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Identification of cultivars of foxtail millet (*Setaria italica* (L.) Beauv.) from morphological and biochemical traits of their seed

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Summary

The objective of this research was to characterise three cultivars of *Setaria italica* (L.) Beauv. "Foxtail millet": Ñandú INTA, Carapé INTA and Yaguané INTA by means of their seeds. The methodologies applied were: description of seed size, shape and weight, chemical changes to the phenol test, ferrous sulphate test and fluorescence tests, and polyacrylamide gel electrophoresis of proteins. ANOVA and comparison means test (Tukey, $p<0.05$) were performed. This study revealed that, with the exception of the fluorescence test, Yaguané, Carapé and Ñandú cultivars could be identified by using the set of methodologies considered.

Introduction

Setaria italica (L.) Beauv. is a summer-annual of good feeding value and palatability possessing rapid establishment with high yields of dry matter in a very short period. It also withstands drought and high temperature conditions (Josifovich and Echeverría, 1968; 1971). In Argentina, most foxtail millet is used as a grazing crop or harvested for hay. Cultivars Carapé and Yaguané are excellent for forage yield (Josifovich and León, 1972; Bruno, 1991). When used as a grain crop, the cultivar Ñandú is preferred (Coscia, 1981). The argentine *Setaria* cultivars Carapé, Ñandú and Yaguané are included as public domain in the National Register of Cultivars. The area allocated to these forage cultivars within the certification scheme represents a negligible part of the whole. Thus, the majority of commercial seed is not under any official controls concerned for varietal identity. According to the National Seeds Law N° 20247, *Setaria italica* seeds are commonly marketed without cultivar identity.

Most forage cultivars show no distinctive morphological characteristics that allow varietal identification by means of their seeds. Whenever differences exist, often they are not enough for accurate characterisation. In most forage cultivars, differences are mainly related to ecophysiological and morphological traits. So differentiation is, in this case, identified as a special production trait. Moreover, as Coles and Wrigley (1976)

emphasised, most morphological features are affected by environmental conditions, and shrivelling and weathering may make comparison with typical samples meaningless.

When verification of cultivars is needed for commercial seed lots, rapid, accurate, trustworthy and reproducible tests are valuable. So much so, that emphasis is being placed on developing variety tests that are repeatable, inexpensive, simple and can be completed in a short time (Payne, 1993).

Some rapid chemical identification techniques reveal chemical differences among seeds or seedlings of different varieties by means of specific treatments. Most of these tests can be placed into one of three general categories. The first category consists of tests using ultra-violet light to reveal differences in seed or seedling fluorescence. In the second category of tests, phenol is employed to cause different colour reactions in seeds, e.g., phenol tests with modifications and ferrous sulphate tests. Tests in the third category use strong chemical acids or bases to bring about different colour changes in seeds. Fluorescence tests and phenol tests are most frequently used for varietal identification (ISTA, 1993).

The fluorescence test has been used for identifying species and cultivars of various Poaceae since Gentner (1929), cited by Payne (1993), reported that roots of *Lolium multiflorum* fluoresce, while roots of *Lolium perenne* are non-fluorescent when viewed on white filter paper under ultra-violet light.

The phenol test was used as a testing procedure for *Triticum aestivum, Oryza sativa*, *Poa pratensis*, *Avena sativa*, *Hordeum vulgare*, *X Triticosecale* and *Lolium* spp. as reported in ISTA (1993). Contrary to the variation that may occur in morphological characteristics, colour reaction is constant for each variety, so it can serve as a basis for grouping of varieties (Vanangamudi, Palanisamy and Natesan, 1988). A standardized phenol method has been developed for testing *Triticum aestivum* seed for varietal purity (Walls, 1965). Many attempts were made to enhance resolution capacity of the phenol colour reaction by using different metallic ions (Banerjee and Chandra, 1977). In these modified phenol tests, treatment with phenol was the same as for the original test; seed pericarp colour development was classified according to a colour chart.

The ferrous sulphate test was successfully applied by Kumar, Chowdhory, Kapoor and Dahiya (1995), who classified genotypes of *Pennisetum glaucum* (L.) R. Br., hybrids and their parental lines.

