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Characterization of phenotypic and nutritional properties of valuable *Amaranthus cruentus* L. mutants

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Abstract: Two selected mutant lines, C26 and C82, with highly significant 1000-seed weight and generated through irradiation treatment of *Amaranthus cruentus* L. 'Ficha', were evaluated and compared to an original nontreated control and reference variety Aztec. The phenotypic traits and biochemical properties of the amaranth seeds were studied. Comparable values for crude protein content, albumins, and globulins fractions and overall coefficients of nutritional quality were found in the evaluated samples. However, C26 showed significantly lower concentrations of celiac-related prolamins and glutelins in comparison with the control sample. Both of the mutagenesis-derived lines showed consistently superior performance of 1000-seed weight across two tested environments during a multiyear evaluation. C82 showed seed weight advantage over control seeds of Ficha and reference variety Aztec and it was released as a new amaranth variety named Pribina in 2013.

Key words: Seed quality, morphological characteristics, mutation breeding, other grain

1. Introduction

Plants, and particularly the horticulture section, are used by people for food, either as edible products or for culinary ingredients, and for medicinal use or ornamental and aesthetic purposes. They are genetically a very diverse group and play a major role in modern society end economy. Fruits and vegetables are important components of traditional food, but are also central to healthy diets of modern urban populations (Bajpai et al., 2014; Kaczmarska et al., 2015; Mlcek et al., 2015; Wojnicka-Poltorak et al., 2015).

Pseudocereals like amaranth (*Amaranthus* spp.) can markedly contribute to the promotion of environmental sustainability, to agrobiodiversity enhancement, to global food production, and to the preparation of healthy foods and food additives.

The genus *Amaranthus*, native to different parts of North, Central, and South America, includes wild (common weed), grain, and ornamental species, mostly with monoecious inflorescences bearing both male and female flowers (Trucco and Tranel, 2011). Some of the grain amaranth species, particularly *A. cruentus* L., *A. hypochondriacus* L., and *A. caudatus* L., are good candidates for plant breeding trials. These species are diploids with chromosome number $2n = 32$, but occasionally it can be 34 (National Research Council, 1989; Chan et al., 1997; Bonasora et al., 2013). Greizerstein and Poggio (1992) proposed that some species with $2n = 32$ are polyploids (basic number $x = 8$) and the chromosome number is $n = 17$. Concerning the allogamy degree, this can vary from 5% up to 30% in individual plants (Hauptli, 1986). It has been reported that *A. cruentus* should hybridize relatively easily (Lanta et al., 2003).

A nutty-flavored grain, amaranth has an attractive chemical composition and very promising nutritional potential when compared to other grains, whether cereals or food legumes, with high impact on human health. Thus, amaranth is a particularly important crop for developing countries (Johns and Eyzaguirre, 2007; Muyonga et al., 2008; Alemayehu et al., 2014). It is characterized by a high protein content of 12.5%–18% (versus ~10% in commercial cereals) with a well-balanced amino acid composition and high lysine and methionine contents (Pospišil et al., 2006; Capriles et al., 2008; Caselato-Suosa and Amaya-Farfán, 2012). The nutritional quality of amaranth proteins resides not only in its amino acid composition but also in

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its digestibility, which is higher than that of cereals and close to that of milk casein (Bejosano and Corke, 1998; Guzmán-Maldonado and Paredes-López, 1999; Repo-Carrasco et al., 2003).

Various gluten-free food products have been developed from amaranth grain in order to supply food for people suffering from celiac disease (Alvarez-Jubete et al., 2010; Ballabio et al., 2011). Celiac disease (gluten enteropathy) is a food intolerance syndrome related to gluten, a complex mixture of proteins called prolamins, contained in wheat, barley, oats, and rye. Because of the lack or extremely low content of these toxic seed proteins in pseudocereals, they are considered to be good substitutes to common cereal-derived products (Saturni et al., 2010; Comino et al., 2013).

Amaranth lipids have a rich spectrum of fatty acids, of which linoleic acid is the most important (Berganza et al., 2003;Ješko and Čertík, 2008). Squalene, which is a cholesterol precursor, may constitute up to 8% of amaranth oil (Lyon and Becker, 1987; Ayorinde et al., 1989; Becker, 1989; Berganza et al., 2003).

