ELECTROPHORESIS AS AN AID FOR IDENTIFICATION OF VARIOUS SPECIES AND CULTIVARS OF GRAIN AMARANTHS.

By

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DECLARATION

This thesis is my own work and has not been presented for a degree in any other University.

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DEDICATED

TO

MY WIFE AGNES

AND

MY DAUGHTERS: JAEL AND NAOMI

AND

MY MOTHER NEREYA.

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SUMMARY

Starch gel electrophoresis was carried out on twelve cultivars of grain amaranths, from the three main grain types: A. caudatus, A. cruentus and A. hypochondriacus. The enzymes used in the study were: peroxidase, esterase, acid phosphatase and leucine aminopeptidase. Acid phosphatase was not useful in identification of the three species because it produced two monomorphic bands in all the three species.

A total of two cathodal bands and four anodal bands of peroxidase isozyme were used to differentiate fully the three species and cultivars within each species of grain amaranths. The peroxidase isozyme showed more variation among species than within species. Peroxidase isozyme could also distinguish fully the cultivars of A. cruentus and A. caudatus but was unable to differentiate the various cultivars of A. hypochondriacus. The peroxidase isozymes produced were monomorphic for all the five cultivars of A. hypochondriacus used in the study.

Esterase produced a total of nine anodal

bands, but had no band at the cathodal side. The three species could be distinguished, although A. cruentus and A. hypochondriacus were relatively difficult to differentiate. The identification of A. caudatus was easy. Esterase isozymes were also useful for identification of various cultivars within species.

Leucine aminopeptidase was also useful for the identification of one of the species of grain amaranths. Only one monomorphic band was observed. The A. cruentus species could be distinguished on the basis of early activity of leucine amino-peptidase when the other two species had not started producing the enzyme. In addition, the A. cruentus could also be distinguished on the basis of the quantity of the enzyme produced, it produced more of the enzyme than the other two species. A. caudatus and A. hypochondriacus were not easy to differentiate because they produced single light staining anodal band.

The results presented here have not fully established the genetic relationship among the three species of grain amaranths. However, there

is some evidence, from the isozymes patterns of peroxidase and leucine aminopeptidase showing that A. caudatus and A. hypochondriacus are a little more related genetically. The results of esterase isozyme also suggest some closeness between A. hypochondriacus and A. cruentus but not A. caudatus. The evidences given here are not conclusive, and therefore more studies on the biochemical approach to evolutionary relationships are needed. But the present study gives a good start on this aspect.

ELECTROPHORESIS AS AN AID FOR IDENTIFICATION OF VARIOUS SPECIES AND CULTIVARS OF GRAIN AMARANTHS.

INTRODUCTION

Grain amaranth was once a major food crop of the Mexicans before Spanish conquest and was just comparable to maize and beans. Its relegation to the status of a secondary crop in the present times is probably a consequence of displacement by larger seeded grains such as maize, and suppression by the church during colonial period in an effort to eradicate the pagan ceremonies of the Aztecs in which amaranth played a central role. Grain amaranth is now cultivated as a minor food crop in Central and South America, Mexico, and parts of Asia and Africa. Currently the grain amaranth is enjoying revived interest as an agronomic crop by diverse groups. Amaranth is attractive because of several reasons. The leaves and grains of amaranths have high quality nutritius amino acid component with very high content of lysine and sulphur-containing amino acids such as methionine. Amaranth possesses the C_4 photosynthetic pathway, it is drought tolerant and besides it is widely adapted in various environments. Although weedy amaranths are used as vegetable crop in many parts of the world, there are however, very few published reports on yield, harvesting techniques and its nutritional value

Also little effort has been made on comparison of grain amaranth with other conventional grain crops.

Vegetable amaranths are popular in West Africa, India, Pennsylvania, Taiwan and parts of China, Philippines and weedy types are widely used in various parts of the world including Kenya as a potherb. The leaves are very rich in protein as well as in vitamins and minerals.

The grain amaranths are grown in India, Central America and the Andean region. The grains have a high protein content of about 16 percent and compares very well with conventional cereals (Appendix 3). The protein of amaranth has very high levels of lysine, much higher than even in opaque-2 (Appendix 4) and Floury-2 mutants of maize. The seeds also have high content of sulphur containing amino acid, Methionine. Since legumes are deficient in sulphur-containing amino acids, and cereals in lysine and grain amaranth in leucine which is abundant in cereals, complementation of grain amaranth in a diet of cereals and legumes would be desirable since the deficiency of one is complemented by the other. The grains can be used as breakfast cereals, or as ingredient in confectionery The grains can be cooked into gruel or milled to produce biscuits, bread, cakes and other baked goods if mixed with wheat flour. The grain is also a potential poultry and livestock feed.

The grain yield are optimistically high (Appendix

1 and 2). After improving of agronomic methods and seed quality by breeding methods grain yield may in future be much higher than it is now.

The genus Amaranthus belongs to the family Amaranthacea. The genus is known at least in part, to be a taxonomically difficult group. This is especially true of the Section Amaranthotyphus where a number of species do not readily exhibit defineable characters. The difficulty is brought about by natural interspecific hybridization and introgression between different taxa. In addition, many species are widespread which adds to their variability and has resulted in different interpretations and nomenclature by different individuals. Identification methods used to distinguish between the various species and cultivars, are largely based on morphological characteristics. But morphological characterization suffer a number of drawbacks. They tend to show a wide range of overlap and some of these are very much controlled by environmental factors. The methods that have been used include: cytogenetics, cytomorphological, morphological and allozyme variation. Recently a combination of morphological and allozyme variation has also been tried. None of these methods is satisfactory on its own. Chemotaxonomic classification has not been tried on the genus. There is however still confusion in the nomenclature, where a given species

may have two or more different names depending on where it is grown. Report of the 2nd Proceeding of Amaranthus conference held in Kutztown, USA 1979, revealed that there is taxonomic problem of distinction between various species of the genus, it was therefore concluded in the meeting that taxonomy of the genus remain a major area for future research emphasis.

Electrophoretic technique for crop identification has been used greatly to supplement the existing methods of crop taxonomy. Using this technique, various taxa, species and cultivars of several agronomic crops have been confirmed and existing doubts and disputes in nomenclature settled. The technique is useful and therefore present study is undertaken to try it in the genus.

The main objectives of the study were:

- to find out if there are variations of isozymes in different amaranthus species and if the variant isozymes could be used to identify the various species and cultivars within species.
- to find out if the relationship between various species and cultivars could be assessed from the isozyme pattern.

2.0. LITERATURE REVIEW

2.1. Breeding and Botanical Aspects of Amaranths.

2.1.1. Breeding of amaranths.

Amaranth offers more genetic diversity in its present undeveloped state than do many conventional crops. Several features of their highly variable breeding systems and overall reproductive biology provide ample choice of breeding methods (Anon. 1984). The breeding objectives are many. Breeders need plants with desirable growth characteristics such as reduced plant size, reduced sensitivity to photoperiod, synchronous flowering, early maturity, reduced lodging, reduced shattering, high seed yield and increased seed size. Also plants are needed which have wider environmental adaptations such as drought tolerance, pest and disease resistance, herbicide tolerance and efficient fertilizer utilization. Finally breeders need plants with good food quality such as white seeds, palatability, high levels of protein and essential amino acids and reduced levels of anti-nutritive factors.

At present not much has been achieved of the above objectives. The major breeding methods currently in use are plant selection and plant introduction. Hybridization to achieve some of the above goals is just starting. Problems involved in hybridization of amaranths are many. Plants have different maturity

periods, no true to type cultivars are available i.e. the varieties used are still very heterozygous (Kauffman, 1985. Personal communication). The flowers are very tiny and largely monoecious.

2.1.2. Botanical aspects of amaranths

Members of the genus Amaranthus are widely distributed throughout the world and it contains about 60 species (Willis, 1973).

The growth habit vary from branched to unbranched, leaf and stem colours range from red to green. The head is consisting of large inflorescence made up of florets. Each floret consist of 3-6 flowers with one staminate flower surrounded by several pistillate flowers (Kauffman, 1980). Amaranths are thus monoecious and largely self-pollinated, although a small percentage of outcrossing of about 5-6% has been observed. In Pennsylvania less than 10% outcrossing had also been observed (Harwood, 1980). The crop is generally wind pollinated with very small proportion of insect pollination.

The chromosome number in the genus Amaranthus is 2n = 32 (A. caudatus; A. cruentus). and 2n = 34 (for A. tricolor). The chromosome number in the genus celosia is 2n = 36 or 72.

2.2 Centres of Origin and Diversity.

The origin of the various species of cultivated amaranths is not easy to trace because the wild ancestors are pantropical and cosmopolitan weeds (Grubben and Sloten, 1981). However, it is thought that the three weedy types A. spinosus, A. hybridus and A. dubius are tropical types, whereas A. retroflexus, A. viridis and A. lividus are more hot-season temperate weeds. Probably all the grain amaranths originated from Central and South America (Sauer, 1977: Grubben and Sloten, 1981), whereas the main vegetable type (A. tricolor originated somewhere in Southeast Asia, probably in India). The most important grain types A. cruentus could have originated in Gautemala, A. hypochondriacus in Peru and Argentina and finally A. caudatus somewhere in the Andean countries.

From that time, several secondary centres of diversity have developed in the main producing areas. The Hindustan centre of origin has become an important secondary centre of diversity for grain amaranth (Grubben and Sloten, 1981), but the crop has a great deal of genetic diversity in South America.

2.2.1. The germplasm collections.

The urgent necessity for establishing a global network of gene banks hardly needs stressing. The

genetic resources of crop plants are rapidly disappearing. For future development of amaranth, a huge gene pool is very important. In the Second Amaranth Conference 1979, an ad hoc meeting was convened to discuss the international cooperation between curators of collection and users of amaranth germplasm. The very important conclusions reached by the meeting was later endorsed by the International Board of Plant Genetic Resources (IBPGR). One of the conclusions was that priority should be given to collection missions in India, Nepal, Bangladesh, Africa, North Mexico, Central America, Andes, Southeast Asia and China. And that on behalf of IBPGR, the germplasm will be stored, evaluated and maintained by the following centres: Colorado will be responsible for collections in Asia, India for collections in Asia and that African collections will be catered for by Nigeria.

2.2.2. Production of grain amaranths.

Production of grain amaranth require optimal edaphic, environmental and agronomic factors just as any other agricultural crop. Field observation have indicated that amaranth is a heavy feeder and will only do well in fertile soils that are freely drained and properly aerated. It does well in soils with high levels of calcium and phosphorus. Amaranth will do well, where the daily

temperatures are above 21°C. A. hypochondriacus and A. cruentus do well at a temperature range of 21-35°C, while A. caudatus perform better at lower temperatures.

Grain amaranths are in general drought tolerant and have been grown in dry-land areas receiving as little as 200 mm of annual rainfall. However, grain amaranths would be ideal crop for regions receiving between 600-800 mm of rainfall annually, though vegetable amaranths need higher rainfall. Many of amaranth species are sensitive to day-length.

Amaranths compete very well with weeds and also does well at high plant density. Optimal population is about 320,000 plants per ha. Where machine harvesting is done a spacing of 75 cm x 30 cm has been recommended (USA). In Kenya, a closer spacing of 40 cm x 20 cm gives higher yield.

2.2.3. Uses of grain amaranths.

Amaranths are used as ornamental plants in many parts of the world for example love-lies bleeding (A. caudatus). The grain and leaves have been used as human food by various people. Vegetable amaranths have also been tried as cattle fodder.

