Research Article

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Pearl millet populations characterized by *Fusarium* prevalence, morphological traits, phenolic content, and antioxidant potential

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Abstract

Background: Pearl millet (*Pennisetum glaucum* L.) has become increasingly attractive due to its health benefits. It is grown as food for human consumption and fodder for livestock in Africa and Asia. This study focused on five pearl millet populations from different agro-ecological zones from Tunisia, and on characterization by morphological traits, total phenolic and flavonoid content, antioxidant activity, and occurrence of *Fusarium*.

Results: Analysis of variance revealed highly significant differences between populations for the quantitative traits. The highest grain weights occurred in the pearl millet cultivated in Zaafrana and Gergis of Tunisia. Early flowering and early maturing populations cultivated in the center (Zaafrana, Rejiche) and south (Gergis) of Tunisia tended to have a higher grain yield. The Zaafrana population showed the highest value of green fodder potentiel (number and weight of leaves/cultivar and the weight of tillers and total plant/cultivar) followed by Gergis and Rejiche. The Kelibia population showed the highest total phenolic and flavonoid content. Rejiche exhibited the greatest antioxidant activity. Trans-cinnamic, protocatechuic, and hydroxybenzoic acids were the major phenolic compounds in all the extracts. Three *Fusarium* species were identified in Tunisian pearl millet populations based on morphologic and molecular characterization. *Fusarium graminearum* and *Fusarium culmorum* occurred most frequently. The average incidence of the three *Fusarium* species was relatively low (<5%) in all populations. The lowest infection rate (0.1%) was recorded in the samples from Zaafrana.

Conclusion: Chemometric analysis confirmed the usefulness of the above traits for discrimination of pearl millet populations, where a considerable variation according to geographical origin and bioclimatic conditions was observed.

Supporting information may be found in the online version of this article.

Keywords: pearl millet; biological activities; *Fusarium* species; Chemometrics

INTRODUCTION

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is an important crop in the arid and semi-arid tropics of Asia and Africa (the Sahelian and part of Sudanian zone from Senegal to Sudan) where it is cultivated for multipurpose grain, stover, and fodder.[1] *Pennisetum glaucum* ($2n = 2x = 14$) is grown in regions with low soil fertility, low annual rainfall (200–600 mm), and high temperature, but it responds positively to more favorable soil and water conditions.[2] The total global millet production in 2016 was estimated at approximately 28 million tons, with Africa and Asia serving as the largest producers, generating 48% and 47% of the total global yield, respectively.[3]

Pearl millets play a critical role in food security, particularly in poor countries in Africa.[4] Pearl millet grain is a source of energy and is rich in micronutrients such as iron and zinc.[5,6] Due to the richness of millets in polyphenols and other biological active compounds, they are also considered to have a role in lowering the rate of fat absorption, slowing the release of sugars, and thus decreasing the risk of several chronic diseases, such as cardiovascular disorders, diabetes, high blood pressure, and impaired vision.[7–10] Pearl millet could replace maize by producing a highly edible oil due to the presence of many unsaturated and essential fatty acids.[11] Moreover, the role of millets in designing modern foods like multigrain and gluten-free cereal products is well known.[12,13]

Pearl millet was introduced in the North African countries in the eighth century.[14] In Tunisia, the production of this staple crop is mainly concentrated in coastal regions, such as the peninsula of Cap Bon (northeast), in Mahdia (center), and in Medenine (southeastern area). The Kairouan region has the highest pearl millet production (50%) in Tunisia.[15,16] Tunisia is endowed with a wide

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repository of plant resources adapted to its different dynamic climate zones.[17]

Recently, limited research has been devoted to Tunisian pearl millet germplasm, and particularly its quantitative aspects,[2] biochemistry,[11,18] and genetic diversity.[19] Tunisian farmers are still only growing traditional landraces of *P. glaucum*. This is well adapted to poor soils and brackish water, making this germplasm a useful basis for future breeding and improvement programs that aim to select high-yielding varieties with high levels of phenolic and flavonoids compounds and tolerant to fungal contamination and environmental conditions.

The *Fusarium* species may infect millet grain. The infections cause a reduction in grain weight and an average decrease of 10% in the β-glucan content in grains, indicating alterations to grain filling, composition, and structure.[20,21] The total loss of millet grain after harvest is estimated to be as much as 15% in many countries and much higher in developing countries.[22]

Mycotoxins are ubiquitous secondary metabolites produced by several fungi such as *Fusarium*, *Aspergillus*, *Penicillium*, *Claviceps*, and *Alternaria* spp., in food and feed,[23–25] which are harmful to humans and domesticated animals. Risks associated with *Fusarium* toxins are usually assessed based on the *Fusarium* species present, as not all species produce all toxins. *Fusarium* spp. from sorghum and millet have only recently become the subject of in-depth research in North Africa. Knowledge about the prevailing *Fusarium* species and possible influencing factors is therefore fundamental in preventing contaminated grains entering the supply chain.

The present investigation aimed to evaluate the morphologic characterization and the distribution of the phenolic fractions, their antioxidants, and the occurrence of *Fusarium* species in pearl millet populations. The phenolic compounds in pearl millet populations are reported for the first time and their relationship with the prevalence of *Fusarium* species is investigated.

MATERIALS AND METHODS

Plant collection

Pearl millet material consisted of 75 *Pennisetum glaucum* cultivars representing five populations collected from the main distribution area of this crop species in the North African coastline of Tunisia (north east, the center east, and the south east). These genotypes were collected in August 2017 from various geographical regions and were cultivated under various agroclimatic conditions (Table 1). At harvesting, the mature spikes were randomly selected, and the weights of 100 grains per population of P. glaucum were determined from North to South (Hawaria, 1 g; Kelibia, 0.9 g; Rejiche, 1 g; Zeaafrana 0.8 g; and Gergis, 1.1 g). The collected grains were dried at 38 °C for 4 days. For long-term storage (more than 6 months), grain moisture content was kept at less than 12% and the grain was stored in sealed plastic containers at 4 °C.

