ELECTROPHORESIS AS AN AID FOR IDENTIFICATION OF DIFFERENT COMMERCIAL CLONES OF PYRETHRUM *Chrysanthemum cinerariaefolium* Vis.

By

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A thesis submitted in part fulfilment for the degree of Master of Science in Agriculture (Plant Breeding) in the University of Nairobi.

FACULTY OF AGRICULTURE

1982

DECLARATION

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

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D E D I C A T E D

TO

MY ELDER BROTHER DANIEL

AND

MY UNCLE MUSA KENTAGOR

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENT..	vi
SUMMARY..	viii
1. INTRODUCTION..	1
2. LITERATURE REVIEW...................................	8
2.1 Botanical aspects of pyrethrum.....................	8
2.2 Breeding of pyrethrum..............................	9
2.2.1 History..	9
2.2.2 Clonal breeding..................................	12
2.2.3 Varietal breeding................................	13
2.3 Clonal multiplication..............................	15
2.4 Identification methods used in pyrethrum...........	15
2.5 The electrophoretic technique......................	17
2.6 The use of electrophoresis for cultivar identification in crops...	22
3. MATERIALS AND METHODS...............................	27
3.1 Materials..	27
3.2 Methods..	27
3.2.1 Preliminary trials...............................	29
3.2.1.1 Sampling and enzyme extraction.	29
3.2.1.2 Starch gel preparation and electrophoresis.....	30
3.2.1.3 Location of enzymes on gels....	32
3.2.2 Confirmatory trials..............................	34
4. RESULTS...	37
4.1 Preliminary tests..................................	37
4.2 Peroxidase bands...................................	39

Page

4.2.1 Description of the bands........... 39
4.2.2 The banding patterns of the commercial clones.................. 46
4.2.3 The banding patterns of the full-sib population..................... 48
4.2.4 The banding patterns of the Japanese clones..................... 50

4.3 Effect of environment on peroxidases......................... 52
4.3.1 Insect infestation................. 52
4.3.2 Differences in soil composition.... 54
4.3.3 Differences in growth conditions................ 54

5. DISCUSSION 60

5.1 Choice of enzyme system and tissue... 60
5.2 The peroxidase bands and their use in identification............... 61
5.3 Distinguishing between closely related clones..................... 65
5.4 Environmental influence on peroxidases....................... 67
5.4.1 Insect pest effect............... 67
5.4.2 Effect of soil differences......... 69
5.4.3 Differences in growth conditions... 70
5.5 Conclusion.......................... 72

REFERENCES.......................... 76

LIST OF TABLES

1. Different combinations of peroxidase bands according to migration position and staining intensity in the 35 clones studied.............................. 42

Page

LIST OF FIGURES

1. Selection stages in the breeding of pyrethrum as currently practised at the National Pyrethrum and Horticultural Research Station, Molo.................... 11

2. Diagramatic representation of electrophoretic banding patterns of acid phosphatases of 12 Kenyan commercial clones......................... 38

3. Different bands and intensities of anodal and cathodal peroxidases of 12 Kenyan commercial clones, 12 full-sib clones with their parents and nine clones of Japanese origin grouped according to migration distance into zones A_I, A_{II}, A_{III} and C_I and C_{II}....... 40

4. Diagramatic representation of electrophoretic banding patterns of anodal and cathodal peroxidases of 12 Kenyan commercial clones, 12 full-sib clones with their parents and nine clones of Japanese origin................ 41

5. Diagramatic representation of electrophoretic banding patterns of anodal and cathodal peroxidases of both healthy (H) and insect infested (I) plants of five commercial colones.... 53

Page

6. Diagramatic representation of electrophoretic banding patterns of anodal and cathodal peroxidases of two commercial clones each grown in three different soils: a prepared soil mixture (S_1), Kabete soil (S_2) and Molo soil (S_3)........................... 55

7. Diagramatic representation of electrophoretic banding patterns of anodal and cathodal peroxidases of 12 Kenyan commercial clones, each grown at the reference collection plot at Molo and in a glass house........ 57

LIST OF PLATES

1. Esterases in young(Y) and mature (M) leaves of three clones. Left to Right: 4729 M. 4729 Y, 4331 M, 4331 Y, 252 Y and 252 M twice............ 84

2. Anodal and cathodal peroxidases in 11 commercial clones. Left to right: 58, 99, 107, 197, 219, 252, 618, 618 (insect attack), 1889, 4729, 4331 and 246................................ 85

3. Anodal peroxidase bands in 12 full-sib clones with their parents. Left to Right: FS_1, 2, 3, 4, 5, 6, 7 8, 9, 10, 11, 12, and parents 473 and 4743............................... 86

Page

4. Anodal peroxidase bands in nine clones of Japanese origin and a commercial clone. Left to Right: J_1, 2, 3, 7, 9, 12, 14, 16, 18 twice, 107 twice and J_7 twice.................... 87

5. Cathodal peroxidase bands in five commercial clones subjected to insect pest infestation (I) and their healthy sprayed replicates (H). Left to Right: 219 H, 219 I, 252 H, 252 I, 1889 H, 1889 I, 4331 H, 4331 I, 4729 H, and 4729 I............... 88

LIST OF APPENDICES

1. Yearly pyrethrum production from 1935 to 1981 in metric tons of dry flowers.............................. 89

2. Yearly pyrethrum production in metric tons of dry flowers and their corresponding producer prices from 1965 to 1981....................... 90

ACKNOWLEDGEMENT

I wish to record my sincere thanks to all the people who helped me in one way or another in the course of this study.

Mr. J.J. Ondieki deserves a lot of thanks for recommending me, while he was the Director of the National Pyrethrum and Horticultural Research Station, Molo, to be sponsored by the Research Division, Ministry of Agriculture to undertake an M.Sc. course in plant breeding at this University. The present Director of the station Mr. J.O. Owuor should be thanked too for showing keen interest in my work. I would also like to thank my colleagues in the plant breeding section of N.P. & H.R.S., Mr. W.G.M. Ottaro for his frequent elderly advice, Mr. J.K. Ikahu and his assistant Mr. M.O. Miseda who together with the T.A. Mr. C. Nginyo and subordinate staff in the section helped in the preparation of the experimental material.

While I carried out the experiments in the laboratory, Professor V.K. Gupta gave very constructive suggestions. This and the role he played in the interpretation of results earn him special thanks. Dr. E.W.M. van Breukelen also gets special thanks for his constructive criticisms during the write up. His speed in reading through

the drafts of the thesis often kept me on my toes and enabled me to finish in time.

Finally, I wish to extend my gratitude to Mrs. R. Abasa of the Dean of Agriculture's Office for her speed and accuracy in typing this work.

ABSTRACT

Pyrethrum, Chrysanthemum cinerariaefolium Vis., is an insecticide producing plant propagated both generatively and vegetatively. With the present emphasis on the selection for improved clones, there is need to establish a quick, inexpensive and reliable method for identification of different clones since earlier used methods have not been satisfactory. In other crops, electrophoresis has proved to be a good technique for cultivar identification.

In the work presented here, starch gel electrophoresis was used to study isozyme patterns of peroxidase, catalase, esterase and acid phosphatase from apical meristems, stems, young leaves and mature leaves. The latter three enzyme systems were found to be inappropriate to use. Peroxidase isozymes on the other hand showed a wide variation in number and staining intensity.

The work presented describes the use of peroxidase isozymes from mature green leaves for identification of twelve Kenyan commercial pyrethrum clones which comprised ten diploids and two triploids. The clones were obtained from the reference collection of clones at the National

Pyrethrum and Horticultural Research Station, Molo and grown in a glass house with limited ventilation.

To determine the possibility of using peroxidase isozymes to distinguish between closely related clones, the banding patterns of 12 full-sibs were included in the study. In addition, the electrophoretic patterns of nine clones of Japanese origin were studied to determine whether their patterns differed from those of the Kenyan clones. Influence of some environmental factors on peroxidases was also studied.

In total, seven different bands were observed on the anodal side and three on the cathodal side of the gel. These bands were distributed over three migration zones on the anodal side (A_I, A_{II} and A_{III}) and two on the cathodal side of the gel (C_I and C_{II}). Differences in intensities and patterns of these bands were used to characterise the clones.

All the 12 commercial clones could be characterised based on their anodal peroxidase isozymes alone. The banding patterns of the two triploid clones were not different from those of the diploids suggesting no effect of their increased chromosome number on the peroxidase isozymes. Among the full-sibs, two were

indistinguishable and some others had close similarities but still there was much variation in this population. This showed that there might be difficulty in identification with increased number of related clones. The clones of Japanese origin did not show a different banding pattern from that of the Kenyan clones. Their bands were distributed over the same migration zones as those of the Kenyan clones. This indicated that the populations from which the Kenyan and Japanese clones have been selected might have had a common genetic source.

In the studies to determine the effect of environmental factors on the peroxidase isozymes, the effect of insect pest infestation (thrips and spider mites), mineral nutrition and climate were observed. The cathodal bands of clones subjected to pests were markedly affected. They generally stained more intensely. The anodal bands were not affected. The cathodal bands may therefore not be useful for identification where there is inadequate pest control. There was no change in either anodal or cathodal banding patterns as a result of growing the clones in soil of different compositions. By growing the twelve commercial clones under a different climate, there was a significant change in the peroxidase isozymes of all clones. However,

all these clones could individually be identified.

It can be concluded that under uniform growing conditions, the present twelve Kenyan commercial pyrethrum clones can be identified satisfactorily by their peroxidase electrophoretic patterns. The technique could also be used to distinguish between a large number of future clones even if they were closely related. This is because of the high degree of polymorphism of the peroxidase bands.

1 INTRODUCTION

Pyrethrum, Chrysanthemum cinerariaefolium Vis., produces a natural insecticide which has been accepted worldwide for domestic purposes. Its rapid paralytic action on a broad variety of insects while being practically non-toxic to mammals, has contributed to its success compared to synthetic insecticides. It also is rapidly degraded by sunlight and air hence reducing environmental hazards. When synergists like piperonyl butoxide are added to pyrethrum, its insecticidal effect is increased by retarding detoxification in the insects. Another important property of pyrethrum is that little insect resistance has been recorded.

Although pyrethrum is essentially grown for its insecticidal properties, a by-product of pyrethrins extraction, pyrethrum marc, is used as a cattle feed.

Commercial use of pyrethrum probably originated in Persia with Chrysanthemum coccineum which was sold as Persian powder in eighteen twenties (Contant, 1976). This genus, being of low flowering ability and pyrethrins content, was replaced around 1840 in Europe with C. cinerariaefolium. This flowers better and it has a high toxic potency. Dalmatia (Yugoslavia), the centre of

origin of this genus, was then the leading world producer. This position was maintained until the first World War when Japan took over the lead. In Kenya pyrethrum was first introduced in 1928. A farmer named Captain G. Walker obtained some seed from Dalmatia and established a commercial field on his farm at Subukia, Nakuru (Chandler, 1948). In 1929 Mr. T.J. Anderson, an entomologist working at the Scott Agricultural Laboratories (now National Agricultural Laboratories), brought some seed from the Ministry of Agriculture at Harpenden, England, and used it for experimental purposes. Both introductions soon reached many farmers and in 1933 the Pyrethrum Growers Association was formed to organize the production and marketing of the crop. Flower production was then less than 400 tons but it rose steadily so that by 1945 Kenya was the world's leading producer at 7,500 tons (see Appendix 1). This position has been maintained since that time although there have been fluctuations in production from time to time. The fluctuations have mostly been a result of changes in market prices and unfavourable weather conditions. The period 1979 to 1981, for example, is marked by a sharp increase in flower production because of a rise in producer prices (see Appendix II). The greater part of this produce is exported with only

two percent used locally. Kenya supplies over 80% of the world's pyrethrum requirements. Other pyrethrum producers are Tanzania, Ecuador, and Rwanda with small contribution from Japan, New Guinea, Brazil, Zaire, Indonesia, India, the U.S.S.R. Taiwan, Rhodesia, Yugoslavia and South Africa in order of declining importance. Pyrethrum, which is Kenya's third export crop after tea and coffee, now has its production and marketing handled by the Pyrethrum Board, one of the country's statutory bodies.

Pyrethrum grows well in deep, well drained soils, preferably of volcanic origin. Double superphosphate is applied at planting time. For flower bud initiation to occur, some degree of chilling is required (Glover, 1955). Such conditions in Kenya are met in highlands over 1800 metres above sea level where rainfall is also over 1000 mm a year. The main producing area is the highlands of Kisii District. Other areas of production are the highlands East and West of the Rift Valley, the foothills of the Aberdares and Mount Kenya, and the higher regions of Kiambu District. Over 95 percent of pyrethrum is now grown by small scale farmers on pieces of land ranging from less than a quarter to one hectare. This is unlike the period before independence when it was grown mainly by

white settler farmers on a large scale, sometimes on more than 10 hectares.

