

A STUDY ON THE ROLE OF CYTOKININS
IN THE DEVELOPMENT
OF STARCH ACCUMULATING STRUCTURES

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DEDICATION

To my parents

TABLE OF CONTENTS

	<u>PAGE</u>
TABLE OF CONTENTS	iii
LIST OF TABLES	vii
LIST OF FIGURES	xii
ABSTRACT	xviii
CHAPTER I INTRODUCTION	1
CHAPTER II CYTOKININ BIOASSAYS	41
2.1 Introduction	41
2.2 Materials and Methods	42
2.2.1 Initiation of soybean callus clones	42
2.2.2 Maintenance of a soybean callus clone obtained from Plant Diseases Division, DSIR, Auckland	43
2.2.3 Bioassays of plant extracts and esti- mation of cytokinin-like activity	44
2.2.4 Soybean hypocotyl bioassay	45
2.3 Results	47
2.3.1 Initiation of soybean callus clones	47
2.3.2 Maintenance of callus clone obtained from DSIR	54
2.3.3 Soybean hypocotyl bioassay	59
2.4 Discussion	61
CHAPTER III CHROMATOGRAPHY SYSTEMS	64
3.1 Introduction	64
3.2 Materials and Methods	64
3.2.1 Chemicals	64
3.2.2 Paper chromatography	64
3.2.3 Column chromatography	65

	<u>PAGE</u>
CHAPTER III (Continued)	
3.3 Results	66
3.4 Discussion	68
CHAPTER IV POTATO	71
4.1 Introduction	71
4.2 Materials and Methods	71
4.2.1 Preliminary experiments with mature <i>S. tuberosum</i> tubers	71
4.2.2 Controlled Growth Room Experiments (Canterbury)	74
4.2.3 Potato Field Trial (Lincoln, mid- Canterbury)	77
4.2.4 Controlled Environment Experiments, Plant Physiology Division, DSIR, Palmerston North	80
4.3 Results	88
4.3.1 Preliminary experiments with mature <i>S. tuberosum</i> cultivar Ilam Hardy tubers	88
4.3.2 Controlled Growth Room Experiments (Canterbury)	95
4.3.3 Potato Field Trial (Lincoln, mid- Canterbury)	97
4.3.4 Controlled Environment Experiments, Plant Physiology Division, DSIR, Palmerston North	111
4.3.4.1 PPD Experiment 1	111
4.3.4.2 Observations on the effects of different growth conditions on the tuberisation response of <i>S. andigena</i> cv 165	141
4.4 Discussion	146
4.4.1 Preliminary Experiments	146
4.4.2 Plant Physiology Division Experiment One	155
4.4.3 Tuberisation response of <i>S. andigena</i> grown under different growth conditions	176

	<u>PAGE</u>
CHAPTER V TUBER FORMATION <i>IN VITRO</i>	180
5.1 Introduction	180
5.2 Materials and Methods	181
5.2.1 Tuberation <i>in vitro</i> . Experiment 1	181
5.2.2 Tuberation <i>in vitro</i> . Experiment 2	182
5.2.3 Tuberation <i>in vitro</i> . Experiment 3	183
5.3 Results	184
5.3.1 Tuberation <i>in vitro</i> . Experiment 1	184
5.3.2 Tuberation <i>in vitro</i> . Experiment 2	186
5.3.3 Tuberation <i>in vitro</i> . Experiment 3	191
5.4 Discussion	193
CHAPTER VI WHEAT	200
6.1 Introduction	200
6.2 Materials and Methods	200
6.2.1 Wheat Field Trial 1	200
6.2.2 Wheat Field Trial 2	204
6.3 Results	209
6.3.1 Wheat Field Trial 1	209
6.3.2 Wheat Field Trial 2	218
6.4 Discussion	254
CHAPTER VII IDENTIFICATION OF CYTOKININS IN WHEAT GRAINS	273
7.1 Introduction	273
7.2 Materials and Methods	275
7.2.1 Trimethylsilylation	275
7.2.2 Permethylation	275
7.2.3 Gas chromatography and combined gas chromatography-mass spectrometry	276

