AN EVALUATION OF FREE AMINO ACIDS AS CHEMOTAXONOMIC MARKERS IN *PROTEA* L. SPECIES

BY

DIANE PROSCH

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Supervisor : Professor J. N. Eloff

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1. INTRODUCTION

One of the major functions of a Botanic Garden should be that of research. When a research laboratory was established at Kirstenbosch National Botanic Garden in 1984, it was decided to start investigating chemical parameters of our indigenous plants, since this is a field that has not received adequate attention in the past. It was hoped to contribute towards the classification of plants by applying chemotaxonomy or chemosystematics, whereby chemical evidence is incorporated with morphological and other information. The genus Protea L. was an obvious choice for the initial material of such research as it is one of the best-known and most prominent genera of our indigenous flora and its taxonomic revision, based mainly on morphological data, was recently completed by Dr. J. P. Rourke (1980) of the National Botanic Gardens, Kirstenbosch. It was hoped that a phytochemical study of Protea species, whereby their free amino acid composition was determined by paper partition chromatography, might be of use in contributing to its taxonomic classification.

The major aim of this study was to determine the influence of various parameters on the free amino acid composition of Protea. If free amino acids are to be used as chemotaxonomic indicators in this genus, it is important to know how factors such as age and type of organ, seasonal variations, geographic locality, flowering or vegetative phase influence the amino acid composition.

Since free amino acids have been found to be genetically

important markers, one would expect that the free amino acid composition of a hybrid would reflect that of the parents. A secondary aim of the project was thus to determine the amino acid composition of Protea hybrids and their phenotypic parents, since natural hybridization between species is known to occur, with P. longifolia and P. laurifolia among "promiscuous" species reported (Rourke, 1980 ; Rycroft, 1958). The experiments on hybrids as well as those to determine the effects of the parameters cited were all carried out concurrently, so it was impossible to weight them in order of importance or to apply the findings of one when testing for another. The results presented in Chapters 3 and 4 thus do not follow any order of importance or relevance.

The first reported systematic application of paper partition chromatography is believed to be the work of Dent, Stepka and Steward in 1947; from a cold 70 - 80 % alcoholic extract of potato tuber tissue they separated out 27 substances which reacted with ninhydrin, of which 21 were identified as amino acids or amides and their derivatives, and 3 -alanine and 4-aminobutyric acid were later recognized (Steward, Thompson and Dent, 1949). Since then numerous such studies have been conducted, usually with the aim of identifying and/or isolating new non-protein amino acids in plants. These have been reported to occur in algae (Madgwick et al, 1970 ; Impellizeri et al, 1975), fungi (Oka et al, 1980), ferns (Virtanen and Berg, & Berg and Virtanen, 1954 ; Meier and Sorenson, 1979), as well as in higher plants. Rosenthal (1982) in his book "Plant Nonprotein and Amino and Imino Acids" gives an extensive and up-to-date

review of these and their plant sources. Alcoholic extracts of leaves or seeds are usually assayed, but the plant material used can be as diverse as roots and root nodules (Miettinen and Virtanen,1952), shoot apices (Steward et al, 1954), nectar (Baker and Baker, 1973 & 1976), cell sap (Hattori and Komamine, 1959), latex (Liss, 1962), fruits (Ellington et al, 1959 ; Gray and Fowden, 1962) and gum exudates (Anderson et al, 1984 & 1985).

As many and new non-protein amino acids have been discovered, interest in their value as chemotaxonomic markers has grown to follow suit. Examples of this are reported for the chemosystematic studies on the Liliaceae, Agavaceae and Amaryllidaceae (Fowden and Steward, 1957), the Aloineae (Riley and Isbell, 1963), on Lathyrus (Bell, 1962), on Vicia (Bell and Tirimanna, 1965), the Cucurbitaceae (Dunnill and Fowden, 1965), on Acacia (Anderson et al, 1984 & 1985), and the Capparidaceae (Nageshwar et al, 1984). Apart from the work of Van Staden in 1966, no chemosystematic work has been conducted on Protea using free amino acids as markers.

In addition to those occurring naturally, Protea hybrids are being cultivated to meet the requirements of the cut flower industry which is expanding rapidly in South Africa although our flowers represent only 0,8% of the market of exporting countries (Anonymous, 1985 a). Of the horticulturally produced cut-flower proteas, 80 - 90 % are either pincushions or Protea spp. (Anonymous, 1985 b). In order to satisfy the overseas market with blooms of top quality with an appealing colour and good stem

length, protea hybrids are being cultivated and propagated at the Protea Research Unit of the Horticultural Research Institute at Tygerhoek, Riviersonderend. A recent report by Mr. G. Brits of Tygerhoek mentions that about 16 protea cultivars, including Protea and Leucospermum hybrids are generally available to protea farmers in South Africa (Brits, 1985). Plant material from 8 Protea hybrids and 3 P. repens varieties cultivated at Tygerhoek, as well as from 2 Protea hybrids growing in the gardens at Kirstenbosch were assayed. Since amino acid composition is genetically determined, it would be expected that hybrids would reflect the free amino acid composition of either or both parents.

Results from analyses of 15 Protea species were used to interpret the possible influence of parameters such as seasonal variations, type and age of plant organ, geographical locality as well as reproducibility of data and the effect of vegetative or flowering phase on free amino acid composition. A total of 40 free amino acids were located by two-dimensional paper chromatography, which included 7 non-protein amino acids and 12 that were unidentified. The latter are of special importance in chemotaxonomy, since the twenty-two protein amino acids and amides are expected to occur universally in all living systems (Charlwood and Bell, 1977), but the presence or absence and characteristic associations of non-protein amino acids can be of taxonomic and nutritional significance in the subdivision of taxa (Bell and Tirimanna, 1965).

Terminology for the parts of the plant assayed is that recommended by J. Pretorius of the Rand Afrikaans University in a paper presented at the First International Protea Research Symposium held in Cape Town in August 1985. Briefly, in the abstract of his paper, he recommends that the Protea inflorescence, borne at the end of a flowering branch, be called a flower head, consisting of many florets (individual flowers), which are sessile on the involucral receptacle and surrounded by the involucral bracts. " The involucral bracts are grouped into series; the bracts of the lower (outer) rows are shorter and scaly and are called scaly bracts, whereas the bracts of the inner rows are longer and showy and are called the inner or colour bracts ". In this work, the term bracts refers to a combination of both the scaly and colour bracts. I use the term vegetative foliage to refer to leaves borne on non-flowering branches.

2. MATERIALS AND METHODS

2.1 Description of plant material assayed

Plant material was collected from several localities around the Western Cape, which will be specified when the results are presented. It was always compared with the same plant material (i.e. the same type of organ from the same species) growing at Kirstenbosch. There are two Protea hybrids growing in the protea gardens at Kirstenbosch viz. Protea eximia x P. roupelliae ssp. roupelliae and Protea laurifolia x P. magnifica; plant material from these hybrids as well as from their phenotypic parent species was assayed. Additional material from Protea hybrids was collected from the Protea Research Unit of the Horticultural Research Institute at Tygerhoek, Riviersonderend. Two collecting trips were undertaken there in March (autumn) and September (spring), so that seasonal and temporal effects on free amino acid composition could be evaluated for some species, in addition to the usual comparison with their phenotypic parent species growing at Kirstenbosch. Voucher specimans of material collected in the field are kept at the Compton Herbarium, Kirstenbosch National Botanic Gardens. Their accession numbers and those of the plants growing at Kirstenbosch and Tygerhoek are noted in an appendix at the end of this thesis. Intra-specific variation was not tested, and plant material from the hybrids and from most field material was usually derived from just one plant; where stands of the same Protea species were cultivated at Kirstenbosch or in the field (e.g. P.repens and P.compacta near Bot River),

the same material from several plants was combined and assayed.

Plant material from the gardens at Kirstenbosch was gathered between 09h00 and 10h00 and assayed immediately thereafter. Material gathered on field collecting trips was placed in plastic bags and transported in polystyrene cooler boxes containing frozen ice-packs. Since such trips usually lasted the whole day, the plant material thus collected was stored in a refrigerator overnight and assayed the following day with no browning of foliage (De Swardt & Pretorius, 1980) being detected. However, browning did occur if plant material was stored in the refrigerator for longer periods.

2.2 Extraction procedure.

The soluble nitrogenous fraction of 30 - 50g fresh plant material was extracted with methanol - chloroform - water (MCW) (12 : 5 : 3 , v/v) after the method of Bieleski and Turner (1966). To compensate for the fact that all plant material assayed had a moisture content of 50% or more of its fresh mass, the actual MCW ratio measured out was 12 : 5 : 1,5 v/v. Since the results were expressed on a dry weight basis, dry mass was determined by weighing a separate sample, with fresh mass as close as possible to that of the samples to be assayed, before and after drying in an oven at 90 - 100 ° C for 24 hours. About 300 ml MCW per 30g fresh plant mass were used.

Plant material was macerated in a Kenwood blender with the appropriate volume of methanol and water, since it had been noted

at an early stage that the chloroform destroyed the plastic material of the blender jug. The extract was decanted through glass wool held in a funnel into an Erlenmeyer flask of appropriate volume. The chloroform was then poured through the glass wool retained material, followed by additional volumes of water (162 ml) and chloroform (90 ml) to extract the lipids and pigments.

2.3 Group separation

This solution was then left to stand overnight to allow the two phases to separate :- the lower chloroform layer contained the lipids and plastids and the upper methanol - water layer contained the free amino acids and amides. It was a personal preference to use Erlenmeyer flasks rather than separatory funnels for this procedure. The methanol-water layer was decanted off, again through glass wool contained in a funnel, into a round-bottomed flask for <u>in vacuo</u> evaporation. The methanol was evaporated off under vacuum and the sample, thus concentrated to approximately half-volume, was applied to a Dowex 50W - X8, 200 - 400 mesh, cation exchange column in the H^+ form. The glass columns were 21,5 cm high with an internal diameter of 2 cm ; the tapering outlet was plugged with glass wool and the Dowex 50 resin filled the lowest 4,5 - 5 cm . The samples were almost neutral with a pH of about 6,0 .

Each column was washed with about a litre of deionised distilled water to remove all non-cationic material, then eluted with 3N NH_4OH. Preliminary experiments, whereby 7,5 ml aliquots were

eluted , evaporated in vacuo and then developed one-dimensionally by descending chromatography on Whatman no.1 chromatography paper in a monophasic butan-1-ol - glacial acetic acid - water (BAW) solvent (90 : 10 : 29 , v/v) showed that both acidic and basic amino acids had adhered to the column and were subsequently displaced ; aspartic acid was the first amino acid to be eluted and isoluecine/leucine the last after about 140 ml of 2N NH_4OH had passed through. They also showed that the water eluates, before and after elution with N NH_4OH, when concentrated in vacuo and separated out by paper chromatography, showed only trace amounts of a few free amino acids, none of which did not appear in the NH_4OH eluate. Therefore, the amino acids were eluted with 180 - 200 ml 3N NH_4OH into a round-bottomed evaporating flask and evaporated under vacuum to a volume of 1 - 2 ml . The exact volume was measured in a 5 ml measuring cylinder, then stored in screw-capped polystyrene tubes in a refrigerator.

2.4 Amino acid separation

Separations were carried out by descending paper chromatography on Whatman no. 1 chromatography paper. As a preliminary step, arbitrary aliquots of the extracts to be assayed were chromatographed one-dimensionally in monophasic BAW (90 : 10 : 29 , v/v) to gauge the presence and concentration of free amino acids present. Thereafter, aliquots equivalent to about 180 to 600 mg dry mass of plant material were applied to sheets of Whatman no. 1 chromatography paper, and separated two-dimensionally with monophasic BAW (90 : 10 : 29 , v/v) in the

first dimension, and with water-saturated phenol in the second dimension, all performed at room temperature. The chromatograms were dried in a fume cupboard, and developed by spraying with 0,25% ninhydrin (indanetrione hydrate) dissolved in 5% methanolic 2,4,6-collidine (trimethyl pyridine) as a locating agent. When heat was applied from a Novex 1250W hairdryer, turned on at full speed and full heat, the free amino acids showed up as spots stained shades of purple and violet, blue, yellow, orange, pink and brown. Their relative concentrations were estimated by allocating a figure based on an arbitrary scale from one to five according to the relative sizes and intensities of the ninhydrin-reacting spots. Amino acids were identified from references in the literature and verified by two-dimensional chromatography of the amino acid purchased from chemical companies or from standard kits supplied by Merck Co. The presence and patterns of free amino acids were thus recorded for each plant, and photocopies of the developed chromatograms were made and filed. Some results were quantified by sending small samples, equivalent to 30 - 60 mg dry mass, through an LKB 4150 automatic alpha amino acid analyser.

Most chemicals used were purchased from BDH or Merck and the amino acids mainly from Fluka with a few from BDH, Merck or Sigma.

3. RESULTS AND DISCUSSION

3.1 Introduction

Results are presented from the two-dimensional paper chromatographic study of **Protea** material. The concentration of free amino acids detected is expressed on an arbitrary scale from 1 - 5, depending on the colour intensity and spot size after development with the locating agent. Tr indicates that merely a trace was detected after 24 hours at room temperature. In some of the Tables, a summation of these arbitrary values is made, to give a total arbitrary concentration value of compounds reacting with ninhydrin. In such cases, those amino acids detected in trace amounts are given an arbitrary value of 0,5.

The amino acids were identified by reference to standard two-dimensional chromatograms, co-chromatography of standards, automatic amino acid analyses and by ninhydrin colour reaction. Some amino acids were isolated and chemically characterised (Van Schalkwyk, 1986).

Isoleucine (Ile) and leucine (Leu) were detected as a single spot since their Rf values for both solvents are very close. However, results from the amino acid analyser printout show that both were present in all species studied and that the Ile concentration was usually double that of Leu. The Rf values for valine (Val) and methionine (Met) were also so similar that they could not be detected separately by the paper chromatography method. Analysis by means of an automatic amino acid analyser showed that Met is

present in very small quantities, so small that they could not be expressed as a percentage from the samples assayed this way, therefore the spot located in the position that these amino acids would be on a paper chromatogram run with the solvents described was interpreted as being mainly valine and is expressed as such in the tabulated results.

A diagrammatic representation of the positions of the free amino acids detected by two-directional paper chromatography is given in Fig. 1, followed by a key to their identity and their colour reaction with the locating agent (Table 1). Abbreviations used in this work are given in brackets. The spots which are not colour-coded in Fig. 1 were shades of purple or violet, which is the usual colour reaction for most free amino acids sprayed with ninhydrin as a locating agent.

Table 1 : Key to the identity of the free amino acids depicted in the preceding diagram (Figure 1), abbreviations used in the text, and the code for their colour reaction with the locating agent.

