Growth, Yield and Grain Nutritional Quality in Three Brazilian Pearl Millets (*Pennisetum americanum* L.) with African or Indian origins

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ABSTRACT

In this study, we are presenting recommendations to the best agricultural use as well as for plant breeding of three millet cultivars namely ENA1 and ENA2, which have African origin, and BRS1501 originally from India. These cultivars were evaluated for growth, yield and grain quality traits. The morphological traits evaluated in this study indicated that the African genotypes ENA1 and ENA2 are better than the Indian genotype BRS1501 for no-till farming or to produce forage with 15% of crude protein at flowering and at harvest to produce stover (around 7% of crude protein content) for livestock feeding. The BRS1501 cultivar exhibited the highest values for total crude protein, albumins and prolams, phytate and mineral contents in grains. ENA1 and ENA2 exhibited the highest values of globulin and glutelin contents. The electrophoretic patterns for storage proteins were similar across the three millets cultivars, except for a higher intensity of two glutelin bands with 21 and 24 kDa in BRS1501. Together, the results allow us to recommend BRS1501 for grain production and ENA1 and ENA2 for biomass production.

Key words: mineral density, SDS-PAGE, phytate, seed storage proteins, amino acids.

INTRODUCTION

Pearl millet (*Pennisetum glaucum* L.) is the sixth most important cereal worldwide and it is a staple food for around 90 million people in the Sahelian region of Africa and northwestern India (Pattanashetti et al. 2016). Pearl millet is a C₄ plant with very high photosynthetic efficiency, dry matter production capacity, short life cycle, and high degrees of tolerance to heat and drought (Bidinger et al. 2009). It is also adapted on saline, acidic and aluminum toxic soils (Bidinger et al. 2009). Therefore, this cereal is cultivated and used as a grain and a forage crop and is the major cereal crop species for people living in the drier...
areas of the Indian subcontinent and in both West and East Africa. Yet, until very recently, pearl millet received comparatively to other crops, little attention from the scientific community (Pattanashetti et al. 2016). Pearl millet has also been shown to be a good option for no-till farming due to its high biomass production even without fertilization (Pimentel et al. 2003). Furthermore, pearl millet can be cultivated in a low-input agrosystem, without costly agronomic practices during its 90 days cycle, producing higher biomass and crude protein content (around 15%) for forage than maize and sorghum (A.C.T. Costa, unpublished data, Pimentel et al. 2003). In Brazil, for instance, there is an important increase in pearl millet use for no-till farming but also in crop rotation, during the dry season, and for stover and grain production for animal feeding. Moreover, its grain is substituting maize in manufacturing chicken feed because of its higher protein content per kilo of grains produced at a lower cost than maize (Pimentel et al. 2003). The grain protein of pearl millet is characterized by a higher quality among cereals because lysine and tryptophan deficiency is diminished when compared to the majority of cereal crop seeds, and it is a “high-energy” cereal that contains carbohydrates, protein, oil and is rich in vitamins B and A, high in calcium, iron, and zinc, and also contains concentrations of potassium, phosphorus, magnesium, zinc, copper and manganese (Bidinger et al. 2009, Pattanashetti et al. 2016).

The main center of origin and domestication of pearl millet is the West African region (Bidinger et al. 2009), but it was later introduced into India, which is nowadays considered a second origin center (Pattanashetti et al. 2016). Nevertheless, there is considerable diversity between African and Indian pearl millet genotypes, which have been evaluated and confirmed by morphological, isozyme and DNA analyses (Pattanashetti et al. 2016). The African genotypes have a higher crop growth rate, they are taller and produce more biomass, with a lower number of fertile tillers than the Indian ones, but both have the same grain yield potential (A.C.T. Costa, unpublished data). For livestock feeding, the African genotypes cultivated in Brazil can be harvested at the flowering stage for forage yield as ray or silage. When harvested at the milk stage the grain of pearl millet has 27 to 32% more protein than maize and its biomass is higher in weight and protein content (Pimentel et al. 2003). On the other hand, pearl millet can be harvested for forage at the end of the cycle because its stover crude protein content is still very close to 7.0%, which is considered the minimum level required for cattle maintenance, but also producing grains to be used for chicken feed (Pimentel et al. 2003). However, studies on pearl millet grain quality traits are still scarce (Saleh et al. 2013).