	<u>PAGE</u>
CHAPTER VII (Continued)	
7.3 Results	277
7.3.1 Zeatin riboside	277
7.3.2 Zeatin	280
7.3.3 Quantification	287
7.4 Discussion	287
CHAPTER VIII GENERAL DISCUSSIONS AND CONCLUSIONS	291
ACKNOWLEDGEMENTS	294
REFERENCES	295
APPENDICES	321

LIST OF TABLES

<u>TABLE</u>		<u>PAGE</u>
2.1	Response of hypocotyl segments to different cytokinins	61
3.1	Soybean callus bioassays of authentic zeatin riboside (A) and zeatin (B) following partition chromatography on Sephadex LH-20 using 35% ethanol as eluant	68
4.1	Potato Field Trial. List of harvest dates, total number of rows harvested and size categories to which excised plant material was allocated	78
4.2	PPD Experiment 1. Harvest schedule detailing the number of pails and plants harvested after a given time in the controlled environment rooms	81
4.3	PPD Experiment 1. Harvest schedule listing the categories excised stolon and tuber material were allocated to, the number of stolons or tubers ground prior to extraction and the fresh weight of material extracted	83
4.4	PPD Experiment 1. Extraction schedule listing the number of stolons or tubers ground prior to extraction, and the fresh weight and dry weight of material extracted	84
4.5	PPD Experiment 1: Starch and Soluble Sugar Analyses. Details of the analyses showing the dry weight, fresh weight and number of stolons or tubers ground per sample, and the dry weight extracted per replicate. The volumes to which the starch hydrolysates were diluted and the aliquots removed for incubation with glucose oxidase are also indicated	85
4.6	PPD Experiment 2. Harvest schedule detailing the number of pails (and plants) harvested after a specified time in each of the controlled environment rooms	88
4.7	Soybean callus bioassay of petroleum ether- and butan-1-ol-soluble components of potato tuber extract. The butanol-soluble components were resolved by partition chromatography on Sephadex LH-20	94
4.8	Field Trial 1. Comparison of the total cytokinin-like activity of stolons from harvest 1 and tubers of diameter 4 to 7.5 mm from harvest 4	111
4.9	PPD Experiment 1. Percentage tuberisation of <i>S. andigena</i> plants under long day conditions in controlled environment rooms	113

<u>TABLE</u>	<u>PAGE</u>	
4.10	PPD Experiment 1. The number of plants harvested, total weight and number of stolons and tubers per plant and the total number of tubers per plant is presented for each harvest	113
4.11	PPD Experiment 1. Range of tuber sizes and the proportion of the total tuber number contributing to approximately 50% of the total fresh weight of tubers	119
4.12	PPD Experiment 1. Starch content of stolons and tubers less than 7.5 mm in diameter	119
4.13	PPD Experiment 1. Soluble sugar content of stolons and tubers less than 7.5 mm diameter	122
4.14	PPD Experiment 1. Estimation of the cytokinin-like activity present in 4 g fresh weight samples of stolon and tuber material	133
4.15	PPD Experiment 1. Estimation of the cytokinin-like activity present in 8 g eq FW of stolon and tuber extracts. The activity was resolved by partition chromatography on Sephadex LH-20 eluted with 20% ethanol	137
4.16	PPD Experiment 1. Comparison of the amount of cytokinin-like activity present in fraction 9 and fraction 12 following the extraction of 4 g eq FW and 8 g eq FW respectively of stolons and tubers. The activity was resolved by partition chromatography on Sephadex LH-20 eluted with 35% ethanol (4 g eq FW) and 20% ethanol (8 g eq FW), respectively	139
4.17	PPD Experiment 1. Contribution from <i>S. andigena</i> tubers of the size categories 10 to 15 mm and 15 to 20 mm diameter to the total number and weight of stolons and tubers per plant	141
4.18	Response of <i>S. andigena</i> to long day and short day conditions	141
4.19	Field harvest dates, storage conditions and length of storage of <i>S. andigena</i> tubers used in all controlled environment experiments	142
4.20	Percentage tuberisation attained by <i>S. andigena</i> plants grown from seed tubers with different storage histories	143
4.21	Growth of <i>S. andigena</i> under differing conditions in the CE rooms (PPD). Plants were subjected to different day/night temperature regimes	144
4.22	Response of <i>S. andigena</i> plants to differing amounts of photosynthetically active radiation (PAR). Seed tubers were planted within one week of harvest. Day and night temperatures were constant at 20°C	145