It has been reported that amaranth leaves, sprouts, and seeds contain flavonoids (e.g., rutin, isoquercitrin, nicotiflorin, vitexin, isovitexin, morin) and phenolic acids (gallic acid, vanillic acid, syringic and ferulic acids) with relatively high antioxidant statuses (Gorinstein et al., 2007; Barba de la Rosa et al., 2008; Paśko et al., 2008; Chlopicka et al., 2012).

Breeding work on grain amaranth is just at the beginning and shows the necessity of further research for drought resistance, grain maturation, and yield improvement (Brenner et al., 2000; Gimplinger et al., 2007). The breeding of new amaranth cultivars has just begun in Europe in the United Kingdom, the Netherlands, Germany, Austria, Denmark, Italy, the Czech Republic, and Poland.

The aim of our research program is to improve the quality and quantity of amaranth production through mutation breeding by γ-radiation. In this context, our research involves the selection of distinct and improved mutants as potential candidates for new varieties. After evaluation of 12 generations of previously induced mutants, two mutant lines, C26 and C82, were selected based on a long-term increased 1000-seed weight over the nonirradiated control form. Here we describe some of our results that preceded the application for DUS testing of the C82 mutant, recently registered as the first Slovak amaranth variety named Pribina.

2. Materials and methods

2.1. Plant material and experimental field

Seeds of *Amaranthus cruentus* L. 'Ficha', found in Peru, were previously treated by a dose of 175 Gy in the Joint FAO/IAEA Programme Agency's laboratories in Seibersdorf, Austria. The seed sample was obtained from the collection of GenBank from the Crop Research Institute Praha-Ruzyně, Czech Republic. Single M_1–M_{11} plants of two mutant lines, C26 and C82, were collected for 1000-seed weight evaluation and for establishment of following generations, and M_{12}–M_{15} seeds were harvested and evaluated in bulk (Figures 1a and 1b).

Field experiments were conducted at two localities: Nitra (290 m above sea level) and Prešov (253 m above sea level), with mean annual precipitation of 600 and 630 mm and mean annual temperature of 9.5 and 8.6 °C for Nitra and Prešov, respectively (Figure 1c). The experimental design was a randomized complete block in a split plot arrangement with 4 replications. The plots had size 2.0 m × 1.5 m, by flat 2.5 m^2 (for 1 experimental variant). Sowing was carried manually at the beginning of May and plants were manually harvested at the end of September each year. The panicles were cut and naturally dried and the seeds were hand-threshed.

2.2. DUS test

On the basis of our results we selected mutant line C82, with long-term significantly increased 1000-seed weight over the nonirradiated control sample, with an obvious tendency of genetic fixation, in order to register it as a new variety. The candidate variety is usually compared to a similar variety of the reference collection (variety of common knowledge, hereinafter referred to as "reference variety") provided by an authorized test center. Comparison was performed for the purpose of determination of distinctness in quantitative and qualitative characteristics (DUS test). Apart from distinctness, the uniformity and stability of the potential new variety were evaluated. Our candidate variety, mutant C82, was compared for distinctness to similar grain variety Aztec, suitable for middle-European cultivation. DUS testing was carried out at an approved centre, the Central Controlling and Testing Institute in Agriculture in Nové Zámky (122 m above sea level), a locality with mean annual precipitation of 600 mm and mean annual temperature of 9.7 °C. The tests were conducted according to UPOV Guidelines TG/247/1 (http://www.upov.int/en/publications/tg-rom/tg001/tg_1_3.pdf) over a standard 2-year period (2012–2013).

