

Molecular genetic characterization of *Amaranthus cruentus* L. mutant lines derived from local and preferred *Amaranthus* cultivar

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ABSTRACT

Objective: Benin's most appreciated *Amaranthus cruentus* L. «local» cultivar is susceptible to saline stress. Molecular diversity and relationships among nineteen gamma-irradiated *A. cruentus* mutant lines and their relative, the «local» cultivar, were studied to identify the best representative sample of those created mutant lines for further saline tolerance breeding.

Methodology and Results: The study was carried out by using a random amplified polymorphic DNA (RAPD) marker. Four RAPD (decamer) arbitrary primers (OPA-02, OPA-08, OPB-05 and OPB-08) out of eleven were able to discriminate all the genotypes sufficiently. Twenty amplicons, all polymorphic, were produced ranging from ~150 bp to ~1200 bp. The mean value of Shannon's information index calculated for genetic diversity estimation was 0.43, and it ranged from 0.27 to 0.62. The genetic similarity coefficient ranged from 0.00 to 1.00. The sets of mutant lines L3 and L6; L12 and L8; L17 and L20; L13, L17 and L20; L17 and L20 appeared to be genetically similar, clustering together inside at 100 % similarity coefficient.

Conclusion and application of findings: Six distinct genotypes (L2, L6, L13, L16, L18 and L23) were identified as representative of the nineteen studied *A. cruentus* created mutant lines. This finding provides a diversity of materials to be considered for saline or other breeding objective. The current study is one step in a whole process of more productive and saline tolerant *A. cruentus* varieties' creation. Mutation and molecular tools used in the current study cleared the way to an effective selection of good source of genes for *A. cruentus* breeding. The next step will be the saline test on the selected mutant lines to identify the most tolerant genotypes that can thrive despite saline effects and fit farmers' and consumers' needs. **Keywords:** Biotechnology, genetic diversity, mutation breeding, molecular genetic markers, Republic of Benin.

INTRODUCTION

Originated in the Americas, the genus *Amaranthus* consists of 60–70 species and including 3 cultivated grain species (*A. caudatus*, *A. cruentus*, and *A. hypochondriacus*) that are nowadays widely studied and cultivated worldwide because of their exceptional nutritional value of both seeds and leaves (Brenner *et al.*, 2000; Costea *et al.*, 2006; Janovská *et al.*, 2012; Gerrano *et al.*, 2017). Amaranth has the potential to enlarge the food base in sub-Saharan Africa thanks to its agronomic and nutritional importance (Akin-Idowu *et al.*, 2016). But salinity has been reported as major problem in amaranth production system. In Benin, for instance, Wouyou *et al.* (2016, 2017a) reported that salinity reduces seeds germination as well as plant root and above ground growth (biomass) of different *A. cruentus* cultivars. Moreover, the most appreciated «local» cultivar showing high agro-morphological characteristics was found to be particularly susceptible to that stress (Wouyou *et al.*, 2016). Comparatively to the costly and temporary solutions such as diminishing the salt rate in the soil through amendment and in irrigation water or washing salt through a good drainage as Dasgan *et al.* (2002) remarked, creating saline tolerant varieties with, at least, the same agronomic traits of interest turns out to be the more economical way to reduce the harmful salinity effect on cultures (Poustini & Siosemardeh, 2004). Genetic variability in plants is caused by diverse factors such as: mutation, random mating and recombination between homologous chromosomes during meiosis (Schreiber *et al.*, 2018; Nwankwo *et al.*, 2019). In amaranth, mutation breeding has been effectively used for cultivar development and generating polygenic variability (Joshi *et al.*, 2018). Using Gamma rays, Gomez-Pando (2014) mutated, in a study, *Amaranthus caudatus* L. and successfully obtained semi-dwarf and dwarf stature, non-branching