Accordingly with rapid tests, instrumental or biochemical procedures have been sought that would be objective, more discriminating, and less affected by growing conditions (Wrigley, Batey, Campbell and Skerritt, 1987). Electrophoretic analysis of seed protein composition is the most universally accepted method of this type and standard procedures have been adopted (Wrigley, Autran and Bushuk, 1982). The electrophoretic patterns of proteins extracts are not influenced by the origin of the seed, the use of fertilizers or growing conditions. Proteins may be regarded as identifiers analogous to fingerprints and proved to be a powerful tool to help characterize and identify varieties of pasture grasses (Gardiner and Forde, 1992).

The aim of this research was to characterise three cultivars of *Setaria italica* (L.) Beauv. Ñandú INTA, Carapé INTA and Yaguané INTA from morphological and biochemical traits of their seeds.

Materials and methods

Plant material
Samples of each of the three cultivars were obtained from INTA germplasm bank. Samples of breeder seed with high genetic intra-cultivar homogeneity were used. First multiplication classes of pure seeds were obtained in the same environment as the original ones. Each sample was homogenised, then the working samples were obtained by the spoon method (ISTA, 1996), and stored in separated envelopes until determinations were made.

a) Spoon method
After preliminary mixing, seeds were poured evenly on the tray without shaking. With the help of a spoon, small portions of seed were removed from not less than five random places on the tray (ISTA, 1996).

b) Morphological description, seeds weight and size
Subsamples of seeds (actually florets) were taken randomly from each cultivar and class. Measurements and observations on seed shape, length and height were made on four replications of 100 seeds for each class and cultivar. A stereoscopic magnifying lens (20x) was used. Measurements were recorded in mm.

Drawings of seeds were made from subsamples taken randomly from each cultivar and class (figure 1). From the working samples, eight replicates of 100 seeds each were counted randomly by hand. Applying ISTA (1996) procedures, the weight per 1000 seeds of the samples was determined for each cultivar.

c) Rapid chemical identification techniques
Testing procedures carried out were: the phenol test (ISTA, 1993), the modified phenol test (Kumar *et al.,* 1995), the sulphate ferrous test (Kumar *et al*., 1995) and the fluorescence test (ISTA, 1993).

Caryopses, instead of seeds, were used for colour tests. To obtain caryopses, seeds were processed with a coffee mill for 2 or 3 seconds, first; then they were rubbed off lemma and palea by hand over friction between forefinger and thumb. Finally each sample was placed into a blower in order to obtain clean caryopses free of glumes and glumeles.

c.1. Phenol test:
Four replications of 50 caryopses each, from each class and cultivar, were imbibed in distilled water for 18 h at room temperature (20°C). The seeds were then transferred to Petri dishes containing filter paper moistened with 3 ml of 1% (v/v) phenol solution, covered, and kept at room temperature. After 4, 8 and 24 h, pericarp colour was evaluated for staining. It was determined that 24 h exposure was necessary for colour stability. Degrees of staining were classified according to Munsell soil colour charts (1994).

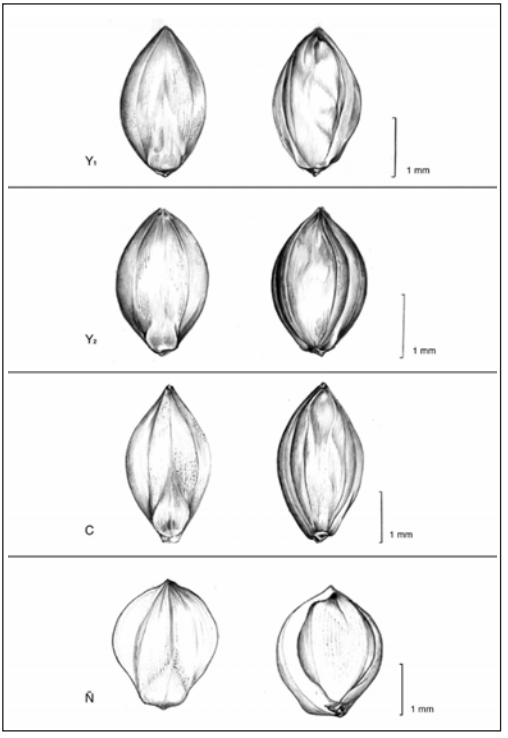

Figure 1. Seed drawings of the three cultivars: Y_1 and Y_2 (Yaguané), C (Carapé) and Ñ (Ñandú). Drawings on the left are abaxial views and on the right are adaxial views.