In Brazil, Indian derived millets such as BRS1501 (Pereira Filho et al. 2003), and African derived such as ENA1 and ENA2, are available and have been cultivated (Pimentel et al. 2003). Thus, in this study, these cultivars were chosen because they represent the two main centers of origin of millet. We have compared the biomass and quality grain traits for these three millet cultivars aiming to present recommendations as to the best agricultural uses for them, as well as to present recommendations for plant breeding. The biomass and grain traits evaluated were biomass at flowering and maturity and the contents of storage protein fractions, phytate and minerals in grain.

MATERIALS AND METHODS

PLANT MATERIAL

The pearl millet cultivars ENA1, ENA2 and BRS1501 were grown in Seropédica, RJ, Brazil (22° 45’ S, 43° 41’ W) during the rainy season. ENA1 and ENA2 were selected from African genotypes (A.C.T. Costa, unpublished data), while BRS1501 was selected from Indian genotypes (Pereira Filho et al. 2003). The experimental design was in blocks
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two glutelin extractions with dodecyl sulphate sodium 0.5% (w/v) and 2- mercaptoethanol 0.6% (v/v) in sodium borate buffer pH 10 (Na\textsubscript{2}B\textsubscript{4}O\textsubscript{7} 12 H\textsubscript{2}O 0.0125 mol L\textsuperscript{-1} and NaOH 0.02 mol L\textsuperscript{-1}). The centrifugations for defatting and for extraction of prolamins and glutelins were performed at room temperature, 25 ºC, while the other extractions for albumins and globulins were performed at 4 ºC. The protein supernatant was retrieved after centrifugation and when two centrifugations were performed, both supernatants were combined and stored at -80 ºC.

Each protein fraction content was determined according to Bradford (1976). Nitrogen content was determined by the Kjeldahl method with a conversion factor of 6.25 for crude protein content (Pimentel et al. 2003).

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Electrophoretic analysis was carried out under denaturing conditions (0.1% (w/v) SDS) in 13% polyacrylamide gels, with 3.8 μg of protein loaded onto each lane for albumins and 5.0 μg for the other storage protein fractions and the running conditions as described by Schmidt et al. (2015, 2016). The gels were prepared and stained with a solution of silver nitrate according to Blum et al. (1987).

PHYTATE CONTENT

Phytate extraction was carried out using a 250 mg sample of flour in 10 mL of 2.4 % hydrochloric acid for 3 h at room temperature with constant agitation. The samples were clarified by centrifugation at 6,000 g for 20 min at room temperature. The supernatant was applied and eluted from an anion-exchange resin (Dowex1x8-400 Sigma) and the phytate determination was based on the colorimetric assay described by Latta and Eskin (1980). The assay was performed with 2.0 mL of Wade reagent (0.03% (w/v) FeC\textsubscript{1}, and 0.3% sulfosalicylic acid) and 3.0 mL of the eluted sample. Phytate contents

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of these solutions were determined at 500 nm using a spectrophotometer.

TOTAL AMINO ACIDS EXTRACTION AND DETERMINATION

The total amino acids were extracted through acid hydrolysis according to Fountoulakis and Lahm (1998) using 22.5 mg of protein of a fine and homogenous flour and 9 mL of 6 N HCl. Prior to the hydrolysis, the oxygen was removed from the tubes by a vacuum pump. For this, we were used hydrolysis tube with side outlet for vacuum, and screw cap for teflon sealing. Afterwards, the samples were incubated at 110 °C for 22 h in a dry bath. After cooling the hydrolysis tubes to room temperature, the tubes were opened and the contents transferred to a volumetric flask and the volume adjusted with Milli-Q water to 25 mL. The samples were filtered in 0.2 μm millex and 200 μL were transferred to a new tube and kept in desiccator with silica gel under vacuum until complete evaporation of the solvent. Samples were reconstituted in 20 μL of 20 mM HCl.