pattern, improved protein content and pigment mutations (color of plants and grains) (Nwankwo *et al.*, 2019). Amaranth species were analyzed to determine genetic diversity among and within them, to obtain the corresponding genetic fingerprints, and to carry out comparisons for differentiating and distinguishing between the genetic variants (Pandey *et al.*, 2019). Diversity in created mutants is studied through techniques including morphological traits, total seed protein, and isozymes. However, molecular markers are now the tools most widely used to assess genetic diversity within and between species (Lanoue *et al.*, 1996; Mondini *et al.*, 2009; Popa *et al.*, 2010; Hamzekhanlu *et al.*, 2011; Patel *et al.*, 2014; Akin-Idowu *et al.*, 2016). Diverse molecular markers such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (ALFP) and Simple Sequence Repeat (SSR) have been used to carry out more accurate research on genetic diversity and phylogenetic relationships among *Amaranthus* species (Žiarovská *et al.*, 2013; Cokran *et al.*, 2019). Random amplified polymorphic DNA (RAPD) technique gained importance in genetic research due to its simplicity, speed, efficiency, relative ease to perform and non-requirement of sequence information (Patel *et al.*, 2014). Patel *et al.*, (2014) emphasized RAPD markers to be easier and quicker to use and are preferred in application where the relationships between closely related breeding lines are of interest (Halldén *et al.*, 1994). To identify the best representative sample of created mutant lines for further saline tolerance breeding, as a step in creation of salinity tolerant *A. cruentus*' varieties, the current study focus on evaluating the genetic diversity and relationships among nineteen gamma-irradiated *A. cruentus* mutant lines and their relative «local» cultivar (control) using the RAPD marker.

MATERIAL AND METHODS

Plant Material

Mutant lines and stabilization: Seeds of «local» and preferred *Amaranthus* cultivar subjected to 200 grays of a mutagenic agent Gamma at the International Agency of Atomic Energy (AIEA) at Vienna were used to generate nineteen (19) mutant lines (L1, L2, L3, L6, L8, L9, L10, L11, L12, L13, L14, L15, L16, L17, L18, L19, L20, L21 and L23) from the «local» cultivar (Lo) used as a control. The obtained mutant lines were self-pollinated until the sixth generation' lines (M₆) using « single-seed descent » technique (Janwan *et al.*, 2013) at the experimental site of the International Institute of Tropical Agriculture (IITA / Benin, (latitude: N 6° 25' 260'' and longitude: E 2° 19' 682''; altitude: 15 meter above sea level) in Abomey-Calavi (Benin Republic) from November 2018 to March 2020. The site is located in southern Benin characterized by a sub-equatorial climate with an annual mean temperatures ranging from 26 to 28°C and an annual rainfall varying between 800 to 1400 mm (Yabi & Afouda, 2012). At each self-pollinated generation, seedlings from individual selected plant were grown in 3 rows on a 3 m long and 1.5 m wide plot. Five plots were used per line. Plants were spaced 50 cm apart within rows and 50 cm between rows with a total of 18 plants per plot. Among those 18 plants grown per plot, the 5 well developed and phenotypically close based on plant height, number of branches and leaves production were identified and their flowers were covered from appearance, against stranger pollen with envelope made of tracing paper. At seeds maturity stage, seeds were harvested per plant per line and dried. Seedlings from one of the five selected plants per plot per line were transplanted onto one plot at the next generation.

Experimental design : Seeds of sixth generation from each 19 mutant lines plus the «local» cultivar (used as control) were manually sown in April 2020 in separated pots

filled of sandy loam in nursery. At the 4–5 leaf stage, seedlings were transplanted to the experimental field (latitude: N 6° 29' 3'' and longitude: E 2° 16' 38'' and located at Abomey-Calavi (Benin Republic)) in given plots. Plots were arranged in a randomized complete block design (Das and Rao, 2015) with three repetitions. An experimental unit (plot) consisted of 10 individuals of a mutant line or «local» cultivar planted in two rows on 2 m long and 1 m-wide raised beds (Akin-Idowu *et al.*, 2016). Plants were spaced 50 cm apart within rows and 1 m between rows, with an inter-plot spacing of 1 m as used by Akin-Idowu *et al.* (2016). The experimental plots were kept weeds-free for the duration of the study and poultry dropping was applied around each seedling one week after transplantation at the dose of 1t/ha according to Souleymane *et al.* (2018). Chemicals pesticides such as Acarius (Abamectine 18g/L) (used at a dose of 300 l/ha) and Mancozeb in wettable powder form (at the dose of 3 kg/ha) were used to control pests and diseases.