2.3. Protein analysis

The protein analyses of seeds in mutant lines were performed during the years 2006–2010 (M_7–M_{11} generations). The total nitrogen content was determined according to a modified Kjeldahl method using a Velp Scientifica system (DK 6 heating digester and UDK 127 basic distillation unit; Velp Scientifica, Italy) followed by titration with H_2SO_4. The fractional composition of protein was performed using the Golenkov method (Michalík, 2002). The nitrogen content in each fraction was determined by the micro-Kjeldahl method. The content of crude protein from total nitrogen

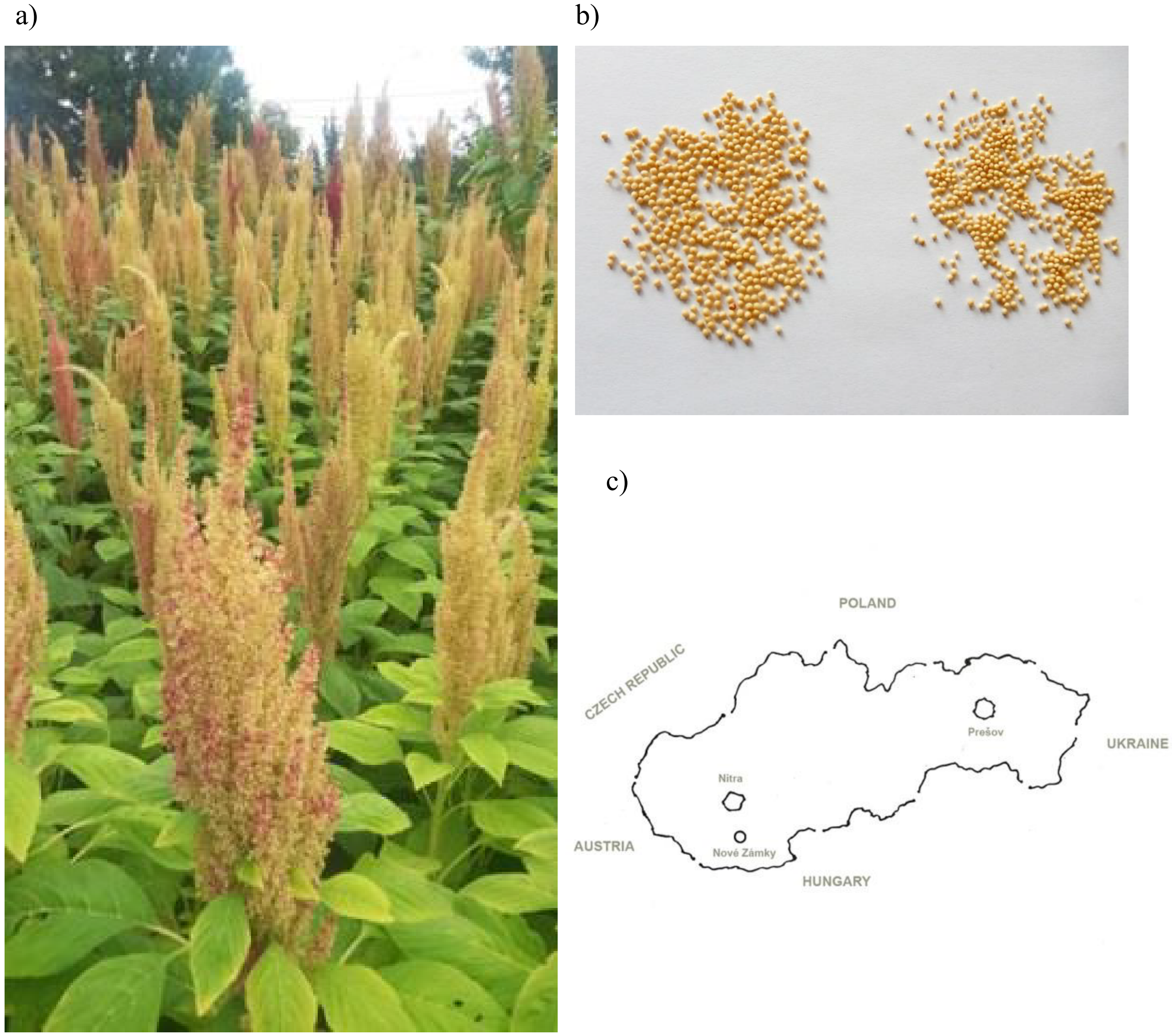

Figure 1. The phenotype of amaranth mutagenesis-derived line C82 (a) exhibiting alternation in seed weight and size (b, left) when compared to commercially accessible whole grain (b, right). Location of the field experiments and DUS tests (c).

was calculated by a conversion rate (% N × 5.7). On the basis of the protein fractions the coefficient of nutritional quality was calculated by the following formula: [(albumins + globulins + rest) / prolamins)] × 100.