2.3. Amaranth Types.

2.3.1. Vegetable amaranths.

Most Amaranthus species have edible leaves and several species are already used as potherbs. However, the species used as vegetable in different parts of the world are: A. tricolor, A. dubius, A. hybridus, A. lividus and A. cruentus.

In West Africa protein from leaves provide as much as 25% of daily protein intake during harvesting season (Grubben, 1976). The species A. hybridus and A. cruentus are among the most important leafy vegetables in southern part of Nigeria. The vegetable yield in Nigeria is between 21.6-50.7 tonns/ha.

In Taiwan and Philippines, the most popular vegetable type is A. tricolor and the vegetable yield is 20 tonns/ha in 25-28 days (Deutsch, 1977). In Pennsylvania and Puerto Rico, the vegetable types grown are A. dubius, A. tricolor and A. blitum (Daloz, 1980). In India extensive studies has been done on A. tricolor and A. dubius as some of the popular vegetables (Mohideen and Rajagopal, 1975).

2.3.2. Grain amaranths.

The main grain species are A. caudatus, A. hypochondriacus and A. cruentus. The former is one of

the major crops in the Andean highlands of Argentina, Peru and Bolivia. The grain is toasted and popped, ground into flour or boiled into gruel. A. cruentus is grown in Mexico and Gautemala both as leafy vegetable and grain crop. It is the most adaptable of all the grain types (Sauer, 1977). A. hypochondriacus is the highest yielding of the grain types. It is cultivated maximally to-day in India in the states of Himachal Pradesh and Uttar Pradesh.

2.3.3. Weedy amaranths.

Of all the 60 or so amaranth species, only a handful are now used as a crop. A few others are now serious weeds in various parts of the world. The main weedy types are A. viridis, A. spinosus, A. retroflexus and A. hybridus. The weedy types are generally indeterminate in growth habit and scatter seeds during dry periods.

These weedy types can be gainfully used as forage crop, and also can be useful to breeders in inter- or intraspecific hybridization. Amaranthus hybridus is thought to be the wild progenitor species of the present-day cultivated grain type A. hypochondriacus (Annon. 1984).

2.3.4. Problems of growing amaranths.

Certain botanical characteristics of grain amaranths

impose agronomic disadvantages. There is very slow seedling growth during the early stages of crop establishment, resulting in poor competitive ability with weeds during this critical stage of development. Besides, this may cause the seeds and seedling displacement if heavy rain preceed germination and seedling establishment. Seed shattering is another serious weakness of grain amaranths. One inherent cause of seed shattering is that individual spikes of maturing plant simultaneously contain ripe and near ripe seeds along with immature ovules at various developmental stages (Schimidt, 1977; Hauptli, 1977). Another undesirable trait is that bracts which subtend individual florets have sharp thorn-like points which can be a source of discomfort to workers handling the crop at harvest. The small seeds are difficult to clean by winnowing or other means commonly available to peasant farmers.

2.3.5. Amaranth grain yield.

Several observations made about grain yield of amaranths indicate that its grain yield is in fact comparable to those of most cereal grains (Appendix 2). Yield of 5-6 tonnes/ha was obtained in Ethiopia (Schimidt, 1977); 3.6 tonnes/ha in Pennsylvania (Vietmeyer, 1982); 3 tonnes/ha in India (Joshi, 1981a) and 3.9 tonnes/ha in Kenya (Gupta, 1985). This is promising and may be higher

in future.

2.3.6. Pests and diseases of amaranths.

Several pests and diseases have been observed at various amaranth growing regions of the world. The most serious diseases in Dahomey are damping-off caused by Pythium aphanidermatum, and wet-rot caused by Choanephora cucurbitarum (Grubben, 1976). In Pennsylvania the soil fungus (Alternaria alternantherae has been reported to cause a lot of leaf damage (Annon. 1984). In Kenya the major diseases observed were Alternaria amaranth; and Albugnia spp. (Gupta, 1985). The disease incidence was observed during wet periods. The Albugnia fungus attacked only A. caudatus species but did not attack the other species.

Insects and birds also cause great deal of crop loss. Caterpillar, Hymenia recurvalis F. and stem-borer (Lixus truncatulus F.) were reported in Dahomey (Grubben, 1976). In Pennsylvania lygus bug(Lygus lineolaris) leaf miners have been observed to cause some damage (Anon, 1984). In Kenya the following pests were observed to cause damage; larvae of Heliothis armigera, aphids, mites, tetranychus spp. (Gupta, 1985).

2.4. Nutritive Aspects of Amaranths.

2.4.1. Nutritional aspects of vegetable amaranths.

Vegetable amaranths provide many important nutritive factors such as protein, vitamins and minerals. Anti-nutritive factors are also present but at levels that are comparable with other conventional vegetables. These anti-nutritive factors are oxalates and nitrates.

Vegetable amaranth contain a significant amount of usable protein which is about 26-33% (Imbamba, 1973; Annon., 1984; Deutsch, 1977; Grubben, 1976). Vitamins A and C have been reported as important components of vegetable amaranth. (Grubben, 1976; Devadas and Saroja, 1980). Vegetable amaranth contain significant amount of minerals such as iron, calcium and others that are important in human nutrition (Oke, 1980).

2.4.1.1. Anti-nutritive factors in vegetable amaranth.

Very many researchers have reported the presence of oxalates and nitrates in vegetable amaranths (Connor et al., 1980; Cheeke and Bronson, 1980; Oke, 1980; Der Maderosian, 1980). Toxicity problems have also been reported in animals fed vegetable amaranth (Marshall et al., 1967). However, Mugerwa and Stafford (1976) have shown that vegetable amaranth were good source of forage for sheep. Most researchers have concluded that there is no danger of feeding on vegetable amaranth

since amounts eaten per day is small and could not constitute any problem, given that vegetable are boiled, crushed and water discarded, which removes much of the nitrate and the soluble oxalate.

2.4.1.2. Leaf protein isolates.

A future promise of vegetable amaranths is the development of leaf-protein concentrates. Leaf protein concentrates (LPC) is one of the several possible new protein products developed for both human and animals. When compared with other species of vegetable amaranth leaf protein is highly extractible and the protein quality of the amaranth leaf nutrient concentrate (determined by amino acid composition, digestibility and nutritional effectiveness) is excellent.

2.4.2. Nutritive aspects of grain amaranths.

2.4.2.1. The physical composition of amaranth grain.

The seeds are very small just about 1000-3000 seeds per gram. Amaranth germ and bran constitute 26% of the seed; the flour 74%. When the whole grain is milled, its protein, vitamins, fat and minerals are concentrated significantly in the bran and germ fraction (Kurien, 1967a). Amaranth germ can contain as much as 30% protein. It also contain about 20% oil. The bran

is high in fibre, protein, vitamins and minerals. It was found that seed contain 15-18% protein; 50-70% starch; 3% fibre; 4-8% fat and 3-4% ash (Becker et al., 1981; Carlsson, 1980) (See Appendix 7).

2.4.2.2. Chemical composition of amaranth grains.

The chemical composition has been worked out by various researchers (Appendix 4). Amaranth has average protein content of 16% which is somewhat higher than that found among commercial varieties of common cereals. In addition, amaranth protein contains relatively high levels of lysine and sulphur-containing amino acid especially methionine in comparison to the other more common grains (Appendix 4). It is however lacking in leucine, which is found in abundant in other cereal grains (Senft, 1981). Thus its protein in combination with that from the more common cereal grains, yields protein which very closely approximates to the FAO/WHO recommended composition (Appendix 5).

Ramachandran and Phansalkar (1956) refering to the seed as 'rajkeera' gave the chemical composition of amaranth seed and biological value as 77. A protein range of 14-18% has been reported by various workers (Elias, 1977; Downtown, 1973; Connor et al., 1980; Carlsson, 1980; Senft, 1981; Hauptli, and Jain 1978 and many others).

The amino acid composition has also been given by various researchers (Appendix 3). Senft (1981) reported lysine content of various amaranths as about 5.0% on the average and that of the sulphur-containing amino acids as 4.4%. Connor et al. (1980) reported similar figures for lysine and Downtown (1973) working on A. edulis found lysine content of grains to be 6.2 g/100 g protein and 5.9 g/100 g protein in the leaves comparing lysine content with that of opaque-2 and floury-2 maize mutants, he found that A. edulis contain 25-30% more lysine than these two mutants (Appendix 6). Thus amaranth's importance is that its essential amino acids component complement those of corn, wheat, and rice and also legumes.

2.4.2.3. The nutritive value of amaranth protein.

The nutritional value of amaranth protein is very good. Protein Efficiency Ratio (PER) ranges from 1.5-2.0 (corrected to casein 2.5) (Betschart et al.,1981). The digestibility of cooked grains is about 90%. Amaranth protein at a biological value of 75 comes closer than any other grain to perfect balance of essential amino acids, which theoretically would score 100 on the nutritionist scale of protein quality based on amino acid composition (Appendix 4). When amaranth flour is mixed with that of maize, the combination almpst reaches the

perfect 100 score, because the amino acids deficient in one are abundant in the other (Senft, 1981).

The fatty acids of amaranth oil comprises of 70% oleic acid and linoleic acid, about 20% stearic acid and about 1% linolenic acid. The oil also contain very high levels of squalene which is a high priced fatty acid normally obtained from shark liver and used in cosmetic Industry (Anon., 1984).

2.4.2.4. Processing of amaranth grains.

Whole amaranth grain can be made into porridge by simply boiling it briefly in water. If toasted or parched lightly the grain becomes a pleasant-tasting food, that can be eaten without further preparation. The whole grain can also be prepared by popping or puffing the whole grain into white kernels that taste like pop-corn. This preparation is common in Mexico and Central America where popped grain is usually used in confections and candiments. Grinding the whole grain to obtain flour for use in other food preparation is also done. Popped amaranth is also used as cereal in various parts of the world.

2.4.2.5. Grain use in other foods.

Amaranth meal or flour is especially suitable for unleavened (flat) breads, where it can be used as

prominent cereal ingredient. The flour is used in Latin America and Himalayas to produce a variety of flat breads (for example tortillas and chapatis).

For making yeast-raised breads, amaranth flour must be blended with wheat meal because it lacks in functional gluten. The high lysine content of amaranth, improves protein quality of the blended food.

Amaranth can be used in many other foods including: soups, pancakes, porridge etc (Anon., 1984).

2.4.2.6. Grain feed.

Unprocessed amaranth grain probably can be used as animal feed, particularly for poultry. It will soon be tested extensively in livestock feeding trials, just as the vegetable types have been tried as fodder for livestock.

2.5. Identification Methods Used in the Genus Amaranthus.

One of the requirements for the effective collection and utilization of the germplasm is the precise and accurate identification of the materials handled (Elias, 1977). The species description and characterization, botanically, cytologically and biochemically is essential to the collection and utilization work and must be one of the early priorities

of any crop development (Harwood, 1980).

2.5.1. Problems of identification in Amaranthus.

The genus Amaranthus is known at least in part to be a taxonomically difficult group (Sauer, 1950; 1977). This is especially true of the section (Amaranthotyphus) where a number of species do not exhibit readily defineable characters to separate them as distinct entities. The difficulty is brought about by natural interspecific hybridization and introgression between different taxa (Sauer, 1950; Grant, 1959b; Edema and Fakorede, 1978). In addition, many species are widespread which in all probability has resulted to their variability and resulted in different interpretations and nomenclature by different individuals (Sauer, 1977; Grubben, 1976). Thus considerable synonymy has existed and is still evident in literature.