Morphological characterization

All cultivars were grown in the 2018 summer season in greenhouses. Each cultivar was seeded in three rows of 2 m length with a spacing of 15 cm between plants and 75 cm between rows, in a light and well-drained sandy soil, under controlled conditions (14 h photoperiod and temperature of 28 °C/24 °C during day / night). Fertilizers were applied at the rate of 150 kg nitrogen (applied in three fractions), 50 kg phosphorus, and 50 kg potassium per ha. The experiment was carried out in a randomized block design with ten replications. A total of 16 morphological descriptors (three qualitative and 13 quantitative characters) were determined and recorded. These were standard pearl millet descriptors (Table 2).[26]

Biochemical characterization

About 2 g of powder was extracted with 20 mL of 80% methanol (0.2 mol L-1 concentration) in mortar-pestle and transferred to test tubes. The mixture was heated at 60 °C for 60 min in a water bath and cooled to room temperature. The mixture was filtered with Whatman filter paper No. 1 and the filtrate was stored at −20 °C during analysis.

Total polyphenol content

Quantitative estimation of polyphenols was carried out using the Folin–Ciocalteu reagent following the extraction protocol described by Dewanto *et al.*[27] Different concentrations of stock solution were prepared. Each was added to 125 µL of Folin–Ciocalteu reagent. After 2 min, 1250 µL of Na_2CO_3 (7 g/100 mL) was supplied to promote an alkaline medium and to start the oxidation–reduction reaction. After adjusting with distilled water to a final volume of 3 mL, these extract solutions were kept in the dark for 90 min at room temperature. The absorbance of each

Table 1. Sampling provenance and bioclimatic characteristics of different agro-ecological zones

Origin/collection site (District)	Geographic position and annual precipitation (mm)	Elevation (m)	Mean monthly temperature (°C)	Latitude	Longitude	Bioclimatic zone
Coastal North: Hawaria/ Kelibia (Nabeul)	North East/HR (500–700)	47/10	5.5–35.3	3 705 304/ 36 846 714	11 012 117/ 1 109 701	SubHumid
Coastal Center: Rejiche (Mahdia)	Center East/ MR (300–400)	5	7.7–31	35.467421	11.042337	Semi Arid Inferior
Continental Center: Zaafrana (Kairouan)	Center West/LR (200–300)	106	5–37.1	35.542069	10.074378	Semi-Arid
Coastal South: Gergis (Mednine)	South East/LR (100–200)	18	6.9–34.3	33.503991	11.089798	Arid Inferior

Rain: LR, MR, and HR = low, moderate, and high rainfall, respectively.

Table 2. Pooled means of morphological traits of pearl millet populations

Pearl millets	Hawaria/ Nabeul	Kelibia/ Nabeul	Zaafrana/ Kairouan	Rejiche/ Mahdia	Gergis/ Medenine	MEAN	LSD (0.05)	CV (%)
Flag leaf length (cm)	23.64	24.91	21.02	23.21	23.2	23.196	17.9	11.54
Flag leaf width (cm)	1.73	1.91	1.98	2.13	1.85	1.92	1.3	0.22
Plant height (cm)	127.6	119.1	139.6	164.5	133.5	136.86	57.6	6.85
Diameter of the stem between the 3rd and 4th node from the top (mm)	0.429	0.399	0.573	0.3256	0.48	0.44132	11.4	6.05
Leaf number/plant	6.1	5.9	8	7.5	6.5	6.8	8.8	3.064
Number of productive tillers per plant	1.1	1.1	1	1	1.1	1.06	0.3	0.2
Appearance of plants at 1 m of height	3	3	3	3	3	3	1.08	0.15
Appearance of plants at 1,5 m of height	2	2	3	2	3	2.4	1.02	0.25
Total weight of plants /cultivar (kg)	12.99	19.45	27.98	20.26	20.98	20.332	11.3	4.63
Leaf weight at harvest per plant (kg)	1.29	2.12	3.15	1.87	2.51	2.188	0.36	0.77
Weight of stems at harvest/ cultivar(kg)	7.34	11.88	17.97	11.99	13.66	12.568	9.2	0.08
Weight of the main panicle (g)	4.16	5.29	6.83	6.43	4.74	5.49	2.36	1.69
Panicle weight per plant (g)	4.33	5.35	6.83	6.43	4.88	5.564	3.2	2.55
Color of grains	4	5	5	5	5	4.8	2.13	1.28
Grain yield (t h^{-1})	1.1	1.3	1.6	1.1	1.2	1.26	0.21	0.02
Days to 50% flowering (days)	63	60	53	55	50	56.2	12.3	6.3

Overall mean values, least significant difference (LSD at $P < 0.05$) and coefficient of variation (CV).

extract solution was read as described at 760 nm. Gallic acid was used as a standard to estimate the total amount of polyphenols.

