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SIGNIFICANTLY LOWER CONTENT OF ANTINUTRITIONAL SOLUBLE OXALATE IN AMARANTH MUTANT LINES DEVELOPED BY RADIATION MUTAGENESIS

Andrea Hricová[1], *Jana Žiarovská*[2], *Milan Suhaj*[3], *Veronika Lancíková*[*1]

Address(es):
[1] Institute of Plant Genetics and Biotechnology, Plant Science and Biodiversity Center, Slovak Academy of Sciences, Department of Reproduction and Developmental Biology, Akademická 2, 950 07 Nitra, Slovak Republic.
[2] Slovak University of Agriculture in Nitra, Faculty of Agribiology and Food Resources, Department of Genetics and Plant Breeding, Slovak University of Agriculture in Nitra, Trieda A. Hlinku 2, 949 76 Nitra, Slovak Republic.
[3] Food Research Institute, Priemyselná 4, 824 75 Bratislava, Slovak Republic.

*Corresponding author: veronika.lancikova@savba.sk

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ABSTRACT

Soluble oxalate with potentially dietary injurious implications for human health were determined in amaranth (*Amaranthus* spp.) gamma-irradiation induced mutant lines by capillary isotachophoresis and compared to their non-irradiated reference samples 'Ficha' (*Amaranthus cruentus* L.) and interspecific hybrid 'K-433' (*Amaranthus hypochondriacus* × *Amaranthus hybridus*) during the cultivation period 2011–2014. The canonical discriminant analysis demonstrated significant differences among mutants and their reference counterparts during multiyear evaluation. Two-way ANOVA approach identified the mutant line D282 as variant with the significantly and long-term lowest soluble oxalate concentration in comparison to respective reference samples as well as other mutant lines. Decrease in a content of this antinutritional factor could be a possible effect of radiation-induced mutation event(s) in the D282 line genome. Therefore, this mutant line might be a good matrix in the further breeding programme of this naturally gluten-free pseudocereal.

Keywords: amaranth, soluble oxalate, nutrition, multivariate analysis

INTRODUCTION

Oxalic acid is a common and widespread constituent of plants, being found mostly at low levels in almost all plant families. It occurs in the form as the free acid, as soluble salts of potassium and sodium, and as insoluble salts of calcium, magnesium and iron **(Noonan and Savage, 1999).** The presence of soluble oxalate in foods may have important dietary implications **(Trevaskis and Trenerry, 1997)**. A high dietary soluble oxalate intake influences mineral and trace element absorption in humans because it binds with $Ca2^{+}$, $Fe2^{+}$ and $Mg2^{+}$ rendering these minerals unavailable. The insoluble calcium oxalate, however, is physiologically inert, its calcium is not ordinarily available and the oxalate remains unabsorbed **(Shirley and Schmidt-Nielsen, 1967; Siener *et al*, 2006)**. The review published by **Noonan and Savage (1999)** highlighted the lack of data available on the oxalate content of common foods. Oxalic acid accumulates in plants especially during dry conditions **(Bressani, 2003)**. In general, oxalate content is highest in the leaves, intermediate in the seeds and lowest in the stems **(Cheeke and Bronson, 1980; Yadav and Sehgal, 2003)**. The mean daily intake of oxalate in English diets has been calculated to be 70–150 mg **(Noonan and Savage, 1999)**.