c.2. Modified phenol test:

For this test, a similar procedure as (*b.1.*) was applied, except in this case, caryopses were exposed to three different imbibitional treatments instead of being imbibed in distilled water. Three groups of four replications of 50 caryopses each**,** from each class and cultivar, were imbibed in: group a) 0.4% (v/v) copper sulphate ($CuSO_4$); group b) 0.6% (v/v) sodium carbonate (Na_2CO_3); and group c) 0.5% (v/v) sodium hydroxide (NaOH). Following incubation for 18 h at room temperature (20°C), the seeds were transferred to petri dishes containing filter paper moistened with 3 ml of 1 % (v/v) phenol solution, covered, and kept at room temperature. In this case and as in *b.1*, observations were carried out after 4, 8 and 24 h, the longest period being necessary for colour stability. Pericarp staining was classified according to Munsell soil colour charts (1994).

c.3. Ferrous sulphate test:

Four replications of 50 caryopses each were imbibed in 5 ml of 1% (w/v) $FeSO_4$ solution and kept in an incubator for 4 h at 35°C (Kumar *et al*., 1995). Following this period, pericarps were examined for staining and colours were classified the same as above, according to Munsell soil colour charts (1994).

c.4. Fluorescence test:
This technique was carried out using four different sample preparations (ISTA, 1996): (1) dry seeds, (2) seeds sprayed with 0.5 % (v/v) NH_4OH and observed immediately and after 1, 3 and 24 h (3) seedlings (constant 20°C temperature, in the dark) with nearly 3 cm root length, (4) and on seedlings as in (3) but sprayed with 0.5 % (v/v) NH_4OH and observed after 1, 3 and 24 h. In (3) and (4) seedling roots were examined after 14 days under germination conditions. White, non-fluorescent, filter paper was used in all situations.

Observations in all four cases were conducted under an UV lamp at 254 nm and 366 nm wavelength with the aim of observing fluorescence or non-fluorescence of caryopses or seedling roots.

d) Storage proteins on SDS-PAGE
Extractions were made from 40 mg of ground seeds of duplicated samples, milled in a cyclonic mill (1 mm–screen). The extraction solution was that of Gardiner and Forde (1992). Immediately before use, it was mixed with 2- mercaptoethanol, dimethylformamide and water in the ratio 3: 1.06: 1.76: 3, thus forming the working buffer. A vortex mixer was used to agitate 40 mg of ground caryopses in 0.4 ml of buffer solution. Homogenization was made with a Heidolph equipment. Prior to centrifugation at 7,300 g for 20 min, samples were re-vortexed and incubated for 10 min. in boiling water. Supernatants were immediately frozen or used immediately for electrophoresis.

Gels were prepared based on the SDS-PAGE discontinuous system of Laemmli (1970). A total acrylamide percentage (%T) 12.5% T was used for the resolving gel. The separation gel mixture contained: (12.5% T) acrylamide, 0.375 M Tris - HCl pH 8.8, 0.1% (w/v) SDS, 0.075% (w/v) APS and 0.05% (v/v) TEMED. The stacking gel mixture consisted of 3.7% T acrylamide, 0.1% SDS, 0.126 M Tris - HCl pH 6.8 (Hames, 1990).

Minislab vertical gels (0.75 mm thick, 7.3 × 10.2 cm, with 10 wells, 13 mm depth) were prepared. Aliquots of 4 µl of protein extract were loaded into each of the wells under electrode buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS (w/v), pH 8.3).

Two vertical minigels were run simultaneously with standards at constant voltage (200V). Electrophoresis run time was about 50 min.