2.4. Statistical analysis

Statistical analysis of the obtained data was carried out by using Statistica 10 software (StatSoft Inc., Tulsa, OK, USA). Multifactor analysis of variance (ANOVA) was performed in order to find statistical differences among the means.

3. Results

3.1. Evaluation of phenotypic traits and 1000-seed weight

A 12-year evaluation of the irradiation effect on amaranth seed weight in the offspring of selected mutagenesis-derived plants documented a long-term significant increase of this principal seed trait that remained unchanged through the process of propagation. These findings led us to apply for registration of the mutagenesis-most influenced mutant line C82 as a new variety, with the significantly highest 100-seed weight among all obtained mutants. The variety is formally released with distinctive or clearly distinguishable one or more important traits documented as uniform and stable when evaluated over multiple locations and years (UPOV Guidelines TG/1/3). Therefore, we investigated the uniformity and stability of previously evaluated yield parameters in two independent locations, Nitra and Prešov, during the 2011–2014 growing seasons.

Generally, the lowest seed weight was recorded for reference variety Aztec grown in Prešov (0.75 g) in 2011, and during this year the highest value of this trait was assessed in candidate variety C82 (0.99 g) grown at the Nitra locality (Table 1). The average 1000-seed weight at Nitra was 0.78 g, 0.85 g, 0.88 g, and 0.97 g for Aztec, Ficha, C26, and C82, respectively. In a similar way, the highest values of seed weight evaluated in Prešov were recorded for mutants C82 and C26 (0.95 and 0.91 g, respectively), whereas Ficha and Aztec showed significantly lower 1000-seed weights (0.86 and 0.76 g, respectively).

Among the four amaranth variants tested during four cropping seasons, mutant C26 was found to be the most stable (Figure 2). The second tested potential breeding line, C82, exhibited the most significant differences over all tested amaranth samples, showing a highest value of 1000-seed weight in 2011. Reference variety Aztec showed significantly lowest seed weight among all samples (Table 1), though exhibiting a slightly higher value in 2014, but still very low compared to the mutants. Similarly, the nonirradiated control Ficha exhibited low values of 1000-seed weight with lowest values at both study sites in 2014.

Table 1. Thousand-seed weight performance of mutagenesis-treated samples and controls tested under two field conditions during the 2011–2014 growing seasons.

Amaranth sample	2011		2012		2013		2014	
	Nitra	Prešov	Nitra	Prešov	Nitra	Prešov	Nitra	Prešov
Aztec	0.78*	0.75*	0.78*	0.77*	0.76*	0.74*	0.78*	0.80*
Ficha	0.85**	0.85**	0.85**	0.89**	0.86**	0.87**	0.84**	0.83**
C26	0.87**	0.90***	0.88***	0.90**	0.89**	0.93***	0.89***	0.91***
C82	0.99***	0.98****	0.97****	0.92**	0.97***	0.94***	0.94****	0.94****

*Significantly different within each column by Tukey test at the 0.01 probability level.