1 - Glycine	(Gly)	(pink ; P)
2 - Alanine	(Ala)	(purple)
3 - Valine	(Val)	(purple)
4 - Methionine	(Met)	(purple)
5 - Isoleucine	(Ile)	(purple)
6 - Leucine	(Leu)	(purple)
7 - Arginine	(Arg)	(purple)
8 - Lysine	(Lys)	(purple)
9 - Aspartic acid	(Asp)	(blue ; Bl)
10 - Glutamic acid	(Glu)	(purple)
11 - Asparagine	(Asn)	(orange ; O)
12 - Glutamine	(Gln)	(violet)
13 - Serine	(Ser)	(grey ; Gr)
14 - Threonine	(Thre)	(grey ; Gr)
15 - Phenylalanine	(Pheala)	(red-brown ; Br)
16 - Tyrosine	(Tyr)	(grey ; Gr)
17 - Proline	(Pro)	(yellow ; Y)
18 - Hydroxyproline	(Hypro)	(pink ; P)
19 - Tryptophan	(Trp)	(blue-purple)
20 - Histidine	(His)	(purple)
21 - Cysteine	(Cys)	(brown-pink ; P)
22 - Cystine	(Cys-cys)	(purple)
23 - Pipecolic acid	(Pipe)	(blue-purple
24 - Ethanolamine	(Etam)	(brown ; Br)
25 - 3 - alanine	(3-Ala)	(blue ; Bl)
26 - 4-Aminobutyric acid	(4- Aba)	(purple)
27 - Phosphoserine	(P-ser)	(purple)
28 - Ornithine	(Orn)	(purple)
29 - 2,3-Diaminopropionic acid	(2,3-Di)	(pink ; P)
U1-U12 - Unidentified amino acids		

3.2 Comparison of free amino acids detectable in old and young foliage

In order to determine whether the age of the leaf influenced free amino acid composition, very young leaves from the tips of the branches were collected and assayed from September onwards, as the leaf buds were noted to be opening from this time, and results compared with those for older vegetative foliage from the previous season. The *Protea* species from which material was assayed are listed in Table 2, and the results are presented in Table 3.

Table 2. List of *Protea* species studied.

No.	Name of species
1	P. burchellii
2	P. eximia
3	P. longifolia
4	P. roupelliae ssp. roupelliae
5	P. eximia x P. roupelliae
6	P. laurifolia
7	P. nitida
8	P. eximia x P. susannae
9	P. magnifica x P. susannae
10	P. repens x P. cynaroides

Table 3. Comparison of levels of free amino acids in old (o) and young (y) foliage. The species studied are given in Table 2. The totals are the summation of the ninhydrin reacting compounds , giving their arbitrary total concentrations .

	1		2		3		4		5	
	o	y	o	y	o	y	o	y	o	y
Gly	2-3	4	2-3	4	2	3	Tr	2-3	2	4
Ala	2-3	4	1	4	3	3	Tr	4	1-2	3-4
Val	1	3	Tr	2		3		2-3	Tr	3-4
Leu/Ile	Tr	3	Tr	2		2		3	Tr	3
Arg		4		Tr	Tr	4		Tr		
Lys		5	Tr			3		Tr		3
Asp	3-4	4	3	2	4	2	3	3-4	3	3
Glu	4	4-5	3	4	4	4	3	5	3-4	5
Asn		2	2	1-2	Tr			2-3		3
Gln	2	2	Tr	2-3	1	2-3		2-3	Tr	3
Ser	3	4-5	2	4	3-4	3		4		4-5
Thre	Tr	2-3		2	Tr	2		2	Tr	2
Pheala		2		2		2				3
Tyr		2-3		Tr		1				Tr
Pro	Tr	2	Tr	1	Tr	2		2	Tr	2
Hypro				1		1				
Trp		2		2						
His		4		1	Tr	3				2
Cys										
Cys-cys										
Pipe	3	2			Tr	2-3				
Etam		2		3	Tr	2		2		2
3-Ala	1	3	1	2-3	Tr	1		2	1	2
4-Aba		1		1-2	Tr	1		3		2
P-ser	2	3	2	2	1	1	2	2-3	2	2
Orn						1				
2,3-Di										
U1		2		1		2-3		1		1
U2				1				Tr		Tr
U3		3		Tr	1	1-2				2
U4		2								
U5			1	1						
U6										
U7				2		2-3				
U8	Tr	1		1				1		1
U9				2		1		1		1
U10										
U11										
U12										
Total	26,5	74	20	53,5	24	56,5	9	47,5	15,5	58,5

Table 3 (contd.). Comparison of levels of free amino acids in old (o) and young (y) foliage. The species studied are given in Table 2. The totals are the summation of the ninhydrin-reacting compounds, giving their arbitrary total concentrations.

	6		7		8		9		10	
	o	y	o	y	o	y	o	y	o	y
Gly	3	2-3	5	3	4	4	3-4	5	4	3
Ala	3	3-4	2-3	2	2-3	3	2-3	4	4	4
Val	Tr	3	1	Tr	Tr			1	3	3
Leu/Ile	Tr	3	Tr	2	1				3	3
Arg	Tr	2	3	5					3	3
Lys		5	2	5	Tr				3	3
Asp	3-4	3-4	4	1	4	3-4	3	5	2	1-2
Glu	3-4	4	4	4	4	4	4	5	4	3
Asn	2	2	1	3	2		1	1-2	2-3	2
Gln	1	3	1		2	1	1-2	4	2	1
Ser	2	5	2	3	4	3	2	3-4	4	4
Thre	Tr	1-2	2	3	1	1	Tr	Tr	3	2
Pheala									2	2
Tyr		Tr	Tr	Tr	Tr				2	2
Pro	Tr	2	Tr	Tr	Tr	2	Tr	Tr	Tr	Tr
Hypro		1						2		1
Trp										
His	Tr	2		2		Tr			2	2
Cys						3	2	2		1-2
Cys-cys							2			
Pipe	2	1								
Etam		2							3	3
3-Ala	Tr	3			1-2	1		Tr	2	2-3
4-Aba	Tr	3	1	2	Tr				3	1
P-ser	2	1	1	1	2	2	1-2	3	3	2
Orn	1	2				2	1	Tr	Tr	2
2,3-Di	1				2-3	2-3	1-2	Tr		
U1			2	2			3		3	2
U2									Tr	1
U3							Tr	Tr	Tr	Tr
U4										
U5						Tr		Tr		
U6										
U7										
U8				1-2					1	1
U9										
U10			4	1-2						
U11			3	3						
U12			3	3						2
Total	28	55,5	43	48,5	33	33	30	39,5	60,5	58,5

Discussion of results

From Table 3, a comparison may be made of the free amino acids detected by two-dimensional paper chromatography in very young and the older vegetative foliage of the plant. Results from the young foliage of species no. 1, 2, 3, 4 & 5 are compared with those from older foliage assayed several months previously, whereas the results for species 6, 7, 8, 9 & 10 are from young and old foliage collected and assayed simultaneously. The total at the bottom of each column is the sum of the concentration values, and is the arbitrary total value of ninhydrin-reacting compounds detected with trace amounts allocated an arbitrary value of 0,5.

As can be seen from the results presented in Table 3, there is a general increase in the concentration of most free amino acids detected in the young foliage and, except for nos. 7 & 8, more free amino acids were detected in young foliage than in old. Only in no.10 were almost the same free amino acids detected in both young and old foliage and in like concentrations. On average, the total concentration of amino acids in young leaves on a dry weight basis was nearly twice as high as that of old leaves (52 vs. 29 concentration units, cf. Table 3).

Gly, Ala, Asp, Glu, and P-ser were detected in both young and old foliage of every species assayed with Glu present in high concentrations throughout. Very high concentrations of Lys were detected in the young foliage of nos. 1, 6 and 7 ; it was detected in the young foliage only of species 1, 3, 5, 6 & 8.

Hypro and Trp, where detected, were present only in the young foliage.

Pipe was only detected in species 1, 3 & 6, where it was detected in both young and old foliage. Etam was not detected at all in species 7, 8 or 9 and except for species no. 10 , it featured mainly in young foliage.

Generally speaking, more of the unknown non-protein amino acids were detected in young foliage than in old ; only U1 was detected in old foliage without being detected in the young foliage as well (Table 3 no.9). U10, U11 and U12, which are often detected together and could be associated in some common metabolic pathway (cf. discussion for Fig. 2, Chap. 3.4 b), were detected in both young and old foliage of species no. 7 , and U12 was detected in the young foliage of species no. 10.

The arbitrary total values of ninhydrin-reacting compounds showed the greatest differences between old and young foliage in those species where the plant material was assayed at different times, usually months apart. The differences in concentration and number of free amino acids detected might thus be due to a seasonal effect correlating with the subtropical summer growth rhythm reported for proteoid species (Specht, 1979 ; Pierce & Cowling, 1984). The generally higher number and concentrations of free amino acids detected in young foliage may be due to the fact that the young, rapidly metabolizing tissue creates a sink for nitrogen reserves, as indicated by higher Asn and Gln levels in the young foliage of all species except no. 10.

3.3 Effect of geographic locality on free amino acid composition

In order to determine the effect of geographic locality on free amino acid composition, a study was made of the free amino acids detectable in Protea species from various localities around the Western Cape and also of those from the same species growing in different garden beds at Kirstenbosch, since these originated from different localities according to plant records. The results, of free amino acid composition detected by two-dimensional paper chromatography, are given in Table 5. Table 4 lists the Protea species and plant organ studied with the locality given within brackets. The original locality of the plants growing at Kirstenbosch with their year of planting at Kirstenbosch in brackets are given below :

1. P. burchellii : Dassenberg - SW/Malmesbury (1980)
2. P. compacta : Farm "Hangnes", Zoetanysberg (1982)
3. P. cynaroides : Garcia Forest Reserve (1979)
4. P. longifolia : Just past Springfontein (1971)
5. P. magnifica L7 : Franschhoek, above Pass (1984)
   P. magnifica L15 : Galgeberg (1980)
6. P nitida : Guerna Kop (1973)
7. P. repens : 7 m W of Salem (1973)
8. P. scabra : Upper Buffels River Valley (1972)
9. P. eximia L2 : Kleinplaat, Forest Reserve (1979)
   P. eximia L7 : Swartberg Pass (1979)
10. P. roupelliae : No information recorded.

Table 4. List of *Protea* species studied and their locality.

No.	Species, organ studied and locality
1 a	P. burchellii Old vegetative foliage (Kirstenbosch)
b	P. burchellii Young vegetative foliage (Kirstenbosch)
c	P. burchellii Vegetative foliage (Slanghoek)
2 a	P. compacta Foliage (Kirstenbosch)
b	P. compacta Florets (Kirstenbosch)
c	P. compacta Foliage (Bot River)
d	P. compacta Florets (Bot River)
3 a	P. cynaroides Foliage (Kirstenbosch)
b	P. cynaroides Foliage (Kleinmond)
4 a	P. longifolia Foliage sub inflorescence (Kirstenbosch)
b	P. longifolia Florets (Kirstenbosch)
c	P. longifolia Foliage sub inflorescence (Bot River)
d	P. longifolia Florets (Bot River)
5 a	P. magnifica Foliage (Kirstenbosch L7)
b	P. magnifica Foliage (Kirstenbosch L15)
6 a	P. nitida Young foliage (Kirstenbosch)
b	P. nitida Young foliage (Tulbagh)
7 a	P. repens Foliage (Kirstenbosch)
b	P. repens Foliage (Bot River)
c	P. repens var. Alicedale Foliage (Tygerhoek)
d	P. repens var. Kouga Foliage (Tygerhoek)
e	P. repens var. Witzenberg Foliage (Tygerhoek)
8 a	P. scabra Vegetative foliage (Kirstenbosch)
b	P. scabra Vegetative foliage (Kleinmond)
9 a	P. eximia Young foliage (Kirstenbosch L2)
b	P. eximia Young foliage (Kirstenbosch L7)
10 a	P. roupelliae Bracts (Kirstenbosch)
b	P. roupelliae Bracts (Mkambati Reserve, Transkei)
c	P. roupelliae Florets (Kirstenbosch)
d	P. roupelliae Florets (Mkambati Reserve, Transkei)

Table 5. Distribution of free amino acids detectable in *Protea* species from various localities. See Table 4 for identity and origin of species assayed.

	1			2				3		4			
	a	b	c	a	b	c	d	a	b	a	b	c	d
Gly	3	4	3	5	2	2	2	3	4	2	2	2	1
Ala	3	4	3	4	3	3	3-4	3	4	3	2	2-3	3
Val	1	3	2	2	3	Tr	3	1		Tr	2	Tr	3
Leu/Ile	Tr	3	2	Tr	3-4	Tr	3		1	Tr	1	Tr	2
Arg		4	1	Tr	3	Tr	4					Tr	3
Lys		5	Tr	2	4		3-4	Tr	1		2-3	Tr	2-3
Asp	3-4	4	4	5	5	2	4-5	3	3	4	3-4	3-4	3-4
Glu	4	5	4-5	5	5	4	5	4	4	4	4	4	4
Asn		2	2-3	1	4-5	Tr	3			Tr	2-3		3-4
Gln	2	2	2	3	4	1	3	1	Tr	1-2	2-3	1-2	3
Ser	3	4-5	4-5	4	4	2-3	3-4	3	2	2-3	2	2	3
Thre	1	2-3	2	1	1	1	Tr	1	Tr	1	1	1	1
Pheala		2			2		2			1-2	1		1-2
Tyr		2-3	Tr	Tr			Tr		Tr		Tr	Tr	Tr
Pro	1	1-2	1	2	2	2	3	Tr		1	3-4	1	3-4
Hypro							1				1		2
Trp		2	1				1				2		2
His		4	1	1	3			1					
Cys								1	2		Tr		
Cys-cys				2	Tr								
Pipe	3	2	1			Tr	1			1			
Etam		2	1	1	2-3		1-2	Tr		Tr			Tr
3-Ala	1	3	1	1	1		1	1		1	2	Tr	1
4-Aba		1	1	1	3	Tr	2	1	4	Tr	3	Tr	3
P-ser	2	3	2	1	2		2	1-2	1	1	2	1	1
Orn				Tr	Tr								
2,3-Di											Tr	1	
U1		2					2						
U2													
U3		3	Tr	Tr	Tr	Tr	Tr						
U4		2					1-2				2		1
U5				2									
U6													
U7													
U8	Tr	1	Tr		1	1	Tr		Tr		Tr		Tr
U9													
U10								2	4				
U11								3	2				
U12								3-4	2				
Total	28,5	74	41,5	45,5	60	22	58	34,5	36	26	43,5	23	49

Table 5 (contd.). Distribution of free amino acids detectable in Protea species from various localities. See Table 4 for identity and origin of species assayed.