<u>TABLE</u>	<u>PAGE</u>	
4.23	A comparison of the response of <i>S. andigena</i> plants grown in different controlled growth rooms at Canterbury (experiment 3). Both light intensity and temperature differed between the rooms	146
5.1	Tuberisation <i>in vitro</i> . Experiment 1. Combinations of sucrose and kinetin with which the modified Murashige and Skoog nutrient agar was supplemented	182
5.2	Tuberisation <i>in vitro</i> . Experiment 2. Culture details listing the number of apical and medial segments incubated on Murashige and Skoog nutrient agar supplemented with different combinations of sucrose and kinetin	183
5.3	Tuberisation <i>in vitro</i> . Experiment 3. Culture details listing the number of whole segments and single-nodal segments of <i>S. tuberosum</i> and <i>S. andigena</i> transferred onto Media 5 and 6	184
5.4	Tuberisation <i>in vitro</i> . Experiment 1. <i>In vitro</i> culture of sprout segments removed from <i>S. andigena</i> tubers.	185
5.5	Tuberisation <i>in vitro</i> . Experiment 2. Tuber formation by medial sprout segments cultured on medium supplemented with different concentrations of sucrose and kinetin	188
5.6	Tuberisation <i>in vitro</i> . Experiment 3. Tuber formation by second transfer shoots obtained from sprout segments cultured <i>in vitro</i>	192
6.1	Wheat Field Trial 1. Harvest schedule listing the dates, developmental stage relative to ear emergence and anthesis, the number of ears dissected in the field per sample, and the number dissected in the laboratory as a "bulk sample" at each respective harvest	201
6.2	Wheat Field Trial 1. Extraction sequence detailing the number of grains extracted, the dry weight of sample extracted, the volume of 80% methanol used for extraction of individual samples over each 24 hour period, and the amount of solvent used to wash the retained residue following each filtration	203
6.3	Wheat Field Trial 2. Harvest schedule listing the date, developmental stage relative to ear emergence and anthesis, the number of ears dissected in the field per sample, and the number dissected in the laboratory as a "bulk sample" at each respective harvest	205

<u>TABLE</u>	<u>PAGE</u>	
6.4	Wheat Field Trial 2. Extraction sequence detailing the number of grains extracted, the dry weight of sample extracted, the volume of 80% methanol used for extraction of individual samples over each 24 hour period, and the amount of solvent used to wash the retained residue following each filtration	206
6.5	Wheat Field Trial 2. Starch and Soluble Sugar Analyses. Details of the analyses showing the dry weight, fresh weight and number of grains ground per sample, and the dry weight extracted per replicate. The volumes to which starch hydrolysates were diluted and the aliquots removed for incubation with glucose oxidase are also indicated	210
6.6	Wheat Field Trial 1. Quantification of cytokinin-like activity in 500 wheat pistils collected at pollination	212
6.7	Wheat Field Trial 1. Quantification of cytokinin-like activity in 500 wheat grains collected at anthesis	216
6.8	Wheat Field Trial 1. Quantification of cytokinin-like activity in 500 wheat grains collected three days post-anthesis	216
6.9	Wheat Field Trial 1. Quantification of cytokinin-like activity in 500 wheat grains collected seven days post-anthesis	218
6.10	Wheat Field Trial 2. Quantification of cytokinin-like activity in extracts of 500 pistils collected at pollination (nine days after ear emergence)	226
6.11	Wheat Field Trial 2. Quantification of cytokinin-like activity in extracts of 500 wheat grains collected at anthesis (10 days after ear emergence)	230
6.12	Wheat Field Trial 2. Quantification of the cytokinin-like activity in extract of 500 wheat grains collected in the "bulk sample" two days post-anthesis (12 days after ear emergence)	233
6.13	Wheat Field Trial 2. Quantification of the cytokinin-like activity in extracts of 100 wheat grains collected two days post-anthesis (12 days after ear emergence)	233
6.14	Wheat Field Trial 2. Quantification of the cytokinin-like activity in extracts of 100 wheat grains collected two days post-anthesis (12 days after ear emergence). The Sephadex LH-20 chromatography eluant was 20% ethanol	235
6.15	Wheat Field Trial 2. Quantification of the cytokinin-like activity collected in fractions 7 to 16 following the treatment of fractions 3 to 9 with β -glucosidase and rechromatography. Extracts of 100 grains from three samples collected two days post-anthesis were treated and bioassayed.	235