Molecular genetic analysis: The molecular genetic analysis was carried out at the Laboratory of Molecular Genetic and Genomes Analysis, in Department of Genetic and Biotechnologies, Faculty of Sciences and Techniques, University of Abomey-Calavi (UAC), Benin Republic.

DNA Extraction: The DNA from fresh young leaves of three randomly selected individual plants of each mutant line and control genotype was extracted by using Mixel Alkyl Triméthyl Ammonium Bromide (MATAB) procedure (Gawel & Jarret 1991) previously modified and adapted to divers tropical crops by Agbangla *et al.*, (2002).

PCR Amplification: In total, eleven (11) RAPD primers for herbaceous species were screened for polymorphism. Four (4) out of them exhibited detectable distinct bands (Table 1) and were considered for the study (Akin-Idowu *et al.*, 2016). DNA from the 19 mutant

lines and the «local» cultivar of *A. cruentus* was amplified in PCR using the chosen primers (Akin-Idowu *et al.*, 2016). DNA dilutions were carried out to obtain necessary concentrations for amplifications. Amplifications were performed in thermal cycler (type *Thermal Blok II and Aplied Biosystems*) using PCR mixture of consisting of 10 × TB, 1.25 mM MgCl₂, 0.3 mM dNTPs, 2.8 μM primer, 0.03 U/μl Taq polymerase, 10 μl MiliQ water. 3 μl DNA was added to each PCR tube containing those reactive. The PCR amplifications reaction performed were programmed for an initial denaturation at 95°C for 5 min, followed by 43 cycles each of 1 min at 94°C for

denaturation, 1 min at 55°C for hybridation and 2 min at 72°C for elongation which was completed by a final incubation of 10 min at 72°C and a conservation phase at 4°C to end the amplifications. After amplification, the PCR products was resolved by electrophoresis in 2 % agarose gel and stained with ethidium bromide (0.5μg/ml). After 25 min of migration, the gel as revealed and the bands were visualized under UV light and photographed using the UV Transilluminator equipment (UVP, LLC Upland, CA). All the PCR reaction was repeated twice to check the reproducibility of the amplification products for each polymorphic primer.

Table 1: List and characteristics of the 10-base nucleotide primers used for the RAPD analysis

Group of the primer	Primer	Percentage of bases (CG%)	Primer's sequence 5'-3'	Reaction type with amaranth samples
B2644-072	OPA-02	70	TGCCGAGCTG	Positive
B2644-073	OPA-03	60	AGTCAGCCAC	Negative
B2644-074	OPA-06	70	GGTCCCTGAC	Negative
B2644-075	OPA-08	60	GTGACGTAGG	Positive
B2644-076	OPA-18	60	AGGTGACCGT	Negative
B2644-077	OPB-04	60	GGACTGGAGT	Negative
B2644-078	OPB-05	70	TGCGCCCTTC	Positive
B2644-079	OPB-06	70	TGCTCTGCC	Negative
B2644-080	OPB-07	70	GGTGACGCAG	Negative
B2644-050	OPN-08	60	ACCTCAGCTC	Positive
B2644-081	OPB-08	70	GTCCACACGG	Positive

Data analysis: The amplified DNA fragments, with the same molecular weight and mobility, were treated as identical bands, and scored in a binary mode, 1 as presence of fragment, 0 as absence (Agbidinoukoun *et al.*, 2017) across all mutant lines and the local cultivar for each primer. The resulting banding matrix was subjected to cluster analysis (Akin-Idowu *et al.*, 2016) under the package UPGMA of R (3.6.2), and Dendro-UPGMA freely available software available at

<http://genomes.urv.es/UPGMA/> (Garcia-vallvé & Puigbo, 2009). A pairwise genetic similarity matrix was constructed using dendroUPGMA based on Jaccards's (1908) coefficient (Akin-Idowu *et al.*, 2016). To assess the relationships among the mutant lines and the control genotype, the similarity matrix was used to construct a dendrogram by the unweighted pair group method with arithmetic average (UPGMA) (Akin-Idowu *et al.*, 2016) using R package (UPGMA).