The taxonomic problems in the genus Amaranthus has been reported by various workers (Grubben, 1976; Annon., 1984; Feine, 1980; Hauptli et al., 1980; Harwood, 1980; Jain et al., 1980 and others). Report of the 2nd Proceeding of Amaranthus held in Kutztown, USA 1979, revealed that there is taxonomic problem of distinction between various species and it was concluded that taxonomy of the genus remain a major area for future emphasis.

2.5.2. Identification methods.

The conventional methods used to distinguish between various species and cultivars in the genus Amaranthus are largely based on morphological characteristics, such as leaf size and shape, inflorescence structure and floral parts, seed shape and colour and others. Several people have used the morphological and physiological characterization to distinguish the various species and cultivars.

Kowal (1954), based on a study of the morphology and anatomy of seeds of 21 species of Amaranthus has regrouped and proposed a new section for the genus, namely Puncticulata. Sauer (1950) gave taxonomic treatment of the species of the genus Amaranthus including their dispersal from Central America into Asia. Recently Pai-chi Huang (1980), used both morphological and physiological characters to group sixty-five accessions collected from Taiwan and other countries into species. He managed to group all the sixty five accessions into 8 different species. Feine (1980), has also used the morphological characterization in the genus very successfully and prepared a provisional key to help identify various species. Yet Anon (1984), still admitted that there is still confusion in the genus and suggested that a monograph of the family is needed. However,

morphological and physiological characterization suffer a number of drawbacks. The method is not rapid and normally take a long time. Secondly, the morphological characters tend to show a wide range of overlap between different species and besides, some of these are very much controlled by environmental factors and therefore not consistent.

Cytogenetic studies have also been tried to help in taxonomizing the genus. Since the knowledge of chromosome numbers has been an aid in revealing the relationships between taxa, it helps in the biosystematic investigation of the genus. Grant (1959b),did some extensive study of the chromosome number of 30 species in the genus. He found that several species shared the same chromosome number (2n = 32; 2n = 34). Kowal (1954), also studied the chromosome number of various sections in the family (Amaranthotyphus Dumort; Blitopsis Dumort and Puncticulatea Kowal). He found that chromosome number between different sections or within one section may be the same. Olusi (1979), tried studying chromosome number of some five species of Amaranthus and found out that chromosome number of members of different species he studied were similar. Pal and Khoshoo (1973a; 1973b) did some cytogenetic study to determine the relationship between the grain amaranths and vegetable amaranths, they observed that

different species may have same chromosome number. Thus it can be noted that the family Amaranthaceae is characterized by small sized chromosomes, this coupled with considerable homogeneity in the chromosome numbers of various species make cytogenetic study less sensitive in distinguishing the various species.

Olusi (1979) tried cytomorphological characterization of the five different species (A. spinosus, A. tricolor, A. lividus, A. viridis and A. hybridus). He counted one hundred pollen grains of each species, investigated pollen size, frequency of occurrence and fertility. A. spinosus and A. viridis showed little variation, with a range of 14-25 microns and 14-23 microns respectively. The percentage fertility was high in all the classes ranging from 82-92% with A. hybridus as the highest and A. viridis as the lowest.

Hauptli and Jain (1978) used a combination of morphological characterization and allozyme variation to study biosystematics between wild and cultivated species of Amaranthus. They used five isozyme systems (alcohol dehydrogenase, esterase, leucine aminopeptidase acid phosphatase and Glutamate oxalate transaminase). They were able to distinguish easily between the weedy species and cultivated species. Jain et al. (1980) used a combination of morphological and allozyme variation

to study the evolutionary pathway of Indian grain amaranths.

It is evident that little or no chemotaxonomy of the genus has been done. And also that despite the above methods of identification, there is still confusion in the taxonomy of the genus.

2.6. Electrophoretic Technique.

Electrophoresis is basically a process of forced diffusion of charged particles within a supporting medium. The proteins are separated based on their differences in net charge, size and shape. The separation takes place at a constant pH and ionic strength, when the electric field is applied. The separation takes place because of the different mobilities of the sample proteins, the separated zones migrate one after the other through the gel. Appropriate histochemical staining techniques are then employed to provide visible 'Finger prints' or 'Zymograms' (Pierce and Brewbaker, 1973).

The charged proteins in the study are sometimes referred to as isozymes. The term isozyme was first coined by Market and Moller (1959) to describe the multiple molecular forms of an enzyme (protein) which exhibit similar or identical substrate specificity occuring within the same organ or tissue. The reason

for occurance of isozyme remain obscure (Shanon, 1968 Scandalios, 1974). Isozymes are direct expressions of gene function during cell differentiation in a developing system. Isozymes occur widely in both plant and animal tissues. Electrophoretic analyses show that isozyme pattern and intensity are specific to the part of the plant or tissue and to the maturity or developmental stage (Pierce and Brewbaker, 1973; Bassiri and Adams, 1978). Certain factors affecting plant metabolism such as mineral nutrition, disease infection, insect infestation (Tuikong, 1982) and cold temperature hardening are known to influence the intensity of the isozyme bands and occasionally cause appearance of different isozymes.

Numerous reports have appeared in literature which discuss the use of isozymes in several scientific studies in both plants and animals alike. These include identification of repeatable differences among taxa, exposition of Mandelian segregation of isozymes, tracing of probable paths of evolution and associating enzyme activity with external factors affecting plant growth (Sheen, 1970).

In genetic studies, electrophoresis has been used to study genetic variability in barley (Kahler and Allard, 1970), genetic polymorphism in safflower (Bassiri,

1977) and in barley (Upadhya and Yee, 1968) and also in maize (MacDonald and Brewbaker 1972). More genetic work has also been reported for genetic linkage in various crops. Kahler and Allard (1970) reported linkage between three loci in barley and even worked out recombination values. Kahler et al. (1981) also reported linkage in barley. Gorman and Kiang (1978) reported linkage in soybeans and Brown and Allard (1969) also reported linkage in maize.

Electrophoresis has also been used in tracing evolutionary pathway of various crops. Jain et al. (1980) tried to trace the evolutionary pathway of Indian cultivars of amaranths. Bassiri and Adams (1978) worked out the evolutionary relationship of various species of bean Phaseolus vulgaris.

The technique has also been used a lot in Plant Breeding studies, especially those dealing with disease resistance, testing performance of hybrid and identification of actual hybrid from a successful cross. In Kenya, (Nyachae, 1979) attempt was made to screen potato clones which were resistant to late blight of potato using electrophoretic pattern of peroxidase isozymes. In beans, Gupta et al. (1981) observed certain peroxidase isozyme bands which appear at specific stages of development in lines susceptible to

bean anthracnose. Okiror et al. (1982), observed electrophoretic differences between bean lines known to be resistant and susceptible to bean anthracnose, with the help of peroxidase and esterase isozyme patterns.

2.7. Electrophoretic Technique as a Tool for Identification of Various Crop Taxa, Species and Cultivars.

Reliable Identification of cultivars using classical methods based on morphological characters has become increasingly difficult because of the large number of lines released and the convergence of these lines on a few of the most desirable characters. Time and resource requirement of growout tests and their dependence on "normal" environmental conditions make such procedures impractical. For this reason biochemical techniques such as electrophoresis are becoming increasingly important in identification of crop taxa, species and cultivars. The method is rapid, besides, a large number of genotypes can be identified using isozyme pattern of one particular isozyme or a combination of isozyme patterns of two or more isozymes patterns or proteins specific to species or cultivars. Isozymes are little affected, by differences in ecological factors (Lee and Ronalds, 1967; Loeschcke and Stegemann, 1966). This is a big advantage over conventional methods of identification which employ mainly morphological

characters some of which are strongly influenced by environmental factors in which the crop is grown. The second advantage of electrophoretic technique is that besides, being consistent over various environments, the method is rapid and only requires a small part of the crop for example seeds or seedling parts can be used satisfactorily, unlike morphological characterization which may probably require all the stages in the life cycle of the crop.

Electrophoresis has been used extensively to study the taxonomic relationship by means of isozyme pattern in various crop taxa and species. In the species Nicotiana, a lot of study has been carried out, that has confirmed the existing well-established taxonomy of the genus (Hart and Bhatia, 1967; Sheen, 1970; Smith et al., 1970).

In wheat, Johnson (1967; 1972), Johnson and Hall (1966), and Johnson et al. (1967), employed electrophoretic technique for assessing the genomic and evolutionary relationships between different Aegilops and Triticum species. Menke et al. (1973), studied the electrophoretic patterns of eleven common American wheat varieties, one durum wheat and one triticale variety. With the help of esterase and peroxidase isozymes the durum wheat and triticale varieties were easily distingui-

shable from all other varieties. However, two common wheat varieties had similar patterns and could not be distinguished. Wrigley and Shepard (1974) also managed to identify Australian wheat cultivars using starch gel electrophoresis of extracted gliadin from a single grain.

In Gossypium, Johnson and Thein (1970) found that protein patterns of 25 species to be largely consistent with the conventional classification of diploid into six genomic groups. Cherry et al. (1970) also found isozyme analysis to corroborate the earlier cytological separations of the genomes in Gossypium with minor deviations. They further found intergenomic isozyme differences to exceed differences among species or varieties within a genomic group.

By use of acid phosphatase and peroxidase isozyme pattern, Bassiri (1977), found close genetic relationship between the common (Carthamus tinctorius L.) and wild (C. oxyacantha BIEB) safflowers. He concluded that either the two species had a common ancestor or that C. oxyacantha was the progenitor of C. tinctorius.

The species and genetic relationship between Phaseolus, species have been studied a lot by conventional methods, but recently chemotaxonomic methods such as electrophoresis has been used to a limited extent. By use of disc electrophoresis Boulter et al. (1967) characterized

and put forward the systematics of several tribes within the Leguminosae. They found that in the tribe Phaseoleae: Phaseolus acutifolius, P. aurens, P. calcaratus and P. vulgaris possessed idential patterns for seed globulin. Bassiri and Adam (1978) using starch gel electrophoresis surveyed the isozymes in several Phaseolus species. They used esterase, acid phosphatase, and peroxidase banding patterns from various tissues of the seedlings from 13 species of beans within the genus Phaseolus. Most species showed unique banding pattern in each isozyme system. They,however, found close banding patterns for the domestic P. vulgaris, wild P. vulgaris and wild P. coccineus indicating close genetic relationships, possibly through species introgression or common descent. Weeden (1984), also used allozyme genotypes to distinguish among white seeded bean cultivars. Out of 90 cultivars of white seeded snap beans, 52 cultivars could be uniquely distinguished by allozyme genotypes alone. The remaining 38 lines could be separated into small groups of 2-5 cultivars each. By use of gel electrophoresis, Bassiri and Rouhani (1977) could identify a large proportion of the 40 cultivars of beans using esterase and peroxidase isozyme patterns.

A lot of electrophoretic study has also been done in Barley. Mc.Daniel (1970) by separating the proteins in diploid tissue (embryonic axis and Scutellum)

of seed of several Hordeum species and varieties by disc gel electrophoresis found that specific qualitative and quantitative protein patterns showed greater variation between cultivars within a species than between species. Kahler and Allard (1970) studied the electrophoretic pattern of esterase in barley seedlings. In their study they concluded that esterase isozyme are useful research tool for determination of the extent and geographic distribution of allelic variability in barley populations. Fedak (1974), combined plant morphological characters and isozymes for identification of Canadian barley cultivars. Bassiri (1976) managed to successfully distinguish most of twelve cultivars of barley using shoot extracts of esterase alone. Hvid and Nielsen (1977) used esterase isozyme successfully to distinguish between various cultivars of barley.