	5		6		7					8		9	
	a	b	a	b	a	b	c	d	e	a	b	a	b
Gly	3	3	3	3	3-4	3	3	2	1	3		4	4
Ala	3	3	2	3	4	3	4	3	4	3	1-2	4	4
Val	1	2	Tr	3	1	2	2	1	2		Tr	2	2
Leu/Ile	Tr	1-2	2	3	1	2	1	1	2		Tr	2	2
Arg		1	5	2								2	Tr
Lys		1-2	5			1-2		Tr			Tr		
Asp	4	4	1	2	4	5	2	3	2	2	2-3	1	2
Glu	4	4	4	5	5	5	3	3-4	4	4	3	4	4
Asn		1	3	4	1	1		1	1			1	1-2
Gln	1	1-2		2	1	1-2	1	Tr		1-2	Tr	1	2-3
Ser	3	4	3	4	3	3	3	2	1	3	1	4	4
Thre	Tr	1	3	2	1	1	1	1	Tr	4	Tr	2	2
Pheala						1	2	1	1			3	2
Tyr			Tr	Tr			1					Tr	Tr
Pro	Tr	1-2	Tr	Tr	1	1	1	2	2		Tr	1	1
Hypro							1						1
Trp													2
His		1	5	2		Tr						2	1
Cys													
Cys-cys												1	
Pipe	2-3	2-3			1	1	2	1	2				
Etam				1		1	1	1	2	2	Tr	3	3
3-Ala	1	1				1	2	Tr	2			3	2-3
4-Aba		Tr	2	2			4	1	1		Tr	2-3	1-2
P-ser	2	2	1	1	2		2	2	2	2	1-2	1	2
Orn		Tr								2			1
2,3-Di		Tr			Tr			Tr		2			
U1			2				1					Tr	1
U2									Tr			1	1
U3												1-2	Tr
U4						1							
U5	1	Tr					1	1	Tr			1	1
U6										4	2		
U7							1	Tr	3			3	2
U8			1-2		1	1							1
U9							1	1	1			1	2
U10			1-2										
U11			3										
U12			3										
Total	27	37,5	51,5	40	30	35,5	40	30	34,5	32,5	15,5	52	54,5

Table 5 (contd.). Distribution of free amino acids detectable in Protea species from various localities. See Table 4 for identity and origin of species assayed.

	10			
	a	b	c	d
Gly	2	2	3	3
Ala	3	4	3	3
Val	2	3	3	3
Leu/Ile	2	3	2	3
Arg	Tr	1	1	2
Lys	Tr	1	1	3
Asp	3	3	3	2
Glu	3-4	2	4	3
Asn	3	4	4	4
Gln	2	3	3	4
Ser	3	4	3-4	4
Thre	1	1	1	2
Pheala		1		1
Tyr		1	2	1
Pro	3	3	3-4	3
Hypro	1		1	Tr
Trp	2	3	2	2
His	Tr	1	1	3
Cys				
Cys-cys	1		1	1
Pipe	Tr		1	Tr
Etam	1	1	2	2
3-Ala	1	2	1-2	2
4-Aba	1	3	2	2
P-ser	2	2	1-2	2
Orn				1
2,3-Di				
U1				2
U2				
U3				Tr
U4				
U5			Tr	
U6				
U7	Tr	1		1
U8	Tr	1	1	1
U9	Tr			
U10				
U11				
U12				
Total	40	50	51,5	61,5

Discussion of results

P.burchellii : Results from the assays of old (a) and young (b) vegetative foliage from P. burchellii growing at Kirstenbosch were compared with vegetative foliage (c) comprising both old and young foliage collected from P. burchellii growing at Twee Heuwels on the Slanghoek - Bains Kloof Road (Table 5 no. 1). More free amino acids were detected and at elevated concentrations in young foliage (b) compared with old (a) from Kirstenbosch, and also when compared with foliage from Slanghoek (c). However, the number and relative concentrations of free amino acids detected in (c) are higher than those in (a). These results are also reflected in the totals representing the arbitrary concentration values for all ninhydrin reacting compounds detected in each species. Glu and Ser were detected in the highest concentrations in (b) and (c), with Gly, Ala, Asp, Glu and Ser being detected in the highest concentrations in all. Possibly due to both old and young foliage comprising (c), Arg, Lys, Tyr, Trp, His, Etam and U3 were detected in (c), albeit in lower concentrations, and in (b). Hypro, Cys, Cys-cys, Orn and 2,3-Di were not detected at all. Pipe was detected in all three samples, elevated in old foliage (a). Locality does not seem to have affected the amino acid composition of (c) when compared with the same species growing at Kirstenbosch.

P. compacta : Foliage (c) and florets (d) of P. compacta were collected and assayed from a locality just before the Bot River en route to Hermanus, and compared with like material growing in

the gardens at Kirstenbosch (Table 5 no. 2). Fewer free amino acids were detected in (c) than in (a) and their concentrations were lower. Glu was detected in the highest concentrations in all, along with Asp in (a), (b) and (d). Lys, Asn and Ser concentrations were high in the florets with Phe detected in the florets (b) and (d) only. Small amounts of Pipe were detected only in the material from Bot River (c) and (d). His, Cys-cys and traces of Orn were detected only in the material from Kirstenbosch. U3 was detected in all and U8 in the florets from both localities, (b) and (d). U1 and U4 were detected only in the florets from Bot River (d) , and U5 only in the foliage from Kirstenbosch (a). In the florets, (b) and (d), the same free amino acids when present are more alike in concentration than in foliage. The greater similarities between the number and concentrations of free amino acids detected in florets compared with that of the foliage is reflected in the arbitrary concentration totals for the organs compared (Table 5 no. 2).

P.cynaroides : Results from the assays of vegetative foliage of P. cynaroides from the quarry site at Kleinmond (b) were compared with those from the vegetative foliage of P. cynaroides growing at Kirstenbosch (a) (Table 5 no. 3). Where the same free amino acids were detected, they were alike in concentration except for 4-Aba, where concentrations were elevated in the Kleinmond material (b). Glu was detected in the highest concentration , followed by Asp, Gly and Ala. An interesting feature of their chromatograms was the presence of U10, U11 and U12, which are usually detected together, but in only a few of the species

assayed, and are not always detected for material assayed at different times throughout the year. They might therefore be indicative of the metabolic state of the plant, and be important indicators of a metabolic pathway common to only a few species. The totals show a very close correlation between the arbitrary total concentration values for ninhydrin reacting compounds.

P. longifolia : Results from assays of foliage and florets, (c) and (d), collected from a locality before Bot River en route to Hermanus were compared with those from the same material growing at Kirstenbosch (a) and (b) (Table 5 no. 4). Glu and Asp were detected in the highest concentrations for all, with elevated concentrations of Lys, Asn, Pro and 4-Aba in the florets (b) and (d). Hypro, Trp, U4 and U8 were detected in the florets only, (b) and (d). 2,3-Di was detected in the florets from Kirstenbosch (b) and in the foliage from Bot River (c). For both foliage and florets, the free amino acids detected and their relative concentrations were similar for both localities, and this is reflected in their arbitrary concentration totals of ninhydrin-reacting compounds detected.

P.magnifica : Results from the assays of foliage from two different P. magnifica stands, in different flower beds at Kirstenbosch and originating from different localities according to plant records, were compared (Table 5 no. 5). The assays were performed at the same time, thus eliminating seasonal effect. More free amino acids were detected in (b), and in general their concentrations were elevated when compared with (a). These results are

reflected in the arbitrary concentration totals of ninhydrin-reacting compounds for both. Glu, Asp, Ser, Gly and Ala were detected in the highest concentrations in both. Of the non-protein amino acids, U5 was detected in both, with like concentrations of P-ser, Pipe and 3-Ala ; Etam was not detected in either. (cf. Table 10 for a quantification by amino acid analyser of the results obtained by paper chromatography for (a) and (b)). Since environmental conditions were the same for all the plants assayed, any differences in free amino acid composition would be genetically determined. These were not too pronounced for the material assayed, and were possibly an indication of the metabolic state of the plants when the foliage was assayed.

P. nitida : Results from the young foliage of a P. nitida tree in the Tulbagh Waterfall Reserve (b) were compared with those from the young foliage of P. nitida growing at Kirstenbosch (a) (Table 5 no. 6). The same protein amino acids were detected in both, albeit at slightly higher concentrations in (b), except for the basic amino acids Arg, Lys and His, which were located collectively as a very large and concentrated spot in (a) and were allocated the maximum value of the arbitrary scale, although individually their values might differ and be somewhat less. Glu was detected in the highest concentrations in both, along with Asn and Ser and the basic free amino acids in (a). Etam was only detected in (b), U1, U8, U10, U11 and U12 were only detected in (a). Like amounts of 4-Aba and P-ser were detected in both, Pipe, 3-Ala, Orn and 2,3-Di in neither. Because U10, U11 and

U12 were not always detected in P. nitida material from Kirstenbosch when assayed at different times throughout the year, differences in free amino acid composition and concentration may be due to the metabolic state of the plant and not necessarily due to geographic locality. Free amino acid composition was similar, although their concentrations were not.

P. repens : Results were compared from assays of the foliage of P. repens growing at Kirstenbosch (a), from a locality near Bot River en route to Hermanus (b), and from 3 varieties (c, d and e) with different flowering times being propagated at Tygerhoek Research Unit (Table 5 no. 7). Glu and Ala were detected in the highest concentrations with that of Asp also high in (a) and (b). Pheala, which was not often detected in foliage, was detected in all except (a). Tyr, Hypro and U1 were only detected in (c). Pipe was detected in all, and Etam and 3-Ala in all except (a). P-ser was detected in all except (b), 4-Aba in (c), (d) and (e), 2,3-Di in (a) and(d), and Orn not at all. Since the varieties were assayed simultaneously, but they, (a) and (b) were assayed at different times of the year, seasonal effects, and those genetically determined, as manifested by the difference in flowering times, may have influenced the relative concentrations detected, since geographic locality has not greatly influenced free amino acid composition.

P. scabra : Results from assays of the foliage of P. scabra from Kirstenbosch (a) and from the quarry site at Kleinmond (b) were compared (Table 5 no. 8). Glu was detected in the highest

concentrations, along with Thre in (a). Pipe and 3-Ala were not detected in either, and Orn and 2,3-Di only in (a). U6 was first detected in the foliage of P. scabra from both localities, and was only detected again in the florets and foliage below the inflorescence of P. cynaroides and in the foliage below the inflorescence of P. eximia assayed in March (cf. Table 8 (a)). The results do not show a convincing correlation between the assays from the two localities, which may be due to seasonal effects, since the material compared was not assayed at the same time.

P. eximia : Results from the assays of young foliage of P. eximia from two different stands at Kirstenbosch, and originating from different localities according to plant records, were compared (Table 5 no. 9). The number and concentrations of free amino acids detected were similar for both. Glu, Ser, Gly and Ala were detected in the highest concentrations for both, with Pheala levels also high. No Pipe or 2,3-Di was detected in either. Apart from Orn and U8 detected in (b) only, the same non-protein free amino acids were detected in both at like concentrations. This is reflected in the similar arbitrary total concentration of ninhydrin-reacting compounds for both.

P. roupelliae : Results from assays of the bracts (b) and florets (d) of an inflorescence of P. roupelliae collected from the Mkambati Game Reserve in Pondoland, Republic of the Transkei, were compared with those of the same material growing at Kirstenbosch (a) and (c) (Table 5 no. 10). The inflorescences

were collected and assayed within three days of each other, which probably accounted for the excellent correlation between the composition of free amino acids detected by paper chromatography in the same floral parts. However, their concentrations differ, as is reflected in the arbitrary total concentration of ninhydrin-reacting compounds detected. Ala, Glu, Asn, Ser, and Pro were detected in all in the highest concentrations. Pheala was only detected in material from Mkambati, and 2,3-Di not at all. U7 and U8 were the only unknowns detected in material from both Mkambati and Kirstenbosch.

The results from all the examples show that, while concentrations may differ, the free amino acid composition was similar in all the species compared for different geographic localities. This was especially significant with regard to the non-protein amino acids detected. Since plant material compared was not always collected and assayed at the same time, the differences that did occur could be attributed to seasonal effects or to the metabolic state of the plant.

3.4 (a) Free amino acids detectable in different organs of the plant

In Table 7, a comparison is drawn between the free amino acids detectable by two-dimensional paper chromatography in various organs of the plant viz. (a) vegetative foliage, (b) foliage on flowering stalk below the inflorescence, (c) involucral bracts, (d) florets, (e) roots, including proteoid roots, (f) young foliage buds. The *Protea* species compared are listed in Table 6.

Table 6. List of *Protea* species studied.

No.	Name of *Protea* species
1.	P. burchellii
2.	P. compacta
3.	P. cynaroides
4.	P. eximia
5.	P. longifolia
6.	P. magnifica
7.	P. pudens
8.	P. roupellii
9.	P. susannae

Table 7. Distribution of free amino acids in various plant organs. The various organs assayed are denoted by a to f ; organs assayed at the same time are bracketed (cf. p.32).

	P. burchellii						P. compacta			P. cynaroides				
	a	b	c	d	e	f	a	c	d	a	b	c	d	e
Gly	3	3	2	1-2	3	4	5	2-3	2	3	3-4	3	1	1
Ala	3	2	2-3	3	2	4	4	2-3	3	3	3	3	2	3
Val	1		2	3	3	3	2		3	1	3	3	3	2
Leu/Ile	Tr		1	3	3	3	Tr	2	3-4		3	2	2-3	1
Arg				3	1	4	Tr	1-2	3		2	2	1	3
Lys			3	4		5	2	3	4	Tr	3	2	1	
Asp	3-4	4	4	4	2	4	5	2	4	3	2	3	5	2
Glu	4	4	4	4-5	4	4-5	5	4-5	4	4	4	4	5	4
Asn		1	3	3	1	2	1	3	4		3-4	4	4-5	3
Gln	2	2	2-3	4	3	2	3	3-4	4	Tr	2-3	2	4-5	3
Ser	3	3	3	3	3	4-5	4	3-4	4	3	4	4		3
Thre	Tr	Tr	1	1	1	2-3	1	1	1	1	2	1	1	2
Pheala			2	2	3	2			2		3	1	1	Tr
Tyr					2	2-3	Tr				4	2	3	Tr
Pro	Tr		2	2		2	2	3	2	Tr	2	1	2	Tr
Hypro											Tr	3	1	
Trp						2					2	2-3	2	
His			2	3		4	1	1-2	3	1	2	3	1	
Cys					2		2							
Cys-cys				1				Tr	Tr	1				
Pipe	3		2	3	2	2					1	Tr		3
Etam				1	1	2	1	1-2	2-3	Tr	1-2	2	1	Tr
3-Ala	1	1	2	1		3	1	1	1	1	1	1		
4-Aba				1	1	1	1	2-3	3	1		2	3	1
P-ser	2			2	1	3	1		2	1	1	1	2	1
Orn				Tr			Tr		Tr		2	2		Tr
2,3-Di														
U1						2					3	2		1
U2											1			
U3						3	Tr		Tr					
U4						2							1	
U5							2				Tr			
U6											1			
U7														
U8	Tr				Tr	1			1	Tr	Tr	1	1	Tr
U9														
U10					2					2	2			
U11										3	2			
U12										3-4	3			
Total	27,5	20,5	38	53,5	40,5	74	45,5	39	57,5	34	68,5	57	48,5	36

Table 7 (contd.). Distribution of free amino acids in various plant organs. The organs assayed are denoted by a to f ; organs assayed at the same time are bracketed (cf. p.32).