<u>TABLE</u>		<u>PAGE</u>
6.16	Wheat Field Trial 2. Comparison of the total cytokinin-like activity detected in fractions 3 to 9 before and after β -glucosidase treatment of extracts of 100 wheat grains collected two days post-anthesis	236
6.17	Quantification of the cytokinin-like activity in extracts of 100 wheat grains collected in the "bulk sample" two days post-anthesis following extraction with either 80% methanol or Bieleski's solvent cocktail	240
6.18	Wheat Field Trial 2. Quantification of the cytokinin-like activity in extracts of 500 wheat grains collected four days post-anthesis (14 days after ear emergence)	240
6.19	Wheat Field Trial 2. Quantification of the cytokinin-like activity in extracts of 100 wheat grains collected four days post-anthesis (14 days after ear emergence)	241
6.20	Wheat Field Trial 2. Quantification of the cytokinin-like activity in extracts of wheat grains collected four days post-anthesis (14 days after ear emergence). The Sephadex LH-20 chromatography eluant was 20% ethanol	245
6.21	Wheat Field Trial 2. Quantification of the cytokinin-like activity in extracts of 500 wheat grains collected seven days post-anthesis (17 days after ear emergence)	249
7.1	Relative intensities of high mass ions in the mass spectrum obtained from direct probe insertion of 1,000 ng trimethylsilylated zeatin riboside	277
7.2	Relative peak heights of ions diagnostic of TMS ₄ -zeatin riboside obtained following the injection of 1,000 ng of trimethylsilylated zeatin riboside standard and multiple ion monitoring of ions at m/z 639, 624 and 536	280
7.3	Relative peak heights of ions diagnostic of TMS ₄ -zeatin riboside obtained following the injection of a 2 μ l aliquot of wheat sample and multiple ion monitoring of ions at A: m/z 639, 624 and 536; and B: m/z 624, 550 and 549	284
7.4	Relative intensities of high mass ions in the mass spectrum obtained from direct probe insertion of 1,000 ng of permethylated zeatin standard	284

LIST OF FIGURES

<u>FIGURE</u>	<u>PAGE</u>
2.1 Responses of callus cultured from soybean cultivars Kitamasume (clone A) and Okuhara to increasing kinetin concentrations and two agar concentrations	49
2.2 Response of callus cultured from soybean cultivars Kitamasume (clones A and B) and Okuhara to increasing kinetin concentrations	51
2.3 Soybean callus bioassays terminated after 14, 21, 28 and 35 days	52
2.4 Comparison of soybean callus bioassays set up with callus passed through different numbers of sub-cultures	53
2.5 Comparison of soybean callus bioassays set up with A 7 mg transplants B 20 mg transplants C callus maintained 7 days on medium devoid of cytokinin prior to starting the bioassay	55
2.6 Comparison of soybean callus bioassays using callus at different ages post-transfer and maintained on either kinetin or 6-benzylaminopurine	56
2.7 Regression lines calculated from the growth of callus on kinetin standards	57
2.8 Response of callus clone Bap to different concentrations of zeatin, zeatin riboside and kinetin	58
2.9 Soybean hypocotyl bioassay. Response of hypocotyl segments from soybean cv Caloria to different kinetin concentrations	60
3.1 Mobilities of zeatin riboside and zeatin (in propan-2-ol - ammonia - water (10:1:1 v/v) on Whatman 3 MM chromatography paper. Compounds were detected by the soybean callus bioassay	67
3.2 Elution volumes of kinetin, isopentenyladenine and 6-benzylaminopurine following partition chromatography on Sephadex LH-20 with 35% ethanol as eluant. Compounds were detected by the soybean callus bioassay	69
4.1 Soybean callus bioassay of paper chromatograms loaded with methanolic extract of mature tuber tissue	89
4.2 Soybean callus bioassay of paper chromatograms loaded with aqueous and butan-1-ol soluble components of mature tuber tissue extract	91