Electrophoretic technique also proved useful in the identification by six bent creeping grass cultivars (Agrostis palustris) and eight Kenturky blue grass(Poa pratensis) using protein banding pattern (Wilkinson and Beard, 1972).

In rice, Gupta and Malik (1980) studied the salt soluble seed protein of seven different genotypes using polyacrylamide gel. The seven genotypes could be identified using a total of 28 bands of proteins in the

genotype. In alfalfa Quiros (1980), used acid phosphatase, esterase and peroxidase isozyme systems to distinguish between 21 alfalfa mother plants. The alfalfa clones identified included 18 tetraploids and 3 hexaploids.

Tuikong(1982) used starch gel electrophoresis to differentiate between twelve Kenyan commercial clones of pyrethrum which comprised of ten diploids and two triploids. By use of anodal peroxidase isozyme alone, he was able to distinguish even closely related clones of pyrethrum, based on seven different anodal bands.

Desborough and Peloquin (1968) used esterase and peroxidase enzymes and protein patterns for the identification of 45 potato cultivars. They obtained five esterase, eight peroxidase bands and more than 20 bands of protein in unique combinations specific for each cultivar.

Electrophoretic patterns have been used on other crops as well. Brown and Allard (1970) used the method to estimate mating system in open pollinated maize population. In pea nuts (Cherry and Ory, 1973), in tomatoes (Rick and Fobes, 1975) and in very many other crops.

In the genus Amaranthus very little

chemotaxonomic reports appear in literature. Jain et al. (1980) used allozyme and morphological characteristics to study evolutionary pathway of Indian grain amaranths. Hauptli and Jain (1978) used a combination of morphological characteristics and allozyme variation to study the biosystematics between wild and cultivated species of Amaranthus.

There is ample evidence pointing to the potential of electrophoretic technique in identification of crop species and cultivars. The technique may be of use in Amaranthus where there is difficulty in systematics and distinction of various species.

3. MATERIALS AND METHODS.

3.1. Materials.

The study included twelve varieties of grain amaranths. Eleven of these varieties were from Rodale Research Centre (USA) sent to Prof. Gupta in 1983 and they have been multiplied at Nairobi University, Kabete Campus. One of the varieties called Jumla, however, is local variety (ex-Nepal) obtained from Katumani Research station (Kenya). The study was based on three cultivars of A. cruentus species, four cultivars of A. caudatus species and five cultivars of A. hypochondriacus species.

Varieties Acc. No.	Botanical Name	Country of origin
674	A. hypochondriacus	-
1008	" "	Taiwan
Jumla	" "	Nepal
1024	" "	Mexico
723	" "	Mexico
A-1113A	A. caudatus	Peru
A-1113B	" "	-
A-982	" "	-
A-713	" "	-
434	A. cruentus	Mexico
1011	" "	Mexico
1034	" "	Dahomey

These varieties had been multiplied and mild

selection for uniformity carried out, at Kabete. No true to type lines were available i.e. the cultivars used were generally heterogenous, homogeneity had not been satisfactorily achieved. The varieties accession numbers will be used throughout the text.

These varieties have been grown in a glasshouse, under controlled environment, where maximum temperature observed was 35°C and minimum 13°C. The experiment took 1 year, and composed of four glasshouse trials and one field trial. The twelve varieties were planted on plastic pots each about 10 litres capacity. The soil mixture was composed of forest soil; balast and horse manure in the ratio 8:1:1 respectively. The plants were watered twice each week.

One trial was conducted in the field, where each variety was replicated three times in a randomized complete block design. The purpose of this trial was to find out if there is environmental effects on the enzymatic patterns.

Samples for enzymatic analysis was taken at various stages of crop development from one week upto the time of senescence.

3.2. Methods.

Various enzymes assayed for, were analysed using various methods, and shall be described separately.

3.2.1. Preliminary trials.

Two preliminary trials were conducted to determine the best stage of analysis for the enzyme systems used and also to find out the best tissue to use and finally to find out if the different environments (glasshouse and field conditions) would give different isozymes patterns.

3.2.2 Enzyme extraction.

From the preliminary experiments, it was found out that: the best band resolution and large number of bands were obtained with seedlings between 1-2 months of age than with younger or older plants, and that leaf extracts of the just fully expanded leaves gave better isozyme patterns than roots, or apical meristems and finally that there was no difference between isozyme patterns from plants grown in glasshouse and those grown under field conditions.

One gram of fresh leaf tissue, from each of the twelve varieties was weighed out separately and thoroughly crushed in a pre-chilled mortar and pestle into which 3 drops of distilled water was added. The crude homogenate was used for the enzymatic analysis.

3.2.3. Starch gel preparation.

The starch was prepared as described by Shaw and

Koen (1968). Tris-citrate gel system was used for the enzymes esterase, peroxidase, acid phosphatase and leucine aminopeptidase, borate gel system was tried also for leucine aminopeptidase There was no difference in enzymatic patterns of leucine amino-peptidase due to the different gel systems used. The tris-citrate gel system was prepared as described by Kahler et al. (1981) 22.0 g of hydrolised potato starch (Connaught lab) was mixed thoroughly with 200 mℓ of gel buffer, this is 11% starch, which proved best for band resolution. The gel buffer was made of 0.015M Tris (hydroxymethyl) amino methane) and 0.003 M citric acid adjusted to pH 8.0. The borate gel system was prepared as described by West and Garber (1967) 22.0 g of hydrolysed starch was mixed with 200 mℓ of 0.03 M borate buffer pH 8.5

The buffer was poured into Erlenmeyer flask and then the starch was added, the flask was swirled thoroughly to suspend the starch evenly in the buffer. The suspension was heated over a medium hot flame with a constant rotary motion. Heating was continued for about 5-7 mins., suddenly the viscosity increased and the suspension became almost opaque. On continued heating and swirling, viscosity dropped again and the liquid became semi-transparent again and bubbles of gas was seen at the bottom of the flask. Heating was stopped short of boiling and the gel was completely de-aerated by

connecting the flask to a suction pump for about 10-20 seconds. Immediately after de-aeration, the gel was poured onto a plexiglass tray (17 x 14 x 0.6 cm) to settle uniformly. The tray was gently shaken to ensure that the edges and corners are uniformly filled. The gel was allowed to cool at room temperature for about 20-25 minutes and then the gel was covered by a wet polythene paper and cooling continued in a refrigerator at about $4^{o}C$ for about 40-45 minutes.

The tray buffer (electrode buffer) contained 0.3 M boric acid and 0.1M NaOH pH 8.6 for all the enzyme systems studied.

3.2.4. Application of the sample to the gel.

The gel was removed from the refrigerator, the polythene paper also removed, and a cut was made 4-5 cm from the cathodal end of the gel. Twelve paper wicks each measuring 6 x 10 mm were cut from Whatman paper No. 3 each to absorb sample from each variety. The crude enzyme extracted from the leaves were then absorbed in the wicks and excess liquid removed by pressing the wicks between filter paper (Whatman paper No. 1). The wicks were then inserted into the slit by a pair of forceps.

3.2.5. Electrophoresis.

The sponge clothes were used to connect the gel

to electrode chambers which contained the tray buffer. The polythene paper was replaced again to prevent evaporation of gel water and to ensure straight boundary of separation. A low voltage of 165V was initially supplied for a period of 20 minutes and then the sample wicks were removed before the power was increased to 300 V. This continued until the borate front had migrated about 9 cm. This took about 4-5 hrs. The electrophoresis was conducted inside a refrigerator at $4^{o}C$.

3.2.6. Slicing of the gel.

After electrophoresis, the gel was removed out of the refrigerator, the thickened ends cut off and then the gel was sliced horizontally into two using a slicer board with an adjustable horizontal fine stainless steel wire (32 gauge) supported on top of the two rails of slicer board. A notch was made in one corner of the gel before slicing to help in the identification of the upper and lower sections. Each gel slab was stained for a different enzyme or same enzyme using a different technique.

3.2.7. Staining of the gels.

Staining of the gels was done to detect or locate areas of enzyme activity. Each enzyme had its own

staining technique.

Areas of peroxidase activity were detected by using the staining method described by (Scandalios, 1964). The solutions used consisted of benzidine solution (1 gm benzidine: 9 mℓ glacial acetic acid: 36 mℓ water) and 3% hydrogen peroxidase. Equal amount of benzidine solution and hydrogen peroxidase solution were mixed together and poured on the gels. Blue bands appeared after 1-2 minutes and fully developed after about 15 minutes after which they turned brown and faded. Drawing and photographs were taken immediately before fading.

Esterase was stained according to the method described by Hvid and Nielsen (1977). The method employ α-naphythyl acetate as a substrate and fast Blue RR salt as a dye coupler. The staining solution consisted of 100 mℓ of 0.2 M phosphate buffer (pH 6.3) used to dissolve 0.15 g Fast Blue RR salt, then 8 mℓ of 1% α-naphythyl acetate (1 gm α-naphythyl acetate: 50 mℓ acetone, 50 mℓ water) was also added to the mixture. The gel was then incubated in the filtered solution for 1-2 hrs at 37°C. in the dark, and then fixed in 50% ethanol. Schematic drawing were made and photographs were taken.

For acid phosphatase the staining method described by Kahler et al. (1981) was followed. The

staining solution consisted of 0.1 g Fast Garnet GBC salt, 5 mℓ of 1 M sodium acetate buffer (pH 5.0); 92.5 mℓ distilled water; 0.5 mℓ 1% magnesium chloride (1 $MgCl_2$); 2 mℓ 1% α-naphthyl acid phosphate, sodium salt (1 gm in 50 mℓ acetone and 50 mℓ water). The GBC salt was dissolved in water, buffer, and $MgCl_2$. Then, the substrate α-naphthyl acid phosphate added to the buffer solution just prior to staining. Phosphate stains in 20-30 minutes at room temperature. The gels were rinsed with distilled water and then fixed in 50% ethanol.

Leucine aminopeptidase (LAP) was stained according to the method described by West and Garber (1967). A solution containing 40 mg L- leucyl-B-naphthyl amide HCl; 50 mg Black K salt in 100 mℓ of 0.2M Tris-maleate buffer (pH 6.0) was used to detect sites of leucine aminopeptidase activity. The gels were incubated in this reaction mixture until the sites were satisfactorily coloured, and were then rinsed thoroughly with distilled water before drawing and photographing done.

4.0. RESULTS

4.1. Results of Preliminary Trials.

The leaves gave better zymograms than any of the other tissues; meristems and stems. Also leaves gave better and more zymograms at the age of two months. There were no differences in isozyme banding patterns between plants grown in glasshouse and those grown under field conditions and therefore subsequent experiments were carried out in the glasshouse.