Routine work on pyrethrum fields includes frequent weeding, pest control, stripping of flowers, and cutting back old stalks at the end of the flowering season. Weeding is still done manually as suitable herbicides have not been found. Picking of flowers is done every two to three weeks because at this stage flowers have the highest pyrethrins content (Head, 1963). The pests that attack pyrethrum are root knot nematodes (Meloidogyne hapla), flower thrips (Thrips tabaci), leaf thrips (Thrips negropilosus) and the red spider mite (Tetranychus ludeni). These pests can cause severe yield loss especially during the dry seasons. Nematode damage can be reduced by raising planting material in uninfected nurseries and transplanting onto fresh land. Thrips and spider mites can be controlled by spraying with Rogor or Metasystox. Diseases have not been so much of a problem in pyrethrum. Out of 23 diseases recorded, True Bud disease caused by the fungus Ramularia bellunensis and False Bud disease, which is a physiological disorder, are the only ones considered to be of economic importance (Robinson, 1963). These diseases are, however, of little importance now because of effective selection against them.

The breeding of pyrethrum in Kenya comprises the selection for improved clones and for improved hybrid or polycross varieties (Parlevliet, 1975). Both breeding programmes result in a large scale multiplication of clones either for commercial use per se or for seed production. During the multiplication, there can be erroneous mixing of different clones. When seeds form on plants in multiplication nurseries, volunteer plants can grow from dropped seed within the rows and also form a source of clonal contamination. This then necessitates a method that can be used to identify clones. To facilitate identification, a reference collection of all commercial clones and other clones of interest to breeding and research has been established at the National Pyrethrum and Horticultural Research Station, Molo.

The identification methods used at present are not satisfactory. Morphological traits especially of the flowers have been used for many years. Chemical analysis of pyrethrins (Head, 1967) using gas liquid chromatography and incompatability between clones (Brewer and Parlevliet, 1969) are methods developed later. The latter method utilises the sporophytic incompatability system which operates in pyrethrum. Identical genotypes give a reciprocal incompatible reaction and different genotypes give a compatible reaction.

This method takes three to four days to produce results. Crude protein content of leaves (Tuikong, 1990, unpublished data) using the Kjeldahl method is a more recently employed method for identification in pyrethrum. It makes use of the wide range in crude protein content of different clones. It is however a method which is highly influenced by environmental conditions.

Apart from the crude protein method, the present methods for identification in pyrethrum are only useful when there are flowers. This is a limitation because flowering is seasonal. It is preferable to have a reliable method that is both quick and not subject to environmental factors like climatic conditions and soil fertility. Electrophoresis is such a method. It has proven useful with several crops. Therefore it would be useful to know more about the potential of the electrophoretic method for clonal identification in pyrethrum. This method would either replace or supplement the existing methods. Therefore the main objectives of this study were:

To determine the possibility of using electrophoresis as an alternative, quick and reliable method for identification of Kenyan commercial pyrethrum clones. This

involves cheking different enzyme systems and different tissues.

To determine whether this same technique can be used effectively to distinguish between closely related clones.

To observe electrophoretic variations that might exist between pyrethrum clones of different origins.

2 LITERATURE REVIEW

2.1 Botanical aspects of pyrethrum.

Pyrethrum, Chrysanthemum cinerariaefolium Vis., is a member of the Compositae family. It is a small perennial herb tending to be woody at the base. It is propagated both by seed and vegetatively from splits (Delhaye, 1968). The flowers which are the harvested part of the crop are produced on branching stems arising from a crown of deeply lobed leaves. The flower head has an outer ring of white ray florets which are female and inner yellow disc florets which are bisexual. Cross pollination is a rule in pyrethrum. This is through a sporophytic incompatability system (Brewer and Parlevliet, 1969) enhanced by protandrism (Brewer, 1968). Pollination is usually by insects. The pyrethrum seed is a dry one-seeded indehiscent fruit or achene. The achene is the seat of pyrethrins where they are formed in the oilglands and secretory ducts (Head, 1966). Other parts of the plant also have pyrethrins but at much lower concentrations.

The pyrethrins are esters which are derived from two acids (chrysanthemic and pyrethric) and three alcohols (pyrethrolone, cinerolone and

jasmolone). Chrysanthemic acid comprises pyrethrins 1, cinerin 1, and jasmolin 1 (commonly termed pyrethrins 1) while pyrethric acid comprises pyrethins 11, cinerin 11 and jasmolin 11 (commonly termed pyrethrins 11). The pyrethrins 1 and pyrethrins 11 together account for the knockdown and kill properties for which pyrethrum is well known.

2.2 Breeding of pyrethrum.

2.2.1 History

The breeding of pyrethrum in Kenya started in the late thirties (Kroll, 1958). Emphasis was then on hybrid varieties. Selection for superior clones started in 1962 (Anon, 1962; Contant, 1963). Earlier, the use of hybrid seed was necessary in order to meet the demand for pyrethrum planting material, because clonal material would take too long to multiply and be supplied to growers (Kroll 1958). However, Contant (1963) argued that future improvement of pyrethrum would be faster with the use of clones. He realised that pyrethrum exhibited a wide genetic and phenotypic variability for many characters and so the best individuals in the population performed by far better than the average of the population as a whole. He then employed a simple and quick selection procedure. With this procedure it was possible for a clone to be established

commercially several years before seed of that clone was available in appreciable quantities after progeny testing. The procedure involved selection of phenotypically outstanding plants. These were split and planted in single line observation trials for one year. The best clones were planted in replicated yield trials which lasted for two to three years. This was followed by multiplication of the top clones for release to growers. The methods used earlier have now been refined with the intention of improving the selection response (Review: Tulkong, in press). Two interrelated breeding programmes are being pursued (Figure 1). One, varietal breeding, deals with the production of improved hybrid or polycross seed for commercial growing and it has four selection stages. The other, clonal breeding, deals with the selection of outstanding plants for yield trials and subsequent clonal multiplication for commercial growing. It has five selection stages. Under both programmes, flower yield and pyrethrins content form the most important selection criteria. Lodging resistance, resistance to bud diseases, splitability and establishment of clones and tolerance to root knot nematodes are secondary but also important criteria.

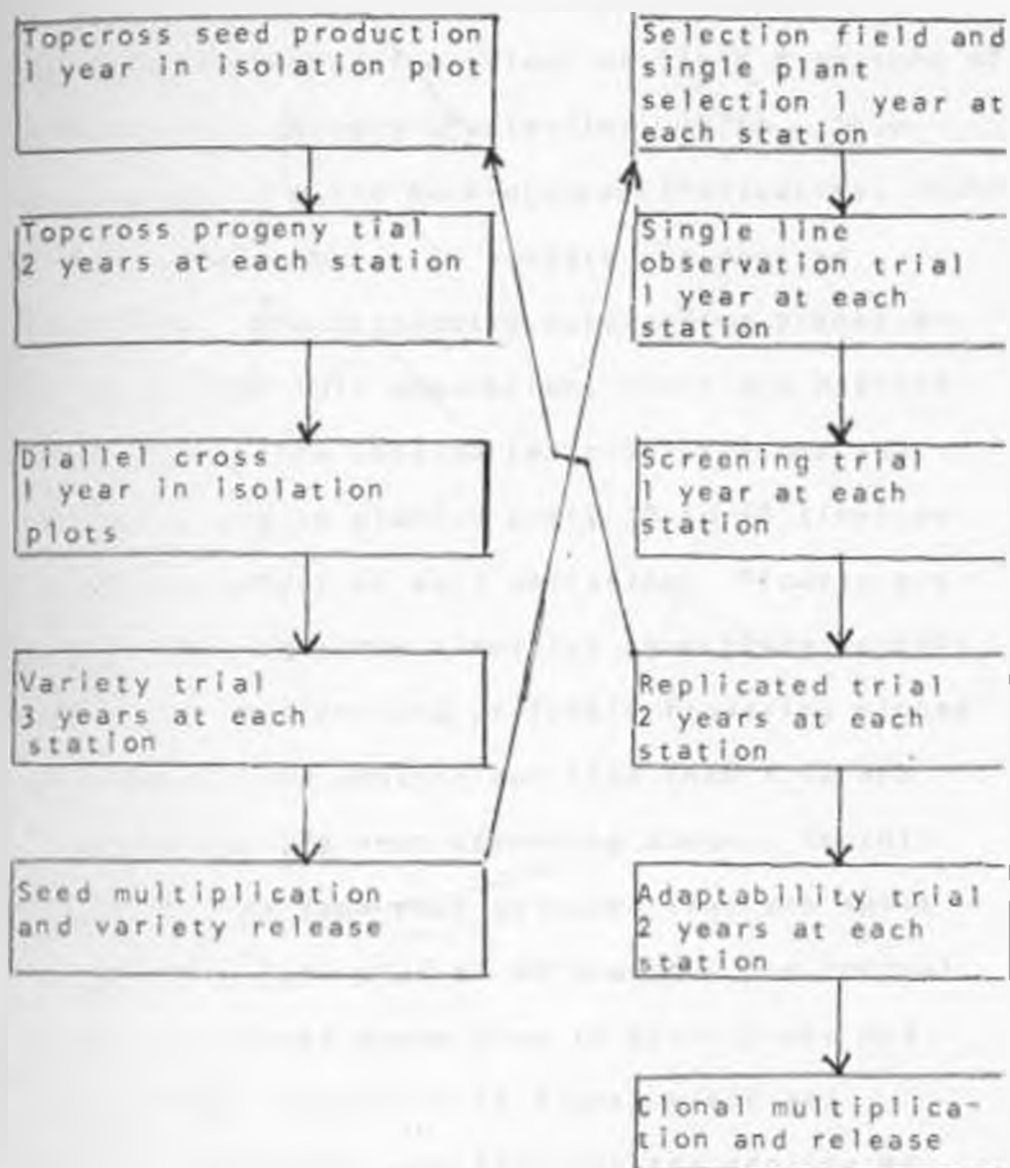


Figure 1.

Selection stages in the breeding of pyrethrum as currently practiced at the National Pyrethrum and Horticultural Research Station, Molo.

2.2.2 Clonal breeding

The clonal breeding programme begins with the establishment of a selection field from seed of a commercial variety (Parlevliet, 1975). This population is quite heterogeneous (Parlevliet, 1974). It gives a fair chance to extract the desired genotypes. Phenotypically outstanding plants are selected from this population, split and planted in a single line observation trial for one year. A control clone is planted every 30 to 50 lines to check the effect of soil variation. Flowers are sampled two to three times for pyrethrins content analysis. Healthy and profusely flowering clones with pyrethrins content not less than 1.8% are selected for the next screening stage. In this stage, plants from each selected line are split to give a single plot of 60 plants. The control clone is planted every five to eight plots and clones that surpass it in flower yield and pyrethrins content are selected for replicated yield trial stage. This stage has three plots of 60 plants each and it lasts for three years. Flower yield and pyrethrins content are measured during this period. These four stages are carried out on the same location. Clones which are better than the control are further tested in adaptability

trials. The adaptability trials are carried out on six to seven locations in the same way as the replicated yield trials. They serve as the last stage before the clones are recommended for release to growers. This stage takes care of genotype-environment interaction effect. It is important that recommended clones have wide adaptation in order to avoid poor performance in some areas. All clonal selections are done in the presence of root knot nematodes, so the clones which do well under such conditions are also tolerant to the nematodes.

A high incidence of natural triploids with a chromosome number of 27 has been observed in the commercial varieties in Kenya (Ottaro, 1978). These triploids tend to be picked during clonal selection because of their large flowers. Some triploids have performed well in the yield trials and they have been recommended for commercial growing.

2.2.3 Varietal breeding

Recurrent selection for general combining ability is employed in the varietal breeding programme. It is a seven year cycle beginning with selected clones from the replicated trial of the clonal breeding programme. The clones are selected because of their desirable agronomic characteristics and they are already well adapted.

They are topcrossed to a tester variety with a broad genetic base. The topcross method is now prefered to the more laborious polycross method used by Contant (1963) because of its simplicity while it gives similar results. Seed is collected from each clone separately and sown for a progeny yield test. Pyrethrins contents are measured for calculations of general combining ability. A diallel cross of the clones with high g.c.a is then made. The hybrid varieties are tested for three years on the same stations as the adaptability trials. The varieties which prove better than the control are recommended for release.

The time taken to release a variety can be reduced by two years when parent clones of the existing commercial variety are included in the topcross seed production and subsequent tests (Parlevliet, 1975). Clones that emerge significantly superior to the parents can also be used as parents of a new variety. Hence testing of hybrids is avoided as it has been found out that the specific combining ability component of variance is much smaller compared to the general combining ability component (Parlevliet and Contant, 1970). This also means that polycross varieties can be released, without a test, based on the breeding

values, of the combining parents (Parlevliet, 1975).

2.3 Clonal multiplication

Newly recommended clones have to be multiplied before they are released to farmers or used for seed production. The Pyrethrum Board of Kenya has established special nurseries for this purpose. Parlevliet (1975) saw mixing of different clones as a serious problem during multiplication and a mixing has been observed in a number of cases. A quick and reliable method for identification of different genotypes would therefore reduce the risk of issuing wrong planting material to farmers.

2.4 Identification methods used in pyrethrum

Morphological traits are often used for identification in crops. Flower morphology in pyrethrum offers the best opportunity of all morphological traits. There can be great differences in size, shape and distribution of the ray florets. However, this does not result in complete identification especially when one deals with many clones because of the higher chances that clones have a similar floral morphology. This method is also limited to flowering periods only. Leaves are difficult to distinguish.