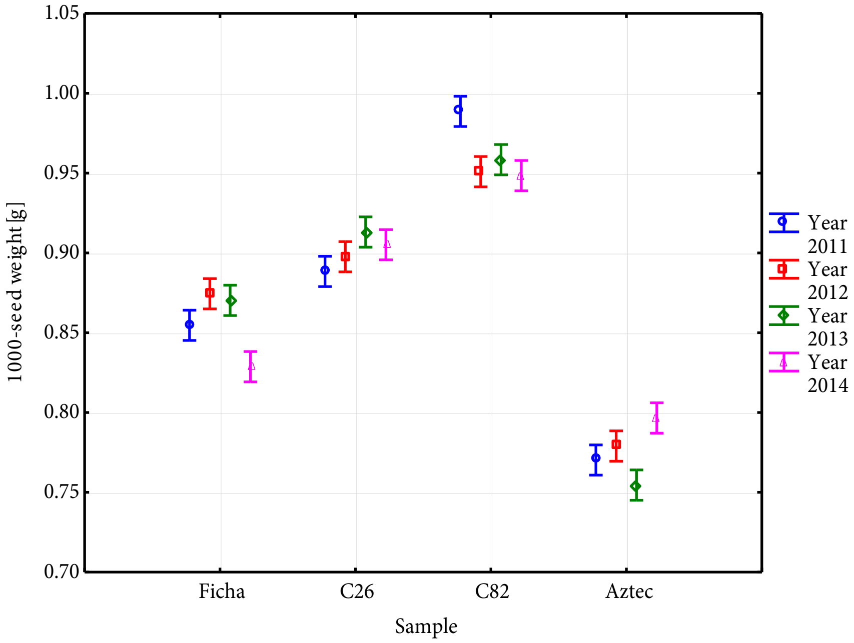

Figure 2. The comparison of stability performance over four growing seasons for 1000-seed weight in amaranth mutant lines C26 and C82 compared to nontreated Ficha and Aztec samples, grown in two different environments. Individual columns represent 95% confidence intervals.

As indicated in Figure 2, Ficha showed similar behaviors in evaluated yield traits as did the mutant line C26, but the values did not reach those of the mutant. As regards the DUS tests conducted during 2012 and 2013, candidate variety C82 showed stability in both environments, whereas the reference variety exhibited statistically lower 1000-seed weight in 2013. This difference could be due to the geographical distance between Nitra and Prešov.

The 1000-seed weights for all experimental groups are recorded in Table 2. Multiple range analysis showed statistically highly significant differences among all tested amaranth seed samples. The potential breeding materials, C26 and C82, had a considerably higher 1000-seed weight (0.90 and 0.96 g, respectively) than the reference variety Aztec (0.78 g) and the nonirradiated control seeds of Ficha (0.86 g). It is evident from Figure 3 that the effect of localities was absent, but genotype × year interaction was observed, especially in the growing season of 2014 (Table 1). Evaluated materials tended to behave differently also at the Prešov locality in 2011 and in Nitra during in 2012.

In parallel, a 2-year DUS trial was carried out by the Central Controlling and Testing Institute in Agriculture in Nové Zámky (2012–2013). Out of the 14 tested traits according to UPOV Guidelines TG/247/1 [betacyanin coloration of cotyledon (1), hypocotyl (2), and petiole (3); presence (4) and shape (5) of blotch on leaf blade; color (6) and type (7) of inflorescence; length of bract relative to utricle (8); inflorescence growth habit (9); betacyanin coloration of stem base (10); shape of stem in cross-section (11); seed color (12); seed shape (13); and 1000-seed weight (14)], they observed differences between candidate variety C82 and reference variety Aztec in 7 characteristics (Table 3). Two of these characteristics (marked with asterisks) are important for the international harmonization of variety descriptions.

Thus, mutant C82 was sufficiently different in the expression of tested characteristics from reference variety Aztec. Successful completion of the DUS trial in two consecutive cropping seasons led to its registration as the first amaranth variety bred in Slovakia, registered in the

Table 2. Multiple range analysis of 1000-seed weight in four amaranth samples tested across two environments during the 2011–2014 growing seasons.

Sample	1000-seed weight (g)
Aztec (reference variety)	0.78*
Ficha (original nonirradiated control sample)	0.86**
Mutant C26	0.90***
Mutant C82	0.96****

*Significantly different by Tukey test at the 0.01 probability level.