<u>FIGURE</u>	<u>PAGE</u>
4.3 Soybean callus bioassay of paper chromatograms loaded with methanolic extract and extract partially purified by Dowex 50 (H ⁺ form) cation exchange chromatography	92
4.4 Soybean callus bioassay of methanolic extract and of extract partially purified by Dowex 50 (H ⁺ form) cation exchange chromatography	93
4.5 Soybean callus bioassay of paper chromatograms loaded with extract of Ilam Hardy "bolter" plants. Tubers were removed from plants under SD conditions, stolons from plants under LD conditions	96
4.6 Soybean callus bioassay of extract of <i>S. andigena</i> cv 165. Tubers were removed from plants under SD conditions, stolons from plants under LD conditions	98
4.7 Soybean callus bioassay of paper chromatograms loaded with aqueous and butan-1-ol soluble components of extract of <i>S. andigena</i> cv 165 tubers.	99
4.8 Potato Field Trial. Fresh weight increase with increasing tuber size	101
4.9 Potato Field Trial. Soybean callus bioassay of paper chromatograms loaded with extract of <i>S. tuberosum</i> cv Ilam Hardy seed tubers	102
4.10 Potato Field Trial. Soybean callus bioassay of extract of <i>S. tuberosum</i> cv Ilam Hardy seed tubers	103
4.11 Potato Field Trial. Soybean callus bioassay of paper chromatograms loaded with aqueous- and butanol-soluble components of stolon tip extracts	104
4.12 Potato Field Trial. Soybean callus bioassay of stolon extract	105
4.13 Potato Field Trial. Soybean callus bioassay of paper chromatograms loaded with aqueous- and butan-1-ol-soluble components of extracts of tubers less than 4 mm diameter	106
4.14 Potato Field Trial. Soybean callus bioassay of paper chromatograms loaded with aqueous- and butan-1-ol-soluble components of extracts of tubers between 4.0 and 7.5 mm diameter	107
4.15 Potato Field Trial. Soybean callus bioassay of extract of tubers 4 to 7.5 mm diameter	108
4.16 PPD Experiment 1. Growth analysis of the population of plants sampled between 21 and 84 days after transfer to controlled environment rooms. Relative rate of tuber weight increase per plant as a function of time after transfer	112