Gels were stained with 0.1% (w/v) coomasie brilliant blue R-250; fixation, staining and destaining were performed as described by Krochko and Bewley (1988).
Aliquots of samples were run parallel to a well with a molecular weight marker mix, (Bio-Rad, USA), containing nine markers ranging from 200.0 to 6.5 kDa.

e - Data analysis
ANOVA and comparison of means test (Tukey; $p<0.05$) were carried out where applicable.

Results and discussion

Figure 1 shows drawings of seed adaxial and abaxial views for each of the cultivars. In all Yaguané cultivar samples, two morphologically different types of seeds were observed.

Besides morphological differences among cultivars, as shown in figure 1, numeric differences were observed also in length and width of seed among the three cultivars (table 1). No differences were detected between breeder´s seed and next generation or first multiplication seed, values presented are discriminatory only by cultivars. Average values for seed width, expressed in mm, showed no significant differences among cultivars. On the other hand, when seed length of each cultivar was analysed, differences were significant. Carapé presented the longest seeds and Ñandú the shortest, while Yaguané was intermediate and distinct from the other two.

Concerning seed weight, significant differences were detected for each variety on 1000 florets weight. Carapé can be separated from Ñandú and Yaguané based on 1000 seed weight (table 2). There was no significant difference between the other two varieties for 1000 seed weight.

The application of the original phenol test showed different degrees of staining, according to the cultivar. The average percentage of stained caryopses was different across each cultivar, but not across classes within a cultivar. Since differences were not detected among classes, only data discriminatory by cultivars are presented.

The modified phenol test (Banerjee *et al*., 1977) was used to increase the resolution of the original phenol technique by means of establishing a wider range of colours, as has been observed in other species. However, for these cultivars, only when NaOH was used for pre-soaking, were wider colours obtained. The other two modified methods produced similar results to the original phenol test (table).

Table 1. Seed sizes, discriminated only for cultivar, measured on seed units. Means followed by different letters are statistically different (Tukey, p>0.05).

Cultivars	Seeds sizes of Carapé, Yaguané and Ñandú cultivars							
	Width (mm)				Long. (mm)			
	x±SD		min.	max.	x±SD		min.	max.
Carapé	1.70 ± 0.218	a	1.50	2.00	2.97 ± 0.087	a	2.80	3.10
Yaguané	1.64 ± 0.198	a	1.50	2.00	2.51 ± 0.283	b	2.00	3.00
Ñandú	1.58 ± 0.253	a	1.10	2.00	2.00 ± 0.022	c	2.00	2.10

Table 2. Seed weight per 1000 seeds (g) for each cultivar, without class discrimination. Means followed by different letters are statistically different (Tukey, p>0.05).

Cultivars	Weight of 1000 caryopses (gr)	
Carapé	2.42 ± 0.035	a
Yaguané	2.47 ± 0.087	a
Ñandú	2.65 ± 0.190	b

Table 3. Matching of results from application of the original phenol test, modified phenol test and ferrous sulphate test. Indications: min. and max. correspond to minimum and maximum staining of caryopses according to reference numbers of Munsell Soil Colour Chart. Colours

Cultivars	Tonalities for original phenol test		Tonalities for modified phenol test						Tonalities for $FeSO_4$ test	
			Cu^{+2}		NaOH		Na_2CO_3			
	min.	max.	min.	max.	min.	max.	min.	max.	min.	max.
Carapé	7.5 YR 5/4 (brown)	10 R 2,5/1 (reddish black)	2,5 Y 3/2 (very dark grayish brown)	10 YR 2/1 (black)	5 YR 7/3 (pink)	2.5 YR 2.5/2 (very dusky red)	10 YR 3/6 (dark yellowish brown)	10 YR 2/1 (black)	2.5 Y 7/4 (pale yellow)	2.5 Y 4/3 (olive brown)
Yaguané	2.5 YR 3/3 (dark reddish brown)	10 R 2.5/1 (reddish black)	5 Y 6/6 (olive yellow)	10 YR 2/1 (black)	10 YR 7/4 (very pale brown)	10 YR 5/4 (yellowish brown)	10 YR 3/6 (dark yellowish brown)	10 YR 2/1 (black)	2.5 Y 7/6 (yellow)	5 Y 6/3 (pale olive)
Ñandú	2.5 Y 6/4 (light yellowish brown)	10 YR 4/4 (dark yellowish brown)	25 Y 6/4 (light yellowish brown)	10 YR 4/4 (dark yellowish brown)	2.5 Y 8/6 (yellow)	10 YR 5/6 (yellowish brown)	2.5 Y 6/4 (light yellowish brown)	10 YR 4/6 (dark yellowish brown)	2.5 Y 6/4 (light yellowish brown)	10 YR 3/3 (dark brown)