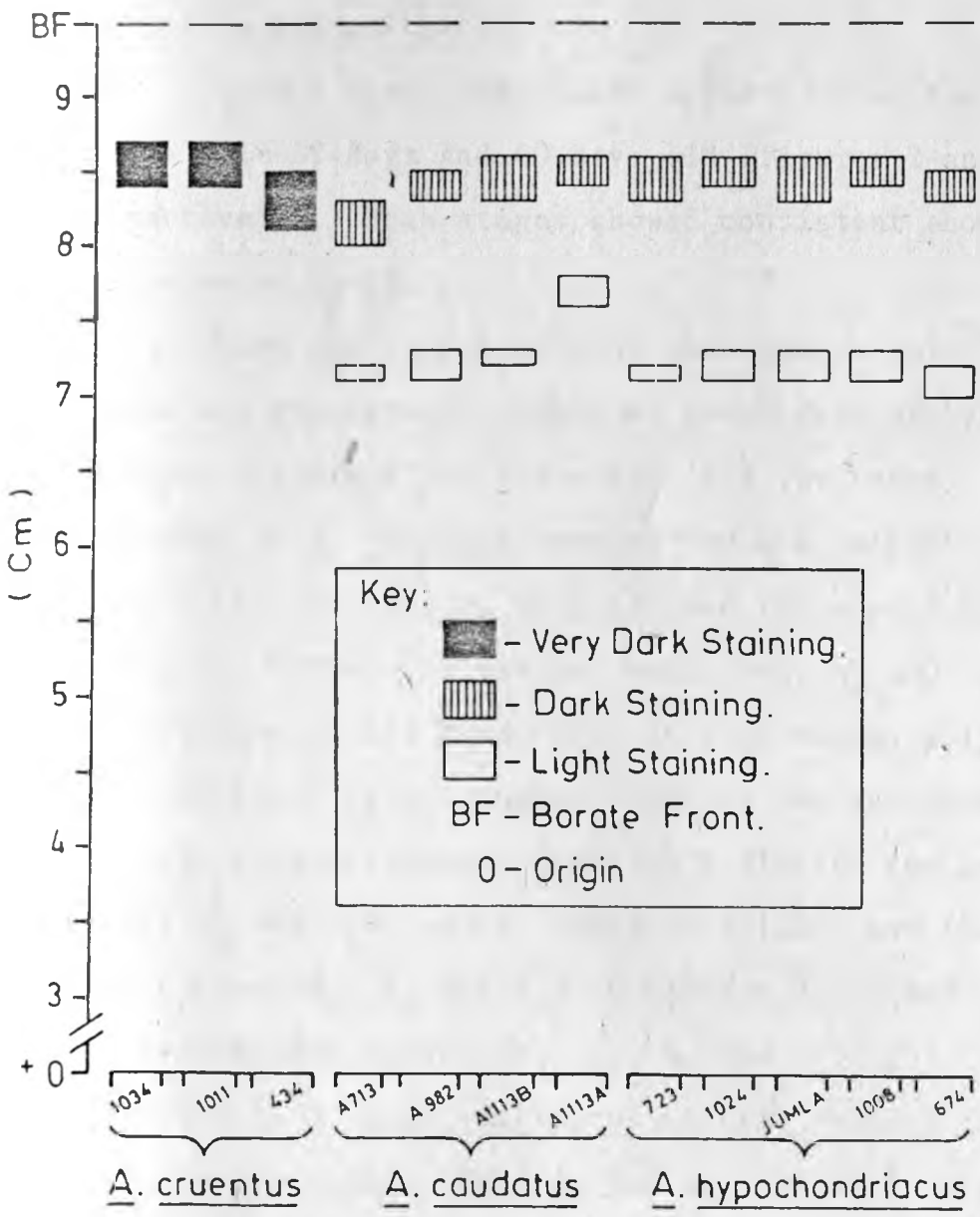
The following four isozyme systems were stained for the preliminary studies: peroxidase, esterase, leucine aminopeptidase and acid phosphatase. The results obtained with acid phosphatase were not useful for identification purpose. Two poorly differentiated bands were observed in the middle of diffused zone throughout the migration distance (Plate 1). There were however differences in the staining intensity among cultivars in these two monomorphic bands but the differences were insignificant as they showed no specific or unique patterns in various species, and cultivars. Therefore this isozyme system was not studied in subsequent experiments. The other 3 isozyme systems were useful for identification purpose and were studied in more details in the subsequent experiments.

4.2. Results of Subsequent Experiments.

4.2.1. Leucine aminopeptidase

This isozyme system showed monomorphism for all the three cultivars at all stages studied. Amaranthus cruentus species at an early stage (2-3 weeks) produced single monomorphic band that showed no difference in all the cultivars of the species. At this stage the other two species do not show leucine aminopeptidase activity. At a later stage (30-60 days) all the species produce single monomorphic band (Plate 2). A. cruentus species produce dark staining single band whereas A. caudatus and A. hypochondriacus produce single but light staining band. At about 70 days and above the intensity of the bands of these two latter species changed. The observation at this stage is that A. cruentus produced dark staining single band; A. hypochondriacus produce medium staining single band and A. caudatus produce light staining band. Ninety days and above, only A. cruentus has single dark staining band, whereas the other two species have two lights staining bands (figure 1) A. cruentus maintained single dark staining band at all stages of development.

Fig.1. Zymogram of leucine amino peptidase 90 days after planting (see Plate 2)



Note: A. cruentus show dark staining band other species show two light staining bands.

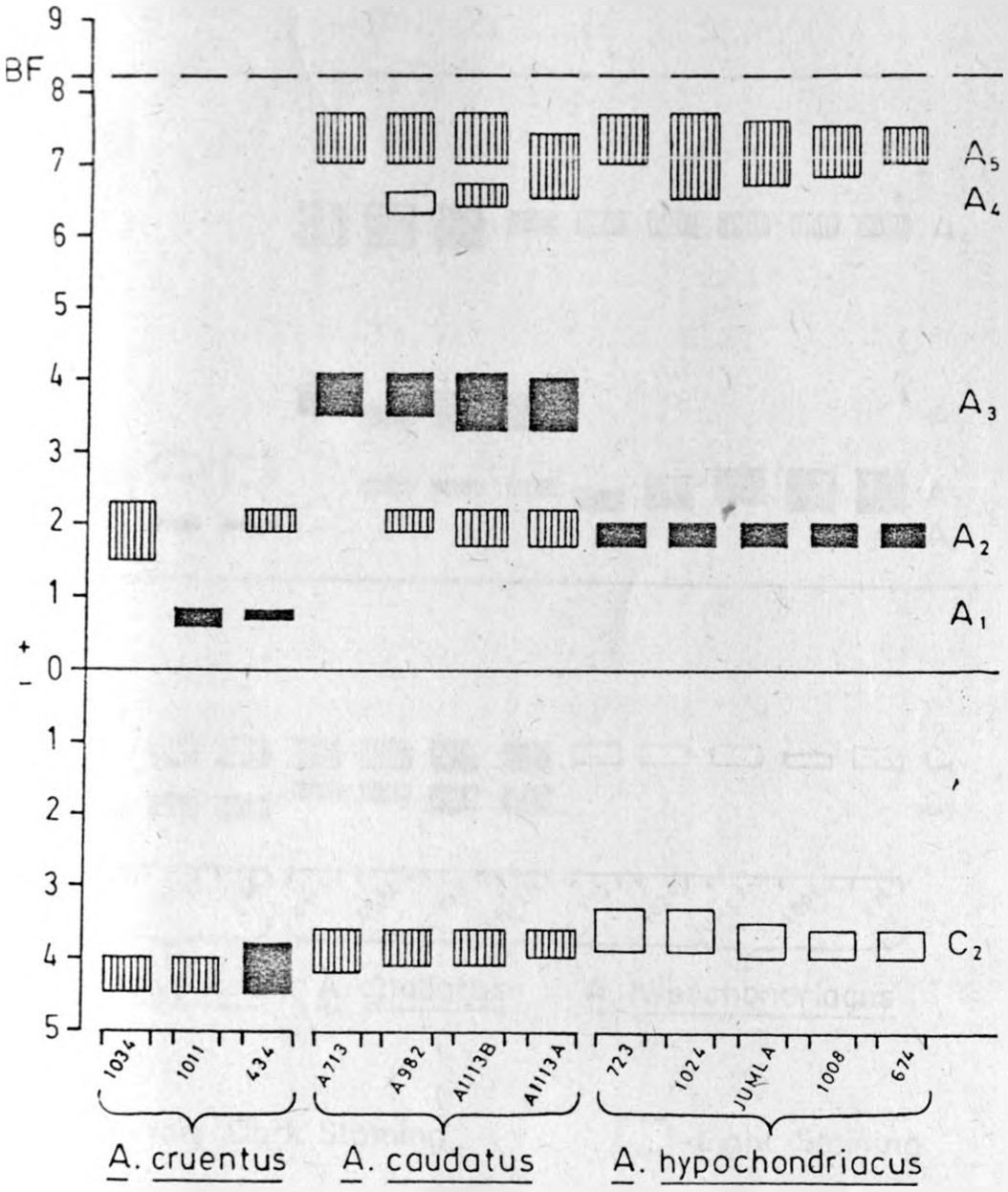
4.2.2. Peroxidase bands.

Useful peroxidase bands appeared when the plants were 52 days and 60 days old (Figures 2 and 3 respectively). Both stages showed consistent anodal and cathodal bands.

When the plants were 52 days old, a total of five anodic and one cathodic bands of peroxidase activity were detected (Figure 2 and Plate 3). All the three cultivars of A. cruentus species had one cathodic band. However, at this stage, only 434 had two anodic bands, A_1 and A_2. Cultivar 1011 had only anodic band, A_1 and 1034 had also one anodic band, A_2. At this stage, all the four cultivars of A. caudatus species had one dark staining cathodic band. Cultivar A-713 had two anodic bands, (A_3 and A_5) while cultivar A-1113A had three anodic bands (A_2, A_3 and A_4). Cultivars A-982 and A-1113B had four anodic bands; (A_2, A_3, A_4 and A_5). All the cultivars of A. hypochondriacus species had one cathodic band and two anodic bands A_2 and A_5. At this stage band A_3 was specific for A. caudatus as it was not present in other species.

At the stage of 60 days a total of four anodic and two cathodic sites of activity were detected (Figure 3 Plate 4). All the A. cruentus cultivars had two or one cathodic band. 1034 had only one cathodic band, C_2

Fig. 2. Peroxidase zymogram of grain amaranths 52 days after planting (see also Plate 3).