<u>FIGURE</u>		<u>PAGE</u>
4.17	PPD Experiment 1. Growth analysis of the population of plants sampled between 21 and 84 days after transfer to controlled environment rooms. Each size category of stolons and tubers is presented as a proportion of the total stolon and tuber weight per plant as a function of increasing plant age	115
4.18	PPD Experiment 1. Growth analysis of the population of plants sampled between 21 and 84 days after transfer to controlled environment rooms. Each size category of stolons and tubers is presented as a proportion of the total stolon and tuber number per plant as a function of increasing plant age	116
4.19	PPD Experiment 1. Increase in dry weight and fresh weight as a function of tuber diameter	117
4.20	PPD Experiment 1. Starch content of stolons and tubers as a function of tuber diameter	118
4.21	PPD Experiment 1. Soluble sugar content of stolons and tubers as a function of tuber diameter	121
4.22	PPD Experiment 1. Soybean callus bioassay of extracts of stolons tips (4 g eq FW)	123
4.23	PPD Experiment 1. Soybean callus bioassay of extracts of tubers 1.5 to 3 mm diameter (4 g eq FW)	124
4.24	PPD Experiment 1. Soybean callus bioassay of extracts of tubers 3 to 5 mm diameter (4 g eq FW)	126
4.25	PPD Experiment 1. Soybean callus bioassay of extracts of tubers 5 to 7.5 mm diameter (4 g eq FW)	127
4.26	PPD Experiment 1. Soybean callus bioassay of extracts of tubers 7.5 to 10 mm diameter (4 g eq FW)	128
4.27	PPD Experiment 1. Soybean callus bioassay of extracts of tubers 10 to 15 mm diameter (4 g eq FW)	129
4.28	PPD Experiment 1. Soybean callus bioassay of extracts of tubers 15 to 20 mm diameter (4 g eq FW)	130
4.29	PPD Experiment 1. Soybean callus bioassay of extracts of tubers 20 to 25 mm diameter (4 g eq FW)	131
4.30	PPD Experiment 1. Soybean callus bioassay of extracts of tubers 40 to 50 mm diameter (4 g eq FW)	132
4.31	PPD Experiment 1. Total cytokinin-like activity in potato tubers following the extraction of 4 g eq FW tissue.	135
4.32	PPD Experiment 1. Soybean callus bioassay of extracts of stolons and tubers of the sizes specified in the figure (8 g eq FW)	136

<u>FIGURE</u>	<u>PAGE</u>
4.33 PPD Experiment 1. Cytokinin-like activity in stolons and tubers expressed as kinetin equivalents (components)	138
4.34 PPD Experiment 1. Cytokinin-like activity in stolons and tubers expressed as kinetin equivalents (total)	140
6.1 Wheat Field Trial 1. Fresh weight and dry weight of grains as a function of days from ear emergence	211
6.2 Wheat Field Trial 1. Soybean callus bioassay of extracts of 500 wheat pistils collected 10 days after ear emergence (pollination)	213
6.3 Wheat Field Trial 1. Soybean callus bioassay of extracts of 500 grains collected 11 days after ear emergence (anthesis)	214
6.4 Wheat Field Trial 1. Soybean callus bioassay of paper chromatograms loaded with extract of 500 grains collected 11 days after ear emergence (anthesis)	215
6.5 Wheat Field Trial 1. Soybean callus bioassay of extracts of 500 wheat grains collected 14 days after ear emergence (3 days post-anthesis)	217
6.6 Wheat Field Trial 1. Soybean callus bioassay of extracts of 500 wheat grains collected 18 days after ear emergence (7 days post-anthesis)	219
6.7 Wheat Field Trial 1. Total Cytokinin-like activity in wheat grains between pollination and seven days post-anthesis (10 and 18 days after ear emergence)	220
6.8 Wheat Field Trial 2. Fresh weight and dry weight of grains as a function of time from ear emergence.	221
6.9 Wheat Field Trial 2. Starch content of developing wheat grains estimated either as the amount per grain or as a proportion of the total fresh or dry weight of the grain	223
6.10 Wheat Field Trial 2. Soluble sugar content of developing wheat grains estimated either as the amount per grain or as a proportion of the total fresh or dry weight of the grain	224
6.11 Wheat Field Trial 2. Soybean callus bioassay of 500 wheat pistils collected at ear emergence	225
6.12 Wheat Field Trial 2. Soybean callus bioassay of 500 wheat pistils collected nine days after ear emergence (pollination)	227
6.13 Wheat Field Trial 2. Soybean callus bioassay of 500 wheat grains collected 10 days after ear emergence (anthesis)	228