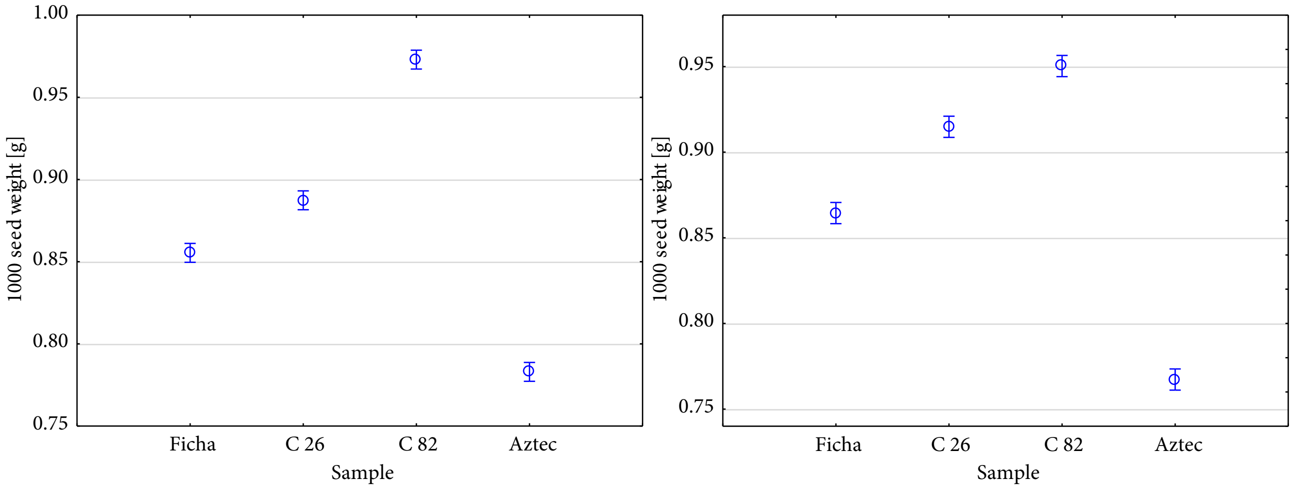

Figure 3. The differences between four evaluated amaranth samples in 1000-seed weight over two environments, Nitra (left) and Prešov (right), during four consecutive growing seasons. Individual columns represent 95% confidence intervals.

Table 3. Differences of phenotypic traits between reference variety Aztec and candidate variety C82.

Characteristic in which the similar cultivar is different	State of expression	
	Reference cultivar Aztec	Candidate variety C82
Young leaf: Distribution of secondary color on upper side	2/central blotch	1/colored basal area
Young leaf: Color on the lower side	3/purple	1/green
Leaf blade: Main color	2/medium green	1/light green
Inflorescence: Color	5/purple	2/green
Inflorescence: Density of glomerules	5/dense	7/medium
Inflorescence: Length of bract relative to utricle	3/long	1/shorter
Seed: Weight of 1000 seeds	3/low	7/high

State Plant Variety Book as Pribina. Currently, as a breeder of a new variety, we have applied for a grant of protection for Pribina to obtain property rights protection.

3.2. Protein analyses

The crude protein content, expressed as percentage from total dry matter of seed samples examined in this study, is given in Table 4. The highest protein content was determined in control seeds of Ficha. However, mutant C82 showed also favorable values, although with no significant difference in comparison with the control.

The content of albumins and globulins in amaranth seed samples is indicated in Table 5. We found similar mean values (53.94% and 54.17%) for albumin and globulin fractions in the seeds of evaluated mutants without statistically significant differences. The significantly highest value (55.25%) of these protein fractions was detected in the nonirradiated Ficha seed sample.

The investigation of prolamins and glutelins revealed that mutation-derived line C26 had the significantly lowest concentration (26.67%) of these storage proteins, followed by mutant line C82 (27.80%). The results showed significant differences in contents of prolamins and glutelins between all tested samples (Table 5). The prolamins, alkali-soluble proteins with imbalanced amino acid composition and low content of essential amino acids, represented only 3.0% in our mutants (data not shown).

Table 4. Crude proteins.

Amaranth sample	Means (%)	Confidence
Ficha	13.66**	12.58–14.74
C26	12.92*	11.91–13.93
C82	13.30**	12.68–14.06

*Significantly different by Tukey test at the 0.01 probability level.

Table 5. The protein fraction composition.

Amaranth sample	Means (%)	Albumins + globulins, confidence interval −95% +95%	Means (%)	Prolamins + glutelins, confidence interval −95% +95%
Ficha	55.25**	52.01–58.50	28.92***	27.55–30.30
C26	53.94*	51.71–56.18	26.67*	24.77–28.58
C82	54.17*	53.49–54.87	.80**	26.45–29.14

*, **, ***: Significantly different by Tukey test at the 0.05, 0.01, and 0.001 probability levels.