Total flavonoid content

The flavonoid amount was carried out by a method based on the formation of complex between phenolic compounds and aluminum trichloride ($AlCl_3$).[27] From the stock solution of millet grains extracts prepared in methanol, different dilutions ranging from 25 to 500 µg mL^{-1} were prepared. Then 0.125 mL of each solution was added to 75 µL of $NaNO_2$ solution (5%) and 0.15 mL of $AlCl_3$ (10%). All was mixed thoroughly for 6 min. The mixture that was obtained was then added to 0.5 mL of 1 M NaOH solution. The assay was carried out by UV-visible spectrophotometry at 510 nm and total flavonoid content was expressed as mg quercetin equivalent (QE) per gram of dry weight (mg QE/g DW) through its calibration curve.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant properties were assessed by a DPPH radical scavenging assay.[28] An aliquot of 2 mL of the extract in methanol, at different concentrations (0.1–2000 µg mL^{-1}) was added to 500 µL of a methanolic solution from DPPH at 0.2 mM. The solution was incubated for 30 min, at room temperature, in the dark, and absorbances at 517 nm were recorded. Butylatedhydroxyanisole (BHA) was used as positive control. All samples were analyzed in triplicate. The percentage of inhibition was determined using the following equation:

DPPH scavenging activity (%) = $[(Ac - At)/Ac] \times 100$where *Ac* is absorbance of control and *At* is absorbance of the sample.

Reversed phase high-performance liquid chromatography (RP-HPLC) evaluation of phenolic compounds from grains extracts

Diluted samples from *P. glaucum* grains were injected into RP-HPLC equipment. The separation of phenolics was performed with an Agilent 1100 series HPLC system. Instrument control and data analysis were carried out using Agilent HPLC Chemstation 10.1 edition through Windows 2000. The separation was carried out on a reverse phase ODS C18 (4 µm, 2509 4.6 mm, Hypersil) column used as stationary phase at ambient temperature. The mobile phase consisted of acetonitrile (solvent A) and water sulfuric acid (0.2%) (solvent B). The flow rate was kept at 0.5 mL min^{-1}. The gradient program was as follows: 15 A/85% B 0–12 min, 40% A/60% B 12–14 min, 60% A/40% B 14–18 min, 80% A/20% B 18–20 min, 90% A/10% B 20–24 min, 100% A 24–28 min. The injection volume was 20 µL and peaks were monitored at 280 nm. Peak identification was obtained comparing the retention time and the UV spectra of the *P. glaucum* phenolics chromatogram with those of pure standards, which were purchased from Sigma (St Louis, MO, USA). Analyses were performed in triplicate.

Identification of associated *Fusarium* species

Field sampling and Fusarium *isolation*

Pearl millet grains were collected randomly from the widely grown pearl millet regions within four agroecological zones of north east, central and south Tunisia during the harvest period (Table 1). The best stage to harvest pearl millet is when the plants reach physiological maturity determined by the black spot at the

bottom of the grain in the hilar region. The ideal moisture content for harvesting grain pearl millet is about 20%. The water activity (a_w) of the grains varied from 0.9 to 0.95 a_w between populations sampled from the south to the north respectively. Samples were labeled with the name of location and Global Positioning System (GPS) coordinates and stored at 4 °C. Isolation of *Fusarium* spp., pearl millet (*Pinnestum glaucum* L.) grains was described by Leslie and Summerell.[29]

Morphological characterization of Fusarium *spp.*

Single macroconidial isolates of *Fusarium* spp. were transferred on potato dextrose agar (PDA) plates for culture characterization. For microscopic characterization, the isolates were grown on Carnation Leaf Agar (CLA) to observing macroconidia morphology, presence / absence of microconidia, and perithecium production. The morphologic identification of *Fusarium* spp. was carried out according to Leslie and Summerell.[29] *Fusarium* species incidence (per cent infection) was determined (approximately 6 g equivalent to 600 grains), using a seed-health test as described by Vogelgsang *et al.*[30]

$$\text{Percent incidence}\,(\%) = \text{number of grains infected with } \textit{Fusarium}\ \text{sp.}/\text{Total no. of grains plated} \times 100$$

$$\text{Frequency}\,(\%) = \text{number of samples with } \textit{Fusarium}\ \text{sp.}/\text{Total no. of samples analyzed} \times 100$$

Molecular characterization of Fusarium *spp.*

Approximately 0.05 g of the freeze-dried fungal tissue was ground into a fine powder in a 2 mL microfuge tube using a mixer-mill. Total DNA from each fungal isolate was extracted using a modified hexadecyltrimethyl ammonium bromide (2% CTAB) extraction procedure.[31] Extracted DNA was resuspended in Tris–EDTA buffer (10 mmol) and stored at −20 °C. The DNA concentrations were estimated by spectrophotometry and / or agarose gel electrophoresis. The DNAs were run in a 0,8% agarose gel to verify the quality and the concentrations. Polymerase chain reactions (PCRs) were performed in a thermal cycler (Master Cycler Eppendorf, Germany).

The PCR DNA analyses were performed separately using the following species-specific primers: Fg16NF/R for *F. graminearum*, FC01F/R for *F. culmorum* and FP82F/R for *F. poae*.[32] The PCRs were performed in a Master cycler gradient in a final volume of 20 µL, containing 10 ng of template DNA, 1.5 mM $MgCl_2$, 0.2 mM each of four dNTPs, 0.5 mM of each primer, and 1 unit of taqpolymerase (Invitrogen) in the corresponding reaction buffer. Thermal cycling conditions for *F. graminearum* involved an initial denaturation step at 95 °C for 2 min, 30 cycles of 94 °C for 30 s, 62 °C for 1 min and 72 °C for 5 min, and a final extension at 72 °C for 5 min. Annealing temperatures of 56 and 57 °C were used for *F. poae* and *F. culmorum* respectively. Fifteen microliters of PCR products were loaded in agarose gel 2%, and were run for 1 h at 120 V, stained with ethidium bromide.

Statistical analysis

All analyses were performed in triplicate and the data were reported as means ± standard deviations (SD). The means were compared using the one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. All analyses were performed using SPSS v.21 software. Differences were considered significant at $P < 0.05$. For the multivariate statistical analyses, principal component analysis (PCA) was applied to examine the inter-relationships between the species that were investigated and to explore the characters significantly contributing to the variation. To check for possible correlations between the different data sets, XLSTAT-Pro 7.5.3 software was used. The correlation coefficient (r) was based on Pearson's correlation and the *P* value was used to show the significance. A probability value of $P < 0.05$ was adopted as the criterion for significant differences.