RESULTS

Amplified bands in the size ranging from ~150 to ~1200 bp were scored to estimate the genetic similarities among the 19 *A. cruentus* mutant lines and their relative «local» cultivar. A total of 20 alleles were generated (Table 2). All RAPD markers used in this study were polymorphic. An average of 5 bands per primer was produced, ranging from a minimum of four (4) bands using primer OPA-02 to a maximum of six (6) bands using primer OPB-08. Based on Shannon's information index, the selected primers showed a relatively low diversity. The diversity index ranged from 0.27 (OPB-08) to 0.62 (OPA-02). The primers OPA-02 showed the most diversity followed by the primer OPA-08 (Table 2). The similarity matrix presented estimated genetic similarities among the set of 19 mutant lines

and the «local» cultivar of amaranth on the basis of the RAPD banding pattern (Table 3). The similarity coefficients ranged from 0.5 to 1 for 75 couple of *Amaranthus* mutants showing high correlations. Mutant lines L3 and L6; L12 and L8; L9, L10; L17 and L20; L13, L17 and L20; L17 and L20 appeared to be genetically similar, clustering together at 100 % similarity coefficient. L23 and «local» cultivar appear also to be genetically similar, clustering together at 90 % similarity coefficient. Sufficient polymorphisms were detected by RAPD markers (Fig. 1). The UPGMA dendrogram from the distance matrix based on Jaccard's coefficient revealed two major clusters and three sub-clusters (Fig. 2). At 30 % height the dendrogram can be observed to have three clusters.

Table 2. RAPD Polymorphism among 19 mutant lines and the «local» cultivar of amaranth.

Markers	Total of Fragments detected	Fragments Polymorphic	Shannon's information Index	Polymorphism (%)	Bands
OPA-02	4	4	0.62	100	300pb-750pb
OPA-08	5	5	0.47	100	250pb-1200pb
OPB-05	5	5	0.37	100	250pb-750pb
OPB-08	6	6	0.27	100	150pb-650pb
Total	20	20	-	400	-
Mean	5	5	0.43	100	-