	P. eximia						P. longifolia					
	a	b	c	d	e	f	a	b	c	d	e	f
Gly	2-3	3	2	1-2	2	4	2	2	2	2	2	3
Ala	1	3	2	2	2	4	3	3	2	2	3	3
Val	Tr		2-3	3	1	2		Tr	Tr	2	2	3
Leu/Ile	Tr		2	1	Tr	2		Tr		1	1	2
Arg					Tr	Tr	Tr				1	4
Lys	Tr									2-3	Tr	3
Asp	3	1	3	4	1	2	4	4	3	3-4	2	2
Glu	3	3-4	4	4	3	4	4	4	3	3-4	3	4
Asn	2		3	3	3	1-2	Tr	Tr	2	2-3	4	
Gln	Tr	Tr	2-3	3	1	2-3	1	1-2	2	2-3	2	2-3
Ser	2	1-2	3	3	2	4	3-4	2-3	2	2	2	3
Thre		Tr	1	1	Tr	2	Tr	1	Tr	1	1	2
Pheala						2		1-2	1	1		2
Tyr						Tr				Tr	1	1
Pro	Tr	Tr	2	3	1	1	Tr	1	Tr	3-4	Tr	2
Hypro				1	1					1		1
Trp			2-3	3		2				2		
His			2	2		1	Tr					3
Cys									Tr	Tr		
Cys-cys		Tr										
Pipe					3		Tr	1			2	2-3
Etam			1	1	Tr	3	Tr	Tr			Tr	2
3-Ala	1		1	1	Tr	2-3	Tr	1	1	2	Tr	1
4-Aba					1	1-2	Tr	Tr	Tr	3	1	1
P-ser	2	1	2	1	Tr	2	1	1		2	1	1
Orn												1
2,3-Di												
U1						1						2-3
U2			1			1						
U3						Tr	1					1-2
U4			1	1						2		
U5			Tr			1						
U6												
U7						2						2-3
U8			1		1	1				Tr	Tr	
U9						2						1
U10												
U11												
U12												
Total	19	15	39	38,5	25	52,5	24	26	20,5	42,5	30,5	56,5

Table 7 (contd.). Distribution of free amino acids in various plant organs. The organs assayed are denoted by a to f ; organs assayed at the same time are bracketed (cf. p.32).

	P. pudens				P. roupelliae				P. susannae			
	a	b	c	d	b	c	d	f	a	b	c	d
Gly	2	2	2	2	Tr	1	2	2-3	1-2	2-3	2	2-3
Ala	3	3	2	3-4	Tr	1-2	2	4	2-3	2-3	2-3	2-3
Val	Tr	2	Tr	3		1	1	2-3	1			3
Leu/Ile	Tr	1		2		Tr	1	3				2-3
Arg	Tr	Tr		3			2	Tr	1			2-3
Lys				3			Tr	Tr	Tr			2
Asp	3	Tr	2-3	5	3	2-3	2-3	3-4	4	4	3-4	3-4
Glu	3-4	4	4	5	3	3	2-3	5	4	4	3-4	4
Asn	1	2	2-3	5		2	2-3	2-3		Tr		3
Gln	2	2	1	3-4		1-2	3	2-3	1-2	2		3
Ser	3	3	3-4	4		2	2	4	2-3	2	2	3
Thre	1	1	Tr	2		Tr	Tr	2	Tr			1
Pheala												
Tyr	1	1	1	5			Tr					
Pro	Tr			1		1	2	2	1	1	3	3-4
Hypro												
Trp	Tr			3								
His				2			1					2
Cys												Tr
Cys-cys												
Pipe	2	1	1	1-2								
Etam	2	2	1	2			Tr	2			1	1
3-Ala	1	Tr	Tr	3		Tr	1	2	1	1		1
4-Aba			Tr	2		Tr	1	3	1			2
P-ser				Tr	2		2	2-3	2	2	1	2
Orn	1			2								
2,3-Di				1								
U1								1				
U2								Tr				
U3	Tr	Tr	Tr									3-4
U4												2-3
U5	Tr		Tr							1		
U6												
U7												
U8	Tr	Tr		1				1				2
U9												
U10												
U11												
U12												
Total	29,5	26,5	23,5	65	9	17,5	29,5	46,5	24	22,5	18,5	52,5

Discussion of results

In Table 7, results are given for the free amino acids detected by two-dimensional paper chromatography in the different organs of the plant. Those organs that were assayed simultaneously are bracketed in the figure. For all species assayed, except P. cynaroides, the highest number of free amino acids were detected in the young foliage (f), then the florets (d), then roots (e) . The arbitrary total concentrations of ninhydrin-reacting compounds also show that most were detected in the very young foliage, followed by the florets and roots, with average total values of 57, 48 and 33 respectively and, on total concentration average, P. cynaroides was again the exception.

Glu was detected in the highest concentrations in all organs for all species assayed. Tyr was detected more often and in elevated concentrations in foliage. Pheala was usually detected in higher concentrations in the inflorescence and young foliage, and Pro concentrations were highest in florets then young foliage. Pipe was detected in the roots of P. eximia but not in any other organ when assayed. Most non-protein amino acids were detected in the very young foliage and florets ; this could be because their concentrations were higher in these rapidly metabolizing plant tissues, leading to an increase in detectability. Seasonal effects may also play a role in the concentration of free amino acids present in the plant organs, so that with an increase in concentration more are detectable.

3.4 (b) The association of certain non-protein amino acids in selected *Protea* species.

When both the cream- and pink-flowered forms of *P. aurea ssp. aurea* were noticed in bloom in the gardens at Kirstenbosch, it was decided to assay the various plant organs of both forms to determine whether the difference in colour form was also manifested in a difference in free amino acid composition. Vegetative foliage, young vegetative foliage, foliage below the inflorescences, bracts and florets of the pink-flowered form, and the vegetative foliage, bracts and florets of the ceam-flowered form were assayed.

U10, which was located as a large brilliant orange spot, U11, bright yellow, and U12, smaller than U10 but also bright orange, were detected on the chromatograms of all parts of the pink-flowered form. There was a trace of U10 on the chromatograms of the foliage and florets of the cream-flowered form ; U11 and U12 were located on the chromatograms of the extracts of the foliage and bracts but not the florets of the cream-flowered form.

Copies of the chromatograms of the foliage and bracts of both forms are shown in Figure 2.

None of these unidentified amino acids was detected in the foliage of *P. aurea ssp. potbergensis* which was in a vegetative phase when assayed shortly thereafter.

It was thought at the time that these amino acids might be connected with the pink colour pigment until they were detected

in the vegetative foliage of both P. nitida and P. cynaroides (cf. Table 5) while both were in a vegetative phase, and also in the roots of P. burchellii (Table 7). It was not always detected in these species for assays done at different times throughout the year. These amino acids might be indicative of a metabolic pathway common to only certain Protea species, and as such be useful as chemotaxonomic indicators.

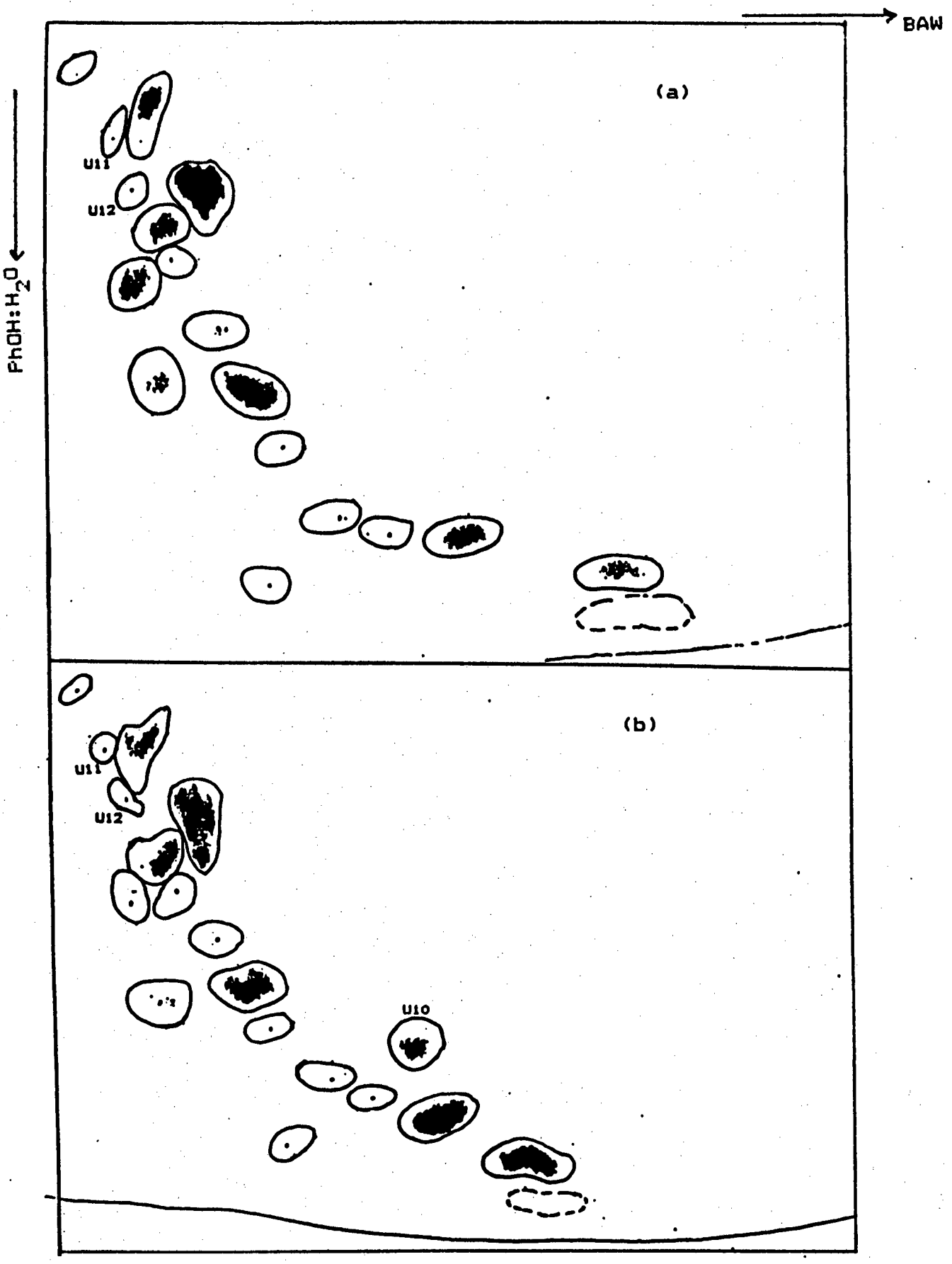

Figure 2. Copies of bidirectional paper chromatograms of extracts of Protea aurea ssp. aurea showing the free amino acid composition of the bracts of (a) the cream-flowered form and (b) the pink-flowered form.

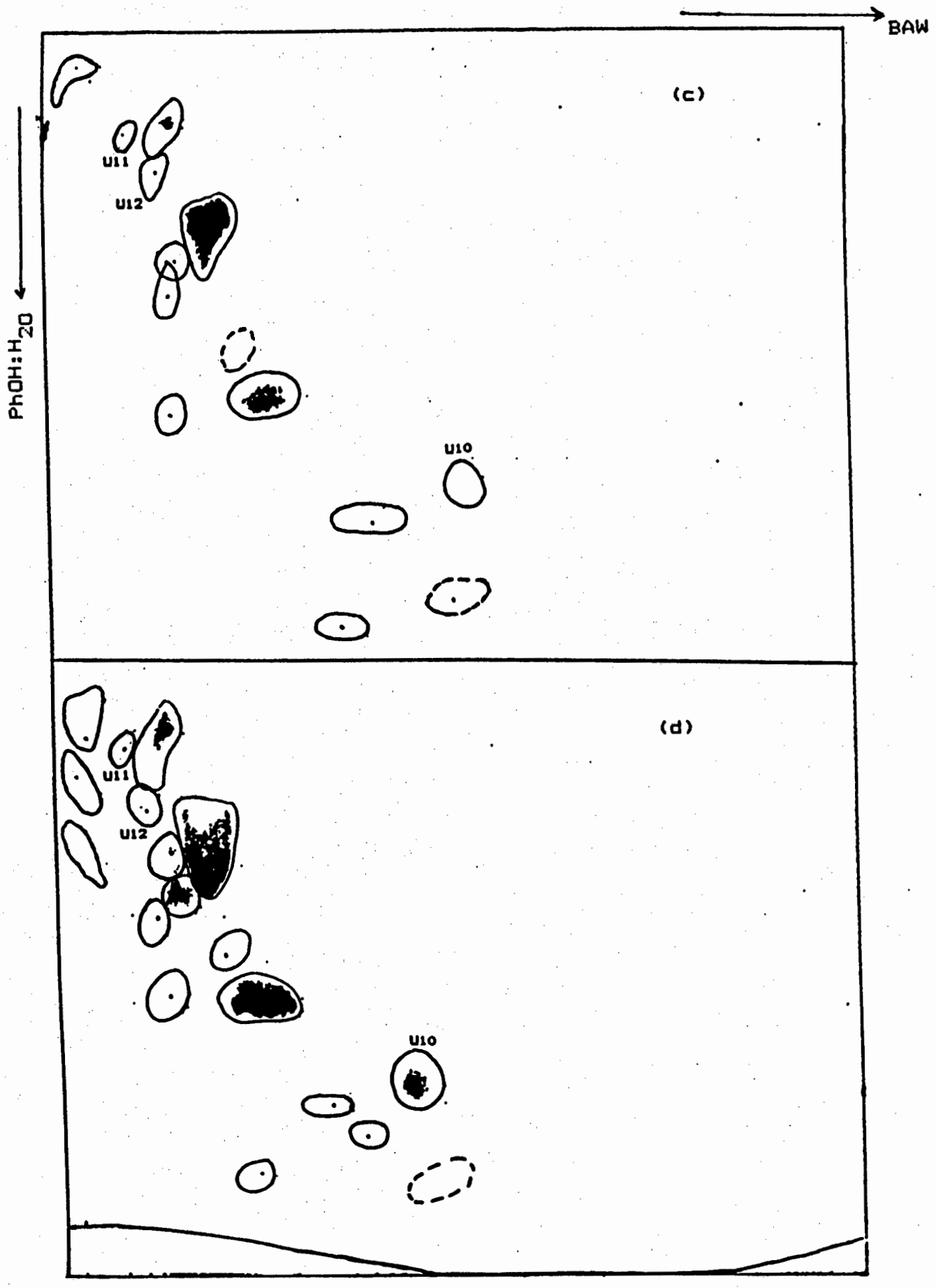

Figure 2 (contd.). Copies of bidirectional paper chromatograms of extracts of Protea aurea ssp. aurea showing the free amino acid composition of the foliage of (c) the cream-flowered form and (d) the pink-flowered form.

3.5 Seasonal effect on free amino acid composition

Results from the seasonal effect on free amino acid composition, as detected by two-dimensional paper chromatography, are presented in Tables 8 (a) and 9. Quantification by automatic amino acid analyser of the results given in Table 8 (a) are presented in Table 8 (b). The month and year of analysis are given in Tables 8 (a) and (b), and the month of analysis in brackets in Table 9, since the plant material was all assayed in the same year. Results given in Table 8 are from the same organ, viz. foliage, whereas the results presented in Table 9 show the seasonal effect on various organs of the plant. Again, in Tables 8 (a) and 9, a summation of the concentrations of compounds which reacted with ninhydrin is made from the paper chromatography results to give an arbitrary total concentration.