A volume of 10 μL was derivatized in 70 μL of borate buffer and 20 μL of derivative agent (AccQ-fluor) according to the manufacturer’s instructions (Waters). The mixture was incubated at 55 °C for 10 min. After cooling down, one μL of each sample was used for separation and quantification in Acquity UPLC system (Waters). Reverse phase separation was performed with Waters BEH C18 column (100 mm x 2.1 mm i.d., 1.7 μm) at 46 °C, with a flow rate of 0.7 mL min⁻¹ ranging between eluents as follows: AccQ-Tag, Eluent B 10% (ACN), 100% Milli-Q water and Eluent B 100% (ACN). The derivatized product was detected at 260 nm. The amino acids histidine, serine, arginine, glycine, aspartate, glutamate, threonine, alanine, proline, cysteine, lysine, tyrosine, methionine, valine, isoleucine, leucine, and phenylalanine were determined based on the amino acid standard H (Prod NCI0180, Waters), as described by Schmidt et al. (2015).

MINERAL CONTENT

A 100 mg sample of flour was used to determine the mineral content according to Badau et al. (2005). The quantification methods were: metavanadate colorimetric assay for phosphorus (P), atomic absorption spectrophotometry for calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn), flame photometry for potassium (K) and turbidimetry for sulfur (S).

STATISTICAL ANALYSIS

The analysis of variance was performed with F (Snedecor) at a level of 5%, for each quantitative trait and, where necessary, the LSD mean test was used at a level of 5%.

RESULTS

The morphological traits of the African genotypes ENA1 and 2 were very different from the Indian genotype BRS1501 (Table I) as stated in the literature (Pimentel et al. 2003). ENA1 and ENA2 were taller, with a lower number of panicles per plant, but the panicles were longer than the panicles of the Indian genotype, BRS1501 (Table I), with

| Cultivars | Plant height (m) | Number of tillers per plant | Number of panicles per plant | Mean size of panicles (m) | Cycle |
|-----------|------------------|-----------------------------|------------------------------|--------------------------|-------|
| ENA1      | 2.05 a           | 3.9 b                       | 1.70 b                       | 0.44 a                   | 90 days |
| ENA2      | 2.19 a           | 4.4 ab                      | 1.75 b                       | 0.50 a                   | 94 days |
| BRS1501   | 1.50 b           | 4.9 a                       | 2.63 a                       | 0.33 b                   | 95 days |

Means followed by the same letter did not differ according to LSD test (α = 0.05).
all the three genotypes exhibiting the same grain yield (Table II). However, ENA1 and ENA2 plants produced higher fresh and dry weights than BRS1501 at flowering (Table II), whereas at harvest, the fresh and dry weights of ENA2 plants were significantly superior than for ENA1 and BRS1501 although all genotypes exhibited the same grain yield (Table II).

The crude protein content in grains was significantly higher in BRS1501 than in both ENA cultivars (Table III), with a higher content of albumins and prolams than in ENA1 and ENA2, whilst the opposite was observed for globulins and glutelins (Fig. 1).

The three pearl millet cultivars used differed only in SDS-PAGE bands for the glutelin protein fraction in which the BRS1501 exhibited higher intensity than ENA1 and ENA2 for two bands of 21 and 24 kDa (Fig. 1). The main prolamin band was identified at 22 kDa (Fig. 1).

The three millets cultivars exhibited similar contents of total amino acids, but the cultivar BRS1501 exhibited a lower proline content than ENA1 and ENA2 (Table IV).

The BRS1501 millet exhibited for all minerals evaluated, except K, higher mineral content than ENA1 and ENA2 (Table V). The amount of Fe and Zn as well as the phytate content was also higher in cultivar BRS1501 than in both ENA cultivars (Table V).

**DISCUSSION**

Pearl millet has been cultivated in Brazil and presents several features in particular the high biomass and crude protein content, which not only is interesting in terms of use in sustainable agro-system, but also for feeding livestock.