The seeds of our mutation-derived amaranth lines do not exceeded the limit stated by WHO/FAO standards (20 mg prolamins (gliadins)/100 g of sample dry matter (Codex Alimentarius Commission, 2007) for gluten-free food and they can be used in special dietary uses for persons intolerant to gluten.

Our results on the overall nutritional quality of the evaluated amaranth seed samples showed that candidate variety C82 exhibits the highest coefficient of nutritional quality, but this difference was not significant compared with the control and line C26 (Table 6). Thus, the mutation-derived amaranth lines have comparable nutritional value to nontreated seeds of Ficha.

4. Discussion

Most of the currently used amaranth species were created by selection on the desired traits from an available germplasm (Weber, 1987; Svirskis, 2003). In these cases, the existing natural variability within species was the basis for a conventional selection process that requires the screening of relatively large populations. In our previous experiment, we used γ-radiation for the development of valuable breeding plant material followed by selection for desired traits (Gajdošová et al., 2007).

According to the UPOV Guidelines, a variety can finally be examined for fulfillment of the DUS criteria (distinctness, uniformity, and stability) required for protection only after a variety has been clearly defined (UPOV Guidelines TG/1/3). A variety is defined by its characteristics resulting from a given genotype or combination of genotypes and can be distinguished from any other varieties by the expression of at least one of the said characteristics. As a result of the characterization of potential new varieties represented by two mutant lines, C26 and C82, considerable differences in seed weight have been found when compared to nonirradiated control accession Ficha. This positively changing yield trait was also demonstrated by bigger seeds (Figure 1b). Moreover, differences in another six phenotypic characteristics were observed in mutant line C82 when compared to the reference variety Aztec, which is satisfactory distinctness regarding the DUS criteria.

Thousand-seed weight is one of the parameters recommended to be tested by the UPOV Guidelines and the most important physical quality trait of amaranth seeds (Gimplinger et al., 2007). Seed size as a major factor controlling seed quantity was also described by Kesavan et al. (2013). They discussed the different factors influencing seed size in cereal crops and *Arabidopsis* and concluded that although several genes are known to be involved in the control of seed size, their interactions and functions are undetermined. Large seeds have some advantages over smaller ones. They improve seedling vigor, and they are favorable for mechanical sowing and grain processing like milling and popping.

Variability of quantitative traits is conditioned by both genetic and environmental factors. The seed/grain yield can be a cultivar property, but it considerably depends on growing conditions, cultivation system, and agricultural practice (Vujacic et al., 2014).

The average of the studied yield parameter across our tested amaranths ranged from lowest at 0.78 g in Aztec to the highest at 0.96 g in candidate variety C82 (Table 1), later registered as new variety Pribina. The mean 1000-seed weight across two environments was 0.87 g reported for both localities. The results revealed that the mutant line C82 possessed ideal characteristics of a stable genotype for an important evaluated yield trait.

Several studies examined this trait in *A. cruentus* and *A. hypochondriacus* under the growing conditions of Central Europe (Jamriška, 1996; Kaul et al., 1996; Fecková et al., 2003; Gimplinger et al., 2007; Vujacic et al., 2014), revealing similar values of seed weight as those that we report in this study for control sample Ficha and reference variety Aztec.

Genotype × environment interactions have been defined as the failure of genotypes to achieve the same relative performance in different environments (Baker, 1988). Statistical analysis of our data did not confirm that average 1000-seed weight was significantly influenced by location, indicating consistent performance when exposed to different environments. A similar response was observed by Varalakshmi and Pratap (2002), García-Pereyra et al. (2011), Kumar and Yassin (2012), and Vujacic et al. (2014)

Table 6. Coefficient of nutritional quality.

Amaranth sample	Means	Confidence interval –95% +95%
Ficha	2018.86*	1633.38–2404.34
C26	2147.12*	1776.80–2517.44
C82	2374.17*	1971.85–2776.48

*Significantly different by Tukey test at the 0.01 probability level.

in testing the stability performance of the grain yield and responsivity of different amaranth species over diverse environments.