Table 8 (a). Concentrations of free amino acid levels in the foliage of two Protea species showing seasonal variation throughout the year.

	P. eximia				P. longifolia			
	6/85	8/85	1/86	3/86	5/85	8/85	12/85	3/86
Gly	2	3	3	1	2	2	3	
Ala	Tr	3	3	3	3	3	3	3
Val			1	1	Tr		3	1
Leu/Ile			1	1	Tr		2	1
Arg			3	2		Tr	4	
Lys			3	2			3	Tr
Asp	3	1	2	1	4	4	2	1
Glu	3	3-4	3-4	Tr	4	4	4	1
Asn	Tr		2		Tr	Tr		
Gln		Tr	1	Tr	1-2	1	2-3	Tr
Ser	Tr	1-2	3	2	2-3	3-4	3	2
Thre		Tr	1	1	1	Tr	2	Tr
Pheala				1-2	1-2		2	1
Tyr			Tr				1	1
Pro		Tr	1	1	1	Tr	2	1
Hypro							1	1
Trp				2				
His			2			Tr	3	
Cys								
Cys-cys		Tr						
Pipe			1	2	1	Tr	2-3	2
Etam			1	1	Tr	Tr	2	1
-Ala	Tr		1	Tr	1	Tr	1	1
4-Aba		Tr	1	1	Tr	Tr	1	1
P-ser		1	1	Tr	1	1	1	1
Orn							1	
2,3-Di								
U1			1	2			2-3	1
U2								
U3						1	1-2	
U4								
U5		Tr						
U6				3				
U7			1	2			2-3	
U8								1
U9							1	
U10								
U11								
U12								
Total	10	16	37	31,5	26	24	56,5	22,5

Table 8 (b). Quantified results, expressed as a percentage of the total ninhydrin-reacting compounds, for seasonal variation of free amino acids detected in P. eximia and P. longifolia by two-dimensional paper chromatography (Table 8 (a)).

	P. eximia				P. longifolia			
	AA% 6/85	AA% 8/85	AA% 1/86	AA% 3/86	AA% 5/85	AA% 8/85	AA% 12/85	AA% 3/86
Gly	6	3	2	1	1		1	1
Ala	3	13	7	17	9,5	7,5	5	21
Val	1			1	1		2	2
Leu	1	2	1	1	1	1	1	3
Ile	1	1,5	1	2	1	1	1,5	3,5
Arg			28	25			36	3
Lys			3	5			4	7
Asp	19	1	1	1	23	20	1	1,5
Glu	18	22	21	1	40	35	18,5	1,5
Asn			2	1				1
Gln	1		1	1	1	1	1	2
Ser	1	2	5	8	6	6	4	10
Thre	1	2	2	2	3	2	3	5
Pheala			1	1	1		1,5	2
Tyr							1	1
Pro				1	1	1	1	2
Hypro	1							
Trp			2	4,5			4	
His							2	1
Cys								
Cys-cys								
Met								
Pipe			3					
Etam	1	6	2,5	5	2	3	4	6
3-Ala	1							
4-Aba	1	1	1	1		1	1	1
P-ser								
Orn								
2,3-Di								
U1								
U2								
U3								
U4								
U5								
U6								
U7								
U8								
U9								
U10								
U11								
U12								

Table 9. Seasonal effects on free amino acid composition of the various plant organs. Assays done in March (3) and September (9), with arbitrary concentration values given for individual amino acids detected and the total of ninhydrin-reacting compounds.

	P. magnifica x *P. susannae*							
	Veg. Fol.(3)	Veg. Fol.(9)	Sub Infl.(3)	Sub Infl.(9)	Br. (3)	Br. (9)	Fl. (3)	Fl. (9)
Gly	3	5	3-4	3-4		3	3	2-3
Ala	3-4	4	4	2-3		3	4-5	3
Val	2	1	2			3	3-4	3
Leu/Ile	2		Tr			2	3	3
Arg							1-2	2
Lys	2		1			Tr	2-3	2-3
Asp	3-4	5	3-4	3		5	4	5
Glu	4	5	4	4	3	4	4-5	4
Asn		1-2	Tr	1	Tr	3	4	3
Gln	2	4	2	1-2		2	4-5	3
Ser	2-3	3-4	3	2		4	4	4
Thre	1	Tr	1	Tr		1	2	1-2
Pheala								2
Tyr						Tr		
Pro	1-2	Tr	1	Tr		3	4-5	3-4
Hypro		2				1	1-2	1-2
Trp								
His								2
Cys							Tr	1
Cys-cys		2		2		2		
Pipe	2							
Etam	1-2					1		1-2
3-Ala	1-2	Tr	Tr			2	1-2	2-3
4-Aba	2		1		1-2	2	4-5	2-3
P-ser	2	3	2	1-2	Tr	1-2		1
Orn	1-2	Tr	2	1	1	2	1-2	1
2,3-Di	1-2	Tr	1	1-2			2	2
U1				3			1	1
U2							2	
U3		Tr		Tr				Tr
U4								
U5		Tr						
U6								
U7								
U8							1	1
U9								
U10								
U11								
U12								
Total	39	39,5	32,5	28	6,5	45,5	60	59,5

Key : Veg. Fol. = Vegetative foliage
Sub Infl. = Foliage below inflorescence
Br. = Bracts
Fl. = Florets

Table 9 (contd.). Seasonal effects on free amino acid composition of the various plant organs. Assays done in February (2) and June (6), with arbitrary concentration values given for amino acids detected and the total of ninhydrin-reacting compounds.

	P. roupelliae					
	Veg. Fol.(2)	Veg. Fol.(6)	Br. (2)	Br. (6)	Fl. (2)	Fl. (6)
Gly	3	Tr	2	1	3	2
Ala	2	Tr	3	1-2	3	2
Val	1		2	1	3	1
Leu/Ile	1		2	Tr	2	1
Arg	Tr		Tr		1	2
Lys	1		Tr		1	Tr
Asp	1	3	3	2-3	3	2-3
Glu	3	3	3-4	3	4	2-3
Asn			3	2	4	2-3
Gln	1		2	1-2	3	3
Ser	1		3	2	3-4	2
Thre	1		1	Tr	1	Tr
Pheala	1					
Tyr					2	Tr
Pro	1		3	1	3-4	2
Hypro			1		1	
Trp			2		2	
His	1		Tr		1	1
Cys						
Cys-cys	Tr		1		1	
Pipe	1		Tr		1	
Etam	1		1		2	Tr
3-Ala	Tr		1	Tr	1-2	1
4-Aba	Tr		1	Tr	2	1
P-ser	2	2	2		1-2	2
Orn	Tr					
2,3-Di						
U1	Tr					
U2						
U3						
U4						
U5	1				Tr	
U6						
U7			Tr			
U8	Tr		Tr		1	
U9			Tr			
U10						
U11						
U12						
Total	26,5	9	40	17,5	51,5	29,5

Discussion of results

Table 8 (a) shows the results obtained by two-dimensional paper chromatography of assays done throughout the year, at roughly three monthly intervals, of two Protea species, P. eximia which occurs naturally among the mountains of the southern Cape, and P. longifolia whose natural habitat is the southwestern coastal region of the Cape between Sir Lowry's Pass and Cape Agulhas (Rourke,1980). Results from assays of foliage below the inflorescence are given for P. eximia, which was in flower from June to January ; foliage assayed in March was below dead flower heads. Results from assays of vegetative foliage are given for P. longifolia, which was flowering when material was collected in May and August.

Results for P. eximia show that most free amino acids were detected in January (1/86) and March (3/86), and the least in June (6/85) ; these results are also reflected in the arbitrary total concentration values of ninhydrin-reacting compounds. Glu was detected in the highest concentrations except in March when only a trace was detected and Ala was the predominant amino acid detected. Ala was detected in high concentrations throughout the year except in June when only a trace was detected. Pipe and Etam were detected in January and March, Gly and P-ser concentrations were highest in August and January, Hypro, Orn and 2,3-Di were not detected at all. Concentrations were generally elevated in the free amino acids detected in January. U6 was detected in high concentrations in the March assay. This

unidentified amino acid was first detected in the foliage of P. scabra (cf. Table 5 no. 8), and subsequently only in the florets and foliage below the infloresecence of P. cynaroides. It may prove to be an important chemotaxonomic or metabolic indicator.

Results for P. longifolia show that most free amino acids were detected in December (12/85) with a general elevation of their concentrations. The arbitrary totals calculated also show that most ninhydrin-reacting compounds were detected in December. As with P. eximia, Glu was detected in the highest concentrations except in March (3/86) when Ala was the predominant amino acid detected. Ala levels were constant for the assays done throughout the year. Of the non-protein amino acids, all were detected throughout the year except Orn, which was only detected in December, and 2,3-Di not at all.

Since P. longifolia had long ceased flowering by December, and P. eximia was nearing the end of its flowering phase at this time, the elevated free amino acid levels in December/January were probably due to a seasonal effect.

Quantification of these results are given in Table 8 (b).

Small samples of the same extracts assayed for the results given in Table 8 (a), equivalent to about 60 mg. dry mass, were analysed by an LKB 4150 Alpha amino acid analyser (lithium buffers). The results, given in Table 8 (b), are expressed as a percentage of the total of all compounds that reacted with

ninhydrin. P-ser could not be accurately determined this way, since a preliminary determination, during which a sample was sent through without ninhydrin being added, showed that a pigment reacted at the same retention time as that for P-ser.

The quantified results for P. eximia show that Asp and Glu were the predominant amino acids detected in June (6/85), followed by Gly and Ala ; Val, Ile, Leu, Gln, Ser, Thre, Hypro, Etam, 3-Ala and 4-Aba were detected in trace amounts. Glu was detected in the highest concentrations in August (8/85) followed by Ala ; Gly was not detected in as high a concentration as depicted by the paper chromatography results in Table 8 (a). Arg then Glu were detected in the highest concentrations in January (1/86), followed by Ala, Ser and Etam ; again, Gly was not detected in as high a concentration as depicted by paper chromatography. Arg then Ala were detected in the greatest concentrations in March (3/86), followed by Ser, Lys, Etam and Trp. The basic free amino acids Lys and Arg, and His were often detected as a large extensive spot by paper chromatography, so that a collective value was allocated to them ; amino acid analyser results for both January and March showed that Arg was the predominant amino acid present and contributed most to the colour reaction with the locating agent, and that His was not included in this composition. Pipe was only detected in a concentration great enough to be expressed as a percentage in January. Gln and Cys-cys (8/85), Tyr (1/86), Pipe and 3-Ala (1/86 and 3/86), were detected in trace amounts by paper chromatography but these were not detected by the analyser in

concentrations great enough to be expressed as a percentage.

The quantified results for P. longifolia show that Glu then Asp were detected in the highest concentrations, followed by Ala and Ser in both May (5/85) and August (8/85). Arg then Glu were detected in the highest concentrations in December (12/85), followed by much lower levels of Ala, Lys, Ser, Trp and Etam ; these results correlate well with those obtained by paper chromatography except that Trp was not detected in this case by this method. Ala was detected in the highest concentrations in March (3/86), followed by Ser, Lys, Etam and Thre. Pipe, which was detected by paper chromatography throughout the year, was never detected by the analyser in concentrations great enough to be expressed as a percentage.

A possible reason for the discrepancy in the values for Gly as denoted by paper chromatography and amino acid analyser is that Gly reacted with ninhydrin as a bright pink spot on paper, which would not be correctly detected by the analyser, which reads the absorbance reaction with ninhydrin in the blue and purple colour region at wavelengths of 440 & 570 nm. Not all the free amino acids detected in trace amounts by the analyser were detected by paper chromatography, and some detected by this method were not always detected by the analyser in concentrations great enough to be expressed as a percentage. Peaks possibly denoting the unknown free amino acids detected by paper chromatography could not be identified on the analyser printout.

In Table 9 the temporal effect on free amino acid composition as detected by two-dimensional paper chromatography is compared for the same plant organs, assayed in March and September, denoted by (3) and (9) respectively, for the hybrid P. magnifica x P. susannae, and in February and June, denoted by (2) and (6) respectively, for P. roupelliae.

P. magnifica x P. susannae : Most free amino acids were detected in the florets in both March and September with very few detected in the bracts assayed in March. Arg was only detected in the florets, and Pipe only in the vegetative foliage assayed in March. Trp was not detected at all. Cys-cys was detected in the foliage and bracts assayed in September. Apart from Pheala, His, Etam and P-ser detected in the florets only in September, and the unknowns U2 & U3, the same free amino acids and at similar concentrations were detected in the florets. Glu and Asp were detected in the highest concentrations for all. The arbitrary totals are identical for ninhydrin-reacting compounds in the florets for both assays, and are almost identical for the vegetative foliage of both assays. Those for the other organs reflect the results obtained by paper chromatography.

P. roupelliae : More free amino acids were detected in February for all organs, with most detected in the florets in June ; these results were reflected in the arbitrary totals of ninhydrin-reacting compounds for all organs. Glu was detected in the highest concentration in all, along with Asp and Asn in the inflorescences. Pro levels were elevated in the inflorescence in

February and were detected in the vegetative foliage. Hypro and Trp were detected in the inflorescence, and Pheala in vegetative foliage only in February. 2,3-Di was not detected at all. Cys-cys and Pipe were detected in all organs assayed in February. Very few non-protein amino acids and no unknowns were detected in June. The best correlation between free amino acids detected and their concentrations was again between the florets.

In order to determine the effect of time and cold-storage on the Protea extracts, which are stored in a refrigerator, chromatograms of some samples, which had been stored in the refrigerator for four months, were compared with their original chromatograms which were run immediately after extraction. The chromatograms were similar and showed practically identical free amino acid detectability. Consequently, one should be able to collect and prepare samples during the growth season and analyze them simultaneously at a later, convenient time.

3.6 Results from two-dimensional paper chromatography compared with those from amino acid analyser (AA %).

Table 10. Free amino acid composition detected by two-dimensional paper chromatography compared with amino acid analyser results. Organs assayed were foliage (Fol.), bracts (Br.) and florets (Fl.).