Pyrethrins content is also used for identification but mostly to confirm identification by morphological traits. This works only when the clones in question are known to differ much in pyrethrins content. Its use is again limited to the flowering period.

Chemical analysis of the pyrethrins has shown that the pyrethrins I/pyrethrins II ratio is fairly constant for each clone and there is some variation between clones. This led Head (1967) to suggest that this ratio can be used for verification of named clonal material. Results from analysis of eighteen clones showed, however, that only six could be correctly characterised. It may find use where distinction on the basis of morphology and pyrethrins content prove inconclusive.

Incompatability is a more reliable method (Brewer and Parlevliet, 1969). In pyrethrum, a sporophytic incompatability system operates. Most clones are strongly self-incompatible. Clones with doubtful identity can then be tested against those in the reference collection. Identical genotypes give a reciprocal incompatible reaction and differing genotypes give a compatible reaction. In a test carried out by Brewer and Parlevliet with different genotypes, only 1 out of 86 crosses gave

a cross-incompatible reaction. This test is reliable but takes three to four days to be completed and it is highly sensitive to environmental influence. The quality of pollen grains is poor during the wet seasons. This makes judgement of test results difficult.

The use of crude protein content of leaves obtained with the Kjeldahl method is also a reliable method (Tulkong, 1980, unpublished). The range of crude protein content between clones is high, varying between 18 and 34%. Differences in protein content is then used to distinquish between clones of doubtful identity. This method is suitable for differentiating clones grown under similar conditions such as in multiplication nuseries. It cannot be used to test a doubtful clone against one in the reference collection because crude protein varies with season and soil fertility.

2.5 The electrophoretic technique

Electrophoresis is basically a process of forced diffusion of charged substances through an electrical field. The sample of the substances is placed on a supporting medium at a suitable distance from each electrode. When migration occurs there is separation of the substances since they move at different rates according to their net

charges. Appropriate methods are then used to fix and fingerprint these substances at the positions to which they have migrated when the run is stopped.

The charged substances in this study are isozymes (synonymous with isoenzymes). These are different molecular forms of an enyzme with similar or identical substrate specificity occuring within the same organism (Markert and Moller, 1959). Here, the isozymes are made to migrate through a starch gel as the supporting medium. An advantage is taken of the different and characteristic net charge of each isozyme which migrates accordingly under an electric gradient. This is followed by histochemical staining to locate the zone of enzyme activity (bands) directly in the starch gel. The occurrence of isozymes has been known since the early fifties with the discovery of a second molecular form of lactate dehydrogenase (Shannon, 1969). The reasons for the existence of isozymes are not clear. It is, however, certain that they are a direct expression of gene function during cell differentiation in a developing system (Scandalios, 1974). Isozymes are known to occur widely in plant and animal tissues. In plants, past work on isozymes has been concentrated on but not restricted to peroxidases, catalases, amylases,

leucine aminopeptidases, esterases, dehydrogenases, phosphatases, phosphorylases, transaminases, and oxidases. One result of interest out of these studies is that isozyme pattern and intensity are specific to the plant part or tissue and to maturity or the developmental stage of the tissue (Peirce and Brewbaker, 1973). However, certain factors affecting plant metabolism such as mineral nutrition, disease infection (Hare, 1966) and cold temperature hardening (McCown et al., 1968) are known to influence the intensity and cause occasional appearances of different molecular forms.

The electrophoretic technique has been used for various scientific studies. Peirce and Brewbaker (1973) suggested in their review work, its possible use in cytogenetic studies, physiological, developmental and population genetics studies, genetic control of polymorphism, relations with diseases and indentification of cultivars, genomes, genera and taxa. Since then, a lot of work has been accomplished in these fields and as a result some early studies have been confirmed and more discoveries made.

A study done as early as 1953 (Bula et al., 1956) on relations of soluble proteins to cold hardiness in alfalfa was confirmed electrophoretically later (Faw and Jung, 1972). Similar

electrophoretic studies covering several enzyme systems have recently been carried out (Krasnuk *et al*., 1975, 1976a, 1976b, 1978a, 1978b). These studies have shown qualitative and quantitative enzymatic differences between cold tolerant and cold-sensitive alfalfa plants. It has therefore been concluded that proteins and enzymes (proteins of known catalytic function) have a role in the biochemical processes that lead to cold tolerance in plants.

In genetics, electrophoresis has been employed in genetic variability and linkage studies in barley species (Kahler and Allard, 1970, Kahler *et al*., 1981), linkage studies in soyabeans (Gorman and Kiang, 1978) and genetic polymorphism in safflower (Bassiri, 1977). Kahler *et al*., (1981) carried out linkage studies of three enzyme systems (6 - phosphogluconate dehydrogenase, glutamate oxolate transaminase and acid phosphatase) and showed that these enzymes together with esterases (Kahler and Allard, 1970) provided useful genetic markers for further population genetic studies in barley species. Similar studies were undertaken by Gorman and Kiang (1978) on soyabeans using alcohol dehydrogenase, amylase and tetrazolium oxidase electrophoretic patterns. As

a result it was possible to determine which loci segregated independently and which were linked. Bassiri (1977) did some investigations on genetic polymorphism in safflower using Iranian and introduced cultivars together with wild ecotypes. His electrophoretic results revealed that there was as much variation between the cultivars as between a cultivar and a wild ecotype. This indicated close genetic relationships between the cultivars and the wild ecotypes.

Electrophoresis has also been applied to plant breeding studies, especially those related to disease resistance. An attempt was made to screen Kenyan potato clones resistant to Late Blight by electrophoretic patterns of peroxidases (Nyachae, 1979). This has not been applied in practice. In beans, Gupta et al. (1981) observed certain peroxidase isozyme bands which appear at particular stages of development of lines susceptible to bean anthracnose. Okiror et al. (1982) again observed electrophoretic differences between bean lines known to be resistant and susceptible to bean anthracnose, with the help of peroxidase and esterase isozyme patterns. These electrophoretic differences were found to be useful in screening for anthracnose resistance at quite an early stage of development of beans. Peroxidase isozymes have

been used as genetic markers in an attempt to screen rice populations for desirable agronomic characters by Pawar and Gupta (1975). They found that peroxidase isozyme patterns fluctuated less in dwarf rice cultivars than in tall cultivars.

2.6 The use of electrophoresis for cultivar identification in crops.

Electrophoresis has become a very important tool for identifying different genotypes in crop species. It is a fast and inexpensive method. Large numbers of genotypes can be positively identified quickly using one or more enzyme systems simultaneously. Individuals that share the same isozyme pattern of one enzyme system can be differentiated by the isozyme patterns of other enzyme systems. In some cases, the enzyme systems are used to complement conventional methods of identification. The most important advantage of the electrophoretic technique is that isozyme patterns specific to cultivars are little affected, if at all, by differences in ecological factors (Lee and Ronalds, 1967). This is because isozymes or proteins are direct expressions of the genetic make-up of the plants. The electrophoretic pattern in a given organism is genetically

controlled and consistent for a given tissue and stage of development.

Several workers have used electrophoresis for cultivar identification. In grasses and cereals, Wilkinson and Beard (1972) were able to distinguish completely between six Creeping bentgrass (*Agrostis palustris*) and eight Kentucky bluegrass (*Poa pratensis*) cultivars using protein bands. Two Kentucky bluegrass cultivars could not be characterised using this method and had to be complimented with morphological observations for complete identification. Menke *et al*. (1973) studied the electrophoretic patterns of eleven common American wheat varieties, one durum wheat and one triticale variety. With esterase and peroxidase isozymes the durum wheat and triticale varieties were easily distinguished from all other varieties. Two common wheat varieties had similar patterns and could not be distinquished. Barley identification has been carried out by several researchers. Kahler and Allard (1970) studied the electrophoretic patterns of esterases in barley seedlings. Fedak (1974) used α-amylase, esterase and acid phosphatase electrophoretic patterns of endosperms of germinating seeds to identify 55 Canadian barley cultivars. It was necessary to complement these

enzyme systems with morphological characters especially of the kernel. The three enzyme systems alone could not provide complete identification because of the narrow genetic base of the Canadian barley cultivars. Contrary to these results, Bassiri (1976) was able to distinquish four Iranian and eight introduced barley cultivars to a large extent using esterase system alone. Peroxidase or acid phosphatase were only needed for complementation. This finding is attributed to the diverse origin of the cultivars used. Other work on the identification of cereal crops is that of Gupta and Malik (1980) on rice. Salt soluble seed proteins of seven different genotypes of rice were analysed electrophoretically using a polyacrylamide gel. The seven genotypes could be identified using the total of 28 bands formed by these genotypes.

The electrophoretic technique has also been used for identification of grain legumes and forage legumes. Identification of 40 broad bean cultivars of different origins with esterase and peroxidase isozyme patterns has been done by Bassiri and Rouhani (1976) in Iran. In their study, they found that the topmost leaves of three to four weeks old plants were more suitable than the lower leaves. Starch gel electrophoresis was

employed and a total of 10 and 17 bands were obtained for esterase and peroxidase systems respectively. Only four cultivars could not be characterised as they had similar banding patterns for both enzyme systems. For common bean cultivar identification, Bassiri and Adams (1978) observed the banding patterns of 34 U.S. major common cultivars belonging to 19 commercial classes, using acid phosphatase, esterase and peroxidase isozymes. Acid phosphatase was not found to be a suitable system because of the large number of monomorphic bands (i.e. bands are present in all cultivars studied) and few polymorphic bands (i.e. bands are present in some cultivars and absent in others. Esterase was also of limited use, but the peroxidase system was useful. Different cultivars belonging to the same commercial class could be identified completely based on all enzyme systems used. The same enzyme systems have been used by Quiros (1980) to distinguish between 21 alfalfa mother plants. The alfalfa clones identified included 18 tetraploids and 3 hexaploids.

Similarly, electrophoretic patterns have been used on other crops as well. Esterase, peroxidase and tuber protein patterns were used by Desborough and Peloquin (1968) to identify potato varieties. Forty five North American

potato varieties were used in the study employing disc electrophoresis. The two enzyme systems were used to identify 36 of the varieties. For complete identification of all the varieties, the additional use of the protein patterns was required. Bassiri (1977) used acid phosphatase and cathodal peroxidase of ten-day old seedlings to distinguish between five Iranian and nine introduced cultivars and seven wild ecotypes of safflower. He concluded that both enzyme systems could be used jointly for complete identification of the 21 genotypes. Electrohoresis has also been used for identification in maize (Brown and Allard, 1970) in peanuts (Cherry and Ory, 1973) in tomatoes (Rick and Fobes, 1975) and others. There is therefore ample evidence to show that it is worthwhile to investigate the use of isozyme patterns to identify pyrethrum clones.

3 MATERIALS AND METHODS

3.1 Materials:

The present study was based on twelve Kenyan commercial pyrethrum clones. They were obtained from the reference collection of clones at the National Pyrethrum and Horticultural Research Station, Molo. They comprise ten diploids and two triploids. The clones are listed below.

433	L/64/197	L/67/618
4729	Ma/65/252	Mo/70/1013
Ma/62/246	Ma/65/99	Ma/63/1889
O/64/219	Sb/65/58	Sb/66/107

The last two are triploids. These clones were selected from either single cross or polycross populations in various years and locations. The naming system of clones used at present indicates the year when selection was done and the location where it was done. For example clone Ma/62/246 was selected at Marindas (Ma) in 1962 and the selection number was 246. For the other clones, Mo stands for Molo, L for Limuru, Sb for Subkia and O for Ol Joro Orok. Clones 4331 and 4729 were selected before 1962 using a different naming system. In this list Mo/70/1013 is the most recently released clone, having been selected in 1970. Reference to clones in this study will be by their selection numbers only. There is no

proper record of the parentage of these clones. It is therefore not known how closely related they are. To determine whether the electrophoretic technique can be used to distinguish between closely related clones, twelve full-sib clones from the variety K200 (clone 4743 x clone L/66/473) were included in the study. They were numbered FS 1 to FS 12. In addition, the electrophoretic patterns of nine clones of Japanese origin were studied (J_1, 2, 3, 7, 9, 12, 14, 16 and 18).

All these clones were raised in a glass house with limited ventilation. The minimum temperature was 10°C and maximum was 36°C during the nine months growing and experimental period. A split of each clone was planted in a black polythene bag filled with humus rich soil obtained from Molo. One tea-spoonful of double superphosphate was thoroughly mixed with soil in each bag (equivalent to 200 kg per hectare). In order to study the effect of different soils, two clones were grown on various soil compositions. Clones 4331 and 4729 were each grown on a soil medium composed of top forest soil, sand and horse manure in the ratio of 2:1:1 and on Kabete soil (reddish brown lateritic soils).

All plants were watered twice a week. They were also frequently sprayed with Diazinon

(2.5 ml in 2 l of water) to control thrips and spider mites. Replicates of clones 219, 252, 1889, 4331 and 4729 were not sprayed for a period of two months. Samples taken from these clones were used to study the effect of the pests on the electrophoretic banding patterns of the enzymes studied. (The effect of the insecticide was considered to be confounded with that of the insect pests). Samples for analysis were not taken until all the clones had attained uniform vegetative growth since there are differences in establishment between clones. This took three months from the time of planting.

3.2 Methods

3.2.1 Preliminary trials.

The use of electrophoresis has not been reported for any study in pyrethrum. Therefore several preliminary studies were needed.