Soluble (oxalic acid and soluble salts) and insoluble oxalate (predominantly the calcium salt) can be extracted from foods, using hot water to extract soluble oxalate, and dilute acids to extract total oxalate, which includes the insoluble oxalate fraction **(Hodgkinson, 1997; Holloway, 1989)**. Oxalic acid is routinely determined by extraction and precipitation of calcium oxalate by **AOAC** methods **(1980)** or by titrimetric, spectrophotometric, high performance liquid chromatography (HPLC) or enzymatic methods **(Trevaskis and Trenerry, 1997)**. Wet chemistry techniques to determine oxalate have been used by **Zarembski and Hodgkinson (1962)** and **Hodgkinson (1997)**. High performance liquid chromatography **(Holloway, 1989; Savage *et al.*, 2000)**, enzymic methods **(Chai and Liebman, 2005a)**, gas–liquid chromatography (GLC) **(Ohkawa, 1985)** or capillary electrophoresis **(Chai and Liebman, 2005b; Trevaskis and Trenerry, 1997)** can be used to quantify the extracted oxalate. **Hönow and Hesse (2002)** developed an HPLC-enzyme reactor method to measure oxalate in foods. In this method, water or acid-soluble oxalate were separated from matrix substances using an anion exchange column; an enzyme reactor was then used to convert the released oxalate to hydrogen peroxide, which was then measured amperometrically. For determination of oxalic acid in some *Algae* **Watanabe *et al.* (1989)** applied an oxalic acid sensor prepared with the combination of an oxalate oxidase membrane and an oxygen electrode.

Amaranthaceae, which are closely related to *Chenopodiaceae*, are widely distributed in the tropical and subtropical regions of the world. The consumption of some wild, marginalized or less known species continues, but there is still a tendency to replace them with cultivated species **(Escudero *et al.*, 1999)**. Having long been neglected by conventional agriculture, today these crops are receiving increasing recognition because of their potential role in ecosystem maintenance, in promoting diversity as well as in generating income and improving the malnutrition (**Baldermann *et al., 2016***).

Amaranth (*Amaranthus* spp.) is an important vegetable and seed crop with high nutritional quality. However, presence of high levels of oxalate in some species of this plant poses a limitation on its usefulness as a human food source or livestock feed **(Gupta and Wagle, 1988; Cheeke and Bronson, 1980; Pisarikova *et al.*, 2006)**. It is inexpensive and a rich source of carotene, protein and ascorbic acid which serves as an alternative source of nutrition for vegetarians in developing countries. Besides nutritional value, it can grow successfully under varied soil and agro-climatic conditions **(Shukla *et al.*, 2003)**. Grain amaranth is a widely adaptable pseudocereal crop that has high protein and calcium concentrations and a lack of gluten. Pseudocereals like amaranth, with high numbers of species exhibiting a high degree of variability, may enhance biodiversity within cereal food supply and deliver essential ingredients as grain and vegetable crops suitable for variable climatic conditions and also for people with allergies.

Several studies performed by different authors showed that oxalate content is variable in different amaranths. Mean total oxalate concentration in the grain of 30 analyzed grain genotypes was 229 mg/100 g, with values ranging between 178 and 278 mg/100 g **(Gélinas and Seguin, 2007)**. In leaves of *Amaranthus tricolor* L. soluble oxalate content was 690 and total oxalate 1270 mg/100g FW (**Gupta *et al.*, 2005**). The fresh leaves of *Amaranthus tricolor* L. were analyzed for their content of oxalic acid. Oxalic acid content of the leaves ranged from 0.91 to

14.92 g/100 g. It was found that blanching and cooking resulted in a significant reduction in oxalic acid **(Yadav and Sehgal, 2003)**. The foliage of 10 promising cultivars of vegetable amaranth from four cuttings was evaluated separately to gather information on different nutritional and antinutritional factors. The lowest oxalic acid content was in AV-45 (0.06 %) followed by AV-151 (0.11 %) **(Gélinas and Seguin, 2007)**. Other investigation of soluble oxalate content in nuts of *Amaranthus caudatus* ranged from 55 to 107 mg/100 g, with a total oxalate ranging from 228 to 236 mg/100 g **(Siener *et al.*, 2006)**. The oxalate concentration for the *Amaranthus retroflexus* species was found within the range of 2.66 to 5.36 % **(Siener *et al.*, 2006; Escudero *et al.*, 1999)**. On average the total oxalate content in a vegetable amaranth *Amaranthus gangeticus* was 91 $g.kg^{-1}$ on a dry weight basis **(Vityakon and Standal, 1989)**. The distribution of oxalate within a plant is uneven. Reports show that the petiole (stalks) of amaranth **(Siener *et al.*, 2006)** contains significantly lower levels of oxalate than the leaves.