RESULTS AND DISCUSSION

The present study was conducted to characterize five pearl millet populations in terms of their agro-morphological traits and to investigate the presence of *Fusarium* species and bioactive compounds in pearl millet populations. The availability of bioactive compounds in food and food grade materials is of supreme industrial importance.

Agro-morphological variation

Morphological characterization and identification of genotypes will be of great significance for varietal improvement, Distinctness, Uniformity and Stability (DUS) testing, genetic conservation, varietal release, and seed-multiplication programs. In this study, morphological data were used to investigate the genetic diversity among five pearl millet populations. The ANOVA revealed highly significant differences between different populations for the quantitative variables (Table 2). Our data demonstrated the highest total weight of plants, leaf weight at harvest per plant, weight of stems at harvest / cultivar, weight of the main panicle and the panicle weight per plant achieved from the pearl millet cultivated in Zaafrana and Gergis, while the lowest ones were from Hawaria. Compared with the populations, phenological traits illustrated that Gergis and Zaafrana were the earlier flowering and maturing populations. Seed color is one of the most important traits of morphological identity of a genotype. It is strongly heritable and determines the quality and acceptance of cultivars. It has an economic value because it constitutes the basis for farmers' variety identification and commercial classification of different varieties of crops.[33] In the present study, seed color was observed using the Munselcolor chart. Based on seed color, the genotypes were grouped as light gray (Hawaria) and dark gray (Rejiche, Kelibia, Gergis, and Zaafrana) (Table 2). Large grains, which are gray and partly corneous, are appreciated by both farmers and consumers.[2] The seed color is also influenced by environmental conditions during ripening besides genetic effect.[2] Thus, this character can be used in a broader classification of genotypes. The flag leaf length and width were more pronounced in Kelibia and Zaafrana pearl millet populations at harvest and grain maturity, respectively. Cereal flag leaves play an important role in the synthesis and translocation of photo-assimilates to the cereal seeds, affecting grain yield.[34] Recent work has shown that flag leaf plays a vital role in grain maturation – i.e. in the supply of carbohydrate and protein fractions that might have a limited role in micronutrient remobilization and transportation to developing grains in pearl millet.[35] All morphological descriptors studied revealed highly significant inter-populations differences, therefore the populations here are considered to be statistically different. This is explained by the fact that populations were selected from different areas, which have distinct environmental and agricultural parameters. Intra-varietal diversity can be very beneficial in the harsh and variable cropping environment because heterogeneous varieties can buffer the risk of yield losses within the population.[36] Among the observed traits,

Table 3. Total phenolic and flavonoid contents and the antioxidant activities of the five populations of *P. glaucum* grain extracts

Pearl millets	Total phenolics (mg GAE/g DW)	Total flavonoids (mg EC/g DW)	DPPH (% inhibition)
Hawaria/Nabeul	96.042	9.103	82.350
Kelibia/Nabeul	136.250	30.385	66.900
Zaafrana/Kairouan	113.333	5.385	81.130
Rejiche/Mahdia	72.083	4.103	84.780
Gergis/Medenine	101.458	12.949	70.580

the mean flowering time and the grain yield of the 75 cultivars averaged across the environments illustrated differentiation among populations of pearl millet. In the case of flowering time, there is a gradient of earlier to later flowering from the south to the north, i.e., from lower to higher mean annual precipitation (Table 1). For example, the coastal south of Tunisia (Gergis), which receives 200 mm of rainfall, produces cultivars with a flowering time below 50 days, whereas the coastal and continental center (Rejiche, Zaafrana), with more than 250 mm rainfall, produces cultivars with a flowering time of 55 days (Tables 1, 2). The Hawaria and Kelibia (coastal north) populations were cultivated in the end of June with precipitation 226–259 mm and flowering time 60–63 days. The grain yield of the Zaafrana population was higher (1.6 t h^{-1}) and coincided with early flowering. According to Pucher *et al.*,[37] early flowering (67 days) appears with low precipitation (350 mm) in central Mali and southern Mauritania. In the current study, early flowering cultivars cultivated in center (Zaafrana, Rejiche) and south (Gergis) Tunisia tended to have a higher grain yield (perhaps due to the escape from terminal drought stress). Similarly, this result is in line with the findings of Bashir *et al.*[38] All populations of pearl millets were vigorous plants at 1 m of height. About 40% of the observed populations (Zaafrana, Gergis) were considered to have a good aspect whereas (60%) have an intermediate aspect at 1.5 m of height. Green fodder potential (at harvesting) was represented by the number and weight of leaves / cultivar and the weight of tillers and total plant / cultivar. The Zaafrana population showed the highest value of green fodder followed by Gergis and Rejiche. The production of fodder pearl millet varied among regions. It depended on cropping practices of farmers.[2] As depicted in Table 2, plant height (164 cm) reached its maximum in the Rejiche, followed by Zaafrana and Gergis. However, Loumerm *et al.*[2] reported that plants grew up to 3 m height with a mean value of 197.87 ± 32.5 cm, except for pearl millets traditionally grown in association with olive trees, which are smaller (range 59–230 cm). Here farmers also choose to grow small plants. Populations from west Africa had on average 227.24 ± 12.56 cm plant height (varying between 129 and 293 cm).[37]

