Electrophoretic characterization of *Amaranthus* L. seed proteins and its systematic implications

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The seed protein profiles of 11 *Amaranthus* taxa (Amaranthaceae) from Spain were studied. These profiles were evaluated as a chemical character to clarify the taxonomic complexity in the genus. Tricine-sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of the *Amaranthus* seed proteins studied showed a range of peptides varying from 64 to 12 kDa, with a larger number of protein bands observed between 25.1 and 12 kDa. For the taxonomic study, 14 bands, some of them subdivided into several isoforms, were considered. The similarity analysis based on the SDS-PAGE profile is a useful character for the discrimination of species in *Amaranthus*, except for *A. cruentus* and *A. hypochondriacus*, for which a hybrid population was found.


ADDITIONAL KEYWORDS: Amaranthaceae – electrophoresis – taxonomy.

INTRODUCTION

*Amaranthus* species have different centres of domestication and origin, being widely distributed in North America, Central America, and the South American Andes, where the greatest genetic diversity is found (Sun, Chen & Leung, 1999; Xu & Sun, 2001). It is estimated that there are 87 species of *Amaranthus*: 17 in Europe, 14 in Australia, and 56 in America (Mujica & Jacobsen, 2003). Because of the paucity of studies on *Amaranthus* systematics, however, the number of species is still tentative. Some species are cosmopolitan, being both introduced and naturalized plants, with a weed-like behaviour, such as *A. retroflexus*, *A. hybridus*, *A. powellii*, and *A. viridis* (El Aydam & Bürki, 1997; Costea, Waines & Sanders, 2001b; Dehmer, 2003; Costea, Weaver & Tardif, 2004). Amongst the cultivated species, *A. cruentus*, *A. hypochondriacus*, and *A. caudatus* stand out and are considered as pseudocereals, with a high seed protein content, a balanced amino acid composition, and a high lysine content (Bressani, 1989; Bressani & García-Vela, 1990; Barba de la Rosa et al., 1992; Stallknecht & Schulz-Schaeffer, 1993; Lehman, 1996; Zheleznov, Solonenko & Zheleznova, 1997; Gorinstein et al., 2001). These *Amaranthus* species are cultivated in different regions of South and Central America, India, and Nepal (Sauer, 1950; Stallknecht & Schulz-Schaeffer, 1993; Zheleznov et al., 1997; Bucaro-Segura & Bressani, 2002).

*Amaranthus* is often difficult to characterize taxonomically, as it has few useful distinguishing features amongst the large number of species. Moreover, hybridization is a common phenomenon in this genus, producing many interspecific hybrids that increase the taxonomic complexity (Greizerstein & Poggio, 1992; Lanta, Havránek & Ondřej, 2003; Wassom & Tranel, 2005). It is also difficult to delimit the distributional areas of the species, as many are cosmopolitan because of their capacity to adapt to the environment as well as the large quantity of seeds produced by the plant (Costea & DeMason, 2001; Costea et al., 2004).

Traditionally, *Amaranthus* has been divided into two sections: *Amaranthus* and *Blitopsis* Dumort (Aellen, 1959; Brenan, 1961; Carretero, 1979). Later, Carretero (1990) divided the genus into three sections and, more recently, Mosyakin & Robertson (1996), based on inflorescence and flower characters, suggested a new classification in which the genus is divided into three subgenera and nine sections.

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In order to clarify the taxonomy of *Amaranthus*, several systematic revisions based on leaf anatomy and morphology (Esparza-Sandoval, Alejandro-Iturbide & Herrera-Arrieta, 1996), pericarp structure (Costea et al., 2001b), and stem morphology and anatomy (Costea & DeMason, 2001) have been developed. Moreover, some systematic studies based on the use of molecular markers have also been carried out (Sammour, 1991; Chan & Sun, 1997; Zheleznov 1992). Employment of molecular markers has also been carried out (Sammour, 1991; Chan & Sun, 1997; Zheleznov et al., 1997; Sun et al., 1999; Xu & Sun, 2001).

Electrophoresis profiles of proteins and isozymes have been successfully used to clarify the taxonomy of families, such as Poaceae (Johnson, Barnhart & Hall, 1967; Duvall & Biesboer, 1989), Cucurbitaceae (Pasha & Sen, 1991), and Fabaceae (Misset & Fontenelle, 1992). Gardiner, Forde & Slack (1986) and Gardiner & Forde (1987, 1988) stated that electrophoresis can also be used to characterize the seed protein profiles of species and cultivars, compare cultivars of different geographical origin, and provide taxonomically useful descriptors that are substantially free from environmental influence. Hence, this method has been used to study cultivated plants, such as *Vitis vinifera* (Altube, Cabello & Ortiz, 1991), *Lolium rigidum* (Bravi et al., 1994), *Hevea brasiliensis* (Leconte et al., 1994), and corn (Aiassa et al., 2003). Moreover, electrophoresis seed protein profiles have already been used in the study of *Amaranthus* species (Gudu & Gupta, 1988; Gorinstein & Moshe, 1991; Zheleznov et al., 1997; Drzewiecki, 2001).