Over the past years a multidisciplinary approach was initiated in cooperation with International Atomic Energy Agency in Vienna with aim to improve quality and quantity of amaranth production through radiation mutagenesis and related biotechnology approaches **(Gajdošová *et al.*, 2007)**. As result, a collection of mutant lines was established and compared to the reference amaranths on the base of some biochemical characteristics and nutritional traits **(Hricová *et al.*, 2011; Kečkešová *et al.*, 2012, 2013)**.

Herein, we present comparative study of soluble oxalate content in selected radiation obtained amaranth mutant lines and their reference non-irradiated controls (*Amaranthus cruentus* 'Ficha' and *Amaranthus hypochondriacus* × *A. hybridus* 'K-433'). Comparative estimation looking for mutants with minimal content of antinutritional soluble oxalic acid was performed by analysis of variance and multivariate statistical approach.

MATERIAL AND METHODS

Plant material and experimental design

Two grain amaranth accessions were used for the gamma irradiation treatment – genotype 'Ficha' (*Amaranthus cruentus*) and interspecific hybrid 'K-433' (*A. hypochondriacus* × *A. hybridus)*, both medium early cultivars with weight of thousand seeds (WTS) 0.85 g and 0.73 g, respectively. Both seed samples were obtained from the collection of Gene Bank of the Research Institute of Plant Production Praha-Ruzyně, Czech Republic. Seeds were treated by a dose of 175 Gy in the Joint FAO/IAEA Programme Agency's laboratories in Seibersdorf, Austria **(Gajdošová *et al.*, 2007)**.

Twenty generations of selected mutant lines with their untreated counterparts ('Ficha' as control A and hybrid 'K-433' as control B) were established in the last two decades. Reference amaranth A was compared with appropriate irradiation-derived mutant lines C15, C26, C27, C82 and C236 and reference B with derived lines D54, D279 and D282 on soluble oxalate content in ground seed samples harvested during the period 2011–2014 from Nitra region. The region is characterized as a warmer and dry area located on the south-western Slovakia (290 m above sea level) with mean annual precipitation 600 mm and mean annual temperature 9.5 °C.

Extraction of soluble oxalate

In order to determine the oxalic acid content by capillary isotachophoresis (CITP) a reported procedure of oxalate isolation by the simple water extraction method was used **(Hönow and Hesse, 2002)**. For the determination a portion of 5 g of the homogenized samples were suspended with 20 ml distilled water and subsequently shaken for 60 min at room temperature (21°C) using a laboratory shaker (Innova 2000, New Brunswick Scientific, Edison, New Jersey, USA) at 3.3 Hz and subsequently the solid phase was removed by filtration on folded paper filter 604 ½ (Schlieser and Schûell, Dassel, Germany).

Capillary isotachophoretic determination

Capillary isotachophoretic separations was performed using an electrophoretic analyser EA 202M (Villa Labeco, Spišská Nová Ves, Slovakia) with ITP Pro 32 (v. 1.0.5) software package for data processing. The method was developed and validated by **Meissner *et al.* (1998)** and **Sádecká *et al.* (2008)**, respectively. Leading electrolyte: 5 mM HCl including 0.1 % MHEC (methyl-hydroxyethylcellulose) adjusted with β-alanine to pH 3.5; terminating electrolyte: 5mM citric acid; sample injection 30 µl, driving current in pre-separation capillary 200 µA, current in analytical capillary 20 µA; conductometric detection. The pre-separation capillary (80 mm x 0.8 mm i. d.) and analytical capillary (160 mm x 0.3 mm i. d.) were made of fluorinated ethylene-propylene copolymer. All analyses were performed in duplicate. Calibration curve for the sodium oxalate was performed in the range of concentrations from 0.5 to 10 $mg.l^{-1}$ with linear regression ($R^2 = 0.996$). From the calibration data limit of detection (0.29 $mg.l^{-1}$) and limit of quantification (0.47 $mg.l^{-1}$) were determined by ISO 11843-2 method using the QC Expert v.2.5 Trilobyte software statistical package.

Statistical analysis

The obtained data were subjected to two-way ANOVA to determine the differences between control variants and mutant lines across 4-year cultivation period. Statistical analysis was carried out by using Statistica 10 (StatSoft, Inc. 2011) software.