Total polyphenol and flavonoid content of pearl millet

The total polyphenol and total flavonoid content of the five pearl millet populations are given in Table 3. Compared with other solvents like methanol and acetone, ethanol is considered as safe and non-toxic;[39] thus, ethanol was selected for extraction of phenolic compounds from pearl millet landraces. A significant variation ($P < 0.05$) in total phenolic content (TPC) was observed among the five populations (Table 3). For instance, the highest value of TPC was found in Kelibia (136.25 mg GAE/g DW) whereas the lowest value was found in Rejiche cultivars (72.083 mg GAE/g DW). This high content of polyphenols of pearl millet has been elucidated previously.[40,41] These results were by far higher than those obtained for other pearl millet cultivars grown in India[42,43] but lower than that obtained from South African pearl millet,[44] and in the same range as those cultivated in Nigeria.[45]

The total flavonoid components (TFC) of different pearl millets differed significantly ($P < 0.05$). The total flavonoid content ranged from 4.103 to 30.385 mg EC/g DW. The Kelibia population exhibited the highest level of flavonoid content (30.385 mg EC/g DW), while the lowest amount was attributed to the Rejiche population (4.103 mg EC/g DW) (Table 3). These TFC content levels were higher than those found by Siroha *et al.*[46]

This variation may be attributed to different intrinsic and extrinsic factors such as plant genetics and cultivars, soil composition and growing conditions, maturity state, post-harvest conditions, and the screening method used for the quantification of TPC or TFC.[47] This quantitative estimation indicated that pearl millet is a rich source of phenolic and flavonoid compounds.

DPPH radical scavenging activity

The free radical scavenging activity of the DPPH radical of the ethanol extracts of different *P. glaucum* populations were evaluated (Table 3). All of the populations that were studied showed free radial scavenging potential exceeding the 50%. Accordingly, all the extracts examined demonstrated significant antioxidant potential. Maximum free radial scavenging potential was shown by the pearl millet cultivated in Rejiche (84.78%) whereas the minimum scavenging potential was found in Kelibia (22.71%). These results were in the same range of those obtained with other Tunisian pearl millet,[41] but they were higher than those obtained with Indian landraces.[42]

Pearl millet phenolic compounds

The amounts of individual phenolic compounds from the five millet grains populations are summarized in Table 4. The qualitative and quantitative composition of phenolics varied significantly ($P < 0.05$). As Table 4 shows, phenolic acids predominated in the five pearl millet populations. The HPLC profiles indicated three major peaks and several minor peaks, revealing the complex nature of pearl millet populations. The flavonoid content ranged from 2.9 μg g^{-1} DM (Gergis) to 24.4 μg g^{-1} DM (Kelibia). Phenolic acid content ranged from 658.3 μg g^{-1} DM (Kelibia) to 1668.6 μg g^{-1} DM (Zaafrana).Trans-cinnamic acid was the major phenolic compound in all the extracts (44–71%) followed by protocatechic acid (19–28%) and hydroxybenzoic acid (0–26%) in all samples. Similarly, Radhouane *et al.*[41] reported that trans-cinnamic, protocatechuic, and hydroxybenzoic acids were the main phenolic compounds of pearl millet extracts. The highest content of trans-cinnamic acid was found in the Zaafrana population, followed by Rejiche, located in the coastal center (Table 4). Kulik *et al.*[48] demonstrated that trans-cinnamic acid inhibited mycotoxin production by *Fusaria* and had strong antifungal activities.

Table 4. Content of individual phenolic compounds (µg/g DM and %) of pearl millet populations

	Hawaria/Nabeul		Kelibia/Nabeul		Zaafrana/Kairouan		Rejiche/Mahdia		Gergis/Medenine	
	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%
Gallic acid	10.90 ± 0.25	0.85 ± 0.02	13.60 ± 0.023	1.99 ± 0.021	9.80 ± 0.16	0.59 ± 0.18	10.10 ± 2.1	0.66 ± 0.21	9.40 ± 0.03	0.67 ± 0.01
Ferulic acid	23.60 ± 0.42	1.84 ± 0.05	30.20 ± 6.21	4.42 ± 0.1	ND	ND	ND	ND	ND	ND
p-coumaric acid	ND	ND	0.60 ± 0.05	0.09 ± 0.12	0.90 ± 0.05	0.05 ± 0.01	1.30 ± 0.03	0.09 ± 0.01	0.90 ± 0.05	0.06 ± 0.01
Gentisic acid	ND	ND	ND	ND	35.40 ± 0.36	2.11 ± 0.15	23.30 ± 10.23	1.53 ± 0.36	19.70 ± 2.21	1.40 ± 0.02
Trans-cinnamic acid	575.80 ± 12.25	44.82 ± 10.23	486.30 ± 5.21	71.23 ± 6.32	731.20 ± 12.3	43.68 ± 8.25	677.10 ± 16.66	44.46 ± 11.23	633.00 ± 11.5	45.01 ± 10.21
Protocatechic acid	365.80 ± 10.36	28.48 ± 9.32	127.60±	18.69 ± 0.15	452.40 ± 11.2	27.03 ± 3.25	414.00 ± 15.26	27.18 ± 12.0	387.10 ± 12.3	27.53 ± 12.21
Hydroxybenzoic acid	253.50 ± 9.25	19.73 ± 5.2	ND	ND	437.60 ± 15.2	26.14 ± 2.41	391.00 ± 14.23	25.67 ± 10.3	352.00 ± 14.3	25.03 ± 0.1212.32
Vanillic acid	48.30 ± 1.05	3.76 ± 1.23	ND	ND	ND	ND	ND	ND	ND	ND
Caffeic acid	ND	ND	ND	ND	1.30 ± 0.05	0.08 ± 0.01	1.70 ± 0.09	0.11 ± 0.08	1.30 ± 0.08	0.09 ± 0.01
Quercitin	2.40 ± 0.05	0.19 ± 0.02	1.20 ± 0.06	0.18 ± 0.05	1.50 ± 0.09	0.09 ± 0.02	0.90 ± 0.06	0.06 ± 0.01	1.70 ± 0.05	0.12 ± 0.06
Catechin	4.30 ± 0.32	0.33 ± 0.05	23.20 ± 0.12	3.40 ± 0.13	3.90 ± 0.14	0.23 ± 0.12	3.60 ± 0.15	0.24 ± 0.05	1.20 ± 0.06	0.09 ± 0.01

ND, Not detected; data are presented as means ± SD (n = 3).