3.2.1.1 Sampling and enzyme extraction

Preliminary results showed that leaves were most appropriate to use for this study. Apical meristems, stems and leaf petioles were tried but were found unsuitable. One gram of leaves was weighed for each sampling from each clone. They

were kept in wet paper towels in a refrigerator ($4^{o}C$) till they were used. Each sample was crushed in a pre-chilled square excavated solid watch glass (embryo block) with a pestle. Each of the samples was put back in the refrigerator until all the preparations were ready for electrophoresis.

3.2.1.2 Starch gel preparation and electrophoresis.

Gel preparation and electrophoresis were done using standard methods described by Shaw and Koen (1968) with some modifications to suit this study. Hydrolysed starch (Sigma No. S-4501), was used with tris-citric acid buffer for gel preparation. The buffer was made from 19 ml of 0.05 M citric acid and 20 ml of 0.19 M tris (Hydroxymethyl) methylamine and the volume was made to 250 ml with distilled water. This gave pH 7.5. Preliminary trials showed that 40 g of starch with this volume of buffer gave a good gel consistency and good seperation of bands. In preparing the gel, a suspension of the starch was made in an erlenmeyer flask. The suspension was heated gently while keeping the flask in constant rotary agitation. Heating made the starch suspension first viscous and opaque. It soon became semi-transparent and less viscous. At this point air bubbles appeared at the bottom of the

flask and heating was discontinued. The gel was then completely degassed with a suction pump and immediately poured on to a plexiglass tray to settle uniformly. It was left to cool at room temperature for 20 minutes and then covered with a polythene sheet and transfered to the refrigerator for further cooling. After 40 minutes, the gel was ready for electrophoresis and was removed from the refrigerator.

For electrophoresis a slit was made in the gel 4 cm from the cathodal end. The crude extracts of the crushed leaf samples were absorbed with Whatman filter paper (No 3) measuring 6 mm x 9 mm. Excess juice was removed from the wicks by pressing them between folded paper towels. The wicks were then inserted in the slit on the gel. It was possible to run the extracts of 13 different samples simultaneously on the gel measuring 190 mm x 149 mm x 6 mm. Sponge cloths were used to connect the gel to electrode chambers which contained a buffer (pH 8.7) made up of 0.1 M boric acid and 0.1 M sodium hydroxide. Electrophoresis was conducted inside a refrigerator (4°C) at 165 Volts for the first 25 minutes to allow for slow separation of bands. The sample wicks were then removed and the voltage increased to 300 Volts. Electrophoresis was stopped as soon as the borate front had

migrated 10 cm from the point of wick insertion (Origin). The duration of electrophoresis was about five hours.

3.2.1.3 Location of enzymes on gels.

Upon completion of electrophoresis, the gels were sliced horizontally into two. Each half was stained separately for a different enzyme system or for the same enzyme using different staining methods. These methods differed in either pH of buffer, substrate used or staining conditions. Esterases, acid phosphatases, catalases and peroxidases are the enzyme systems that were tried in the preliminary studies. During these trials samples of barley and maize were included as control because they posses these enzymes (Scandalios, 1974). Different staining procedures tried for esterases were similar to those described by Shaw and Koen (1968), Brown and Allard (1969) Krasnuk *et al*. (1976) with some modifications, Hvid and Nielsen (1977) and Okiror *et al*. (1982). These methods showed that esterases are present in pyrethrum. They were however diffused throughout the migration zone.

Acid phosphatase was stained according to the methods of Shaw and Koen (1968), Krasnuk *et al*. (1976) with some modifications and Kahler *et al*.

(1981). Only the method of Kahler _et al._ (1981) resulted in clear resolution of bands. 0.1 g fast garnet GBC salt was dissolved in 5 ml distilled water and 0.5 ml 1% $MgCl_2$. The substrate (2.0 ml 1% naphthyl acid phosphate sodium salt — 1 g dissolved in 50 ml acetone and 50 ml H_2O) was then added to the buffer solution just prior to staining. The brown phosphatase bands appeared in one and half hours at room temperature. Three monomorphic bands with no differences in staining intensity were obtained.

Catalases were localised using the method of Shaw and Koen (1968) and that of Robinson (1966). No bands were observed for this enzyme system.

Three staining techniques were attempted for fingerprinting peroxidases: the methods used by Robinson (1966) with modifications, Krasnuk _et al._ (1975) and Gupta _et al._ (1981). The first method consisted of covering the gel with 0.5% hydrogen peroxide (100 vols) for about a minute. It was then washed three times with water and immersed in 95 ml of a 1% potassium iodide solution acidified with 5 ml of glacial acetic acid. Blue bands appeared within three minutes on the anodal gel only. They were fully developed after 20 minutes. The second method utilized guaiacol as the hydrogen donor.

The gels were incubated in 10 ml of 0.1 M guaiacol for 30 minutes at 35°C with subsequent transfer to 0.3% hydrogen peroxide. Brown anodal and cathodal bands appeared after about 20 minutes. The bands were not as sharp as those obtained with the acidified potassium iodide. Benzidine was used as the hydrogen donor for the third method. Equal amount of benzidine solution (made by dissolving 1 g benzidine in 9 ml of glacial acetic acid and 36 ml of water) and 3% hydrogen peroxide was poured on the gels. Blue bands appeared on both anodal and cathodal gels within two minutes and they were fully visible after 10 minutes. This method was easy and gave results quickly but the bands were not clear and sharp. They also turned brown and faded soon after they attained maximum visibility.

The modified method of Robinson (1966) was adopted during the following trials. It was fairly quick and gave clear, stable bands. Since the dye coupler used in this method was not specific to the cathodal peroxidases, benzidine was used to locate the cathodal bands.

3.2.2 Confirmatory trials

Results of the preliminary trials showed that peroxidase was the most suitable enzyme to be used

in electrophoretic identification of pyrethrum clones. This enzyme system was then studied in detail. Enzyme extracts from different developmental stages of the leaf tissue were run.

In all further experiments, one gram of leaves per clone was used and electrophoresis was conducted as described earlier. Clone 58 was arbitrarily chosen as standard. Samples of it were placed at both ends of the slit in the gel. This was to facilitate visual scoring of band staining intensities. When the bands were fully developed, the gels were washed with water and placed on a gel viewer. Fixing the bands was done by covering the gel with distilled water. Nine gels were run in total for the commercial clones. The full-sib clones and the Japanese clones were each run four times. The result of each experiment was photographed with colour film and also marked on graph paper. These results were later used to determine the means of staining intensities and migration positions of bands on the gel. The means were then used to draw the diagrams presented in the results.

Included in the work with peroxidases were studies of the effect of different soil compositions on isozymes of replicates of clones 4331 and 4729. The electrophoretic patterns of clones 219,252,

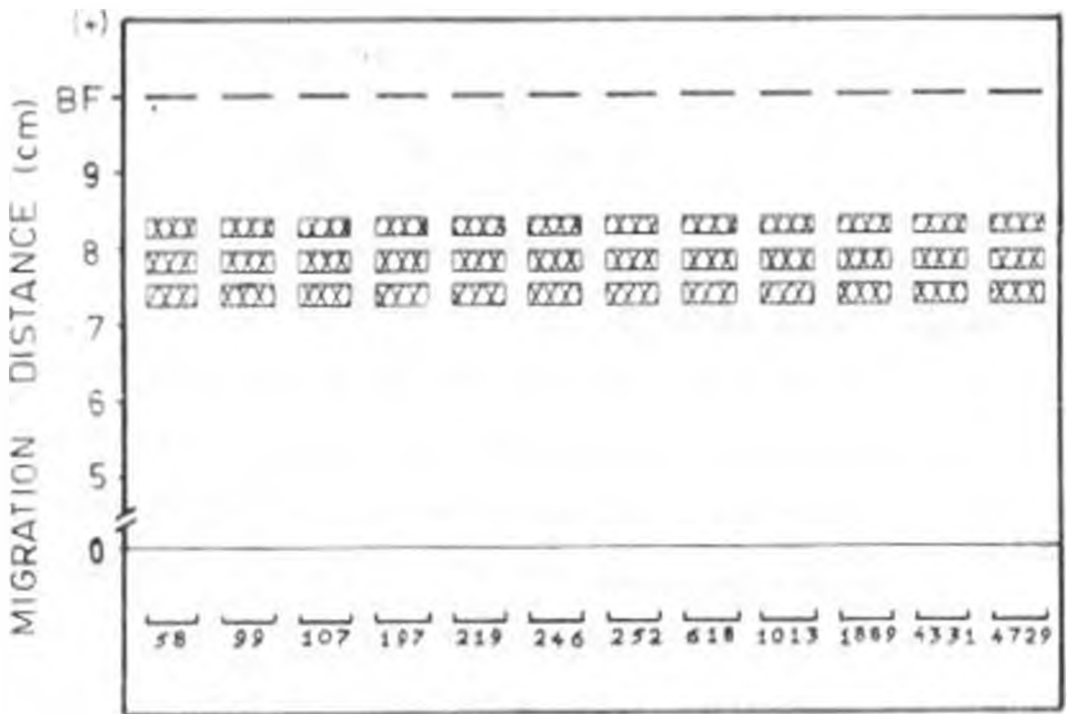
1889, 4331 and 4729 were compared with those of their replicates subjected to insect pest attack. Finally, samples of the twelve commercial clones were obtained from the research station at Molo and their peroxidase isozymes were studied. This study was meant to provide more information on the environmental effect of peroxidase isozyme patterns of these clones.

4 RESULTS

4.1 Preliminary tests.

Preliminary trials with the catalase, esterase, peroxidase and acid phosphatase enzyme systems showed that only the peroxidase system was useful for clonal identification in pyrethrum. This observation was made after trials with different staining techniques on apical meristems, stems, young leaves (1 to 2 weeks old) and mature green leaves. Catalases were not observed on any of the gels ran with sample extracts from the different tissues using two staining methods. The presence of esterases was observed using five staining methods on sample extracts from young leaves and apical meristems. They were not differentiated into bands but diffused throughout the migration zone (Plate 1) and so they were not used. Acid phosphatase isozymes were obtained using extracts from mature and young leaves. There were three monomorphic bands which did not exhibit any differences in staining intensities (Figure 2). Therefore these bands could not be used for identification. Peroxidase bands were further studied for use in identification. Enzyme extracts were obtained from leaves only as other

Figure 2. Diagramatic representation of electrophoretic banding patterns of acid phosphatases of 12 Kenyan commercial clones.



KEY

— Medium staining bands

0 — Origin

BF — Borate buffer front

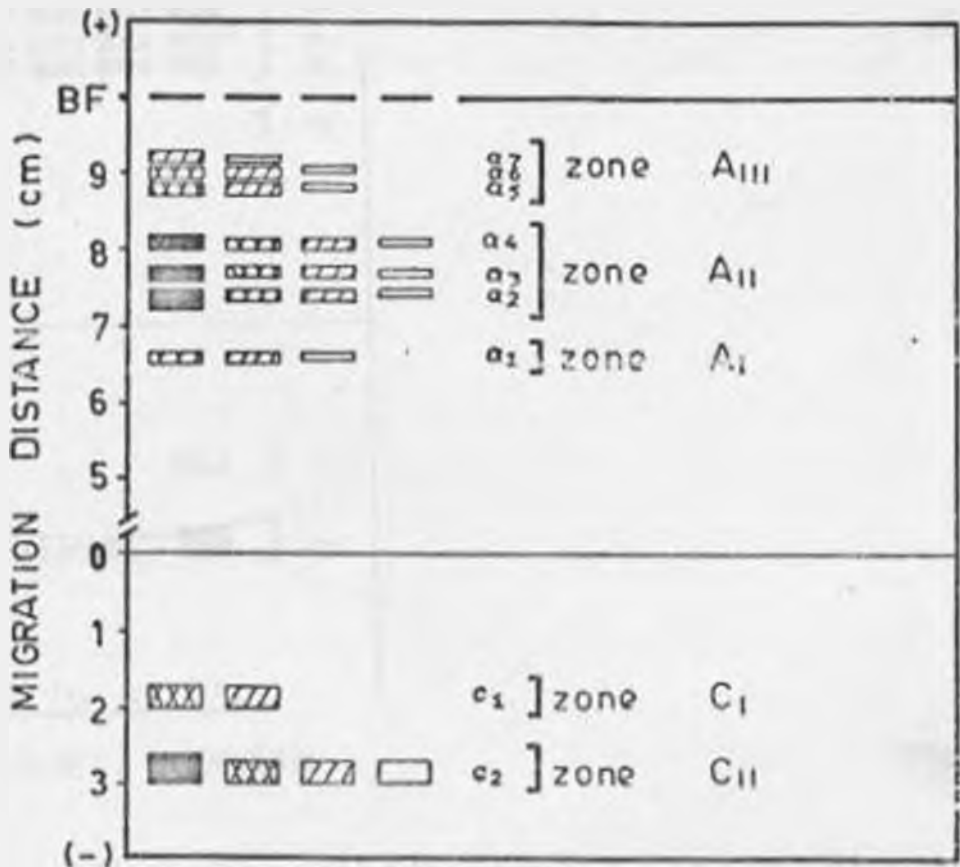
tissues were not suitable. Trials with samples from different developmental stages of the leaf tissue showed that mature green leaves of over four weeks old were most suitable. Younger leaves gave faint bands or no bands at all.