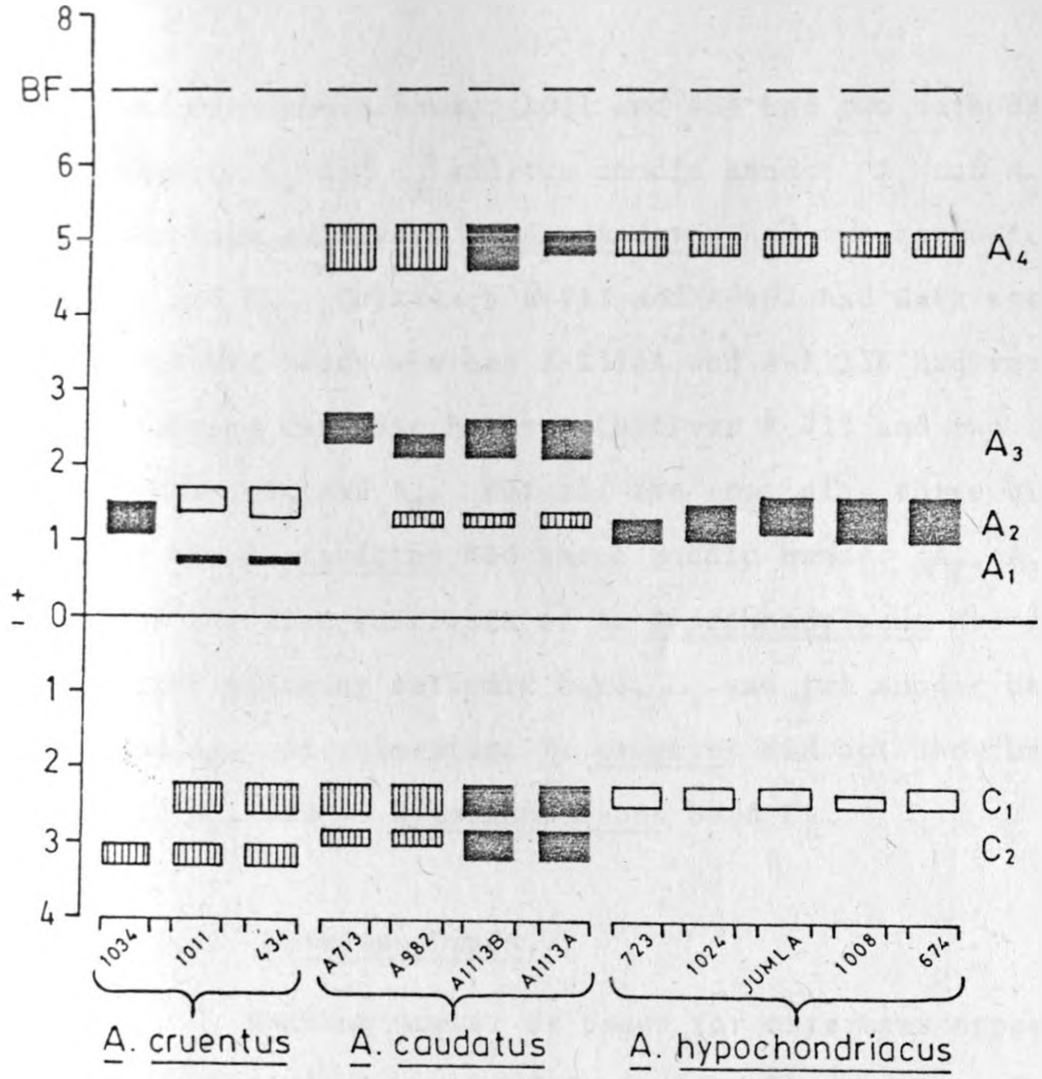


Key:

- Very Dark Staining
- Light Staining
- Dark Staining
- BF - Borate Front
- 0 - Origin

Note:
- Band A_3 is specific to A. caudatus
- Fastest band A_4 and/or A_5 is missing in A. cruentus
- A. hypochondriacus is lacking band. A_3 but has band A_5.

Fig 3. Peroxidase zymogram of grain amaranths 60 days after planting (See plate 4).



Key:

-Very Dark Staining -Light Staining

-Dark Staining BF-Borate Front 0-Origin

Note: - Band A_3 specific to A. caudatus

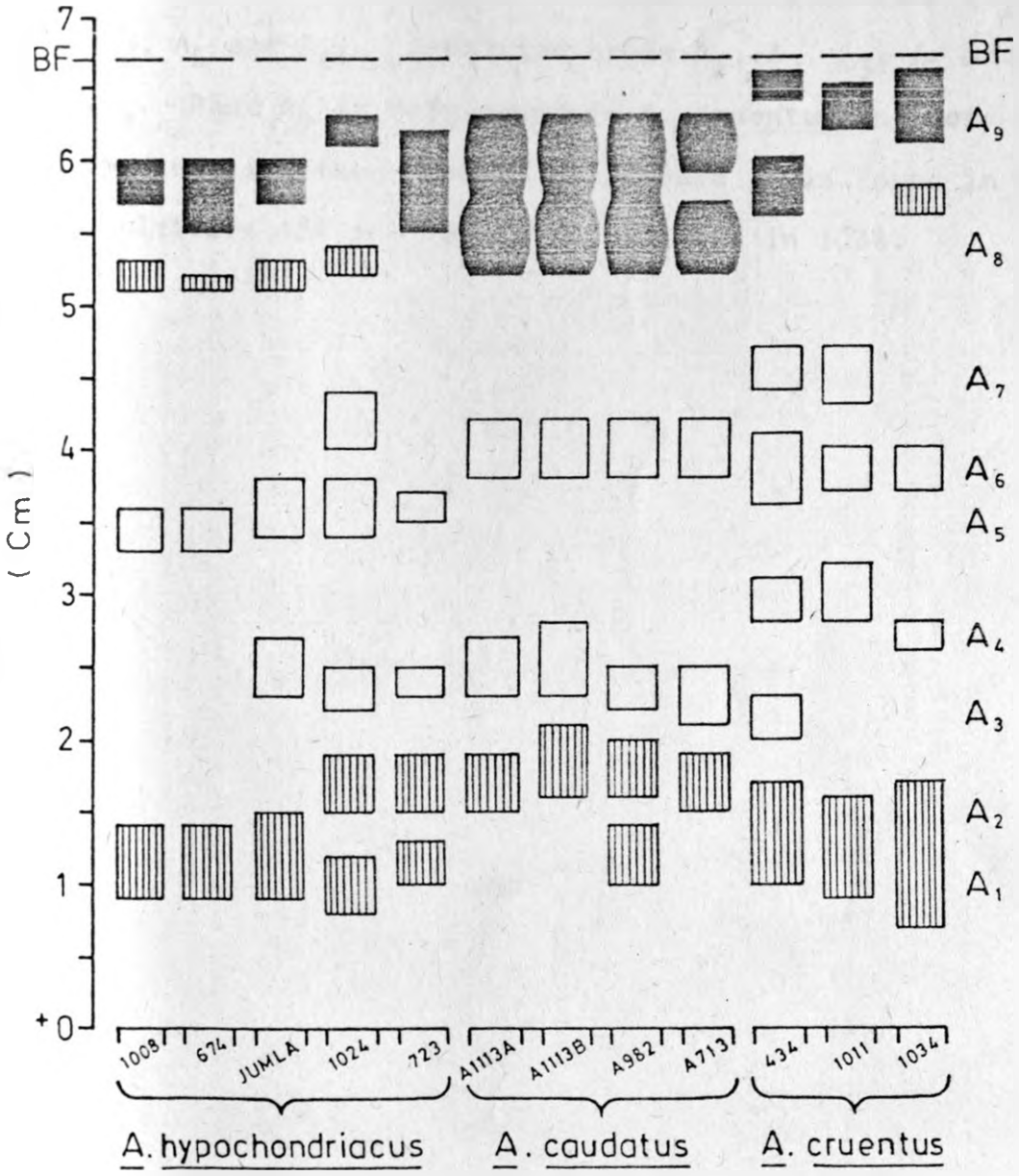
- Fastest band, Band A_4 is missing in A. cruentus
- A. hypochondriacus are lacking band A_3 but has band A_4 and has only band C_1 which is light staining.

and one anodic band. 1011 and 434 had two cathodic bands; C_2 and C_1 and two anodic bands; A_1 and A_2. All the four cultivars of A. caudatus had two cathodic bands: C_1 and C_2. Cultivars A-713 and A-982 had dark staining cathodic bands whereas A-1113A and A-1113B had very dark staining cathodic bands. Cultivar A-713 and two anodic bands: A_3 and A_4. But all the remaining three cultivars of the A. caudatus had three anodic bands: A_2, A_3 and A_4. All the five cultivars of A. hypochondriacus had only one light staining cathodic band, C_1 and two anodic bands A_2, and A_4. At this stage A. cruentus did not show bands A_3 and A_4, and A. hypochondriacus band C_2.

4.2.3. Esterase bands.

Maximum number of bands for esterases appeared when the plants were 60 days old. There was no esterases activity on the cathodic side, all the bands appeared on the anodic side only. A total of 9 anodic bands were detected (Figure 4 and Plate 5). Two cultivars of A. hypochondriacus 1008 and 674 had same number of bands(A_1, A_5, A_8 and A_9). Jumla cultivar had 5 bands: (A_1, A_3, A_5, A_8 and A_9). Jumla had band A_3 which the former two varieties did not have. Cultivar 1024 had seven bands(A_1, A_2, A_3, A_5, A_6, A_8 and A_9). Cultivar 723 had five bands: (A_1, A_2, A_3, A_5 and A_9).

Fig. 4. Esterase zymogram of grain amaranths 60 days after planting (see Plate 5).



Key:

- Very Dark Staining. - Light Staining.
- Dark Staining. BF - Borate Front 0 - Origin

Note: Band A_4 is specific to A. cruentus band A_8 and A_9 joined and very conspicuous specific to A. caudatus.

Cultivars A-1113A and A-1113B had similar bands (A_2, A_3, A_5 and A_8). A-982 had bands A_1, A_2, A_3, A_5 and A_8. Band A_4 is only found in A. cruentus and not present in other species while Band A_7 is found in the cultivars 434 and 1011 and not found in 1034.

5.0. DISCUSSION.

5.1. Acid Phosphatase.

Acid phosphatase was not useful for identification purposes because it had two poorly differentiated monomorphic bands which were common to all the 12 varieties investigated. Jain et al. (1980), studying the morphology and allozyme variation of Indian amaranths also found monomorphism of acid phosphatase at all the loci they studied. Tuikong (1982) also found monomorphism of acid phosphatase bands in the commercial varieties of pyrethrum grown in Kenya. He suggested that may be acid phosphatase has an important biochemical role in pyrethrum that is uniform in all the varieties and that no isozyme variation is permissible. However, Kahler et al. (1981) working with barley and Quiros (1980) working with alfalfa used acid phosphatase successfully for identification of varieties they studied.

5.2. Leucine aminopeptidase

This isozyme was very useful in distinguishing A. cruentus species from the other two species investigated at all the stages of crop development. But due to monomorphism observed in the A. cruentus species, it could not, however, be used to distinguish between cultivars of A. cruentus, neither could it help to

differentiate the cultivars of A. caudatus and A. hypochondriacus. This is because, at an earlier stage about 14-21 days leucine aminopeptidase produced only one monomorphic band with same intensity to all the cultivars of A. cruentus. At this stage leucine amino-peptidase activity is only present in A. cruentus species and not in other species. At about 30-60 days all the species produced significant leucine amino-peptidase activity, but the A. cruentus showed dark staining band whereas A. caudatus showed only light staining band. Thus A. cruentus species could be distinguished from the other two species on the basis of their earlier production of the enzyme and also on the quantity of the enzyme produced even at later stages. The distinction of the two species A. caudatus and A. hypochondriacus was not very easy although at about 70 days A. hypochondriacus produce medium staining single band and A. caudatus produce light staining band. Thus on the basis of quantity of leucine aminopeptidase produced at this stage the two species could also be identified.

Jain et al. (1980) found a lot of homogeneity in grain amaranths with respect to their banding pattern in various enzyme systems, including leucine amino-peptidase. West and Garber (1967) used leucine amino-peptidase, successfully to distinguish various species

of Phaseolus. Results of this study indicate that A. cruentus could be distinguished using this isozyme system, but not A. caudatus and A. hypochondriacus. Other enzyme systems have to be used to identiy them.

5.3. Peroxidase.

Peroxidase zymograms were very useful in identification of various species and to some extent cultivars within species (Figures 2 and 3 and Table 1). The combination of anodal and cathodal bands at 52 days and 60 days, helped in characterization of three Amaranthus species and the cultivars of A. cruentus and A. caudatus.

Using anodal zymogram, at 60 days, A. cruentus species could be distinguished from the other species because they only had A_1 and A_2 bands, but did not have bands A_3 and A_4 (Figure 3). At 52 days, band A_4 appeared in cultivars A-982 and A1113A but it was faint and not very consistent and therefore was not useful in identification of species although it may be useful in the distinction of cultivars.