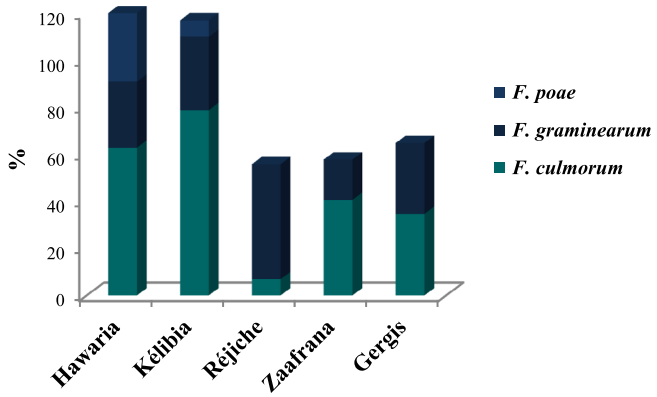

Figure 1. Distribution of different detected *Fusarium* species in Tunisian Pearl millet samples collected at five regions (n = 240).

The coastal center pearl millet populations represent a good source of trans-cinnamic acid, specifically, and of phenolic acids, generally. They are followed by the populations from the continental center (south-east). The RP-HPLC metabolite spectra in the different grain matrices were compared to identify common and unique metabolites, reflecting a great variability and richness in the pearl millet grains of the different populations. Accordingly, five metabolites were commonly detected in the five populations (gallic acid, trans-cinnamic acid, protocatechic acid, quercitin, and catechin). Three phenolic acids (*p*-coumaric acid, gentisic acid, and caffeic acid) were specifically detected in the center and south-east regions. Ferulic acid was only detected in the coastal north region represented by Hawaria and Kelibia. Taken together, these results show the strong effect of the environmental factor on the chemical composition of the pearl millet grains.[41]

Fusarium species spectrum in Tunisian pearl millet samples

This is the first study to document the distribution and prevalence of *Fusarium* species in the regions that are most commonly cultivated with pearl millet (*P. glaucum* L.) in Tunisia. Three different *Fusarium* species were detected in Tunisian pearl millet populations based on morphologic and molecular data. Morphological characteristics such as mycelium appearance, growth rate, pigment, and spodorochia formation varied among *Fusarium* species. The PCR assay used for the identification of *Fusarium graminearum*, *F. culmorum*, and *F. poae* isolates was quick and precise compared to conventional methods. *Fusarium graminearum* and *F. culmorum* (Smith, Saccardo; no teleomorph known), were the predominant species (31% and 45% of all detected *Fusarium* species, respectively) in four agroecological zones of Tunisia (Kelibia, Hawaria, Zaafrana, and Gergis) (Table 1). However, *F. poae* (Peck, Wollenweber; no teleomorph known) was the third occurring species, with the lowest frequency at 7%. In Kelibia, Hawaria (coastal North), Zaafrana (continental Center), and Gergis (coastal South) populations, the highest frequencies occurrences of *F. culmorum* were 79%, 63%, 41%, and 35% respectively. However, the Rejiche (center east Tunisia) samples showed that *F. culmorum* occurred least frequently (7%). We observed that *F. graminearum* occurred frequently in the Rejiche population (49%), followed by Gergis (30%), Kelibia (31%), Hawaria (28%), and Zaafrana (17%). *Fusarium poae* was recorded only in Hawaria and Kelibia pearl millet

Table 5. Mean incidence of *F. graminearum* (FG), *F. culmorum* (FC) and *F. poae* (FP) pearl millet samples collected from five regions in Tunisia

Populations/*Fusarium* species	Mean incidence of *Fusarium* species (%)		
	FG	FC	FP
Hawaria	3.5 ± 1.3	1.5 ± 1.8	1.2 ± 0.2
Kelibia	2.8 ± 0.3	2.8 ± 0.2	0.3 ± 0.1
Rejiche	2.4 ± 0.7	0.2 ± 0.5	0.7 ± 1.5
Zaafrana	3.1 ± 1.7	2.4 ± 1.3	0.1 ± 1.2
Gergis	1.8 ± 1.9	1.2 ± 0.7	0.2 ± 1.7