4.2 Peroxidase bands.

4.2.1 Description of the bands.

After examining the gels from all the runs, the banding patterns were established according to their mean staining intensities. The bands stained from dark and thick to faint and thin. These bands were grouped according to their migration distances from the point of sample insertion (origin). The migration distance of a band is read from the edge closest to the origin. A total of seven different bands were observed on the anodal side of the gel (a_1 to a_7) and two on the cathodal side (c_1 and c_2). These bands are presented in Figures 3 and 4. Results in Figure 4 are also presented in another form in Table 1. These bands occured such that 23 different combinations of mobility and intensity were observed on the anodal side of the gel and six on cathodal side. The anodal bands were divided in three zones designated as A_I, A_{II} and A_{III} and the

Figure 3. Different bands and intensities of anodal and cathodal peroxidases of 12 Kenyan commercial clones, 12 full-sib clones with their parents and nine clones of Japanese origin grouped according to migration distance into zones A_{I}, A_{II}, A_{III} and C_{I} and C_{II}.



KEY :

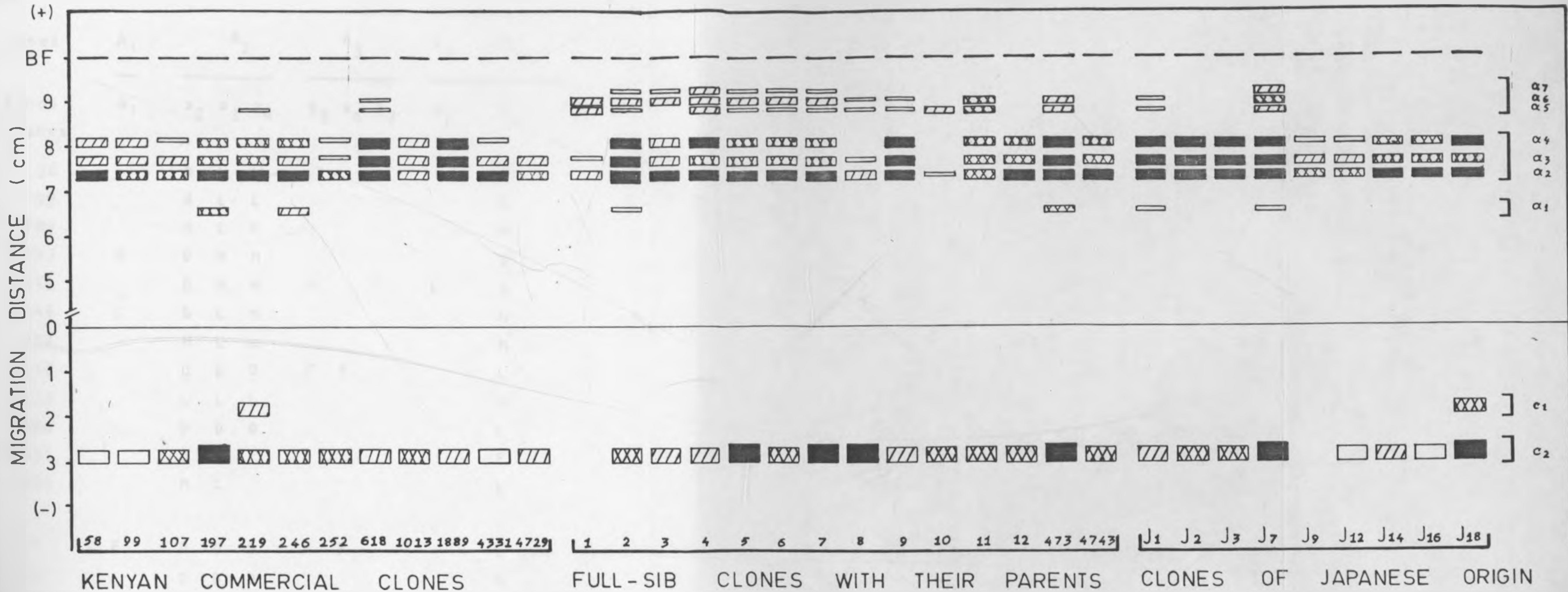
Staining intensity :

- Dark
- Medium
- Light
- Faint

0 — Origin

BF — Borate buffer front

Figure 4 Diagramatic representation of electrophoretic banding patterns of anodal and cathodal peroxidases of 12 Kenyan commercial clones, 12 full-sib clones with their parents and nine clones of Japanese origin.



KEY :

Staining intensity — See Figure 3.

0 — Origin.

BF — Borate buffer front.

Table 1. Different combinations of peroxidase bands according to migration position and staining intesity in the 35 clones studied.

		Anode							Cathode	
	Zones	A_I	A_2			A_3			C_I	C_{II}
	Bands	a_1	a_2	a_3	a_4	a_5	a_6	a_7	c_1	c_2
	Clones									
Kenyan commercial clones	58		D	L	L					F
	99		M	L	L					F
	107		M	L	F					M
	197	M	D	M	M					D
	219		D	M	M	F			L	M
	246	L	D	L	M					M
	252		M	L	L					M
	618		D	D	D	F	F			L
	1013		L	L	L					M
	1889		D	D	D					L
	4331		D	L	F					F
	4729		M	L						L
Clones of Japanes origin	J 1	F	D	D	D	F	F			L
	J 2		D	D	D					M
	J 3		D	D	D					M
	J 7	F	D	D	D	L	M	L		D
	J 9		M	L	F					
	J 12		M	L	F					F
	J 14		D	M	M					L
	J 16		D	M	M					F
	J 18		D	M	D				M	D

Key: D - Dark

M - Medium

L - Light

F - Faint

Table 1. cont.

		a_1	a_2	a_3	a_4	a_5	a_6	a_7	c_1	c_2
Full-sib clones and their parents	FS 1		L	F		L	L			
	FS 2	L	D	D	D	F	L	F		M
	FS 3		D	L	L		L	F		L
	FS 4		D	M	D	L	L	L		L
	FS 5		D	M	M	F	L	F		D
	FS 6		D	M	M	F	L	F		M
	FS 7		D	M	M	F	L	F		D
	FS 8		L	F		F	F			D
	FS 9		D	D	D	F				L
	FS 10		F			L				M
	FS 11		M	M	M	M	M			M
	FS 12		D	M	M					M
	4/3	M	D	D	D	L	L			D
	4743		D	M	M					

cathodal bands in two zones designated as C_I and C_{II}. Bands within zone A_{III} had the greatest relative mobility and they were in the range of 8.7 to 9.3 cm from the origin whereas bands from zone A_I had the lowest relative mobility being about 6.5 cm from the origin (the borate front had moved 10.0 cm). The C_{II} bands had the greatest relative mobility on the cathodal gel and the C_I bands had the lowest relative mobility being in the range of 2.6 cm to 3.0 cm and 1.7 cm to 2.0 cm respectively.

Bands in zones A_I and A_{III} were mostly light and faint. Only six clones had zone A_I bands. Clones 197 and 4743 had medium a_1 bands and 246 and FS_2 had light a_1 bands while J_1 and J_7 had faint bands at the same position. Zone A_{III} bands were less common among the commercial and Japanese clones, but twelve of the fourteen clones in the full-sib population had them. The Japanese clones with zone A_I bands also had A_{III} bands. Two commercial clones (219 and 618) had zone A_{III} bands which were all faint. Of all the clones, only two (FS_2 and J_7) had bands on all the seven anodal migration positions.

Zone A_{II} bands were found in clones from all three groups. These bands were useful for identification. They were distributed over three positions: a_2, a_3 and a_4. The dark a_2

band was the most common one, appearing in 24 clones. This band was rather thick in one clone (FS_2). The medium staining band at the same position was less frequent and appeared in 7 clones. The light a_2 band was found in three clones (1013, FS_1 and FS_8) and the faint a_2 band in only one (FS_{10}). At position a_3 a dark band occured in nine clones and a medium band occured in 12 clones while a light band was found in 10 clones. Five clones had the faint a_3 band. Eleven clones had the dark a_4 band, of which five were Japanese (J_1, J_2, J_3, J_7 and J_{18}), four were from the full-sib population (FS_2, FS_4, FS_9 and parent 473) and two were commercial clones (618 and 1889). Ten clones had a medium band at the same position and four had a light band. The faint a_4 band occured in five clones.

Generally, all clones had bands a_2 while some lacked either a_3 or a_4 or both of them.

There were not many cathodal bands. One band (c_1) was found in zone C_I with variation in staining intensity. Another band, also with varying staining intensity, was found in zone C_{II} on migration position c_2. Band c_1 was observed in only two clones (219 and J_{18}). Clone 219 had a light band and J_{18} had a medium band. In zone C_{II} a thick dark c_2 band was found in seven clones (197, FS_5, FS_7, FS_8, 473, J_7

and J_{18}). A thin c_2 band occured in three staining intensities: medium, light and faint. Thirteen clones had a medium staining band and eight had the light band whereas only five (58, 99, 4331, J_{12} and J_{16}) had a faint band. Two clones (FS_1 and J_9) did not have any of the cathodal bands and two (219 and J_{18}) had both the c_1 and c_2 bands.

With the help of the nine bands observed, attempts can be made to identify the clones on the basis of the band combinations appearing in each of them. However, the light and faint bands were generally not as important for indentification as the dark and medium bands. The light and faint bands sometimes showed variation between some samples of the same clone. In some gels, the light bands appeared as faint and the faint bands did not appear. Such variations were rarely observed among the dark and medium bands.

4.2.2 The banding patterns of the commercial clones.

The identification of the 12 commercial clones was done with emphasis on the anodal bands. The clones also showed differences in cathodal bands but these bands were not useful because they are subject to environmental changes (paragraph 4.3.1). Based on the anodal peroxidase bands, clones 197,

246, 219, 618, 219, 618, 1013 and 1889 had distinct banding patterns. Looking at Figure 4, Table 1 and Plate 2, clones 197 and 246 had a_1 bands which differed in staining intensity. These clones also differed in their a_3 bands which were medium and light respectively. Clones 219 and 618 were the only ones with bands in zone A_{III}. In this zone, clone 219 had a faint a_5 band and clone 618 had faint a_5 and a_6 bands. These two clones also differed in bands of zone A_{II}. Clone 219 had a dark a_2 and medium a_3 and a_4 bands, while clone 618, like clones 1013 and 1889, had bands at zone A_{II} which did not differ in staining intensities at positions a_2, a_3 and a_4. All A_{II} bands of clone 1013 were light and those of 618 and 1889 were dark. Among the other six clones, the light and faint a_3 and a_4 bands caused some close similarities. These bands sometimes did not stain well and so they were variable between some of the samples of the same clone. Cases like these were noted when, in some samples, the light bands were faint and the faint ones did not appear. When band a_4 of clone 99 was light, this clone was indistinguishable from clone 107. Similarly, when band a_3 of clone 107 was faint, the electrophoretic pattern of this clone was similar to that of clone

252. When band a_4 of clone 107 did not appear it was indistinguishable from clone 4729. However, all these clones can quite easily be distinguished from each other by simultaneously running two or three samples of the same clone because one of the samples may give band(s) which are not so faint. So, the use of more samples makes the process of distinguishing between clones with light and faint bands easy

Under standardised environmental conditions, the commercial clones could also be identified to some extent using the cathodal bands. Clones 197 and 219 were easily recognisable. Clone 197 had a thick dark c_2 band and clone 219 was the only one with a c_1 band. Three clones (58, 99, and 4331) had faint c_2 bands whereas clones 618, 1889 and 4729 had light c_2 bands. The rest of the clones (107, 246, 252 and 1013) had each a medium staining c_2 band.

All the twelve commercial clones could be distinguished individually using the anodal peroxidase bands alone. Several clones had the same cathodal bands. However, these bands could be used to differentiate between clones which had close similarities in their anodal bands.

4.2.3 The banding patterns of the full-sib population.

One important aspect of this study was to

find if electrophoresis can be used to distinquish closely related clones. Twelve full-sibs were used in the study. Their parents were included for future research on the inheritance of peroxidase isozymes in pyrethrum.

Comparison of the peroxidase patterns showed that two of the full-sibs were indistinguishable, a few had close similarities and others had unique banding patterns. FS_5, FS_6 and FS_7 had the same anodal banding patterns (Figure 4, Table 1 and Plate 3). FS_6 differed from the other two clones by the cathodal band c_2 which was medium staining. The c_4 band of clones FS_5 and FS_7 was thick and dark. FS_1 and FS_8 were similar in their a_2 and a_3 bands which were respectively light and faint. These clones differed slightly by their a_5 and a_6 bands. FS_1 had light bands and FS_8 had faint bands at these positions. The remaining clones (FS_2, FS_3, FS_4, FS_9, FS_{10}, FS_{11} and FS_{12}) had quite distinct anodal bands. FS_2 was the only clone with band a_1. FS_3 had no a_4 band and unlike FS_{12}, which did not have any of the zone A_{III} bands, it had a light a_6 and a faint a_7 band in this zone. All three zone A_{III} bands of clone FS_4 were light and its zone A_{II} bands (a_2, a_3 and a_4) were dark with the exception of a_3 which was medium staining. All zone A_{II} bands

of clone FS_9 were dark and those of FS_{11} were medium. In zone A_{III}, FS_9 had a faint a_5 band and FS_{11} had medium a_5 and a_6 bands. FS_{10} had a single band in each of zones A_{II} and A_{III}. These were a faint a_2 band and a light a_5 band.