Comparing the morphological traits (Tables I and II) of the African genotypes, ENA1 and ENA2, to the Indian genotype BRS1501, chosen for this study, the African genotypes exhibited higher plant height and larger size of the panicles, but lower number of tillers and panicles per plant than the Indian genotype (Table I). However, the shoot fresh and dry weights at flowering, which can be used for no-till agriculture or for forage, as ray or silage, and at harvest, to be used as stover for livestock, were

| Cultivars | globulins | albumins | prolams | glutelins | CP* |
|-----------|-----------|----------|---------|-----------|-----|
| ENA1      | 48.2 a    | 10.7 b   | 21.4 b  | 19.7 a    | 11.4 b |
| ENA2      | 48.1 a    | 11.5 b   | 21.2 b  | 19.2 a    | 12.0 b |
| BRS1501   | 43.9 b    | 17.1 a   | 25.6 a  | 13.4 b    | 17.6 a |

Means followed by the same letter did not differ according to LSD test (α = 0.05).

* Total N x 6.2.
higher for the two African genotypes than for the Indian genotype (Table II).

Another interesting finding was that although changes were observed among the genotypes, grain yield was the same for the three genotypes cultivated in the rainy season (Table II). However, when these genotypes were grown during the dry season, A.C.T. Costa (unpublished data) obtained higher grain yield for ENA2 than for ENA1 and BRS1501, which is probably because ENA2 was also shown to be more tolerant to rust, the principal disease for pearl millet cultivation (Pereira Filho et al. 2003), than ENA1 and BRS1501. The Indian genotype BRS1501 exhibited more panicles per plant than the African ENA1 and ENA2, which have a larger panicle (Table I), and possibly explains the similar results obtained for grain yield for all three genotypes under the conditions tested in this work (Table II).

The data current available in the literature show the same pattern presented in this work for grain crude protein content, where Indian pearl millet cultivar exhibited higher grain crude protein content than African millet cultivars. African pearl millets were evaluated by Nkama et al. (2005) and the grain quality trait varied from 71.9 to 120.5 g kg$^{-1}$, whilst Abdalla et al. (1998) in a distinct study with six African pearl millets detected grain protein content variation between 113 and 121 g kg$^{-1}$. In the case of Brazilian pearl millet cultivars with Indian origin, Bastos et al. (2005) reported crude protein contents between 128.6 and 176.6 g

\begin{table}[h]
\centering
\begin{tabular}{lccc}
\hline
amino acid & ENA1 & ENA2 & BRS1501 \\
\hline
His & 17.8 a & 19.91 a & 18.82 a \\
Ser & 35.7 a & 38.57 a & 37.91 a \\
Arg & 35.18 a & 39.16 a & 34.36 a \\
Gly & 24.59 ab & 25.9 a & 21.75 b \\
Asp & 57.27 a & 64.57 a & 57.66 a \\
Glu & 22915.55 a & 25581.2 a & 21892.88 a \\
Thr & 29.93 a & 26.53 a & 27.23 a \\
Ala & 55.63 a & 60.44 a & 51.94 a \\
Pro & 47.19 a & 50.36 a & 43.08 b \\
Cys & 5.87 a & 7.07 a & 6.3 a \\
Lys & 26.67 a & 29.29 a & 25.60 a \\
Tyr & 28.9 a & 29.43 a & 26.13 a \\
Met & 43.91 a & 47.34 a & 40.76 a \\
Val & 44.08 a & 46.76 a & 43.59 a \\
Ile & 33.38 a & 35.56 a & 30.64 a \\
Leu & 76.02 a & 82.43 a & 69.53 a \\
Phe & 42.80 a & 42.16 a & 37.12 a \\
\hline
\end{tabular}
\caption{Total amino acid content (mg g$^{-1}$ protein) in grains of three pearl millets cultivars.}
\end{table}

Means followed by the same letter did not differ according to LSD test ($\alpha = 0.05$).
kg⁻¹, much higher than the previous reports. Stover crude protein content (N tissue content. 6.25) of ENA2 were around 6.77%, very close to 7.0%, which is considered the minimum level required for cattle maintenance (Pimentel et al. 2003). In addition, the crude protein content of the ENA2 grains were high (130 g kg⁻¹) for a crop without N fertilization (Pimentel et al. 2003). Pearl millet has a deep and profusely developed root system, which exploits the deeper soil layers and can assimilate leached nutrients such as N to increase its N content (Bidinger et al. 2009). It is another common practice in Brazil to cultivate pearl millet during the dry winter after soybean cultivation in the summer to use and recycle the fertilization residues from the preceding culture (Pereira Filho 2003).