In order to find possible tendencies in the samples and the discriminant power of the variables the canonical discriminant analysis was used. The presentation and visualisation of the results was done by Unistat v. 5.6 (Unistat, London, United Kingdom).

RESULTS AND DISCUSSION

This study examined the antinutritional soluble oxalic acid of five amaranth mutant lines of *A. cruentus* L. (C15, C26, C27, C82 and C236) and three mutants of hybrid *A. hypochondriacus* × *A. hybridus* (D54, D279 and D282) derived by radiation mutagenesis of genotype 'Ficha' (*A. cruentus*) and interspecific hybrid 'K-433' (*A. hypochondriacus* × *A. hybridus*). Comparative study on soluble oxalate content in ground seeds was performed on plant material grown during the period 2011 – 2014 in Nitra region.

To gain deep insight into the differences among amaranth samples we applied a multivariate analysis on oxalic acid concentration data. The data were sub-divided according to the observation years 2011 – 2014 as comparative variables. The canonical discriminant analysis confirmed differences among compared amaranth reference and mutant samples as it is visible on the plot of canonical functions (Figure 1). First discriminant function was able to explain up to 94 % of total variance concerning mainly the soluble oxalate variation in 2012 and 2013 years, which were the most discriminating time factors for distinguishing the compared groups of amaranths. The significance of shown differences on plot of discriminant functions is indicated by transposition of group samples as it was previously demonstrated by factor analysis. Moreover, significant differences demonstrated by canonical discrimination were confirmed by classification procedure which resulted in 100 % correctly categorised all amaranth samples into their relevant groups.

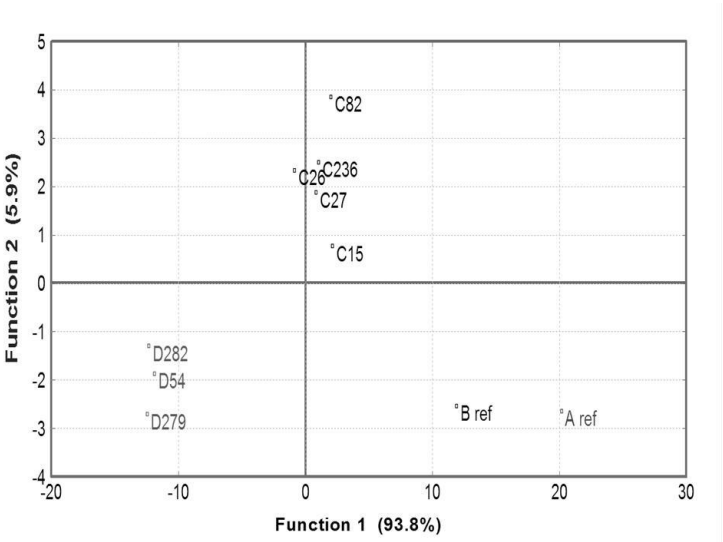

Figure 1 Differentiation of amaranth mutant lines from their reference samples by canonical discriminant analysis

Multiyear comparison of soluble oxalic acid content in all examined amaranth samples is shown in Table 1a. The content of this undesirable antinutritional factor ranged in tested amaranth seeds from 2.2 to 14.04 $mg.kg^{-1}$. **Llelaboye and Pikuda (2009)** determined several antinutritional factors such as tannins, saponins and oxalic acid in seeds of three less known crops – *Jatropha cucas, Trichosanthes cucumerina* and *Citrullus vulgaris*. Among the seeds rich in oxalic acid, greatly affecting the mineral content, were the *Citrullus vulgaris* seeds (40.65 mg/100g). The obtained values of oxalic acid in our study was found in lower levels in comparison to these data as well as some results reviewed for amaranth **(Siener *et al.*, 2006).**

During the period of observation the biggest variability in oxalate content was found within C82 mutant line. In the case of comparison according to year of production the largest oxalate content was found in the year of 2012 and 2013. Considering the nutritional aspect the minimal content of oxalate (2.2 $mg.kg^{-1}$) was found in seeds of mutant line D282 in the 2011 year.