Many of the full-sibs had some cathodal bands in common. Five clones (FS_2, FS_6, FS_{10}, FS_{11} and FS_{12}) had the medium band c_3, while FS_5, FS_7 and FS_8 had the thick dark band c_2. FS_3, FS_4 and FS_9 possesed the light c_2 band. One clone (FS_1) had no cathodal band. No full-sib had the c_1 band.

In this study, the full-sib clones showed similarities in their banding patterns, but there was also much variation.

4.2.4 The banding patterns of the Japanese clones.

Japanese clones were also included in this study in order to determine whether their electrophoretic patterns differed from those of the Kenyan clones. Three clones (J_1, J_7 and J_{18}) had unique anodal banding patterns among this group. (Figure 4 and Plate 4). J_1 and J_7 were the only clones with bands in the three anodal zones, J_7 being the only one with bands at all the seven positions of the three zones. J_1 had no a_7 bands. The other clone with unique pattern (J_{18}) did not posses any

bands in zone A_I and A_{III}. It had a medium a_3 band between dark a_2 and a_4 bands in zone A_{II}. Three pairs of clones could not be differentiated on the basis of the anodal bands. They were J_2 and J_3, J_9 and J_{12}, and J_{14} and J_{16}. Both J_2 and J_3 had dark a_2, a_3 and a_4 bands and each of clones J_9 and J_{12} had medium a_2, light a_3 and faint a_4 bands. The other indistinguishable pair, J_{14} and J_{16}, had a dark a_2 band and medium a_3 and a_4 bands.

Considering the cathodal bands as well, two clones (J_2 and J_3) were indistinguishable The other two pairs with similar anodal bands had different cathodal bands. J_9 had no cathodal band while J_{12} had a faint c_2 band. J_{14} and J_{16} differed by having respectively a light and a faint c_1 band. The clones which had unique anodal banding patterns (J_1, J_7 and J_{18}) also differed by their cathodal bands. J_{18} was the only clone with both a c_1 and a c_2 band.

An interesting observation was that the bands of these Japanese clones were distributed over the same migration zones as those of the Kenyan clones. However, each of the nine clones had an a_4 band. This is not the case in the Kenyan pyrethrum used in this study.

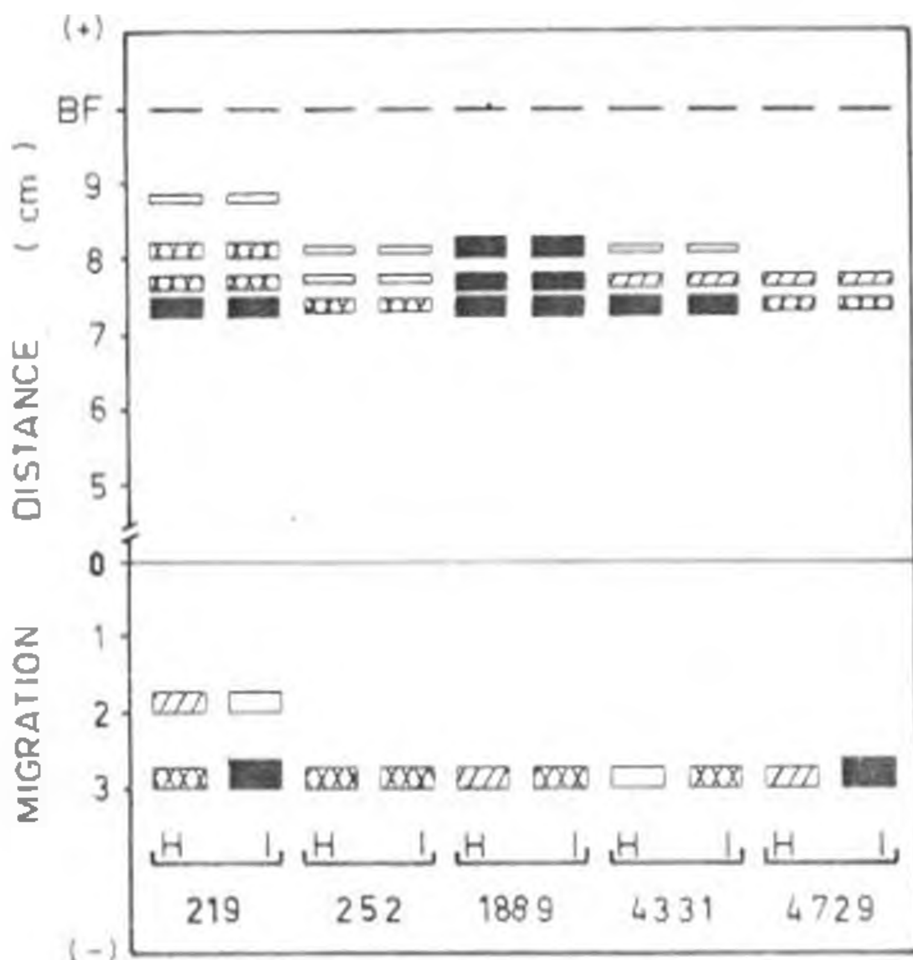
4.3 Effect of environment on peroxidases.

The effect of the environment on the peroxidase bands was studied by observing the combined effects of insects and insecticide used to control these insects, the effect of soils of different compositions and the variation in bands of samples of clones grown under environmental conditions different from the glass house conditions.

4.3.1 Insect infestation.

In the five clones tested, there were no differences in anodal bands between plants infested with insects and their healthy sprayed replicates (Figure 5 and Plate 5). The cathodal bands of insect infested plants were however markedly affected. The c_2 band of clone 1889 changed from light to medium when subjected to insect pest attack and the c_2 band of clone 4331 changed from faint to medium. Each of clones 219 and 4729 developed a thick dark c_2 band while their healthy replicates had respectively thin, medium and light c_2 bands. The c_1 band of clone 219 which was of medium staining intensity in the healthy replicate, was found to be light in the samples of the pest infested plant. Clone 252 was not affected by the insect infestation. It has been observed in the field and also in the

Figure 5 Diagramatic representation of electrophoretic banding patterns of anodal and cathodal peroxidases of both healthy (H) and insect infested (I) plants of 5 commercial clones.



Key See Figure 3

glass house that spider mites and thrips prefer some clones to others. Clone 252 was slightly attacked by these pests while the other clones had a rapid and heavy built up of insects on them. The change in cathodal peroxidase bands as a result of insect pest attack suggeststhat these bands may not be useful as markers for identification.

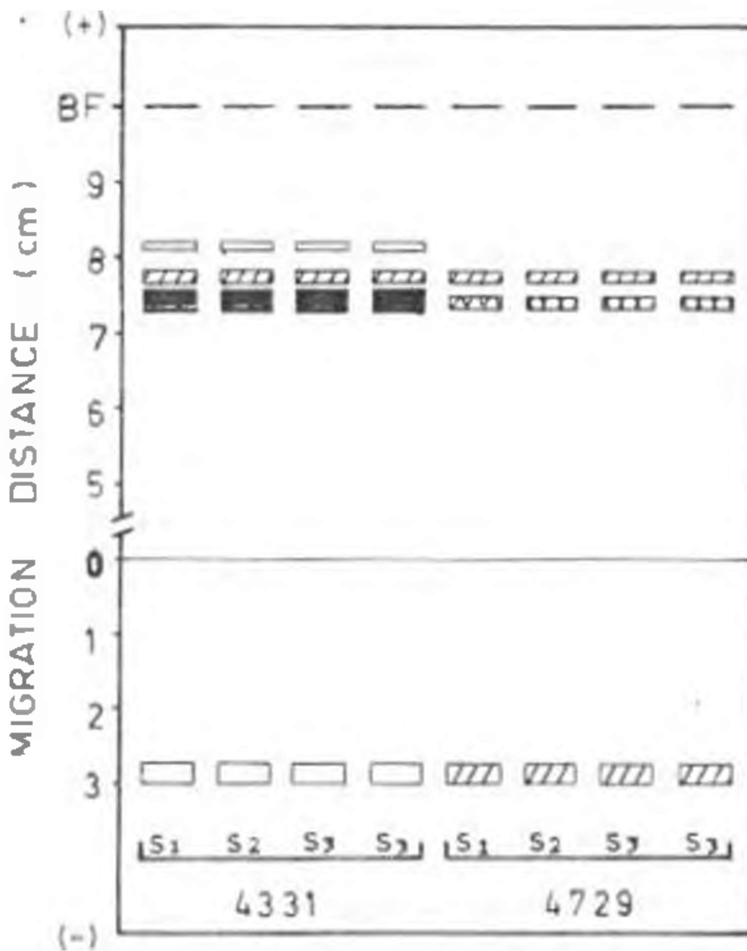
4.3.2 Differences in soil composition.

Two clones (4331 and 4729) were grown on three different soil compositions. The replicates of these clones which were used for the study had the same patterns as those obtained during the runs for identification (Figure 6). The peroxidase isozyme patterns of pyrethrum clones were not changed as a result of growing them on different soils. This shows that mineral composition in soil may not be an important factor in the peroxidase system in pyrethrum as it did not affect isozymes.

4.3.3 Differences in growth conditions.

The isozyme patterns of the twelve commercial clones grown at Molo were rather interesting when compared with the results obtained from glass house raised material. They differed significantly as none had the same banding

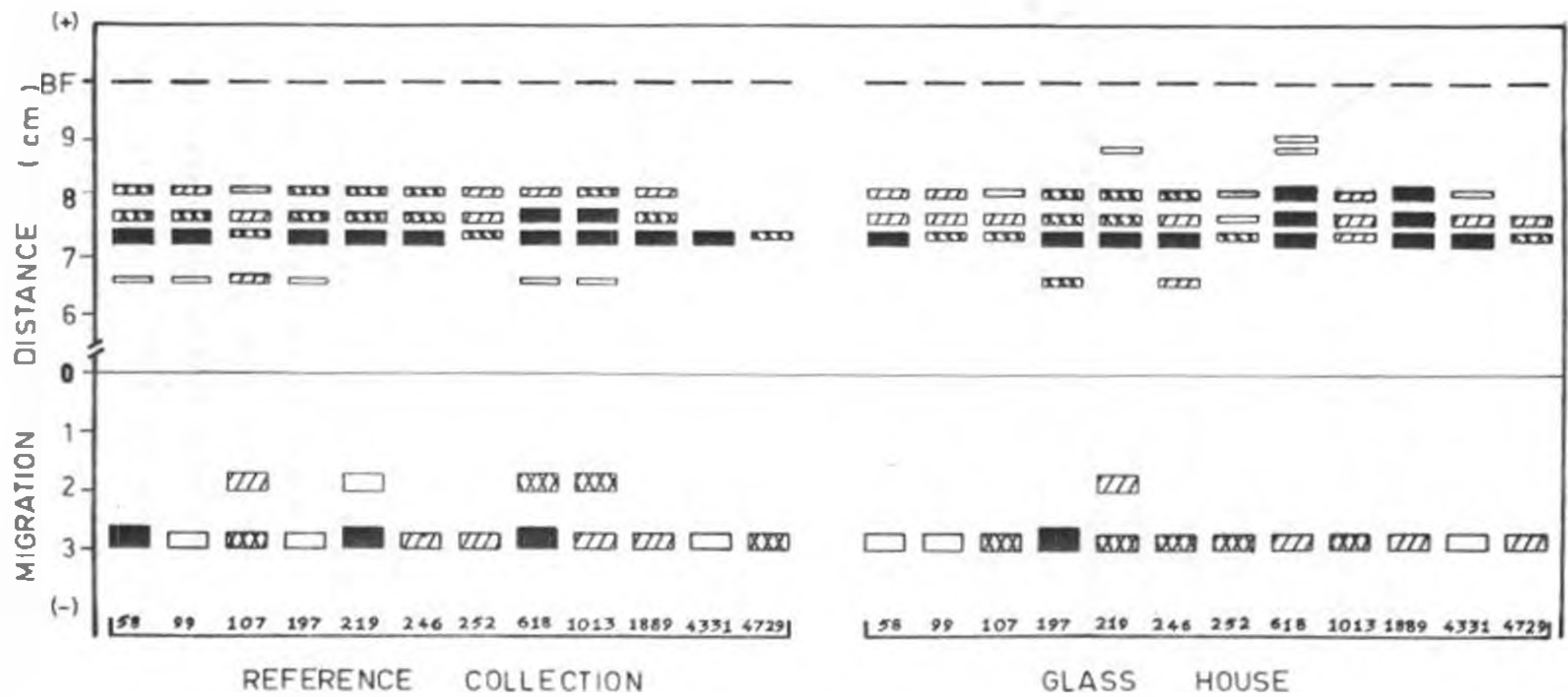
Figure 6. Diagramatic representation of electrophoretic banding patterns of anodal and cathodal peroxidases of two commercial clones each grown in three different soils: a prepared soil mixture (S_1), Kabete soil (S_2) and Molo soil (S_3).