In this work, 11 taxa distributed in Spain were studied. According to Mosyakin & Robertson (1996), the examined taxa belong to two of the three subgenera, both monoics: subgenus *Amaranthus* and subgenus *Albersia* (Table 1). Subgenus *Amaranthus* is represented by two subsections which are included in section *Amaranthus*: subsection *Amaranthus* (A. retroflexus) and subsection *Hybida* (A. cruentus, A. hypochondriacus, and A. powelli ssp. bouchonii). Subgenus *Albersia* is represented by members of section *Blitopsis* (A. blitum, A. viridis, and A. deflexus), section *Pentamorion* (A. muricatus), and section *Pyxidium* (A. albus, A. blitoides, and A. graecizans ssp. sylvestris).

The aim of this work was to evaluate the inter- and intraspecific variability of these species and their taxonomic relationships using, as characters, the electrophoresis profile of seed proteins.

**MATERIAL AND METHODS**

**PLANT MATERIAL**

The samples of *Amaranthus* seeds were taken from wild populations and some herbarium specimens. Voucher specimens of the populations studied are deposited in the Herbarium of the Department of Plant Biology and Ecology of the University of Seville (see Table 1 for details of localities and voucher information).

**MATERIALS**

Standards for electrophoresis were obtained from Amersham Pharmacia (Uppsala, Sweden). Acrylamide, N,N-methylenebisacrylamide and Coomassie Brilliant Blue G-250 were obtained from Serva (Heidelberg, Germany). Sodium dodecysulphate and N,N,N′-tetramethylethylenediamine were purchased from Sigma Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

**ELECTROPHORESIS**

*Amaranthus* seed proteins were extracted with 0.2% NaOH (1 : 10, w/v) by stacking at 1000 r.p.m. for 30 min at room temperature. Extracted proteins were recovered by centrifugation at 7.152 *g* for 15 min. Tricine-sodium dodecysulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Schägger & von Jagow (1987) with slight modifications. The separating gel consisted of 15% T and 2.6% C, where the composition of the acrylamide mixture is defined by the letters T (total percentage concentration of acrylamide and bisacrylamide) and C (percentage concentration of the crosslinker relative to the total concentration T). The stacking gel consisted of 4% T and 3% C. The lengths of the separating and stacking gels were 6 and 2 cm, respectively, with a gel thickness of 1 mm. Electrophoresis was performed at a constant voltage of 60 V for stacking and 120 V for separation. Protein bands were fixed in a solution containing 20% methanol and 8% acetic acid for 15 min, before staining with 0.25% Coomassie Brilliant Blue G in 45% methanol, 10% acetic acid for 24 h. Destaining of the gel was performed in 10% acetic acid. Fourteen protein bands were analysed and quantified with Chemigenius-2 (Syngene, UK) using the program Genetools (version 3.05) for gel analysis.

**PROTEIN DETERMINATION**

The method described by Bradford (1976) was employed for protein determination using bovine serum albumin as standard.

**CLUSTER ANALYSIS**

Selected protein bands were used for the construction of a distance matrix. Cluster analysis of *Amaranthus* taxa was performed using the NTSYS-pc program,
employing the Bray–Curtis index of dissimilarity (Bray & Curtis, 1957). The dissimilarity index was transformed to the index of similarity ($1 - \text{dissimilarity index} \times 100$). A dendrogram was produced from the distance matrix using the unweighted pair group method with arithmetic average (UPGMA).

**RESULTS AND DISCUSSION**

According to the Osborne classification, *Amaranthus* seed proteins are composed of albumins, globulins, and glutelins in similar proportions and prolamins in minor amounts (Abougch, Martínez & Añón, 2003).
The SDS-PAGE profile of the *Amaranthus* seed proteins studied showed a range of peptides varying from 64 to 12 kDa (Fig. 1). The most abundant peptides were in the ranges 64–49.2 kDa and 36.8–32.8 kDa, although the largest number of protein bands were observed between 25.1 and 12 kDa. Fourteen bands were considered for the taxonomic study, although some were subdivided into several isoforms based on the differences observed in Rf.