Previous work by Chandna and Matta (1990) revealed that the pearl millet seed storage proteins are very conserved, with little or no variation among genotypes, which has been confirmed in our study. Another similar pattern also observed by both our study and by Chandna and Matta (1990) is related to the similarity between the contents of prolamin and glutelin, even though our data indicated a prevalence of globulins (Fig. 1), while Chandna and Matta (1990) reported that prolamin and glutelin were co-dominating protein fractions. It seems that at least part of this difference in prevalent fractions could be attributed to the differences between the extraction methods used. The present study also showed low diversity for grain storage proteins separated on SDS-PAGE, in which pearl millet cultivars with different origins exhibited similar patterns of protein distribution, even though for total protein content as well as percentages of different fractions, they have shown more diversity. Yet, the main prolamin band identified in the present study with 22 kDa (Fig. 1) is in accordance with the results reported by Gomez-Martinez (2012). Future studies should perhaps employ more detailed analysis of Pearl Millet storage proteins by using 2D-PAGE, as has been done for a wide range of crops species (Arruda et al. 2013, Vilhena et al. 2015, Schmidt et al. 2016).

The amino acid content was also shown to be similar to the one reported previously in the literature (Ravindran 1992). Proline is known for its high concentration in proteins of prolamine fraction in cereals (Shewry and Halford 2002). However, in this study it was not possible to establish a relationship between the cultivar with more prolamin content (BRS1501) and the cultivars with more proline (ENA1 and ENA2). Moreover, the cultivar BRS1501 exhibited higher total protein content, and this characteristic is not coincident with the percentage of the amino acid lysine, which exhibited no difference among cultivars, suggesting that there is little difference in the content of lysine among pearl millet cultivars. The identification of a high-lysine genotype is particularly important because cereal grains are deficient in this essential amino acid (Azevedo et al. 2006, Schmidt et al. 2016), and consequently would have a major impact in terms of the nutritional aspect of pearl millet grains.

| Substance | ENA1 | ENA2 | BRS1501 |
|-----------|------|------|---------|
| phytate   | 8.4 b| 8.7 b| 10.5 a  |
| P         | 4.4 b| 3.8 c| 4.9 a   |
| K         | 3.3 a| 3.0 a| 3.9 a   |
| Ca        | 0.3 b| 0.3 b| 0.4 a   |
| Mg        | 1.1 b| 1.1 b| 1.3 a   |
| S         | 0.9 b| 0.9 b| 1.3 a   |
| Cu        | 6.1 b| 5.9 b| 9.5 a   |
| Fe        | 53.8 b| 50.8 b| 97.1 a |
| Mn        | 13.4 b| 12.1 b| 22.6 a |
| Zn        | 42.4 b| 39.0 b| 71.4 a |

Means followed by the same letter did not differ according to LSD test (α = 0.05).
The breeding of cereals for a higher content of lysine is a very important goal particularly for developing countries, and the best example of a successful project is the one undertaken by the International Maize and Wheat Improvement Center (CIMMYT), that has achieved quality protein maize (QPM), which is used in several countries (Azevedo and Arruda 2010). In a similar manner, pearl millet breeding programs should also consider and give more attention to this aspect not only for lysine, but possibly for other essential amino acids.

It is interesting that in the study by Badau et al. (2005), ten African pearl millets were evaluated and none of them was shown to be superior in all minerals, i.e., whereas one was higher for Ca another was for Fe and so on. Overall, the range of mineral concentrations observed for the three pearl millet cultivars studied (Table V) were in line with previously reported data for other millet cultivars. For instance, the concentrations observed in this study are within the same range of those reported by Abdalla et al. (1998) and/or Badau et al. (2005) for all minerals, except K, once Abdalla et al. (1998) reported about 0.09 g kg\(^{-1}\) for this mineral. On the other hand, Buerkert et al. (1998) reported K concentration similar to our data (between 3.0 and 3.71 g kg\(^{-1}\)).