	P. eximia				P. magnifica				P. repens			
	Fol (6)	AA % (6)	Fol (8)	AA % (8)	Fol L7	AA % L7	Fol L15	AA % L15	Br.	AA %	Fl.	AA %
Gly	3	10	4	8	3	6	3	4	2	1	2	1
Ala	2	3,5	4	19	3	8	3	9	3	3	2-3	3
Val	1		1	1	1	1	2		4	8	3	3,5
Leu		1		1		1		1		2		1
Ile	1	1	1	1	Tr	1	1-2	1	4	9	3	2
Arg							1	9	Tr		1	2
Lys							1-2		2	1	2	1
Asp	5	19	1	1	4	6	4	3	2	1	2	1
Glu	5	16	5	34	4	45	4	22	3-4	10	4	18
Asn	4	1					1	1	4	32	4	22
Gln	1	1	1	1	1	2	1-2	2	2	3	3	8
Ser	4	4	4	4	3	7	4	9	3	6	3	5
Thre	Tr	1	1	3	Tr	2	1	3	2	3	1-2	3
Pheala		1	1	1		1		1,5			Tr	
Tyr											Tr	
Pro					Tr	1	1-2	2	3-4	9,5	4-5	21
Hypro		1							1		2-3	
Trp								5	2	2	2	2
His						2	1	2	Tr	2	1	1
Cys												
Cys-cys		1									Tr	
Met				2		4		2				
Pipe			2		2-3		2-3					
Etam			2	2					1	2	1	2
3-Ala	3		2		1		1	1	1		1	
4-Aba							Tr	1	1	1	3	1
P-ser	2		1		2		2		2		2	
Orn			Tr				Tr					
2,3-Di							Tr					
U1												
U2												
U3									Tr		Tr	
U4												
U5	2		1		1		Tr		1		1	
U6												
U7			2						1		1	
U8									1		1	
U9											Tr	
U10												
U11												
U12												

Table 10 (contd.). Free amino acid composition detected by two-dimensional paper chromatography compared with amino acid analyser results. Organs assayed were bracts (Br.) and florets (Fl.) from localities Mkambati (Mk.) and Kirstenbosch (Kir.).

	P. roupelliae							
	Br. (Mk)	AA %	Br. (Kir)	AA %	Fl. (Mk)	AA %	Fl. (Kir)	AA %
Gly	2	2	2	2	3	1	3	1
Ala	4	10	3	6	3	4	3	5
Val	3	6	2	5	3	5	3	4
Leu		1		1		1,5		1
Ile	3	3	2	2,5	3	3	2	2
Arg	1	2	Tr		2	6	1	2
Lys	1		Tr	6	3	2	1	2
Asp	3	1,5	3	3,5	2	1	3	4
Glu	2		3-4	19	3	4	4	21
Asn	4	26	3	16	4	28	4	22
Gln	3	7	2	4	4	11	3	7
Ser	4	12	3	8	4	7	3-4	6
Thre	1	2	1	2	2	2	1	2
Pheala	1	1			1	1		
Tyr	1				1	1	2	
Pro	3	5	3	8	3	9	3-4	11
Hypro			1		Tr		1	
Trp	3	6	2	1	2	2	2	1,5
His	1	4	Tr	3,5	3	6	1	3
Cys								
Cys-cys		1	1		1		1	
Met				1				
Pipe			Tr		Tr		1	
Etam	1	3	1	3	2	2	2	1
3-Ala	2		1		2		1-2	
4-Aba	3	6	1	3	2	2,5	2	3
P-ser	2		2		2		1-2	
Orn					1			
2,3-Di								
U1					2			
U2								
U3					Tr			
U4								
U5							Tr	
U6								
U7	1		Tr		1			
U8	1		Tr		1		1	
U9			Tr					
U10								
U11								
U12								

Discussion of results

Amino acid analyser results for each amino acid are expressed as a percentage of the total area of the nitrogenous compounds that reacted with nitrogen. In the figure they are given under the abbreviated heading AA % , and are compared with the results given by two-dimensional paper chromatography. Their totals do not add up to 100 % as there were several small unidentified peaks on the printout of results, which could have been due to pigments in the extract, or of amino acids with concentrations too low to be expressed as a percentage.

The results for P. eximia are a comparison of the free amino acids detected by assays done in June and August, denoted by (6) and (8) respectively. The P. magnifica samples were from plants growing in different garden beds, L7 and L15, in the protea garden at Kirstenbosch and, according to plant records, originate from different localities. Results for P. roupelliae are given for the bracts (Br) and florets (Fl) of an inflorescence picked in the Mkambati Game Reserve in Pondoland, Republic of Transkei ; this is abbreviated as (Mk), compared with the same material collected two days later from P. roupelliae growing at Kirstenbosch and abbreviated (Kir).

As found by Lewis and Stock (1978), the main amino compound detected by the amino acid analyser was ammonia, and the concentrations of free amino acids present was very low. Results for ammonia are not given, and the values for the amino acids

detected are calculated as a percentage of the total minus the ammonia peak area.

On paper chromatograms Ile and Leu are located as one spot and are allocated only one concentration value. However, the analyser gives values for individual amino acids, and in most cases the concentration of Ile was at least double that of Leu. Met and Val also had very similar R_f values and could not be identified separately by two-dimensional paper chromatography. The analyser results in Table 10 show that a small amount of Met was detected in P. eximia foliage in August, in P. magnifica foliage from both localities, and in P. roupelliae bracts from Kirstenbosch. P-ser values could not be accurately determined since a pigment was present with the same retention time as p-ser (L. Powrie pers. comm.).

Possibly due to a slight change in buffer compositions, since the results are given for assays done at different times, and depending on the ammonia concentration detected, the correlation between the automatic amino acid analyser values and concentrations allocated to the amino acids located by paper chromatography were not as good as were expected. The only correlation between the two sets of results was an indication of which amino acids were present in the greatest and lesser concentrations. Often an amino acid detected in trace amounts by paper chromatography was not detected by the analyser in concentrations great enough to be expressed as a percentage. An advantage of the analyser results was that the relative

concentrations of Ile and Leu could be quantified and the presence of Met detected ; in addition, the identity and relative concentrations of the basic amino acids, located as an extended spot by paper chromatography, could be determined. However, the possible identity of the unknowns could not be determined from the analyser printout.

4. Free amino acids present in Protea hybrids and their phenotypic parents

The free amino acid composition of Protea hybrids growing in the gardens at Kirstenbosch, and of some cultivated at Tygerhoek Research Institute for commercial purposes were determined and compared with that of their phenotypic parent species. The purpose of this study was to determine which free amino acids occur, and to test an hypothesis that since the free amino acids in hybrids are genetically determined from the parents, they are useful markers for identifying hybrid parents. This study could also indicate the taxonomic value of free amino acids in general. If the free amino acid composition of the hybrids cannot be explained from the free amino acid composition of its parents, it could mean that its expression is determined to a greater extent by environmental or other factors rather than from genetic determination.

The ability to hybridize is a measure of close relationship, so that this characteristic becomes taxonomically more valuable or critical when it can be used differentially (Stace,1980). An axiom of genetics is that genes located on the same chromosome are genetically linked, so that combinations of alleles which are present in the parental genotypes appear more frequently in progeny of hybrids than do new combinations (Stebbins,1971). Thus if the parents of a hybrid are homozygous for the alleles producing the enzyme responsible for the synthesis of a specific amino acid, one would expect the hybrid to contain all the amino

acids present in both parents. However, due to genetic or other influences, the characters of a hybrid may not always be intermediate between both parents, but much nearer to one parent than the other, or even indistinguishable from one and , as well as possessing the compounds of both parents, hybrids sometimes anabolize new substances present in neither parent (Stace, 1980).

In Tables 11 & 12 the Protea species and hybrids studied are listed. In Table 12, the identification number (I.D. no.) of the parent species as listed in Table 11 is given, e.g. hybrid no. 1 (H1) is a cross between parent species no. 2 and no. 7 as listed in Table 11 and its I.D. no. is thus 2/7.

Tables 13 - 16 show the results obtained from two-dimensional paper chromatographic studies of Protea hybrids and their phenotypic parent species. The results show the free amino acids and their concentrations detected in foliage (Tables 13 & 14) and in florets (Tables 15 & 16). In Tables 13 and 15, the I.D. nos. of the parent species are given below the number denoting the name of the hybrid. In Table 15, the results in column H7 (a) are for the hybrid no. H7 (Table 12) assayed in March, and those in column H7 (b) are for the same plant assayed in September.

From the concentrations of the free amino acids detected in the hybrid and parent species, correlation coefficients were determined between the parent species of each hybrid, between the hybrid and each parent, and between a theoretical hybrid and the hybrid, where the free amino acid composition and concentrations for the theoretical hybrid were calculated from the sum of the

concentration value of each amino acid detected in the parent species divided by two. Even when an amino acid was detected in only one parent species, its concentration was divided by two, so that every free amino acid detected in either or both phenotypic parent species of each hybrid was considered to be present in the theoretical hybrid. These correlation coefficients are presented in Table 23.

To make it easier to interpret the results presented in Tables 13 - 16, the free amino acids detected in the foliage and florets of some Protea hybrids and their phenotypic parent species are presented separately in Tables 17 - 22. An asterisk was placed next to the amino acid concentrations which could not be reconciled with the hypothesis that free amino acids in the hybrid are derived from either or both parents.

Table 11. List of *Protea* species studied.

No.	Name of *Protea* species
1	P. burchellii
2	P. compacta
3	P. cynaroides
4	P. eximia
5	P. laurifolia
6	P. longifolia
7	P. magnifica
8	P. neriifolia
9	P. pudens
10	P. repens
11	P. roupelliae
12	P. susannae

Table 12. List of *Protea* hybrids studied.

No.	Name of hybrid	I.D. no.
H1	P. compacta x P. magnifica	2/7
H2	P. cynaroides x P. repens	3/10
H3	P. eximia x P. roupelliae	4/11
H4	P. eximia x P. susannae	4/12
H5	P. magnifica x P. burchellii	1/7
H6	P. magnifica x P. laurifolia	5/7
H7	P. magnifica x P. susannae	7/12
H8	P. neriifolia x P. minor	8/(6/9)
H9	P. repens x P. neriifolia	8/10

Table 13. Distribution of free amino acids in Protea hybrid foliage. The numbers indicate amino acid concentration on an arbitrary 5 point scale. Table 12 gives the identity of the hybrids studied (H1 - H9).

	H1 2/7	H2 3/10	H3 4/11	H4 4/12	H5 1/7	H6 5/7	H7 7/12	H8 8/6/9	H9 8/10
Gly	4-5	3	2	3	2-3	4	3		4
Ala	3	4	1-2	4	3-4	4-5	3-4	3-4	3-4
Val	1-2	3	Tr	2-3	1-2	3	2	Tr	Tr
Leu/Ile		3	Tr	2	Tr	2	2	Tr	Tr
Arg		3				2-3			
Lys	2	3		2	Tr	Tr	2		2-3
Asp	4	1-2	3	3-4	4	5	3-4	3-4	5
Glu	5	3	3-4	4	4-5	5	4	4-5	5
Asn	4	2				2			4
Gln	2-3	1	Tr	2	Tr	3	2	1	2
Ser	4	4	2	2-3	3	4	2-3	2	4
Thre	1	2	Tr	1	1	1-2	1		2
Pheala		2							
Tyr		2				Tr			
Pro	2	Tr	Tr	2	Tr	2	1-2	1-2	1
Hypro		1		1					
Trp	1					1			
His		2							2
Cys	1	1-2							
Cys-cys	1			1				1	2
Pipe	2-3				1-2	3	2		2
Etam		3				1-2	1-2		1-2
3-Ala	1	2-3	1	1-2		2	1-2	Tr	1
4-Aba	1	1		1-2	1-2	2	2		
P-ser	2	2	2	2		2-3	2		2
Orn	1-2	2		1-2	1		1-2	Tr	Tr
2,3-Di	2			1-2	2	1	1-2	1-2	
U1	1	2				Tr			2
U2		1							
U3	Tr	Tr				Tr			
U4									
U5	1		1-2			Tr			Tr
U6									
U7									
U8		1				Tr			Tr
U9						1			
U10									
U11									
U12		1							

Table 14. Distribution of free amino acids in foliage of *Protea* species. The numbers indicate amino acid concentration on an arbitrary 5 point scale. Table 11 gives the identity of the species studied.

	1	2	3	4	5	6	7	8	9	10	11	12
Gly	2-3	5	3	2-3	3	2	3	1*	2	3	Tr	2
Ala	3	4	4	1	3	3	3	2-3	3	4	Tr	3
Val	1	2	2	Tr	Tr		2		Tr	3		1
Leu/ Ile	Tr	Tr	2	Tr	Tr		1-2		Tr	2		
Arg		Tr			Tr	Tr	1		Tr	2		1
Lys		2	2	Tr			1-2			2		Tr
Asp	3-4	5	2	3	3-4	4	4	4	3	2	3	4
Glu	4	5	4	3	3-4	4	4	4	3-4	4	3	4
Asn		1		2	2	Tr	1		1	1		
Gln	2	3	2	Tr	1	1	1-2	1	2	2		2
Ser	3	4	3	2	2	3-4	4	1-2	3	3-4		3
Thre	Tr	1	2		Tr	Tr	1	Tr*	1	2		Tr
Pheala			1							1		
Tyr		Tr										
Pro	Tr	2	1	Tr	Tr	Tr	1-2	Tr	1	1		1
Hypro									Tr			
Trp			1									
His		1			Tr	Tr	1		Tr	2		
Cys		2	2							2		
Cys-Cys												
Pipe	3		Tr		2	Tr	2-3		2	2		
Etam		1	1			Tr			2	2		
3-Ala	1	1	1	1	Tr	Tr	1		1	1-2		1
4-Aba		1	1		Tr	Tr	Tr			2		1
P-ser	2	1	1	2	2	1	2	2	1	2	2	2
Orn					1		Tr			1		
2,3-Di					1		Tr		Tr	Tr		1
U1										2		
U2			Tr							Tr		
U3						1			Tr			
U4												
U5		2		1			Tr		Tr			
U6												
U7										2-3		
U8	Tr		1						Tr			
U9												
U10												
U11			2									
U12			3									

* = Detectable in pink-flowering form only

Table 15. Distribution of free amino acids in *Protea* hybrid florets. The numbers indicate amino acid concentration on an arbitrary 5 point scale. Table 12 gives the identity of hybrids studied.

	H1 2/7	H3 4/11	H4 4/12	H5 1/7	H7a 7/12	H7b 7/12	H9 8/10
Gly	3	2	4	3	3	3	4-5
Ala	4	3	3	4	4-5	3	5
Val	4	3	3-4	3	3-4	3	4-5
Leu/Ile	3	2	3	3	3	3	4
Arg	4	2	3	4	1-2	2	3
Lys	4	2	3	3	2-3	2-3	5
Asp	5	3	5	5	4	5	5
Glu	5	4	5	5	4-5	4-5	5
Asn	5	4	4-5	4	4	3-4	5
Gln	5	4	4	4	4-5	3	5
Ser	5	4	4-5	4	4	4	5
Thre	1-2	1	2-3	2	2	2	3-4
Pheala	2	Tr	2			2	2
Tyr		Tr	Tr				
Pro	5	3	4	4	4-5	4	5
Hypro		1	1-2	1	1-2	1-2	2
Trp	2	2	2-3				2
His		3	3	3-4		2	5
Cys	3		1	1	Tr	1	Tr
Cys-cys		Tr					
Pipe							1-2
Etam	1	2	2	2	2	1-2	2
3-Ala	1	1	2	3	1-2	3	3-4
4-Aba	3	2	2	4	4	3	4
P-ser	3	2	1	1-2	Tr	1	2
Orn	3	Tr	2	4	1-2	1	2
2,3-Di	3		2	1	2	2	2
U1	3	1			1	1	3
U2					2		1
U3	Tr		1	Tr		Tr	Tr
U4			2				
U5		Tr					
U6							
U7							
U8	1	1	1-2	1	1	1	2
U9	1			1			
U10							
U11							
U12							

Table 16. Distribution of free amino acids in Protea florets. The numbers indicate the amino acid concentrations on an arbitrary 5 point scale. Table 11 gives the identity of the species studied.