A similarity analysis based on the SDS-PAGE profile of seed proteins was carried out. The software used displayed a single tree from all those possible (Fig. 2). Two major clusters (A and B) with about 45% similarity were obtained. The first cluster (group A) includes *A. powellii* ssp. *bouchonii*, *A. graecizans* ssp. *sylvestris*, and *A. retroflexus*, and the remaining taxa studied form group B. Within group B, two subgroups (C and D) were distinguished with 58% similarity. Group C includes five species, with *A. deflexus* as the most dissimilar taxon (63%). The other taxa of this group form two well-defined clusters: *A. muricatus* and *A. viridis* with 79% similarity, and *A. blitoides* and *A. blitum* with 72% similarity. With respect to the last group (group D), *A. albus* clearly differs from *A. cruentus* and *A. hypochondriacus* (72%), whose populations appear to be partly mixed.

By contrast with the complexity of the identification of *Amaranthus* species using morphological characters, our results showed that the *Amaranthus* taxa studied are well defined based on the seed protein profile; in particular, species such as *A. blitoides* showed a similarity higher than 95% in the populations studied. Thus, except for *A. hypochondriacus* and *A. cruentus*, the UPGMA dendrogram arranges the studied species in single clusters, indicating that the data provided by the electrophoresis profile of seed proteins are useful in the discrimination of *Amaranthus* taxa at the specific level. The fact that the populations of *A. cruentus* and *A. hypochondriacus* are mixed could be the result of two different mechanisms. Firstly, a hybridization phenomenon, which is very frequent in these species (Costea, Sanders & Waines, 2001a). In this case, population C3 of *A. cruentus* could be a hybrid population between *A. cruentus* and *A. hypochondriacus*. This is possible as this population shares the same peptide band kDa with *A. hypochondriacus* populations, which was not observed in the other *A. cruentus* populations. Sammour (1991) also observed an intermediate protein profile between *A. viridis* and *A. hybridus* (subgenus *Albersia*) in a population of *A. viridis*, indicating a hybridization process between the two taxa. The second explanation for the protein profile of this population could be the hybrid origin of *A. hypochondriacus* from *A. cruentus* and *A. powellii*, as proposed by Sauer (1993) and supported by some molecular studies (Transue et al., 1994; Chan & Sun, 1997).

Although the dendrogram obtained from the seed protein profile (Fig. 2) discriminates between species, it does not group these species according to the infrageneric classification shown in Table 1. Thus, relationships established by the electrophoretic profile of seed proteins do not match the established relationships based on morphological or traditional characters. For example, *A. blitum* and *A. blitoides*, morphologically different and assigned
to different sections (Mosyakin & Robertson, 1996), show a similar protein profile, indicating a closer relationship. In addition, A. albus, A. cruentus, and A. hypochondriacus are in the same group (D), although they are assigned to different sections and even different subgenera: A. albus belongs to subgenus Albersia section Pyxidium, whereas A. cruentus and A. hypochondriacus are included in subgenus Amaranthus section Amaranthus. Although, from a morphological point of view, A. albus is very different from A. cruentus and A. hypochondriacus, recently, Costea & DeMason (2001) found similarity between them using leaf anatomical features, as they observed intermediate characters between both subgenera in A. albus. The fact that species belonging to subgenus Albersia are distributed between the three main groups (A, C, D) is congruent with their heterogeneous taxon composition, and supports the opinion of Mosyakin & Robertson (1996) that this subgenus needs a taxonomic revision in order to delimit new sections.

In agreement with Sammour (1991) and Zheleznov et al. (1997), a correlation between the electrophoresis profile of seed proteins and the karyological data is observed in Amaranthus. Thus, included in group A are taxa (A. powellii ssp. bouchonii, A. graecizans ssp. sylvestris, and A. retroflexus) with basic numbers x = 16 and 17 (Pastor, 1992). In group D, all taxa show x = 16, whereas, in group C, all species show x = 17, except for A. blitoides, which has x = 16 (Pastor, 1992). This could mean that the seed protein profile is one of the phenotypical differences determined by the basic number.

In conclusion, seed proteins in Amaranthus are useful characters to discriminate between species. In addition, these characters show low environmental and evolutionary variability. The profile obtained by SDS-PAGE of seed proteins provides interesting information that will doubtless help in the clarification of this complex genus. Our results show the close relationships between species belonging to different taxonomic groups, such as sections or subgenera, and allow a hybrid population to be distinguished. A more detailed study is necessary in order to clarify the infrageneric classification of this genus.

Figure 2. Clustering based on the seed protein profiles of 11 taxa of Amaranthus according to the Bray–Curtis similarity index (1 – dissimilarity index × 100). V-1, V-2, V-3, A. viridis; P-1, A. powellii ssp. bouchonii; M-2, M-1, M-3, A. muricatus; D-3, D-2, D-1, A. deflexus; G-1, A. graecizans ssp. sylvestris; B-3, B-2, B-1, A. blitoides; R-2, R-3, R-1, A. retroflexus; Bl-2, Bl-1, A. blitum; A-2, A-3, A-1, A. albus; C-3, C-2, C-1, A. cruentus; H-1, H-2, A. hypochondriacus.

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