The pearl millet cultivars that showed the best concentration of Fe and Zn in the work of Govindaraj et al. (2013), were those that also exhibited Fe and Zn concentrations close to those obtained in this study (Table V), where hybrids cultivated in India exhibited ranges between 30 and 80 g kg\(^{-1}\) Fe and the best parental line exhibited 102 mg kg\(^{-1}\) Fe. When Zn is concerned, Govindaraj et al. (2013) reported values in hybrids ranging between 31 to 70 mg kg\(^{-1}\), whereas the best parental line exhibited 84 mg kg\(^{-1}\) Zn. The Fe and Zn concentrations in the genetic material are important for plant breeding since their genetic controls are predominantly additive and the environmental effect is less expressive. In addition, there is a positive correlation between Fe and Zn concentrations, indicating the effectiveness of simultaneous selection (Govindaraj et al. 2013).

The mineral concentration in grains is an important issue because more than two billion people, mainly in the developing world, suffer from micronutrient malnutrition, also known as “hidden hunger” (WHO/FAO 2000). Biofortification is one of the best approaches for addressing this global problem since it enhances the nutritional value of foods (Pimentel et al. 2003), whatever technique is used to produce such biofortified lines. One example of this major current efforts in the area of biofortification is the Harvest Plus program, from The Consultative Group on International Agricultural Research (CGIAR), that has been developing and distributing varieties of food staples (rice, wheat, maize, cassava, pearl millet, beans, and sweet potato) that contain higher Fe, Zn and provitamin A contents (Khush et al. 2012). Therefore, the data available on mineral content variability for the pearl millet lines studied is essential when recommending the ones to be used in breeding programs.

Phytate is mainly considered a common anti-nutritional factor in cereals (Badau et al. 2005, da Silva et al. 2011) and its detrimental effects include the reduction of protein and mineral absorption by the human body system and other monogastric (Cowieson et al. 2011). The reduction of protein and mineral absorption occurs due to a strong chelation of metals/minerals by phytic acid. However, some beneficial effects have also been reported, such as a reduction in blood glucose, cholesterol and triglycerides, which may reduce the risk of cancer and heart disease (Saleh et al. 2013). In addition, the detrimental effects of phytate are commonly reduced by food processing (Saleh et al. 2013). Although the data on phytate content for BRS1501, ENA1 and ENA2 (Table V) were shown to be within the same range as that observed by Badau et al. (2005), it was clear that the two distinct origins
led to different contents and therefore some genetic variability exist that can be exploited in breeding programs.

The morphological traits evaluated in this study (Tables I and II) indicated that the African genotypes ENA1 and ENA2 are better than the Indian genotype BRS1501 for no-till farming or to produce forage with 15% of crude protein at flowering and at harvest (Table II) to produce stover (around 7% of crude protein content) for livestock feeding (A.C.T. Costa, unpublished data, Pimentel et al. 2003). These behaviors would be expected since these cultivars retain their ancestral morphology, i.e., the African ones, ENA1 and ENA2, are higher, low-tillering and large-panicled, while the Indian one, BRS1501, is smaller, high-tillering and small-panicled (A.C.T. Costa, unpublished data). Bidinger et al. (2009) also reported that pearl millet, from both origins, is considered one of the most water stress adapted cultivated species. However, while the grain yield was the same for the three genotypes (Table II), the crude protein content was higher for BRS1501 (Table III), which can be indicated for the production of chicken feed.

It is interesting that the agronomic and quality traits were convergent since the cultivar preferably chosen for grain production for manufacturing chicken feed, based on grain crude protein content, BRS1501, was also superior in amount of grain protein (Table III), and mineral concentration (Table V), whereas the cultivars of preference for biomass at flowering and harvest (Table II), ENA1 and ENA2, produced quality grains lower than BRS1501 for crude protein content and concentration mineral.

Despite their different origins, African (ENA1 and ENA2) or Indian (BRS1501), all these pearl millet cultivars exhibited very similar electrophoretic pattern for protein storage, even though they differed in content for the distinct protein fractions. Based on the results, BRS1501 is better and more appropriate for grain production, since it exhibited higher content of total crude protein and minerals than ENA1 and ENA2, which are indicated for biomass production. Future studies must consider such differences and try to better understand the regulation of the metabolic pathways involved, especially when amino acids and proteins are concerned, to explore any genetic diversity existent and to produce better lines for commercial use.

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