grains, at 2% and 7% (Figure 1). However, the average incidence of the three *Fusarium* species was relatively low (<5%) in all populations (Table 5). Hawaria showed the higher infection rate (3.5%) but Zaafrana samples recorded the least infection rate (0.1%) (Table 5). The importance of *Fusarium* species in cereal grains creates economic problems.[49] This increase in *Fusarium* growth and grain contamination depend on different variables including method of agriculture practices, relative humidity, atmosphere, conditions of storage and grain moisture content.[50] Indeed, the temperature and relative humidity were higher in the harvesting period of the pearl millet populations from July–August (Rejiche, Zaafrana, and Gergis) to September (Kelibea, Hawaria). Our findings agree with the study by Kosiak *et al.*[51] who revealed that *F. graminearum* and *F. culmorum* are closely related species. *Fusarium graminearum* (teleomorph *Gibberellazeae*) is the most frequently encountered and the most virulent species worldwide,[52,53] even though, many studies showed variations in *Fusarium* species identification between countries.[50,54–56] *Fusarium* was known as an important toxigenic fungus worldwide.[57] Houissa *et al.*[58] affirmed the multi-toxin co-occurrence in Tunisian pearl millet across the same agroecological zones of sampling in our study and during the 2014 and 2015 campaigns. In the north coastal zone (Hawaria, Kelibia), *Alternaria* and *Fusarium* metabolites were common ($P < 0.05$). Farmer samples sourced from the coastal north zone were the most contaminated (149 μg kg^{-1} mean and 1909 μg kg^{-1} maximum).[58] Accordingly, the results of the current study showed the prevalence of three species of *Fusarium* with high frequency (*F. graminearum*, *F. culmorum*, and *F. poae*) in the north coastal area (Fig. 1). None of the samples deriving from the continental center (Zaafrana) and the coastal center (Rejiche) and south contained *Fusarium* metabolites (Beauvericin; Diacetoxyscirpenol; Equisetin; Monoacetoxyscirpenol) at detectable levels.[58] The change in *Fusarium* species and mycotoxin contamination within the agroecological zones and years of sampling was predicted because of the climate difference from north to south of Tunisia and the high genetic diversity between pearl millet populations.[19] Previous studies showed that fungal geographic worldwide distribution and mycotoxins patterns were strongly influenced by climate change and environmental conditions (temperature, humidity, and precipitation).[59–61] In fact, little consideration has been given to grain contamination by fungi or mycotoxins relating to the phenolic profile, which has been claimed to exert an effect against fungal contamination.[62,63] Recently, some reports have suggested that phenolic compounds act as antifungals in grains, preventing the occurrence of mycotoxins.[64] Pearl millet grain in the continental center and the coastal center illustrated that the low prevalence of *Fusarium* species may be related to its phenolic profile. Data on the occurrence and percentage incidence of *Fusarium* species would be very helpful for predicting the extent of post-harvest infection, colonization, and the subsequent deterioration of pearl millet populations.

Chemometric analyses

Principal component analysis of pearl millet grains associated parameters

Principal component analysis was performed to examine the feasible clusterings of 16 morphological parameters that were significantly different among five regions. The five populations were differentiated according to the first two principal components (PC1 and PC2), which accounted for 84.08% of total variance (Fig. 2(a)). The first principal component (PC1) explained 61.80% of the total variance, and the separation of samples between Rejiche province and the other provinces could be observed. Moreover, Gergis and Zaafrana formed a cluster that was positively correlated with this axis. These two populations were characterized by the highest values of leaf number / plant, diameter of the stem between the third and fourth node from the top, number of productive tillers per plant, total weight of plants / cultivar, leaf weight at harvest per plant, weight of stems at harvest / cultivar, weight of the main panicle, panicle weight per plant, and the earlier mean flowering time (Fig. 2 (a)). The second axis, which explained 22.28% of the total variance, revealed two principal clusters. The first, which was negatively correlated with PC2, included Hawaria and Kelibia from the coastal north region of Tunisia and was characterized by late-flowering time and the lowest values of morphological parameters (Fig. 2(a)).

Similarly, based on biochemical parameters (total polyphenols and flavonoid contents), individual phenolic compounds, the antioxidant activity and the average incidence of *Fusarium* species (Fig. 2(b)) were investigated. The results showed that the first two components, F1 and F2, accounted for 87.39% of the total observed variation. The first principal component (F1) explained 63.74% of the total variation and was positively correlated with DPPH, *p*-coumaric acid, gentisic acid, trans-cinnamic acid, protocatechic acid, hydroxybenzoic acid, and caffeic acid. The second principal component, F2, explained 23.65% of the total variation and was positively correlated with ferulic acid, vanillic acid, and quercitin. The PCA biplot based on the two first axes, F1 and F2, showed three major groups (Fig. 2 (b)), where pearl millet populations from the continental center and coastal south were clustered together and were specifically characterized by the highest antioxidant activities, high amounts of gentisic acid, trans-cinnamic acid, protocatechic acid, hydroxybenzoic acid, caffeic acid, and the lowest fungal incidence. Pearl millet cultivars from Hawaria and Kelibia were distinguished at the left side of the plot and were characterized by high total polyphenol and flavonoid content (Fig. 2(b)). This