The species A. caudatus is also distinguished from A. hypochondriacus and A. cruentus on the basis of band A_3. This band is specific to A. caudatus (Figure 3 and Table 1).

Using anodal bands, the A. hypochondriacus species could be distinguished from the rest (A. cruentus and A. caudatus) because it only possesses two anodal bands A_2 and A_5 (Figure 2 and Table 1) but does not have bands A_1 and A_3 or alternatively the A. hypochondriacus species possess bands A_2 and A_4 according to Figure 3 and Table 2 but they do not have bands A_1 and A_3.

Cathodal bands could also be used for the identification of species, based on the number of bands and their staining intensity. At 52 days, all the three species had one only cathodal band(C_2). A. hypochondriacus had light staining cathodic band, C_2 whereas the other two species had medium to dark staining band, C_2 (Figure 2). At 60 days, A. hypochondriacus had only one light staining band, C_1 whereas the other two species possessed either two or one but medium to dark staining bands (Figure 3). A. hypochondriacus could be distinguished from the other species with the help of this band C_2 as it was absent in this species but present in all other species (Figure 3).

The distinction of cultivars within species was also possible to some extent using either cathodal or anodal bands alone or a combination of both. The three A. cruentus cultivars could be characterised fully using peroxidase zymograms at 52 days and 60 days.

Table 1: The peroxidase banding pattern and staining intensity in the 12 varieties of grain amaranths (60 days), based on migration distance of the bands from the origin.

		Anode				Cathode	
Banding Number		A_1	A_2	A_3	A_4	C_1	C_2
Species.	Variety						
A. hypochondriacus	674		D		M	L	
	1008		D		M	L	
	Jumla		D		M	L	
	1024		D		M	L	
	723		D		M	L	
A. caudatus	A1113A		M	D	D	D	D
	A1113B		M	D	D	D	D
	A 982		M	D	M	M	M
	A 713				M	M	M
A. cruentus	434	D	L			M	M
	1011	D	L			M	M
	1034		D				M

Key: D - Dark staining; M - Medium staining; L - Light staining.

At 60 days, 1034 could be distinguished from the other two cultivars because it had only one dark staining anodal band, A_2, whereas the other two cultivars had two anodal bands A_1 and A_2. Also at this stage (60 days), 1034 could be distinguished from the rest because it has only one cathodal band (C_2) whereas the other two cultivars had two cathodal bands (C_1 and C_2) (Figure 3). Using the zymograms at 52 days, 1034, 1011 and 434 could be distinguished from one another. At this stage 1034 has only one medium staining anodal band(A_2)and 1011 has only one medium staining anodal band A_1 whereas 434 has two anodal bands A_1 and A_2 (Figure 2). Thus the three cultivars of A. cruentus could be distinguished using peroxidase zymogram.

The four cultivars of A. caudatus could only be partly identified. A-713 could easily be identified from the rest because it does not have band A_2 whereas all the A. caudatus species have this band. A-982 could be identified from A-1113A and A1113B because at 60 days it has medium staining band, A_4 whereas these two have dark staining band (Figure 3). However at 52 days, A-982 and A-1113B are indistinguishable (Figure 2). Thus using peroxidase isozyme, only A-713 and A-982 could be properly distinguished, A-1113A and A-1113B are generally indistinguishable, although at 52 days when A_4 appear, A1113A could be distinguished from A1113B

(Figure 2). But this is conclussive identification because band A_4 is not consistent and not sharply differentiated.

In the available literature, no work has been reported on peroxidase in the genus Amaranthus. This study reveals, the importance and potential of peroxidase in differentiation of species in the genus. The study has shown that the three grain species of Amaranths can be distinguished from one another very easily using peroxidase isozyme. However, peroxidase has also been used a lot in the identification of various crop species (Sheens 1970; Hart and Bhatia, 1967; Bassiri and Adams, 1978) and cultivars (Tuikong, 1982; Hvid and Nielsen, 1977; Quiros, 1980; and many others).

5.4. Esterases.

Esterase zymogram showed many bands but most of these bands were monomorphic and of little use in identification (Figure 4 and Table 2). However identification of various species was possible. Differentiation of A. hypochondriacus from A. cruentus was to some extent difficult, for they had many bands in common. But if we combine the two enzyme systems (Peroxidase and estarase) then identification can be confirmed (Figures 3 and 4).

Table 2: The esterase bands and band intensity in the 12 varieties of grain amaranths (60 days) based on migration distance of the bands from the origin.

		Anode								
Band Number		A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9
Species variety	674	M				L			M	D
	1008	M				L			M	D
A. hypochondriacus	Jumla	M		L		L	L		M	D
	1024	M	M	L		L			M	D
	723	M	M	L		L	L			D
A. Caudatus	A-1113A		M	L			L		D	
	A-1113B		M	L			L		D	
	A-982	M	M	L			L		D	
	A-713	M					L		D	D
A. cruentus	434	M		L	L		L	L	D	D
	1011	M			L		L	L	D	D
	1034	M			L		L		D	D

Key:

D - Dark staining
M - Medium staining
L - Light staining.

The A. cruentus could be distinguished on the basis of band A_4, the band is light staining and is specific to only the A. cruentus species. The A. caudatus species could be distinguished easily on the basis of their faster band which is unique shaped i.e. bands A_8 and A_9 are joined together to form a unique shape (Figure 4) except in A-713 variety where these two bands are a little separated. The A. hypochondriacus could be distinguished on the basis of the light staining band A_5. This band is specific to only the A. hypochondriacus varieties. Although the three species could be distinguished from one another, it is only A. caudatus which is easy to identify from the other two species using esterase isozymes. For A. cruentus, the distinguishing band A_4 is not very conspicuous, the same applies to A. hypochondriacus for band A_5 is not very conspicuous either.

The cultivars of various species could be characterised to some extent using esterase enzyme system. The cultivars 674 and 1008 of A. hypochondriacus are distinguishable because they do not have band A_3 whereas the other cultivars of A. hypochondriacus have this band. Jumla is separated from cultivars 1008 and 674 because it has band A_3 and does not have bands A_2 and A_1. Whereas 1024 and 723 are separated from Jumla because

they have band A_2 which Jumla does not have. Cultivar 723 is separated from 1024 at this stage of 60 days because it does not have band A_8 which 1024 has.

Cultivars of A. caudatus also share some common bands A_2, A_3, A_6 and A_8 and A_9, A-1113A and A-1113B are indistinguishable using esterase isozyme system at this stage. A-982 could be distinguished on the basis of band A_1, it has this band which the others do not have. A-713 could be distinguished on the basis of disjointed band A_8, and A_9 whereas all the other A. caudatus at this stage have jointed band A_8 and A_9.

Banding pattern of 434 and 1011 of A. cruentus are almost similar, but could be distinguished on the basis of band A_3 i.e. 434 is the only A. cruentus species which has band A_3 the others do not have it. Cultivar 1011 could be identified on the basis of band A_3 which it does not not have, but it has band A_7 which 1034 does not have. Cultivar 1034 could be distinguished from the other cultivars on the basis of missing bands A_3 and A_7 (Figures 4 and Table 2).

5.5. Assessment of Relationship Between the Species Investigated.

Electrophoretic banding homology is considered a powerful approach in estimating genetic relationship

between two or more species. (Johnson et al., 1967, Johnson and Thein, 1970). However, in this study, the genetic relationship between the three species was difficult to estimate because of two reasons. The 12 varieties investigated did not have any established interspecific hybrid between any two or more of the studied three species. Besides, the three studied species show a very large number of polymorphic bands with very few monomorphic bands that suggestion of genetic relationship was made impossible (Figure 1, 3, and 4). Although the data presented here do not elucidate evolutionary relationships among the three species investigated, they do provide basic information and criteria to distinguish between species.

5.6. Seasonal Variation in Banding Pattern of Esterase Isozyme.

The peroxidase, Acid phosphatase and Leucine amino-peptidase, showed constant banding pattern in the long and short rainy seasons. However, esterases showed less intense band staining during the rainy season but more intense and distinct sharp bands during short rains. This may not be very surprising as there is also temperature change during these two periods. Long rains is generally available during cold season whereas short rains come during hot seasons. Temperature affected peroxidase banding pattern

in tobacco (De Jong et al., 1968) and therefore this reduced banding pattern of esterase could be due to temperature effect.

Summarising the discussion of the study the distinguishing stages and distinguishing bands of the various isozymes studied are given below. Leucine amino peptidase could be used to distinguish A. cruentus at all stages of crop development due to large quantity of the enzyme produced which other species produced only to a limited quantity. The single band was monomorphic for all the species.

Peroxidase isozymes were distinctive at two stages viz: 52 and 60 days. At 52 days band A_3 present in all the A. caudatus cultivars was a distinguishing feature. The A. cruentus cultivars identified because they were lacking bands A_3, A_4 and A_5. The A. hypochondriacus were identified on the basis of missing band A_3. At 60 days, the distinguishing bands were A_3 and C_2. All the A. hypochondriacus varieties did not have band C_2, the remaining species had this band, also the A. hypochondriacus did not have band A_3 at this stage.

Esterase isozymes were critical at 60 days stage. The distinguishing band for A. caudatus was band A_8 and A_9 which formed unique shape when joined together. Thus

on the basis of this shape, A. caudatus species could easily be separated from the rest.

The results of the peroxidase and leucine amino-peptidase isozymes indicate that the species A. caudatus and A. hypochondriacus are somewhat genetically related. According to these two isozymes A. cruentus is a little distantly related. The results of esterase isozyme suggested some weak relationship between A. cruentus and A. hypochondriacus. From these evidences, it is difficult to make conclusion on the evolutionary pattern in the grain amaranths. However, the study gives a starting point of the biochemical approach to evolutionary studies in the genus.

6.0. CONCLUSIONS

The variation of peroxidase, esterases and leucine amino peptidase were greater among species than within species i.e. there were large interspecific variation in these isozymes than intraspecific variation. This stresses the usefulness of these isozyme variants in chemotaxonomic studies in the genus Amaranthus. On the basis of these large number of polymorphic bands, the three species were easily identified. The results agree with the present species classification in the 3 grain amaranth species.

There was minor variation in isozyme banding patterns of peroxidase and esterase in the cultivars within species. The variation was significant and could be used to distinguish some cultivars from others. However, some very closely related cultivars were indistinguishable using electrophoretic banding pattern alone. Such cultivars may better be identified using a combination of conventional taxonomic methods and electrophoretic technique. Thus electrophoretic technique would supplement the existing conventional methods in the systematics of the genus Amaranthus.

While the present study has not fully established the evolutionary relationship between the 3 species investigated, it does provide basic information for further work towards

molecular approach to taxonomic and evolutionary problems in the genus Amaranthus. What may be needed is to test more species of the genus, if they confirm the result of this finding, then rapid, inexpensive method of differentiating between species of the genus is available.

The most important aspect of the study was the identification of various species and this has been shown that the 3 species viz: A. cruentus, A. caudatus and A. hypochondriacus have specific differences within themselves at various stages of development which make their identification easier. Also to be noted is that in some cases, single isozyme system may not be enough to distinguish all the species and a combination of two or more isozyme systems may be necessary so as to distinguish fully all the species.

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Plate 1: Zymogram of acid phosphatase 75 days after planting. The first 3 from left are A. cruentus, next 4 are A. caudatus and the last are A. hypochondriacus.

