sejnen and Z: Jbal Zaghouan



Figure 3: Bivariate correlation matrix between functional traits in control (upper-right triangle) and under phosphorus (lower-left triangle). Blue and red colours indicate positive and negative significant correlations, respectively. Only values of significant correlations (P < 0.05) were shown.



Figure 4: Contribution of line, population, treatment, bioclimatic stages factors and their interactions in the total explained variance for the analyzed functional traits.



Figure 5: Population differences in forage quality traits in two contrasting phosphorus availability treatments. Each line shows the norm of reaction of each population for a given trait. A: Control conditions; B: Under phosphorus deficiency. D: Douar El Haj Wniss, E: Enfidha, F: Fayedh, H: Haouaria, K: El Kef, R: Raouad, S: Sejnen and Z: Jbal Zaghouan



Figure 6: Redundancy analysis (RDA) plot showing the relationship between forage quality traits (response variables) and the environmental factors (explanatory variables) retained after forward selection $D_{-}C$, BIO12 and BIO15) ($D_{-}C$: Distance to coastline, BIO12: Annual Precipitation, BIO15, Precipitation seasonality) and the forage quality traits. Populations (= samples) are marked as black up triangle.

ID	Location	Latitude	Longitude	Distance to coastline (Km)	Bioclimatic stage
F	Fayedh	35°4'4.1"N	9°40'32.1"E	116.3	Arid
Е	Enfidha	36°7'8.04"N	$10^{\circ}27'44.90''E$	0.55	Lower semi-arid
Ζ	Jbal zaghouan	36°22'6.80"N	$10^{\circ} 5'20.00"E$	39.1	Upper semi-arid
Η	Haouaria	37° 2'32.54" N	$10^{\circ}59'38.58"E$	2	Sub-humid
R	Raoud	36°57'0.00"N	$10^{\circ}14'14.00"E$	0.2	Upper semi-arid
\mathbf{S}	Sejnen	37°4'38.90"N	9° 9'51.10"E	9.8	Humid
Κ	El Kef	36° 6'36.80"N	$8^{\circ}37'39.00"E$	165.7	Lower semi-arid
D	Douar El Haj Wniss	$35^\circ\!40'56.40"\mathrm{N}$	$8^\circ 53' 49.00'' \mathrm{E}$	193.2	Upper semi-arid

Table 1: Geographical coordinates of the Tunisian populations of Brachypodium hybridum and their climatic regions

Table 2: ANOVA results of the applied linear mixed models (LMMs) testing the effects of population, line, treatment and their interaction on the analyzed functional traits. Values in bold indicating significance at p < 0.05.

	Population	Population	Line	Line	Treatment	Treatment	Population*Treatment	Populat
	F value	Р	F value		F value	Р	F value	Р
NDF	18.014	< 0.001	7.251	< 0.001	20.591	< 0.001	0	1
ADF	42.099	< 0.001	7.616	< 0.001	61.781	< 0.001	0	1
ADL	55.679	< 0.001	20.99	0.753	191.788	< 0.001	0	1
RFV	56.494	< 0.001	7.998	< 0.001	39.606	< 0.001	46.482	< 0.001
Hemicellulose	7.949	< 0.001	5.837	< 0.001	1.490	0.74	0	1
Cellulose	21.656	< 0.001	8.569	< 0.001	34.626	< 0.001	0	1
IVDMD	0.845	0.55	1.216	< 0.001	187.398	< 0.001	52.519	< 0.001
Ash	1.266	0.276	6.429	< 0.001	69.615	< 0.001	0	1
CP	66.180	< 0.001	4.749	< 0.001	0.9	0.34	0	1
EE	18.047	< 0.001	4.180	< 0.001	11.778	0.27	8.074	< 0.001
NFC	6.211	< 0.001	7.312	< 0.001	1.841	0.175	0.32	0.94
TDN	42.099	< 0.001	7.616	< 0.001	61.781	< 0.001	0	1
RFQ	28.320	< 0.001	9.765	< 0.001	34.33	< 0.001	0.218	0.981
ME	42.099	< 0.001	7.616	< 0.001	61.781	< 0.001	0	1

Table 3: Partitioning the variance associated with the pure climate effect (Climate | Geography), the pure Geography (Geography | Climate), and the joint effect of climate and geography (Climate [?] Geography), based on a redundancy analyses (RDA) for the 14 forage quality traits in the Tunisian *Brachypodium hybridum* populations under phosphorus deficiency.

	df	Variance	F	$\Pr(>F)$	Proportion of variance
Full Model:	3	72.12	1.732	0.001	0.56
Geography +					
Climate					
Pure Climate:	2	31.16	1.323	0.001	0.19
(Climate					
Geography)					
Pure Geography:	1	29.62	1.623	0.001	0.26
(Geography					
Climate)					
Climate [?]		12.71			0.11
Geography					
Total		145.61			0.44
unexplained					
Total variance		248.23			1