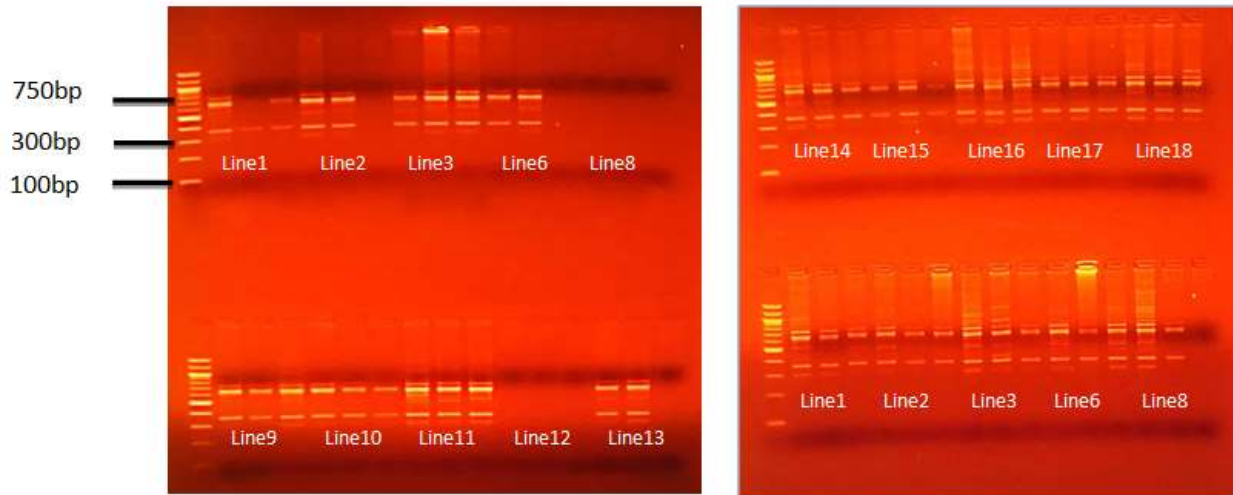


Fig. 1: Electrophoresis profile of DNA from 19 *A. cruentus* mutant lines and their relative local cultivar (control) in 2% agarose gel generated by the marker OPA-02. Amplicons size range from 300pb to 750pb for this marker (100 bp ladder marker DNA was used)

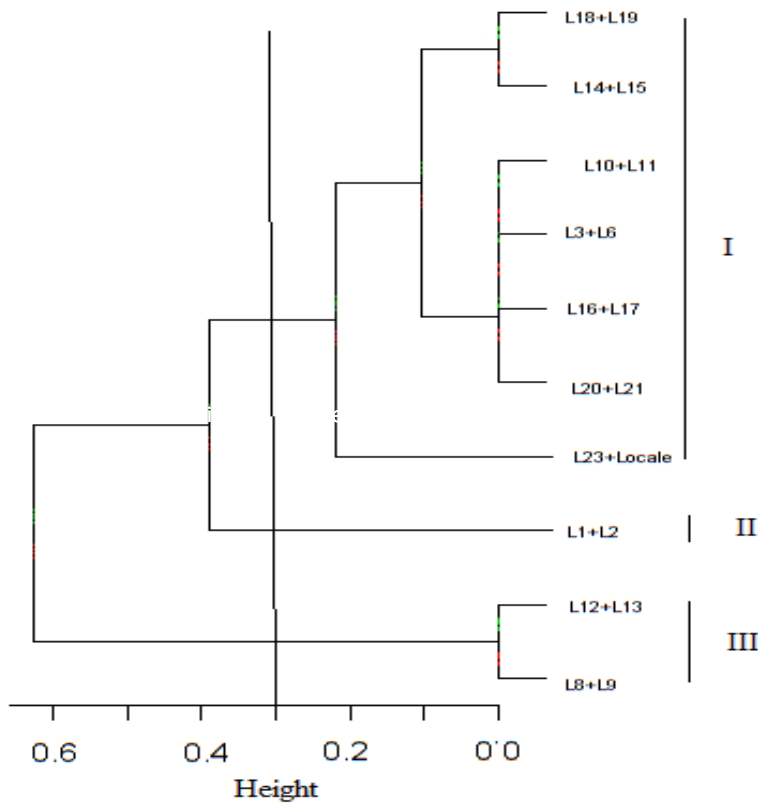


Fig. 2: UPGMA-based dendrogram derived from RAPD analysis of 19 amaranth mutant lines and the «local» cultivar

DISCUSSION

Amaranth became rediscovered and is intensively studied through the molecular based polymorphism analysis (Pandey *et al.*, 2019). In many studies (Lee *et al.*, 2008; Ray & Roy, 2009; Solano & Porfirio, 2010; Lymanskaya, 2012; Nwankwo *et al.*, 2019), the random amplified polymorphic DNA (RAPD) marker like other molecular markers has been successfully used for genetic diversity evaluation and phylogenetic relationships studies between *Amaranthus* species. Then, molecular analysis have helped resolve the distinctness in the genetic makeup of crop plants when morphological investigations have failed (Transue *et al.*, 1994; Guillermo *et al.*, 2014; Nwankwo *et al.*, 2019). In this study, nineteen mutant lines and their relative «local» cultivar were evaluated for molecular diversity using DNA. Of the eleven primers screened in this study, four were able to discriminate all the genotypes sufficiently generating twenty fragments all polymorphic and ranging from ~150 to ~1200 bp in size with an average of 5 amplicons per primer. The same percentages of RAPD polymorphism (100%) were found by Patel *et al.* (2014) at the intraspecific level for *A. cruentus* and *A. caudatus* in a similar study for RAPD assessment on genetic variation for a total of 58 individuals comprising 15 parental genotypes and their 43 F1 hybrids of grain *Amaranthus* with four discriminating selected primers. The total number of amplified fragments varied from four to six for all the primers (OPA02, OPA08, OPB05 and OPB08). The selected primers sufficiently revealed clear and reproducible RAPD profiles as found by Ray & Roy (2009) and Akin-Idowu *et al.* (2016) in their studies. The values of the similarity index among the nineteen mutant lines and the «local» cultivar (control) ranged from 0.00 to 1.00 indicating a significant relationship. Pandey *et al.* (2019) found similar results at intra-specific level accessing genetic diversity and relationships