Key: See figure 3

pattern as its replicate (Figure 7). Within each of two pairs of clones (58 and 197, 219 and 246) it was difficult to differentiate on the basis of the anodal bands. Clones 58 and 197 had dark a_2 and medium a_3 and a_4 bands like clones 219 and 246 but they also had light a_1 bands which the latter pair of clones did not have. The a_1 band was a new band for clone 58 while clone 197 in the glass house had a medium a_1 band. The a_2, a_3 and a_4 bands of clones 58 and 197 were similar to those of clone 197 grown in the glass house. These two clones differed by their cathodal band which was a dark c_2 band for clone 58 and faint c_2 band for clone 197. Clones 219 and 246 also differed by their cathodal bands. Clone 219 had a faint c_1 band and a dark c_2 band while clone 246 had a light c_2 band. These bands were different from those of the glass house material where the c_1 band of clone 219 was light and the c_2 band was medium, whereas the c_1 band of clone 246 was also medium. On the anodal side, band a_2 of clone 219 disappeared and clone 246 did not show the light a_1 band. Clones 99 and 1889 had similar bands in zone A_{II}. These were a dark a_2, medium a_3 and light a_4. Their differences arose because of a new faint a_1 band of clone 99 which clone 1889 did not possess and also because of their cathodal bands.

Figure 7. Diagramatic representation of electrophoretic banding patterns of anodal and cathodal peroxidases of 12 Kenyan Commercial clones, each grown at the reference collection plot at Molo, and in a glass house.



KEY— See Figure 3

The cathodal bands were a faint c_2 for clone 99 and a light one for clone 1889. Clones 58, 107, 618 and 1013 had new bands in zone A_I. Three of these clones, 58, 618 and 1013, had faint a_1 bands and clone 107 had a light a_1 band. In zone A_{II} clone 107 had bands similar to those of its replicate in the glass house and clone 618 also had similar bands in this zone except the a_4 band but the bands of clone 1013 were all different in this zone. They were dark a_2 and a_3 bands and a medium a_4 band. These were different from the light a_2, a_3 and a_4 bands observed in the glass house material. A new cathodal band was also observed for clones 107, 618 and 1013. Clone 107 had a light c_1 band and clones 618 and 1013 had medium c_1 bands. The c_2 bands of clones 107 were similar for the Molo and glass house materials, but clones 618 and 1013 had respectively dark and light c_2 bands while those of their glass house replicates were respectively light and medium. There were no changes in the a_2 bands of clones 252, 4331 and 4729. On the other hand, clone 4331 did not have its light a_3 and a_4 bands and clone 4729 did not have its light a_3 band. Clone 252 had light a_3 and a_4 bands unlike its glass house replicate which had both these bands faint. The cathodal bands of clones 252 and 4729 were also not the same as those of their glass

house counterparts. They had only c_2 bands which were light in clone 252 and medium in clone 4729. The c_2 band of clone 4331 did not change.

Although none of the clones grown at Molo had the same banding patterns as their replicates grown in the glass house, they could all be distinguished from each other.

5 DISCUSSION

5.1 Choice of enzyme system and tissue.

This study has shown that pyrethrum clones can be distiguished on the basis of their electrophoretic banding patterns. Four enzyme systems (catalases, esterases, acid phosphatases and peroxidases) in four tissues (apical meristems, stems, leaf petioles and leaves) were studied.

Catalases were not observed in any of the tissues, while esterases were found in apical meristems and 1 to 2 week old leaves. Extracts from mature green leaves (over 4 weeks old) as well as young leaves had acid phosphatase bands but peroxidase bands were consistently found only in mature green leaves and not in young leaves or apical meristems. Esterases were not used for identification because they were not differentiated into bands but diffused throughout the migration zone. Although acid phosphatase bands were observed, they were not useful for identification because of their monomorphic nature and lack of differences in staining intensity. Only peroxidase bands were found to be useful for identification purposes during the preliminary trials.

The variation of esterases and peroxidases

in different tissues can be seen as resulting from the functioning of specific series of alleles in each tissue. A similar explanation was given by Upadhya and Yee (1968) for variation of isozymes in different tissues of barley seedlings. This explanation does not, however, apply to the acid phosphatase isozymes which were monomorphic and they were observed in both young and mature leaves unlike esterases and peroxidases which occured only in young and mature leaves respectively. The acid phosphatases may be having an essential biochemical role in the pyrethrum plant and may not allow for variations. Quiros (1980) found the same banding patterns for leucine aminopeptidase isozymes in alfalfa clones and could therefore not use this for identification of alfalfa clones.

5.2 The peroxidase bands and their use in identification.

The peroxidase bands occured in three migration zones on the anodal side (A_I, A_{II} and A_{III}) and two on the cathodal side of the gel (C_I and C_{II}). In total, there were seven different bands on the anodal and two on the cathodal side of the gel. These bands varied in staining intensity from dark and thick to faint and thin. The presence and intensity of any of the bands in

a clone was used as a tool to distinguish between the clones. Zone A_{II} bands were most useful for identification. These were the bands designated a_2, a_3 and a_4, mostly dark and medium staining bands. The zone A_I bands (a_1) and zone A_{III} bands (a_5, a_6 and a_7) consisted mainly of light and faint bands which were sometimes variable between different samples of the same clone. Such variations involved the disappearance of a faint band or the appearance of a faint band instead of a light one. This was an observation rarely made among the dark and medium bands. However, clones with light and faint bands could still be distinguished from each other by simultaneously running two or three samples of the same clone since one of the samples may give slightly different band(s). Similar variations were also observed among the light and faint cathodal bands and rarely among the medium and dark bands.

The nine different bands obtained from the 35 clones studied were used for identification on the basis of the various band combinations which appeared in these clones. The anodal peroxidase bands alone were sufficient for complete identification of the 12 commercial clones. Identification was done by first considering clones

with distinctly different banding patterns. This was followed by looking for differences in number and intensity of bands, especially those in zone A_{II}, among the other clones. It was also possible to consider first clones which had similarities and then later find what bands could be used to differentiate them. For example the dark a_2 band was common among clones 58, 197, 219, 246, 618 1889 and 4331 (Figure 4 and Table 1). Clones 99, 107, 252 and 4729 had a medium a_2 band while only clone 1013 had a light a_2 band. Thus, clone 1013 is different from all the other clones and also the clones with the dark a_2 band can be distinguished from those with the medium band. Further differentiation is then achieved by similarly using bands a_3 and a_4 and bands in zone A_I and A_{III}.

Such differentiation as for the commercial clones also was possible for the full-sibs and the Japanese clones. An interesting observation on the clones of Japanese origin was that their type of banding patterns did not differ from that of the Kenyan clones. Their bands were distributed over the same migration zones as those of the Kenyan clones. This may be taken as an indication that the populations from which the Kenyan and Japanese clones have been selected might have had a

common genetic source. It was also noted that no band considering differences in intensity occured which was common to all the 35 clones studied. This was in contrast to the electrophoretic findings in the identification of potatoes (Desborough and Peloquin, 1968), peanuts (Cherry and Ory, 1973), wheat (Menke et al., 1973) and safflower (Bassiri, 1977). This high degree of polymorphism in peroxidase isozymes of pyrethrum supports a report by Parlevliet (1974) showing the existence of a considerable genetic variation in the Kenyan pyrethrum population. The observed monomorphic nature of acid phosphatase bands, however, contradicts this finding. This could be due to the different biochemical roles in the plant played by each of the enzymes.

Included in the commercial clones were two triploids (107 and 1889). Their banding patterns were similar to those of the diploids. This observation suggested that their increased chromosome number did not have a corresponding peroxidase isozyme effect. Similar observations were made by Quiros (1980) on alfalfa clones. The three hexaploids he used in his studies on identification had similar patterns to those of the 18 tetraploids in three enzyme systems studied (peroxidase, acid phosphatase and leucine

aminopeptidase). Triploid pyrethrum clones have a distinguishing feature in their large flower size compared with the diploids.

5.3 Distinguishing between closely related clones.

An important part of this study was to determine the possibility of using electrophoresis to establish differences between closely related pyrethrum clones. The peroxidase enzyme system failed to distinguish effectively between the 12 full-sibs used in the study. Two of them (FS_5 and FS_7) could not be distinguished from each other and other clones had close similarities. For example FS_6 differed from FS_5 and FS_7 only by the cathodal band c_2 (Figure 4, Table 1 and Plate 3) and FS_1 and FS_8 differed only by their a_5 and a_6 bands. However, most of the clones had unique banding patterns.

When the two parents were also considered, FS_{12} had similar bands to those of 4743. Only this full-sib was without zone A_{III} bands. The others resembled clone 473 closely in that they posessed these bands. Although none of the parents had band a_7, six of the full-sibs did. Similar observations were made about a_2 and a_4 bands. The parent clones had dark a_2 bands but two of the progenies (FS. and FS_8) had light bands and one (FS_{10}) [illegible]

band at that position. It was also observed that bands a_3 and a_4 were missing in FS_1 and FS_8. FS_1 also lacked band c_2. All these bands were present in the two parents.

A study involving several pairs of parent clones and their progenies could lead to a conclusion on the inheritance of these isozymes. It was generally observed that there was a wide variation in number and intensity of these bands among the full-sibs such that some had close similarities and others were distinctly different. The variation was in agreement with observations made by Miller *et al.* (1972). They used the electrophoretic technique to identify alfalfa hybrids from crosses and found that crosses between cultivars with the A protein and cultivars with the B protein produced seeds that contained both proteins but at reduced concentrations. They also observed a wide variation in number and density of bands. Their data indicated that the proteins were controlled by a pair of codominant genes at a single locus. This was also in accordance with the results of Brown and Allard (1969) on the inheritance of isozyme differences among the inbred parents of a reciprocal recurrent selection population of maize. They demonstrated that each of five enzyme systems

(alcohol dehydrogenase, cathodal esterase, anodal esterase, phosphatase and peroxidase) was governed by codominant alleles at a single Locus. Further, Menke et al. (1973) reported findings which showed that isozymes are generally inherited as major genes in Mendelian fashion.

The observed banding patterns of the 12 full-sibs cannot be used to draw a conclusion on the inheritance of the peroxidase isozymes in pyrethrum. On the observed similarities between some of the full-sib clones, it appears that some difficulties could arise using peroxidase to distinquish between closely related clones. These cases of identical clones are, however, not expected to be frequent considering that many of the full-sibs in this experiment were individually characterised.

5.4 Environmental influence on peroxidase.

In seperate experiments to determine the effect of enviromental factors on the peroxidase isozymes, the effect of insect pest infestation (thrips and spider mites), mineral nutrition and climate were studied.

5.4.1 Insect pest effect.

Examination of the results of the insect

infestation experiment revealed that the anodal bands were not affected but the cathodal bands were markedly affected by insects (Figure 5 and Plate 5). Four out of five clones showed an increased intensity in band c_2 after attack. There was no effect on the bands of clone 252. This clone seemed to show some resistance to these pests as it was only slightly attacked while the others had a heavy infestation. The observed increase in staining intensity of band c_2 of the other clones was an indication of altered activity of the peroxidase isozymes. The effect of the insect pests and hence the biochemical changes (disease symptom development) the isozymes underwent is not an isolated observation. Many workers have reported studies concerning peroxidases and how they have been linked with diseases in crops. Johnson and Cunningham (1972) showed in wheat that inoculated leaf rust resistant lines had higher peroxidase than the healthy controls nine days after inoculation. Okiror _et al_. (1982) showed that in beans anthracnose inoculation induced qualitative and quantitative changes in peroxidase isozymes. These changes did not affect all isozymes equally, showing that not all isozymes are involved in disease development. This also agrees with the present finding that only

cathodal isozymes are influenced by the pest attack. Whether the changes observed in the cathodal peroxidase isozymes in pyrethrum were due to injury or disease development as a result of the pest attack, is not known. Shannon (1969) has recorded findings in sweet potatoes where similar changes in peroxidase isozymes were obtained by wounding and by inoculation with the Black rot fungus, *Ceratocystis fimbriata*. The change in cathodal peroxidase bands as a result of insect infestation suggests that these bands may not be useful as markers for identification. For the cathodal bands to be reliably used as markers for identification, they should be consistent under a particular environment. This can only be achieved through proper control of the pests and this can be difficult under field conditions. Whenever proper control of these insects is possible, the cathodal bands can be used in addition to the anodal bands for identification.

5.4.2 Effect of soil differences.

In the experiment with different soil compositions, it was found that the different soil types used did not have any effect on the peroxidase isozymes (Figure 6). This was unlike the results obtained by Wilkinson and Beard (1972) in

Kentucky Blue grass where they noted the importance of uniform mineral nutrition for all experimental material. Mineral nutrition may then have different effects on different crops. In pyrethrum, it seems that peroxidase isozymes can be used as genetic markers for identification irrespective of soil composition. This is particularly so for the soils studied.