Plate 2: Zymogram of leucine amino peptidase at 60 days after planting. The first 3 are A. cruentus the next 4 are A. caudatus and last 5 are A. hypochondriacus.

Plate 3: Zymogram of peroxidase at 52 days after planting. The first 3 from left are A. cruentus, next 4 are A. caudatus and the last 5 are A. hypochndriacus. See also schematic diagram figure 2.

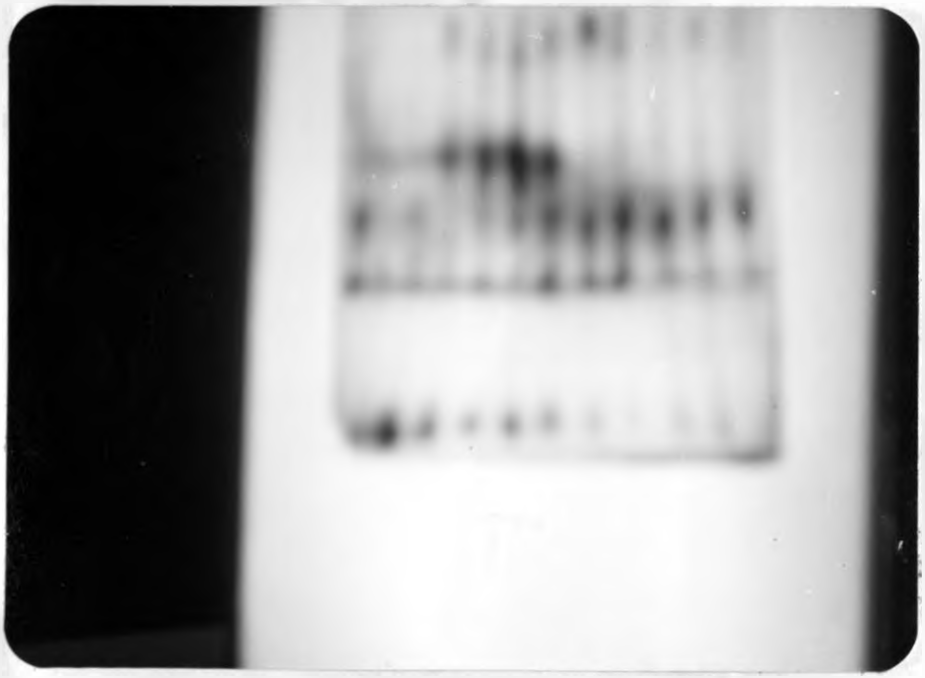
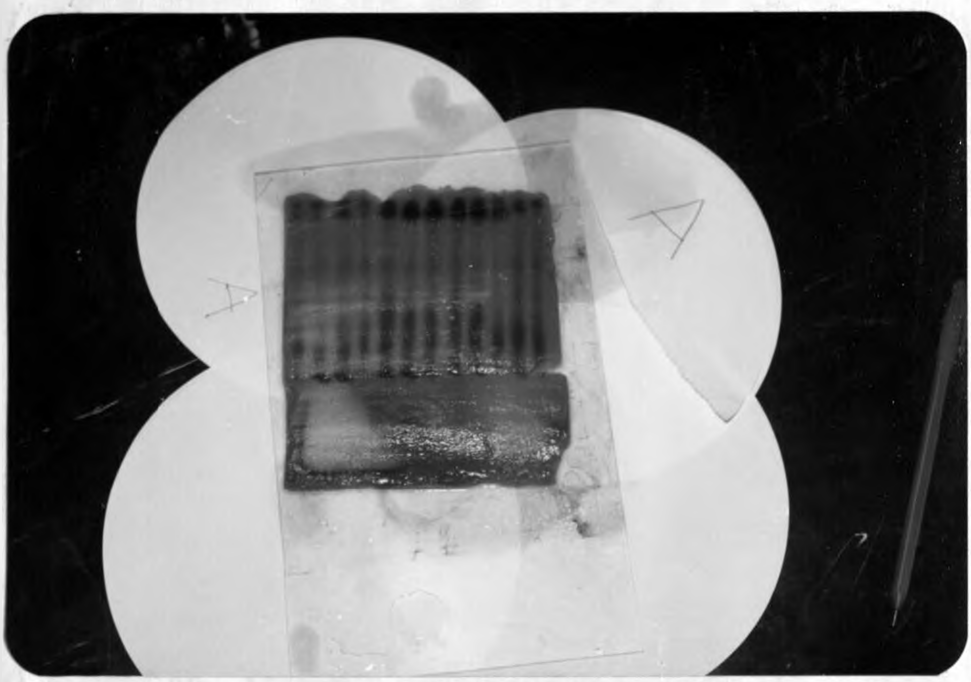


Plate 4: Zymogram of peroxidase at 60 days after planting. The first 3 from left, are A. cruentus, the next 4 are A. caudatus and the last 5 are A. hypochondriacus. See schematic diagram figure 3.

Plate 5: The zymogram of esterases at 60 days after planting. The first five (from right) varieties are A. hypochondriacus, next four A. caudatus and last three are A. cruentus. See also schematic diagram figure 4.



Appendix 1: Average yield of grain amaranths in various countries.

Country	Grain Yield (kg/ha)
U.S.A[a]	683-3900
Ethiopia[b]	4,480
Pennsylvania[c]	1,800
India Uttar (Pradesh and Himachal Pradesh)[d]	3,000
Kenya[e]	3,210
Mexico[f]	4½ bushels/ Acre
Rodale [g]	1,307

a, b, c, d, e, ...g refer to the sources of information.

a = After USDA, Agric. Statistics, 1974.

b = Schimidt, D; Debre Zeit Ethiopia 1977. 1st Proc. Amaranth Seminar.

c = Vietmeyer, 1982. Ceres (FAO) 15(5): 43-46.

d = Joshi, BD. 1981a. AGP/PGR/48, (FAO) Rome, Italy.

e = Gupta, V.K. 1985. Amaranth progress report (Kenya)

f = Senft, J.P. 1981.

g = Kauffman, C. 1981.

Appendix 2: Yield of grain amaranths as compared with the yield of other common cereals.

	USA Average Yield (kg/ha)		World Average Yield (kg/ha)
Barley	2000	(1825)*	1910
Oats	1700		1660
Corn	4500		2400
Rice (rough)	5100		2300
Rye	1350		1740
Wheat	1800	(3230)*	1560
Soybean	1580		1370
Amaranth	683-3900	(4380)*	
Triticale	(3060)*		

Source: USDA, Agric. Statistics, 1974

( )* - figures marked this way are obtained from Schmidit, 1977 1st. Proc. Amaranth Seminar.

Appendix 3: Protein content and quality of grain amaranth as compared with other common grains.

Grain	% Protein	Limiting amino acid	Protein score
Amaranth	15	Leucine	67
Barley	9	Lysine	58
Buckwheat	12	Leucine	83
Corn	9	Lysine	35
Oats	15	Lysine	62
Rice	7	Lysine	69
Soybean	34	Methionine/ cysteine	89
Wheat	14	Lysine	47

Source: Senft, J.P. 1981. Rodale Research Report 80-3: pp 8, 10-11.

Appendix 4. The dry matter, ash, calcium, phosphorus, crude fibre, ether extract, crude protein and amino acid percentage compositions of A. edulis compared with those of other feed grains. (Data expressed on a dry matter basis).

Analyses	A. edulis[a]	Maize* (Yellow) (dent)	Sorghum* (milo)	Wheat*	Barley*
Dry matter	88.4	89.0	89.0	89.0	89.0
Ash	4.4	1.2	1.9	1.8	2.7
Calcium	0.25	0.02	0.04	0.06	0.09
Phosphorus	0.71	0.35	0.33	0.41	0.47
Crude fibre	5.8	2.2	2.20	3.4	5.0
Ether Extract	6.9	4.4	3.1	1.9	2.1
Protein (N x 6.25)	16.5	10.0	12.4	14.3	13.0
Lysine	0.94	0.20	0.30	0.51	0.60
Histidine	0.41	0.20	0.30	0.30	0.30
Arginine	1.54	0.51	0.40	0.80	0.60
Threonine	0.59	0.40	0.30	0.40	0.40
Glycine	1.19	-	-	1.00	0.40
Valine	0.71	0.40	0.60	1.60	0.70
Methionine	0.32	0.10	0.10	0.20	0.20
Isoleucine	0.60	0.51	0.60	0.60	0.60
Leucine	0.99	1.11	1.60	1.00	0.90
Tyrosine	0.60	-	0.40	0.51	0.40
Phenylalamine	0.73	0.51	0.51	0.70	0.70
Cystine	0.34	0.10	0.20	0.20	0.20

Sources: - * Maize, sorghum, wheat, and barley analyses from National Academy of Sciences - National Research Council (1964).

a - Connor et al., 1980. See bibliography.

Appendix 5: Combinations of grains to

Quality	TRP	MET CYS.
FAO/WHO	1.0	3.5
Amaranth	1.4	4.4
Corn	0.6	3.2
50 Amaranth/50/ Corn	1.0	3.8

Source: Senft, J.P. 1981

improve protein

THR	ISL	VAL.	LYS	PHE TYR	LEU
4.0	4.0	5.0	5.5	6.0	7.0
2.9	3.0	3.6	5.0	6.4	4.7
4.0	4.6	5.1	1.9	10.6	13.0
3.5	3.8	4.4	3.5	8.5	8.9

Appendix 6. Amino acid composition of Amaranth seeds and leaves compared with that of opaque-2 and Normal maize.

	Amaranthus[a]		Maize[b]	
Amino acid	Leaves	seeds	Opaque-2	Normal
Lysine	5.9	6.2	3.39	2.00
Histidine	2.2	2.8	3.35	2.85
Ammonia	1.5	1.9	3.41	3.28
Arginine	5.7	10.6	5.10	3.76
Aspartic acid	9.2	8.9	8.45	6.17
Glutamic acid	10.8	17.2	19.13	21.30
Threonine	4.5	3.7	3.91	3.48
Serine	4.8	5.7	4.99	5.17
Proline	5.3	4.1	9.36	9.67
Glycine	5.5	7.3	4.02	3.24
Alanine	7.0	4.2	6.99	8.13
Valine	6.1	4.4	4.98	4.68
Cystine	0.7	1.6	2.35	1.79
Methionine	1.3	2.3	2.00	2.83
Isoleucine	5.3	3.9	3.91	3.82
Leucine	9.2	5.9	11.63	14.29
Tyrosine	4.1	4.1	4.71	5.26
Phenylalanine	5.5	4.3	4.96	5.29
% Protein	26.4	14.5		

Sources: a Downtown J.S.W. 1973
b Harpstead, D.D. 1971 Scientific America 225:34

Appendix 7: Proximate composition of amaranth seeds.

Sample	% moisture	% N	% Protein	% Fat	% Fibre	% Ash.
Commercial (A. cruentus)	6.71	3.00	17.55	7.71	3.44	2.80
Commercial (A. cruentus)	6.23	3.11	18.19	8.04	5.34	3.81
P.1.337611	9.82	2.97	17.37	7.71	3.36	3.77
No N, H_2O						
P.1.337611	9.44	2.95	17.26	7.48	4.39	3.32
7516N						
A.cruentus x A. hypochondriacus	9.60	2.75	16.09	8.03	4.25	3.04

Source: Becker, R.R., Wheeler, E.L., Lorenz, K., Stafford, A.E., Grosjean, O.K., Betschart, A.A. and Saunders, R.M. 1981.