	1	2	4	7	8	10	11	12
Gly	1-2	2	1-2	3-4	1	2	3	2-3
Ala	3	3	2	3-4	2-3	2-3	3	3
Val	3	3	3	3	2-3	3	3	3
Leu/Ile	3	3-4	1	3	3	3	2	2-3
Arg	3	3	Tr		3	1	1	3
Lys	3-4	3-4	Tr	1	1-2	2	1	2
Asp	4	4-5	4	4	4	2	3	4
Glu	4-5	5	4	4-5	4	4	4	4
Asn	3	4-5	3	4	4	4	4	3
Gln	4	4	3	3	3	3	3	3
Ser	3	4	3	4	3-4	3	3-4	3
Thre	1	1	1	1	1	1-2	1	1
Pheala	2	2				Tr		
Tyr				Tr		Tr	2	
Pro	2	2	3	4	4	4-5	3-4	3-4
Hypro			1	2		2-3	1	
Trp			3	2	2	2	2	
His	3	3	2	2		1	1	2
Cys	1	Tr		Tr	1			Tr
Cys-cys						Tr	1	
Pipe	3						1	
Etam	1	2-3	1	2		1	2	1
3-Ala	1	1	1	2-3	1	1	1-2	1-2
4-Aba	1-2	3	Tr	2-3	2-3	3	2	2
P-ser	2	2	1	Tr		2	1-2	2
Orn	Tr	Tr			1			
2,3-Di								
U1								1
U2								
U3						Tr		2-3
U4								2
U5				2		1	Tr	Tr
U6								
U7						1		
U8		1	1	1		1	1	2
U9						Tr		
U10								
U11								
U12								

Table 17. Free amino acids detected in the foliage and florets of the hybrid P. compacta x P. magnifica and its phenotypic parent species. (Identities of the numbers are given in Tables 11 & 12)

	Foliage			Florets		
	2	2/7	7	2	2/7	7
Gly	5	4-5	3	2	3	3-4
Ala	4	3	3	3	4	3-4
Val	2	1-2	2	3	4	3
Leu/Ile	Tr	*	1-2	3-4	3	3
Arg	Tr	*	1	3	4	
Lys	2	2	1-2	3-4	4	1
Asp	5	4	4	4-5	5	4
Glu	5	5	4	5	5	4-5
Asn	1	4	1	4-5	5	4
Gln	3	2-3	1-2	4	5	3
Ser	4	4	4	4	5	4
Thre	1	1	1	1	1-2	1
Pheala				2	2	
Tyr	Tr					Tr
Pro	2	2	1-2	2	5	4
Hypro						2
Trp		1*			2	2
His	1	*	1	3	*	2
Cys	2	1		Tr	3	Tr
Cys-cys		1*				
Pipe		2-3	2-3			
Etam	1			2-3	1	2
3-Ala	1	1	1	1	1	2-3
4-Aba	1	1	Tr	3	3	2-3
P-ser	1	2	2	2	3	Tr
Orn		1-2	Tr	Tr	3	
2,3-Di		2	Tr		3*	
U1		1*			3*	
U3		Tr*			Tr*	
U5	2	1	Tr			2
U8				1	1	1
U9					1*	

Table 18. Free amino acids detected in the foliage and florets of the hybrid *P. eximia* x *P. roupelliae* and its phenotypic parent species. (Identities of the numbers are given in Tables 11 & 12)

	Foliage			Florets		
	4	4/11	11	4	4/11	11
Gly	2-3	2	Tr	1-2	2	3
Ala	1	1-2	Tr	2	3	3
Val	Tr	Tr		3	3	3
Leu/Ile	Tr	Tr		1	2	2
Arg				Tr	2	1
Lys	Tr			Tr	2	1
Asp	3	3	3	4	3	3
Glu	3	3-4	3	4	4	4
Asn	2			3	4	4
Gln	Tr	Tr		3	4	3
Ser	2	2		3	4	3-4
Thre		Tr		1	1	1
Pheala					Tr*	
Tyr					Tr	2
Pro	Tr	Tr		3	3	3-4
Hypro				1	1	1
Trp				3	2	2
His				2	3	1
Cys						
Cys-cys					Tr	1
Pipe						1
Etam				1	2	2
3-Ala	1	1		1	1	1-2
4-Aba				Tr	2	2
P-ser	2	2	2	1	2	1-2
Orn					Tr*	
2,3-Di						
U1					1*	
U5	1	1-2			Tr	Tr
U8				1	1	1

Table 19. Free amino acids detected in the foliage and florets of the hybrid P. eximia x P. susannae and its phenotypic parent species. (Identities of the numbers are given in Tables 11 & 12)

	Foliage			Florets		
	4	4/12	12	4	4/12	12
Gly	2-3	3	2	1-2	4	2-3
Ala	1	4	3	2	3	3
Val	Tr	2-3	1	3	3-4	3
Leu/Ile	Tr	2		1	3	2-3
Arg			1	Tr	3	3
Lys	Tr	2	Tr	Tr	3	2
Asp	3	3-4	4	4	5	4
Glu	3	4	4	4	5	4
Asn	2			3	4-5	3
Gln	Tr	2	2	3	4	3
Ser	2	2-3	3	3	4-5	3
Thre		1	Tr	1	2-3	1
Pheala					2	
Tyr					Tr*	
Pro	Tr	2	1	3	4	3-4
Hypro		1*		1	1-2	
Trp				3	2-3	
His				2	3	2
Cys					1	Tr
Cys-cys		1*				
Pipe						
Etam				1	2	1
3-Ala	1	1-2	1	1	2	1-2
4-Aba		1-2	1	Tr	2	2
P-ser	2	2	2	1	1	2
Orn		1-2*			2*	
2,3-Di		1-2	1		2	
U1						1
U3					1	2-3
U4					2	2
U5	1					Tr
U8				1	1-2	2

Table 20. Free amino acids detected in the foliage and florets of the hybrid *P. magnifica* x *P. burchellii* and its phenotypic parent species. (Identities of the numbers are given in Tables 11 & 12)

	Foliage			Florets		
	1	1/7	7	1	1/7	7
Gly	2-3	2-3	3	1-2	3	3-4
Ala	3	3-4	3	3	4	3-4
Val	1	1-2	2	3	3	3
Leu/Ile	Tr	Tr	1-2	3	3	3
Arg			1	3	4	
Lys		Tr	1-2	3-4	3	1
Asp	3-4	4	4	4	5	4
Glu	4	4-5	4	4-5	5	4-5
Asn			1	3	4	4
Gln	2	Tr	1-2	4	4	3
Ser	3	3	4	3	4	4
Thre	Tr	1	1	1	2	1
Pheala				2		
Tyr						Tr
Pro	Tr	Tr	1-2	2	4	4
Hypro					1	2
Trp						2
His			1	3	3-4	2
Cys				1	1	Tr
Cys-cys						
Pipe	3	1-2	2-3	3		
Etam				1	2	2
3-Ala	1	*	1	1	3	2-3
4-Aba		1-2	Tr	1-2	4	2-3
P-ser	2	*	2	2	1-2	Tr
Orn		1*	Tr	Tr	4*	
2,3-Di		2	Tr		1*	
U3					Tr*	
U5			Tr			2
U8	Tr				1	1
U9					1*	

Table 21. Free amino acids detected in the foliage and florets of the hybrid P. magnifica x P. susannae and its phenotypic parent species. (Identities of the numbers are given in Tables 11 & 12)

	Foliage			Florets			
	7	7/12	12	7	7/12 (a)	7/12 (b)	12
Gly	3	3	2	3-4	3	3	2-3
Ala	3	3-4	3	3-4	4-5	3	3
Val	2	2	1	3	3-4	3	3
Leu/Ile	1-2	2		3	3	3	2-3
Arg	1	*	1		1-2	2	3
Lys	1-2	2	Tr	1	2-3	2-3	2
Asp	4	3-4	4	4	4	5	4
Glu	4	4	4	4-5	4-5	4-5	4
Asn	1	*		4	4	3-4	3
Gln	1-2	2	2	3	4-5	3	3
Ser	4	2-3	3	4	4	4	3
Thre	1	1	Tr	1	2	2	1
Pheala						2*	
Tyr				Tr			
Pro	1-2	1-2	1	4	4-5	4	3-4
Hypro				2	1-2	1-2	
Trp				2			
His	1			2	*	2	2
Cys				Tr	Tr	1	Tr
Cys-cys							
Pipe	2-3	2					
Etam		1-2*		2	2	1-2	1
3-Ala	1	1-2	1	2-3	1-2	3	1-2
4-Aba	Tr	2	1	2-3	4	3	2
P-ser	2	2	2	Tr	Tr	1	2
Orn	Tr	1-2			1-2*	1*	
2,3-Di	Tr	1-2	1		2*	2*	
U1					1	1	1
U2					2*		
U3						Tr	2-3
U4							2
U5	Tr			2	*	*	Tr
U8				1	1	1	2

Table 22. Free amino acids detected in the foliage and florets of the hybrid P. repens x P. neriifolia and its phenotypic parent species. (Identities of the numbers are given in Tables 11 & 12)

	Foliage			Florets		
	8	8/10	10	8	8/10	10
Gly	1	4	3	1	4-5	2
Ala	2-3	3-4	4	2-3	5	2-3
Val		Tr	3	2-3	4-5	3
Leu/Ile		Tr	2	3	4	3
Arg		*	2	3	3	1
Lys		2-3	2	1-2	5	2
Asp	4	5	2	4	5	2
Glu	4	5	4	4	5	4
Asn		4	1	4	5	4
Gln	1	2	2	3	5	3
Ser	1-2	4	3-4	3-4	5	3
Thre	Tr	2	2	1	3-4	1-2
Pheala		*	1		2	Tr
Tyr						Tr
Pro	Tr	1	1	4	5	4-5
Hypro					2	2-3
Trp				2	2	2
His		2	2		5	1
Cys		*	2	1	Tr	
Cys-cys		2*				Tr
Pipe		2	2		1-2*	
Etam		1-2	2		2	1
3-Ala		1	1-2	1	3-4	1
4-Aba			2	2-3	4	3
P-ser	2	2	2		2	2
Orn		Tr*	1	1	2*	
2,3-Di			Tr		2*	
U1		2	2		3*	
U2			Tr		1*	
U3					Tr	Tr
U5		Tr*				1
U7			2-3			1
U8		Tr*			2	1
U9						Tr

Table 23 Correlation coefficients (corr.coeff.) between parent species (identity of the numbers are given in Table 11), between the hybrid (identity of the numbers are given in Table 12) and each parent, and between the theoretical hybrid and hybrid (as in Table 12).

Hybrid no.	Corr. coeff.(r) between parents	Corr. coeff.(r) between hybrid & parent species	Corr. coeff.(r) between theoretical hybrid & hybrid
Florets:			
H1	2 & 7 : 0,59	H1 & 2 : 0,64 H1 & 7 : 0,51	0,67
H3	4 & 11: 0,81	H3 & 4 : 0,86 H3 & 11: 0,83	0,89
H4	4 & 12: 0,59	H4 & 4 : 0,83 H4 & 12: 0,74	0,86
H5	1 & 7 : 0,44	H5 & 1 : 0,63 H5 & 7 : 0,62	0,78
H7(a)	7 & 12: 0,57	H7a& 7 : 0,73 H7a& 12: 0,67	0,92
H7(b)		H7b& 7 : 0,70 H7b& 12: 0,68	0,86
H9	8 & 10: 0,78	H9 & 8 : 0,72 H9 & 10: 0,70	0,77

Table 23 (contd.) Correlation coefficients (corr.coeff.) between parent species (identity of the numbers are given in Table 11), between the hybrid (identity of the numbers are given in Table 12) and each parent, and between the theoretical hybrid and hybrid (as in Table 12).

Hybrid no.	Corr. coeff.(r) between parents	Corr. coeff.(r) between hybrid & parent species	Corr. coeff.(r) between theoretical hybrid & hybrid
Foliage:			
H1	2 & 7 : 0,77	H1 & 2 : 0,76 H1 & 7 : 0,78	0,81
H2	3 & 10: 0,49	H2 & 3 : 0,41 H2 & 10: 0,62	0,64
H3	4 & 11: 0,77	H3 & 4 : 0,82 H3 & 11: 0,86	0,88
H4	4 & 12: 0,71	H4 & 4 : 0,51 H4 & 12: 0,79	0,74
H5	1 & 7 : 0,91	H5 & 1 : 0,79 H5 & 7 : 0,80	0,81
H6	5 & 7 : 0,82	H6 & 5 : 0,85 H6 & 7 : 0,89	0,90
H7	7 & 12: 0,83	H7 & 7 : 0,77 H7 & 12: 0,75	0,81
H8	8 & 6 : 0,90 8 & 9 : 0,81	H8 & 8 : 0,80 H8 & 6 : 0,79 H8 & 9 : 0,74	0,83 0,83
H9	8 & 10: 0,56	H9 & 8 : 0,74 H9 & 10: 0,52	0,72

Discussion of results

From the results presented in Tables 13 - 16, the free amino acid composition of the foliage and florets of *Protea* hybrids and that of their phenotypic parent species is compared. Since free amino acid composition is genetically determined, it would be expected that the presence and association of certain free amino acids in the hybrids would be reflected in either or both parents.

The identity of *P. minor*, hybrid no.H8 in Table 12, was uncertain, therefore both *P. pudens* and *P. longifolia*, which have both been described as *P. minor* (Rourke,1979), were assayed as parent species.

A very low number of free amino acids was detected in *P. roupelliae* (Table 14 no. 11) when it was assayed with the hybrid (Table 13 no.H3) and *P. eximia* (Table 14 no.4) in July, 1985 ; many more amino acids were detected in its foliage when assayed seven months later. However, the results are presented for the assays done at the same time ; this was not always possible for all the species and plant organs assayed. Column H7 (a) in Table 15 gives the results of florets assayed in March, and column H7 (b) of those assayed in September for hybrid no. H7 as identified in Table 12.

Glu, Asp, Ala, Ser, Val, and Pro were detected in the foliage of all hybrids assayed (Table 13). Glu, Asp,and Ala were detected in the foliage of all parent species (Table 14). There was an increase in the number as well as a general elevation of the concentrations of free amino acids detected in the florets of the

hybrids and their parent species studied. In the florets, Gly, Ile/Leu, Arg, Lys, Asn, Gln, and Thre were detected in all hybrids (Table 15) in addition to Ala, Val, Asp, Glu, Ser, and Pro which were detected in the foliage of all hybrids, with a noticeable elevation in the concentrations of Pro. Apart from Arg, all the protein amino acids detected in the florets of all hybrids were detected in those of all parent species too.