When the ferrous sulphate test was performed, three categories were distinguished with regard to final caryopses staining colours, similar to the original phenol test. The percentages of stained caryopses differed between the tests (table 4).

None of the three cultivars showed fluorescence, for any of the four sample preparations, when observed under U.V. light.

Table 4. Tinction percentages of caryopses for original phenol and ferrous sulphate test. Means followed by different letters are statistically different (Tukey, p>0.05).

Cultivar	Percentage of stained caryopses original phenol test	Percentage of stained caryopses ferrous sulphate test
Carapé	99.63 ± 0.744 a	94.00 ± 1.633 a
Yaguané	42.15 ± 3.182 b	66.15 ± 2.700 c
Ñandú	1.25 ± 1.280 c	73.70 ± 2.656 b

All three cultivars presented a similar banding profile for SDS-PAGE. Evaluation of protein profiles was visual, scoring only differences in position, thickness and presence or absence of bands. Each of the samples gave a polypeptide profile of 10 main bands (figure 2), 0: sample loading point, 9: tracking dye. Molecular weight standards were simultaneously run with caryopses extracts. Comparison among patterns of MW standards and samples revealed that bands 1 to 8 corresponded to polypeptides of an approximate molecular weight range from 66.2 to 6.5 kDa. In this scheme, bands 3 and 4 established differentiation of Carapé from Ñandú and Yaguané. Although all cultivars presented both bands, bands 3 and 4 in Carapé were in a slightly different location than in Ñandú and Yaguané, giving a definite distinction. These bands were located between 31.0 and 21.5 kDa. These results are in accordance to Afzal, Kawase, Nakayama and Okuno (1996), which established that variation in electrophoregrams of total seed proteins was observed in the range from 30 to 20 kDa.

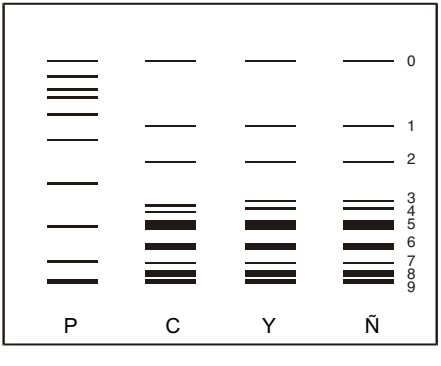

Figure 2. Scheme of SDS-PAGE of: MW standards (P), Carapé cv. (C), Yaguané cv. (Y) and Ñandú cv. (Ñ.).

Conclusions

This study provides evidence that morphological differences in shape, size and weight among caryopses of the cultivars studied existed.

The phenol test, a quick and simple tool, allowed cultivar identification by means of colour percentages. Modified phenol test results, showed that degree of staining depended on the metallic ion and on its accompanying anion. This test, when using NaOH as an imbibing agent, constituted a complementary technique. The ferrous sulphate test also proved useful and provided enhanced resolution.

Differentiation among the three cultivars was not possible by the fluorescence test. Electrophoregrams of total seed proteins allowed differentiation of Carapé from the other two cultivars based on differences in thickness and location of two main bands. No differences between Ñandú and Yaguané could be detected by this methodology. Considering that foxtail millet is a highly autogamous species (de Wet, 1995), it is reasonable to assume that genetic heterogeneity between samples of the same cultivar is not an issue. Consequently, results from this research might be extended to seeds corresponding to the three studied cultivars.

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