among 7 *Amaranthus* species. Ranging from 0.27 to 0.62, the mean value of Shannon's information index calculated for genetic diversity estimation was 0.43 indicating a relatively low diversity between all the studied genotypes as commented by Zavinon *et al.* (2019) in their study for phenotypic diversity in pigeon pea landraces. This finding is in the same alignment with the high similarity noted in the current research and could be explained by the parental relationship between the 19 created mutants lines and the relative local cultivar. In this study, the similarity coefficients ranged from 0.5 to 1 for 75 couples of mutant lines and local cultivar pointing out high correlations. As the mutant lines couples L3 and L6 or L12 and L8, the lines L9, L10, L17, L20 and L13 appear to be genetically similar at 100 % similarity coefficient. L23 and locale accession appeared also to be genetically similar, clustering together at 90 % similarity coefficient. Similar high genetic similarity values (0.58 to 0.98) were found by Singh *et al.* (2013) for RAPD analysis in a comparative study of Inter Simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) loci in assessing genetic diversity in *Amaranthus* species. The dendrogram showed that the mutant lines L1 and L2 were most similar but substantially different from the mutant lines L6, L13, L16, L17 and 23. It was also observed that the mutant lines L16 and L17 were similar. On the whole, the dendrogram reveals the RAPD markers' effectiveness in detecting sufficient polymorphisms to discriminate the studied mutants (Akin-Idowu *et al.*, 2016). Results in the current study provide with information that allow bringing the set of the nineteen *A. cruentus* created genotypes down to a representative sample. The mutant lines L2, L6, L13, L16, L18 and L23 can then be identified as representatives of the evaluated mutant lines (L1, L2, L3, L6, L8, L9, L10, L11,

L12, L13, L14, L15, L16, L17, L18, L19, L20, L21 and L23). In the same alignment with Guillermo *et al.* (2014) findings, molecular analysis helps resolve, in the current study, the distinctness in the genetic makeup of the *A. cruentus* evaluated mutants. Combining traditional breeding techniques and biotechnological methods like molecular

markers, as Kaminski *et al.* (2003) reported, turns out to give new possibilities for obtaining the high quality, uniform and distinctive breeding material to generate F1. Mutation and molecular tools used in the current study cleared the way to an effective selection of good materials for *A. cruentus* breeding objectives.

CONCLUSION AND APPLICATION OF RESULTS

In conclusion, using specific primers, the RAPD analysis was relevant in the current study for it was able to detect sufficient polymorphisms to discriminate the studied mutants. It was helpful to clearly reveal genetic diversity among the mutant lines and the «local» cultivar. Six distinct genotypes (L2, L6, L13, L16, L18 and L23) were identified as representative of the nineteen studied *A. cruentus* created mutant lines. This finding provides a diversity of materials to be

considered for saline breeding objective. The current study is one step in a whole process of more productive and saline tolerant *A. cruentus* varieties' creation. Mutation and molecular tools used in the current study cleared the way to an effective selection of good source of genes for *A. cruentus* breeding. The next step will be the saline test on the selected mutant lines to identify the most tolerant genotypes that can thrive despite saline effects and fit farmers' and consumers' needs.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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