The data on soluble oxalate in amaranth seeds collected during the 4-year period of observation were subjected to analysis of variance by two-factor ANOVA (Table 1b). Statistical differences in soluble oxalic acid amount were determined between amaranth reference and mutant samples ($p = 0.000$) and growing season ($p = 0.000$). Two-way ANOVA statistical approach revealed high significant

differences between reference amaranths and respective mutant lines ($p < 0.05$, $p < 0.01$). Hybrid mutant lines (D54 – D282) showed highly significantly lower oxalate content in comparison to both non-irradiated control samples and to all *A. cruentus* lines (C15 – C236). Most interesting results were observed in the mutant line D282 that was identified as variant with the significantly and long-term lowest soluble oxalate concentration in comparison to respective reference samples as well as other mutant lines. This beneficial long-term suppression of oxalic acid in the D282 seeds could be a consequence of gamma-ray mutagenesis.

Table 1 Soluble oxalic acid content in seeds of eight amaranth mutant lines and two non-irradiated control samples harvested during the 2011–2014 period in Nitra region

a)

Amaranth variants	Soluble oxalic acid [mg.kg^{-1}]							
	2011		2012		2013		2014	
	x_{mean}	s_x	x_{mean}	s_x	x_{mean}	s_x	x_{mean}	s_x
A control	10.45	0.05	10.34	0.31	14.04	0.29	8.47	0.18
C15	4.77	0.22	8.58	0.13	9.38	0.32	6.94	0.10
C26	4.13	0.05	10.03	0.21	9.26	0.66	7.4	0.27
C27	6.02	0.25	7.39	0.03	9.63	0.30	5.22	0.37
C82	4.75	0.13	11.97	0.06	11.30	0.55	7.67	0.30
C236	5.36	0.24	8.18	0.06	6.17	3.3	9.14	0.06
B control	10.05	0.5	7.89	0.03	12.16	0.59	6.53	0.34
D54	4.42	0.18	5.59	0.02	4.16	0.13	5.23	0.90
D279	3.64	0.24	6.71	0.03	5.21	0.27	3.50	0.23
D282	2.20	0.25	2.42	0.01	3.47	0.13	2.88	0.07

b)

Amaranth variants	Means [mg.kg^{-1}]	α = 0.05	α =0.01
D282	2.74	a	a
D279	4.77	b	a, b
D54	4.85	b	b
C27	7.07	c	c
C236	7.22	c	c, d
C15	7.42	c, d	c, d
C26	7.71	c, d, e	c, d
C82	8.93	d, e	d, e
B control	9.16	e	d, e
A control	10.83	f	e

(a – f) Different letters represent significant differences by LSD test at respective probability level

The response to gamma-ray irradiation treatment and radiation genetic effect can be observed and different quantitative and qualitative characteristics and traits can be positively modified what leads to improvement of existing or development to the new varieties. Mutation-based breeding involves the development of new crop varieties by generating and utilizing genetic variability through chemical and physical mutagenesis (**Gomez Pando *et al*., 2013**). Currently, over 3 200 registered mutant varieties covering 232 different crops have been developed using induced mutagenesis (**Mehlo *et al*., 2013*;* IAEA, 2015;** reviewed in **Oladosu *et al*., 2016**). Many of these varieties have significantly contributed to an improvement in the socio-economic conditions of farmers. Thus, mutation breeding has big potential for agriculture and is important approach of modern plant breeding.

CONCLUSION

Analysis of gamma irradiation-derived mutants revealed differences in the content of one of the antinutritional factors – soluble oxalic acid. The hybrid mutant line D282 was found as variant with the significantly lowest soluble oxalate concentration in seeds in comparison to its reference counterpart as well as other mutant lines of the current report. This mutant line was able to hold this anti-nutritional quality aspect during four years of cultivation. Decrease in a content of the antinutritional substance, that generally limit the nutrient availability to the human body, could be a possible effect of radiation-induced mutation event(s) in the D282 line genome. Thus, a further task would involve unravelling the genetic basis of the reported (mutant) trait.

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