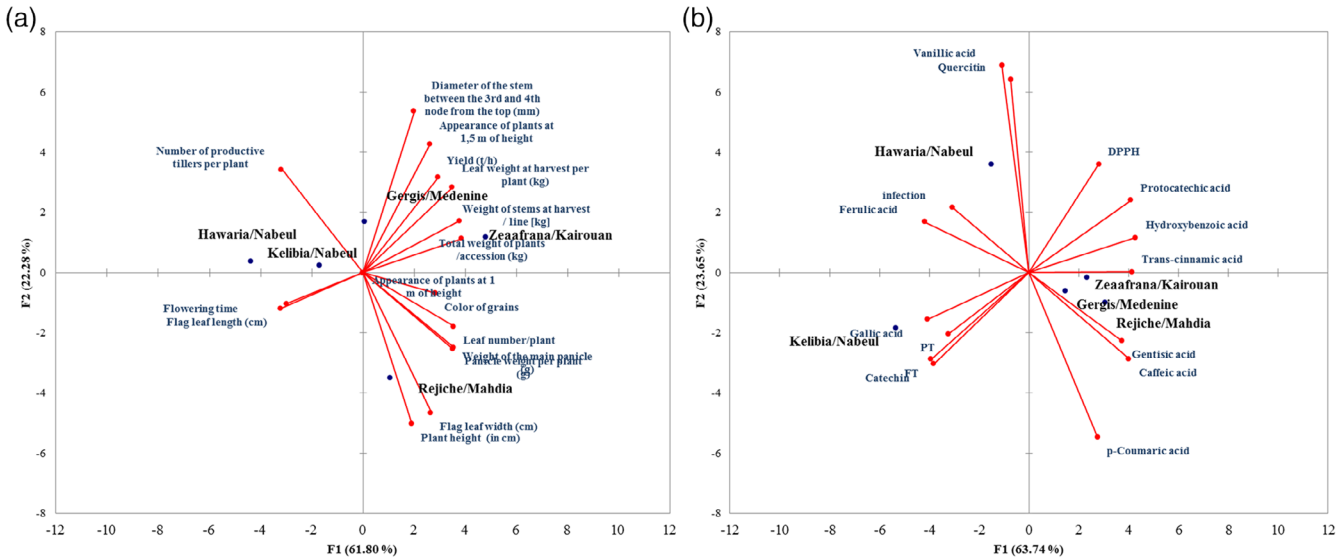

Figure 2. Principal component analysis (PCA) of pearl millet population based on morphological descriptors (a) and biochemical features and *Fusarium* infection parameters (b).

analysis showed the phytochemical distinctiveness of the pearl millet populations that were investigated. Moreover, populations were structured according to geographical origin and bioclimatic conditions. The classification pattern is consistent with the view that geographical and environmental origins may explain the observed variability.

Correlation analyses

To identify potential metabolites correlated with the biological activities (antioxidant and fungal incidence) of the pearl millet grain extracts, a correlation analysis was performed between the relative content of the identified metabolites (%), the total polyphenol and flavonoid content, the antioxidant activity (IC50) and the average incidence of *Fusarium* species (Fig. 3). Since the biological activities could be attributed to synergistic, cumulative or antagonistic effects of the identified compounds, all metabolites (majors asminors) should be considered.[65] A correlation matrix generated 225 pairs of correlations, which showed correlations between different sets of metabolites, the antioxidant activities, and the incidence of *Fusarium* species (Fig. 3). As shown in Fig. 3, the fungal incidence of *Fusarium* species were negatively correlated with antioxidant activity and with several metabolites, namely, *p*-coumaric acid, gentisic acid, trans-cinnamic acid, protocatechuic acid, hydroxybenzoic acid, and caffeic acid. In fact, these metabolites were previously known to have strong antifungal activities and to inhibit the mycotoxin produced by *Fusarium*.[48] Compounds derived via the phenylpropanoid / shikimic acid pathway (cinnamic acid, its precursor benzoic acid, ferulic acid and its precursor *p*-hydroxycinnamic acid) were found to be more abundant in heads of FHB-resistant wheat than in those of susceptible wheat following *Fusarium* inoculation.[66] Moreover, *Fusarium* incidence was positively correlated with the antioxidant capacity of pearl millet cultivars ($P < 0.05$). With five pairs of significant correlations ($P < 0.05$), *Fusarium* incidence was positively correlated with groups of metabolites (gallic acid, ferulic acid, vanillic acid, caffeic acid, quercitin, and catechin; $P < 0.05$). These findings are consistent with the view that the fungal prevalence is due to a large spectrum of metabolites that could play an important role in conferring these biological properties. Similarly, antioxidant activity (DPPH assay) was correlated with a range of metabolites. The highest positive correlations were found with trans-cinnamic acid, protocatechic acid, and hydroxybenzoic acid ($r > 0.69$, $P < 0.05$), while the highest negative ones were revealed when correlated with catechin ($P < 0.05$; Fig. 3). We can therefore conclude that the mechanisms underlying these biological activities involved a complex of compounds acting synergistically or antagonistically within the extracts.[67,68]

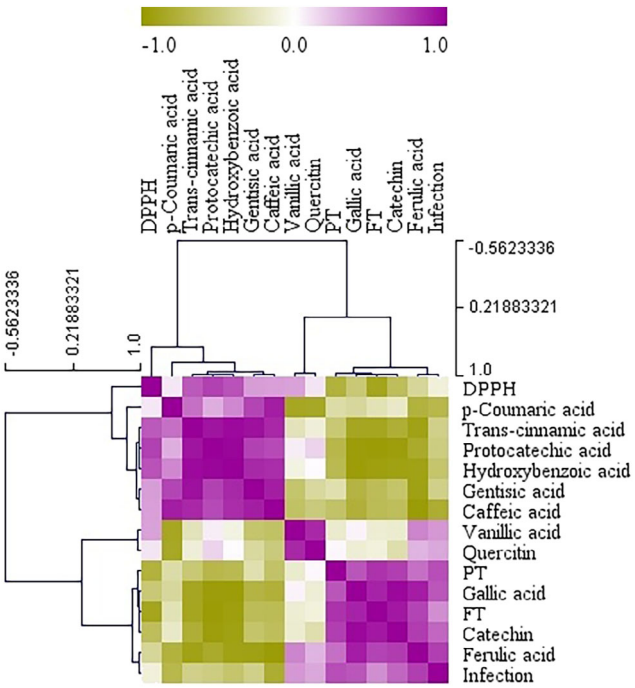

Figure 3. Heat-map matrices of the correlation between the metabolites, antioxidant and fungal incidence of the pearl millet population's grains. The value of the correlation coefficient is represented by the intensity of the mustard color ($-1 < r < 0$) or purple color ($0 < r < 1$), as indicated on the color scale.

CONCLUSION

In conclusion, a detailed characterization of the North African pearl millet population was assessed and the distinctiveness of pearl millet samples was highlighted. Principal component and correlation analyses showed that several phenolic compounds were correlated with antioxidant mechanisms. This study brings new insights into the functionality of the antioxidant pearl millet grain compounds, such as the potential to protect against fungal contamination. These findings would be of great value for assessing possible health hazards in humans and animals on consumption of such susceptible food grains and could help to redirect selection through the less susceptible region where conservation is required.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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