Pipe was detected in the florets of H9 only, but was detected more frequently in the foliage of the hybrids, including that of H9. On the other hand, Etam was detected in the florets of all hybrids assayed, but was detected less frequently in the hybrid foliage. These trends were noted for the parent species as well, where Pipe was detected more frequently in foliage than in florets, and Etam was detected more frequently in florets than in foliage.

Of the unidentified amino acids, U8 was detected in all hybrid florets, and U3 in all except those of H3 ; both were detected less frequently in the hybrid foliage. The same applied for U8, but not U3, in the parent species. U5 was detected more frequently in the foliage than in florets of the hybrids, but was common only to the florets and foliage of parent species no.7.

Once the identification of the unknowns is determined, along with the role of the non-protein amino acids detected, their presence or absence may be just an indication of the metabolic state of the plant at the time the material was collected and assayed. However, their presence and/or accumulation in the plant, and the

organ in which they are found, may be genetically determined, and thus be of chemotaxonomic use.

More free amino acids were detected in the florets of hybrids than in the florets of the parent species. This was not always the case in foliage. On average, more free amino acids were detected in florets than in foliage (average no. in florets was 22,9 vs. 17,1 in foliage ; average no. in hybrid florets was 26,5 vs. 19 in hybrid foliage), thus making florets a better choice as organ for analysis.

For an easier visual inspection of the data as discussed above and presented in Tables 13 - 16, the free amino acid composition and concentrations detected in the foliage and florets of hybrids and their phenotypic parent species are compiled in Tables 17 - 22. As an additional means of their analysis, correlation coefficients were determined for the amino acid concentrations between (i) the phenotypic parent species of each parent, (ii) the hybrid and each parent species, and (iii) a theoretical hybrid and the hybrid. The free amino acid concentrations for the theoretical hybrid were determined from the sum of each amino acid concentration detected in the parent species divided by two, even for an amino acid detected in only one parent.

In determining the correlation coefficients (r) from the results shown in Tables 13 - 16, trace amounts were allotted a concentration value of 0,5 , and a concentration of e.g. 3 - 4 was taken to be 3,5. Where an amino acid was not detected, a value of 0 was allotted. This was not considered to be an

altogether satisfactory method, because it is possible that if larger quantities of plant material had been used, those free amino acids not detected by the methods used might have been detected in proportionally lower concentrations. However, the interpretation of the results presented throughout this thesis are based on the presence (i.e. detection by the methods used) or absence of free amino acids in the plant material assayed, so that this assumed far greater significance than was allowed for in determining the correlation coefficients. In all cases, the same quantity of plant material was analyzed to validate comparisons.

In Tables 17 - 22, an asterisk is placed next to the amino acid concentrations which could not be reconciled with the hypothesis that the free amino acids in the hybrid are derived from the parents. In some cases, an amino acid was detected in the parent species but not in the hybrid. For example, His was detected in the foliage and florets of both parent species (Table 17), but was not detected at all in the hybrid. Often, an amino acid was detected in both florets and foliage in the hybrid, but only in one parent species and in different organs for each parent. An example of this can be seen in Table 20, where Orn was detected in the hybrid foliage and florets, but only in the foliage of parent species no.7 , and only in the florets of parent no.1 . In this case, the hybrid was also obviously able to produce or accumulate higher concentrations of this amino acid than its parents.

In most cases, the asterisk emphasized free amino acids that were detected in the hybrid only and not in either parent. This could indicate genetic control of the production or accumulation of the relevant amino acid(s) independent of the parent species.

From Table 23 the following interpretations of the data presented in Tables 17 - 22 can be made. In florets, the highest r values were between the hybrid and the theoretical hybrid, and these values were greater than r values between the hybrid and its parent species, which in turn were greater than r values between the parent species. H9 was an exception, where r between the parent species was almost equal to r between the theoretical hybrid and their hybrid, and only slightly better than the correlation between the hybrid and parent species. The close similarity between the r values for H9 thus implies that free amino acids would not be suitable markers for detecting chemotaxonomic differences in that plant.

There was a very good correlation in florets between the theoretical hybrid and hybrid except for H1. The correlation between the florets of each hybrid and its parent species was very similar for each parent except H1. This also casts doubt on the suitability of using free amino acids as a means of identifying the parents of Protea hybrids. This point was proved when an attempt was made to identify the parents of H8. P. minor, one of the parents of hybrid H8, could either refer to P. longifolia or to P. pudens as both were earlier known as P. minor. When the r value for the foliage of the theoretical

hybrid between H8 and both sets of parents was calculated, exactly the same value (0,83 , Table 23) was obtained. This makes it impossible to decide whether the other parent of H8 was P. longifolia or P. pudens.

In foliage, r values between the theoretical hybrid and the hybrid were greater than those between the parents, except for H5 and H8 (parents 8 & 6). There was an identical correlation between H8 and the theoretical hybrid determined from parent species 8 and 6, or 8 and 9 (0,83). However, as stated previously, free amino acids are not much use chemotaxonomically where r values are high between the parent species or between the hybrid and its parent species (e.g. H1 and H6, Table 23).

On average, r values between parent species is greater for the foliage (0,76) than for florets (0,63), thus implying that florets would be the better organ to use for chemotaxonomic evaluation, owing to greater differential differences.

As can be seen from the data presented in Tables 17 - 22, and from the correlation coefficients in Table 23, there is generally a high correlation between the parent species of the hybrids assayed, as well as a close correlation between the hybrids and their parent species. This augurs well for the commercial Protea breeder, but not for the chemotaxonomist. One would expect that hybridization should usually only take place between closely related species. This certainly holds as far as the occurrence of free amino acids in Protea is concerned. It thus appears that the presence and concentrations of free amino acids in Protea

hybrids and their phenotypic parent species are too similar for them to be worthy markers for species identification.

5. CONCLUSIONS

Paper chromatography proved to be a cheap and effective method for determining qualitatively the free amino acid composition of the material assayed, provided that several chromatograms covering a range of plant material concentrations were run, but, in addition, the quantification of these results by automatic amino acid analyser was necessary for relevant conclusions to be drawn.

More free amino acids, with a general elevation in their concentrations, were detected in young foliage buds when compared with foliage at least a year old (52 vs. 29 average total concentration units, Table 3). Differences in free amino acid composition were detected in the various organs assayed ; most free amino acids were detected in very young foliage, then florets and roots, with a general elevation in concentration when compared with those detected in foliage (average total concentration units of 57, 48, 33 and 29 respectively, Table 7). These differences were also apparent between florets and bracts (average total concentration units of 48 and 32 respectively, Table 7), and between vegetative foliage and foliage below the inflorescence (average total concentration units of 27 and 23 respectively, Table 7) for plant material collected and assayed simultaneously.

Free amino acid composition was similar in all the species compared from different geographic localities, although differences in their concentrations did occur. A better

evaluation could be made, when the free amino acid composition of more than one type of organ (e.g. foliage and florets) was compared. Since plant material compared was not always collected and assayed at the same time from each locality, the differences that did occur were attributed to seasonal effects.

Results from the simultaneous assays of roots and young foliage (Table 7) do not show differences in free amino acid composition great enough to indicate seasonal storage and translocation of nitrogen reserves, in the form of free amino acids, from roots to young shoots, as was detected in grasses by Weinman (1940 and 1942). However, additional research using more samples may prove this to be indeed so.

The greatest effect seemed to be a seasonal response, with an increase in the number and concentration of free amino acids in foliage, from about the end of August to February, irrespective of whether the plant was in a flowering or vegetative phase. This effect correlates with the subtropical summer growth rhythm reported for proteoid species by Specht (1979) and Pierce and Cowling (1984). Nowacki *et al* (1984) also found an increase in the levels of free amino acids in sour cherry buds during spring development, which was not correlated with changes in protein content. As far as is known, the possible seasonal or geographical variations of free amino acids of the same species has only been investigated by Murakami and Hatanaka (1983), who found no qualitative differences in the fern *Asplenium unilaterale*. Sciutio *et al* (1980) reported differences in basic

metabolism, as reflected in free amino acid composition, which are related to the different stages of the life cycle in certain red algae species. In the Protea species studied, seasonal effect on the plant's metabolism could determine the sporadic detection of certain non-protein amino acids such as U6 in P. cynaroides (Table 7) and in P. eximia (Table 8 (a)), and U10, U11 and U12 (cf. Chap 3.4 (b)), which were not always detected in the foliage of P. nitida or P. cynaroides (unpublished results).

In the study of Protea hybrids and their parent species, the genotypic parents of the hybrids studied were in most cases unknown. Since geographic locality did not seem to play an important role in influencing free amino acid composition, it was assumed to be adequate to compare hybrids with their phenotypic and not necessarily their genotypic parent species. On average, more free amino acids were detected in florets than in foliage (22,9 vs. 17,1 ; 26,5 vs. 19 in hybrids), thus indicating that florets would be a better organ for analysis.

Correlation coefficients (r) were determined for the free amino acid concentrations (i) between the parent species of each hybrid; (ii) between the hybrid and each parent species; (iii) between a theoretical hybrid, possessing all the free amino acids detected in both parents and at a concentration of half of their sum, and each hybrid. In general, the r values were greatest between the theoretical hybrid and the hybrid, and least between the parent species. Average r values for free amino acid concentration between parent species were greater for the foliage

(0,76) than for florets (0,63), also indicating that florets would be the better organ for analysis, due to greater differences. However, generally similar r values for the hybrid and each parent species indicated an equal genetic contribution from each parent, with similar free amino acid composition in the hybrid and parent species.

With few exceptions, Glu was detected in the highest concentrations in all species, irrespective of type or age of organ assayed, then Asp, Ala, Gly and Ser. In roots, Glu was detected in the highest concentration, followed by Asn, with much lower Asp concentrations detected, possibly indicating the active translocation of nitrogen reserves (Weinman, 1940 & 1942 ; Ta and Joy, 1985). Pipe was detected in the roots of all species studied, even when it had not been detected in any other organ (cf. P. eximia Table 7).

Most protein amino acids were present in higher concentrations than non-protein amino acids, and were therefore detected more often than the latter. Tyr was detected more often and in elevated concentrations in foliage, while Pro concentrations were highest in young foliage and florets. The accumulation of Pro in various plant organs is widely reported. Hong-Qi et al (1985) reported the accumulation of Pro in the anthers of Petunia hybrida during floral development until anthesis, and Nowacki et al (1984) reported that levels of Pro and Ser increased with development of flower buds ; their results confirmed the theory that Pro is stored as a source of soluble nitrogen as well as a

translocation compound for the rapid growth of tissues, which appears to be applicable to Protea florets and young foliage buds as well. Pro accumulation in leaves during water deficiency stress (Patel and Vora, 1985) and other environmental stresses as cited by Syvertson and Smith (1983), were not reflected in the results from Protea foliage.

The possible identification of U3, U5 and U10 is made from references in the literature. U3 also had an R_f of 0,85 in phenol-water and stained a grey-green colour with locating agent, when located in large concentrations, as with dimethyl histidine, isolated from the red alga, Gracilaria secundata by Madgwick et al (1970). It was usually detected in only trace amounts as denoted by a faint purple spot after 24 hours at room temperature. U5 is tentatively identified as homoserine from its position with regard to other already identified amino acids, as depicted by Fowden (1958). It was detected mainly in the foliage and bracts of Protea, and has a function in common with Asn, viz. the translocation and mobilization of carbon and nitrogen reserves, in pea leaves (Murray 1983 ; Melcher, 1985 ; Ta and Joy 1985 ; Ta, Joy and Ireland, 1985). U10 meets the identification criteria of α-(Methylenecyclopropyl)glycine, first located and isolated from litchi seeds by Gray and Fowden (1962).

From the results presented, it would seem that the metabolic state of the plant influences the composition and concentrations of the free amino acids detected, in other words amino acids are actively used or produced as metabolites. Consequently, amino

acids may not be very dependable as taxonomic indicators in Protea. If free amino acids are to be used as chemotaxonomic indicators, factors that may influence the metabolic state of the plant will have to be minimised, and the following points should be considered :

(a) use the same type of organ ;

(b) use material of the same age ;

(c) collect material at the same time of year to eliminate seasonal variations ;

(d) geographic locality does not seem to affect amino acid composition ;

(e) plant material compared must be either all in the flowering phase or all in the vegetative phase.

Most of the requirements set above would be met if florets were assayed for amino acids. In general, the seasonal variations observed in amino acid composition of florets were minimal compared with that observed in leaves and other organs. Consequently, it is proposed that florets would be the most suitable organ for the analysis of amino acids as chemotaxonomic markers in Protea.

6. SUMMARY

Material from 28 Protea species and hybrids was assayed, and a total of 40 free amino acids were located by two-dimensional paper chromatography, including 7 non-protein amino acids and 12 ninhydrin positive compounds that were unidentified. Extraction of the amino fraction was with methanol-chloroform-water, and group separation by a cationic exchange column of Dowex 50 resin in the H^+ form was effective in separating both basic and acidic amino acids. Individual amino acid concentrations were determined by allocating arbitrary concentration values, depending on spot size and colour intensity after development with the locating agent, after two-dimensional, descending paper partition chromatography, with some results quantified by means of an alpha automatic amino acid analyser. Quantification by amino acid analyser of the results obtained by two-dimensional paper chromatography was useful in determining the identity of the free amino acids detected, but the correlation between the concentrations as calculated for both methods was not as good as was expected.

In evaluating the importance of free amino acids as chemosystematic indicators, the effect of factors such as age and type of organ assayed, seasonal variations, geographic locality, and vegetative or flowering phase on their composition was determined. The results showed that free amino acid composition and concentrations are indeed influenced by seasonal effects and the type of organ assayed. Age was an important factor in foliage especially, with more free amino acids being detected and

at higher concentrations in very young foliage when compared with old (52 vs. 29 average concentration units). Geographic locality did not appear to affect free amino acid composition.

There was a difference in the number and concentrations of free amino acids detected in the various plant organs assayed, these being highest in very young foliage and in florets. The average arbitrary total concentration units determined for ninhydrin-reacting components present, gave 57 concentration units for young foliage and 48 for florets.

There was a generally good correlation between the free amino acid composition of like organs assayed from the same species from various geographic localities, with any discrepancies being due to a quantitative rather than a qualitative difference.

The study of the free amino acid composition of several Protea species and hybrids was undertaken in order to determine whether the free amino acids present in hybrids reflect all those of the parent species. If they were genetically stable markers they could be useful as chemotaxonomic markers, and as such contribute towards the natural classification of this genus. The free amino acid composition of the Protea hybrids studied reflected in general that of their phenotypic parent species, although some amino acids were detected in hybrids but not in either parent and vice versa.

With few exceptions, glutamic acid was detected in the highest concentrations in all organs assayed, followed by aspartic acid,

alanine, glycine and serine.

Seasonal variations and type of organ assayed had a marked effect on amino acid composition, and if this is not taken into consideration, amino acids would have limited value in the chemotaxonomy of Protea. Because the free amino acid composition of florets was shown to be scarcely affected by the parameters tested, florets would be the most reliable organ to assay as a source of free amino acids as taxonomic indicators in Protea.

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