5.4.3 Differences in growth conditions.

The isozyme patterns of the twelve commercial clones grown at Molo differed significantly from those of their replicates grown for the experiment in the glass house (Figure 7). None of them had the same banding patterns as their replicates grown in the glass house. Two clones, 58 and 197, were indistinguishable by their anodal bands. Clones 219 and 246 had similar anodal bands but differed by their cathodal bands. Clones 99, 107, 618 and 1013 each had new bands in zone A_1. There were no changes in a_2 bands of clones 252, 4331 and 4729 but other bands differed. The other clones had various band combinations which also differed from those of their glass house replicates. Such differences in isozyme patterns between the plants of the same genotype grown at different locations

have been reported by other workers. Wilkinson and Beard (1972) noted the importance of growing experimental material of Kentucky blue grass (*Poa pratensis*) under uniform moisture, temperature, light and mineral nutrition prior to leaf sampling. McCown *et al*. (1969) had earlier observed prominent changes in peroxidase isozymes, while studying effects of environmental changes on 10 enzyme systems in *Dianthus*. The hardy cultivars developed additional bands under winter conditions. Henke *et al*. (1973) compared the esterase isozymes of three wheat varieties grown at two locations and got results which were not significantly different and they could not be attributed to environmental effects. But they recommended examination of more varieties and environments.

In pyrethrum, the peroxidase isozymes underwent qualitative and quantitative changes when grown under glass house conditions. It was however, observed that all the clones could individually be distinguished under glass house and field conditions. This observation was also in agreement with results obtained by Loeschcke and Stegemann (1966) showing that when ecological factors affected electrophoretic patterns, the banding pattern for each cultivar was unique under a given set of conditions. The extent

to which an individual clone is affected by change in climate therefore depends on the genetic make-up of that clone. Alteration of the expression of banding patterns can be said to result from changes in expression of alleles controlling each of the bands. Having noted the effect of thrips and spider mites on cathodal bands, these insects might have contributed to the alteration of the cathodal bands obtained in this experiment. Soil effect can be discounted in this experiment on the grounds that both the glass house and Molo plants grew on similar soils and the experiment with soils of different compositions has shown that it has no effect.
It seems therefore that in pyrethrum, temperature is a more important factor affecting peroxidase isozymes. This is supported by the observation that in the glass house all plants had a healthy growth but none of them flowered. It is known that chilling conditions, which were not met in glass house conditions, are necessary for flower initiation (Glover, 1955).

5.5 Conclusion.

The method presented here for identification of pyrethrum is not limited to the twelve commercial clones but it can be used for additional clones

which may have similar or unique patterns of peroxidase isozymes. Whether the clones being identified have some similarities or are distinctly different depends to some extent on the parentage of these clones. Differences between clones which have a common parent may be difficult to establish but the problem of identification can be solved considerably by running two or three samples of each clone simultaneously on the same gel. This procedure facilitates the visual scoring of staining intensity of individual bands of the clones. In case the electrophoretic technique fails to differentiate between any pair of clones, conventional methods for identification can be employed as a supplement. Differences in pyrethrins content can be used first whenever the plants are in flower and when the plants to be distinguished are suspected to have large differences in their pyrethrins content. In the absence of flowers, differences in crude protein content of leaves obtained by the Kjeldahl method can be used.

The large number of polymorphic peroxidase bands observed makes pyrethrum particularly suitable for studies on its genetics and physiological variations as reflected in these bands. The peroxidase bands can be used to

measure the variation present in synthetic or hybrid pyrethrum populations being grown commercially and also for clonal selection. It has already been observed that the full-sib population used in this study was quite variable.

On identification, an important step has been reached. Where the identity of clones is doubtful, peroxidase isozyme patterns offer a reliable and quick method as the electrophoretic technique gives results in less than eight hours. With the development of the tissue culture technique for rapid multiplication of pyrethrum clones, enzyme electrophoresis will be a handy aid in checking the genotypic purity of the clones being multiplied. Similarly, electrophoresis will be useful in mutation breeding programmes in pyrethrum research.

Summarising, a simple, reliable and quick method for identification of pyrethrum clones has been developed. The technique, electrophoresis of peroxidase isozymes in mature green leaf tissue, can be used effectively to differentiate the present 12 Kenyan pyrethrum commercial clones. Considering the polymorphic nature of these peroxidase bands, the technique could be used to distinguish between a large number of future clones

even if they are closely related. It will also be possible to carry out further research in pyrethrum with the help of its electrophoretic isozymes.

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Plate 1. Esterases in young (Y) and mature (M) leaves of three clones. Left to Right: 4729 M, 4729 Y, 4331 M, 4331 Y, and 252 M twice.

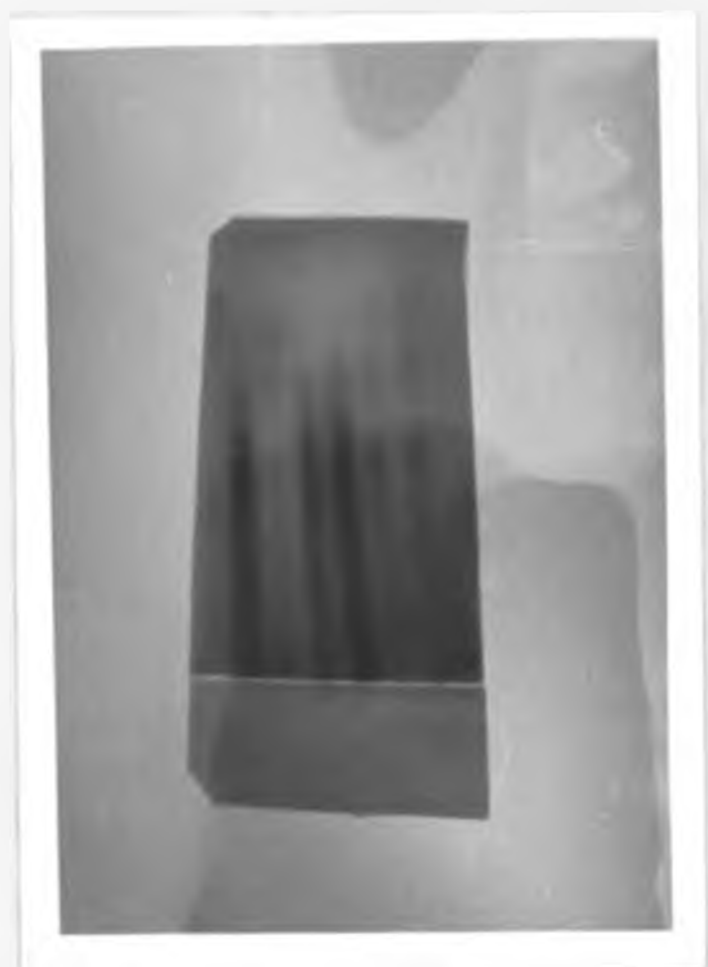


Plate 2. Anodal and cathodal peroxidases in 11 commercial clones. Left to Right: 58, 99, 107, 197, 219, 252, 618, 618 (insect attack), 1889, 4729, 4331 and 246.

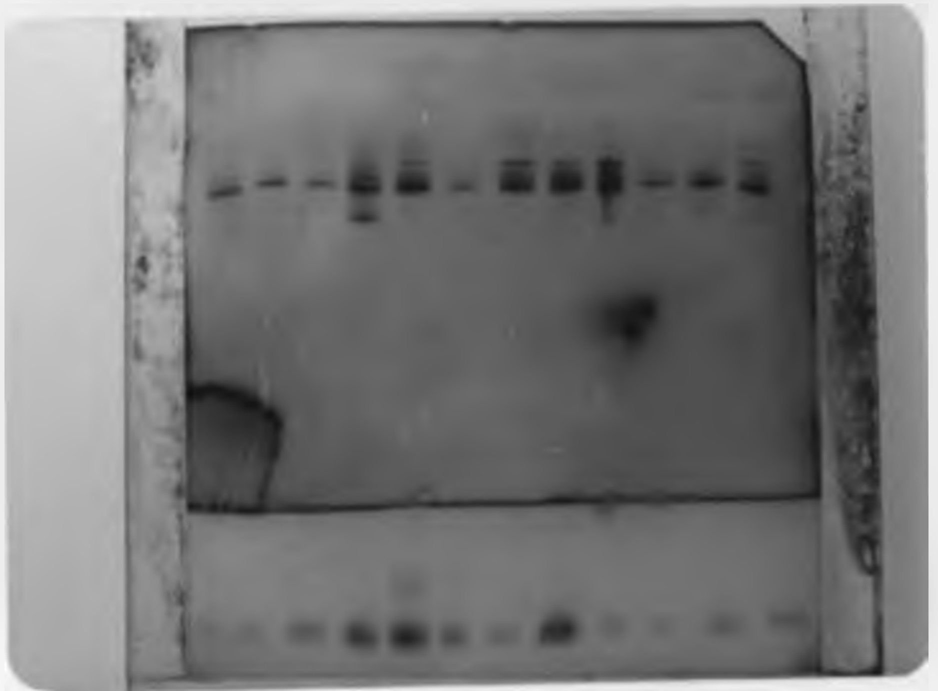
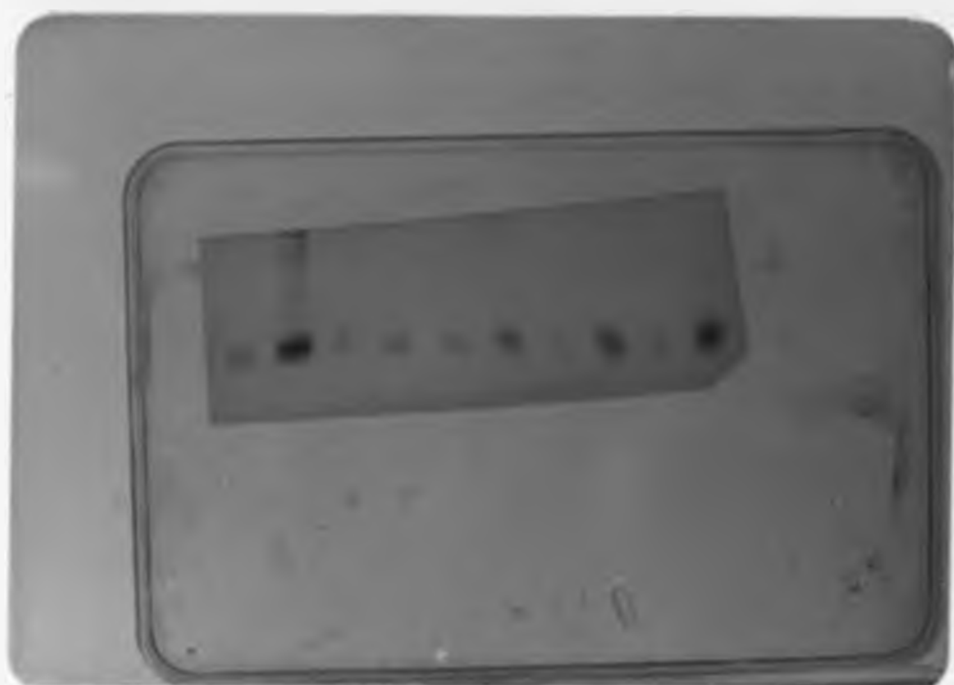


Plate 3. Anodal peroxidase bands in 12 full-sib clones with their parents. Left to Right: FS 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and parents 473 and 4743.

Plate 4. Anodal peroxidase bands in nine clones of Japanese origin and a commercial clone. Left to Right: J., 2, 3, 7, 9, 12, 14, 16, 18 twice, 107 twice and J7 twice.

Plate 5. Cathodal peroxidase bands in five commercial clones subjected to insect pest infestation (I) and their healthy sprayed replicates (H). Left to Right: 219 H, 219 I, 252 H, 252 I, 1889 H, 1889 I, 4331 H, 4331 I, 4729 H and 4729 I.



Appendix 1: Pyrethrum production from 1935 to 1981 in metric tons of dry flowers.

Period	flower production	Period	flower production
1934/35	327	1958/59	4912
1935/36	1095	1959/60	6604
1936/37	1005	1960/61	9312
1937/38	1894	1961/62	10931
1938/39	2915	1962/63	8511
1939/40	5954	1963/64	5269
1940/41	5856	1964/65	6256
1941/42	5557	1965/66	7876
1942/43	4173	1966/67	10698
1943/44	6652	1967/68	11237
1944/45	7528	1968/69	7423
1945/46	6848	1969/70	6005
1946/47	3970	1970/71	9748
1947/48	1582	1971/72	14414
1948/49	1541	1972/73	10698
1949/50	2211	1973/74	13722
1950/51	2266	1974/75	15034
1951/52	2781	1975/76	14267
1952/53	2356	1976/77	11428
1953/54	2591	1977/78	8437
1954/55	3527	1978/79	7450
1955/56	3477	1979/80	10423
1956/57	3933	1980/81	15702
1957/58	4596		

Source: Pyrethrum Board of Kenya production figures.

Appendix II: Yearly pyrethrum production in metric tons of dry flowers and their corresponding producer prices from 1965 to 1981.

Period	Flower production	Producer price Ksh./Kg of Pyrethrum
1964/65	6256	368
1965/66	7876	423
1966/67	10698	411
1967/68	11237	375
1968/69	7423	311
1969/70	6005	333
1970/71	9748	375
1971/72	14414	379
1972/73	10698	408
1973/74	13722	430
1974/75	15034	472
1975/76	14267	530
1976/77	11428	630
1977/78	8437	905
1978/79	7450	1200
1979/80	10423	1200
1980/81	15702	1200

Source: Pyrethrum Board of Kenya production figures.