The nutritional value of pseudocereals is mainly connected to their proteins, which are generally higher than in common cereals and rich in essential amino acids with high importance for the food and pharmaceutical industries (Bressani, 1994; Gorinstein et al., 2002). The protein content of various *Amaranthus* spp. seeds ranges according to different authors from 11% to 18% (Bressani et al., 1987; Oleszek et al., 1999; Gorinstein et al., 2001; Palenčárová and Gálová, 2009). Studies on crude proteins in *A. cruentus* L. reported about 16% (Mendoza and Bressani, 1987; Bressani and García-Vela, 1990; Gimplinger et al., 2007). The seeds of eight amaranth groups (*A. cruentus* and *A. hypochondriacus*) grown in Hungary and Austria were investigated and, as a result, the difference between the lowest (14.23%) and highest (17.40%) protein content was relatively large, suggesting that breeding might be a potential tool to increase the protein level (Tömösközi et al., 2009).

The 5-year protein analyses of our amaranth seed samples showed that mean protein contents ranged between 12.92% and 13.66%. We observed a significant decreasing of crude protein content in mutation line C26 (12.92%), probably as a consequence of increase in seed size. However, higher seed weight had no negative influence on seed protein content in mutant C82, where the protein content reached 13.37% and was comparable with the control sample (13.66%).

Amaranth grain is a highly nutritional and well-balanced food providing multiple medicinal benefits (Alvarez-Jubete et al., 2010; Caselato-Sousa and Amaya-Farfán, 2012). Its proteins contain mainly globulins and albumins, and very little or no storage prolamin proteins, which are abundant storage proteins in cereals and cause celiac disease (Gorinstein et al., 2002; Gálová et al., 2012). The fractional composition of amaranth proteins indicates their high digestibility and the bioavailability of presumably easily digestible albumins and globulins (about 50%) with counterbalanced amino acid composition (Barba de la Rosa et al., 1992, 2009; Bressani, 1994; Zheleznov et al., 1997; Gamel et al., 2004). Our results confirmed the favorable content of albumins and globulins (53.94%–55.25%) and lower content of prolamins and glutelins (26.67%–28.92%) in the screened amaranth seeds. Similarly, Palenčárová and Gálová (2009) detected the average contents of albumins and globulins in a collection of various amaranth genotypes in the range of 47.83%–59.83%, prolamins of 2%–3.99%, and glutelins of 22.67%–29.62%. While no significant differences were found in the overall coefficient of nutritional quality among our analyzed samples, mutation line C26 differs in the content of prolamins and glutelins, which is significantly lower in comparison to control seeds. Moreover, we found that prolamin content in our mutation-derived breeding material represented only 3.0% (data not shown), whereas in wheat prolamins constitute about 37.4% and in barley about 32.7% of total seed proteins (Gálová et al., 2012). The prolamin fraction plays a decisive role in the diet of people with gluten intolerance because it contains celiacally active protein components. Therefore, the low prolamin contents in our mutant lines fulfill the WHO/FAO standards.

The mutants of several crops have been generated through radiation mutagenesis in the last decade (Encheva et al., 2003; Adekola et al., 2007; Tabosa et al., 2007; Chen et al., 2008; Fu et al., 2008;; Gómez-Pando et al., 2013), including amaranth (Gómez-Pando et al., 2009). Changes were registered for branch number, pedicel length, plant height, life cycle duration, leaf morphology, stem, foliage and seed color, seeds per head, seed size, seed yield, 1000-seed weight, tolerance to pathogens and soil salinity, and chemical composition such as protein and oil content. In addition, some chlorophyll and anthocyanin mutations were reported. This is an indication of the possibility to improve many quantity and quality traits using radiation-induced mutations.

Herein, we present the characterization of the breeding lines C26 and C82 previously generated by radiation mutagenesis. Multiyear phenotypic characterization of an important yield parameter (1000-seed weight) was performed in two fields. Both investigated mutants showed seed size advantage over nonirradiated control seeds of Ficha, as well as reference variety Aztec, with predictable performance of this yield trait. In addition, mutant line C82 was registered as a new amaranth variety, Pribina, after successful completion of a DUS trial.

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