<u>FIGURE</u>	<u>PAGE</u>
6.14 Wheat Field Trial 2. Soybean callus bioassay of 500 wheat grains collected 12 days after ear emergence (2 days post-anthesis)	229
6.15 Wheat Field Trial 2. Soybean callus bioassay of 500 wheat grains collected in the "bulk sample" 12 days after ear emergence (2 days post-anthesis)	231
6.16 Wheat Field Trial 2. Soybean callus bioassay of extracts of 100 wheat grains collected 12 days after ear emergence	232
6.17 Wheat Field Trial 2. Soybean callus bioassays of extracts of 100 wheat grains collected 12 days after ear emergence. <ol style="list-style-type: none"> 1. Extracts were resolved by partition chromatography on Sephadex LH-20 2. Soybean callus bioassay of the first zone of activity in "1" (fractions 3 to 9) after hydrolysis with β-glucosidase and further separation on Sephadex LH-20 	234
6.18 Wheat Field Trial 2. Soybean callus bioassay of extracts of 100 wheat grains from the "bulk" sample collected 12 days after ear emergence. <ol style="list-style-type: none"> 1. Samples were extracted with 80% methanol 2. Samples were extracted using Bielecki's procedure 	237
6.19 Wheat Field Trial 2. Soybean callus bioassay of extracts of 500 wheat grains collected 14 days after ear emergence (4 days after anthesis)	239
6.20 Wheat Field Trial 2. Soybean callus bioassay of extracts of 100 wheat grains collected 14 days after ear emergence (35% ethanol)	242
6.21 Wheat Field Trial 2. Soybean callus bioassay of extracts of 100 wheat grains collected 14 days after ear emergence (20% ethanol)	243
6.22 Wheat Field Trial 2. Soybean callus bioassay of extracts of 500 wheat grains collected 17 days after ear emergence (7 days post-anthesis)	244
6.23 Wheat Field Trial 2. Soybean callus bioassay of extracts of 500 wheat grains collected 17 days after ear emergence <ol style="list-style-type: none"> 1. Soybean callus bioassay of fractions 3 to 9 after hydrolysis with β-glucosidase and further separation on Sephadex LH-20 2. Soybean callus bioassay of the remaining non-hydrolysed fraction 	246
6.24 Wheat Field Trial 2. Soybean callus bioassay of extracts of 500 wheat grains collected 21 days after ear emergence (11 days post-anthesis)	247

<u>FIGURE</u>		<u>PAGE</u>
6.25	Wheat Field Trial 2. Soybean callus bioassay of extracts of 500 wheat grains collected 28 days after ear emergence (18 days post-anthesis)	248
6.26	Wheat Field Trial 2. Total cytokinin-like activity in wheat grains from ear emergence to maturity. The data are expressed as kinetin equivalents per grain	250
6.27	Wheat Field Trial 2. Total cytokinin-like activity in wheat grains from ear emergence to maturity. The data are expressed as kinetin equivalents either per gram dry weight or per gram fresh weight	251
6.28	Wheat Field Trial 2. Changes in the activity of the cytokinin-like compounds in developing grains of wheat between ear emergence and maturity. The data are expressed as kinetin equivalents per grain	252
6.29	Wheat Field Trial 2. Changes in the activity of the cytokinin-like compounds in developing grains of wheat between ear emergence and maturity. The data are expressed in kinetin equivalents per unit dry weight	253
7.1	FID trace from gas chromatography of 1,000 ng trimethylsilylated zeatin riboside standard.	278
7.2	Total ion current trace from combined gas chromatography - mass spectrometry of 1,000 ng trimethylsilylated zeatin riboside standard	279
7.3	Multiple ion monitoring of ions characteristic of TMS ₄ -zeatin riboside. 1,000 ng TMS ₄ -zeatin riboside standard injected	281
7.4	Multiple ion monitoring of ions characteristic of TMS ₄ -zeatin riboside. 2 µl aliquot of trimethylsilylated wheat sample injected	282
7.5	FID trace from gas chromatography of 1,000 ng permethylated zeatin standard	283
7.6	Multiple ion monitoring of ions characteristic of Me ₃ -zeatin. 100 ng permethylated zeatin standard injected	285
7.7	Multiple ion monitoring of ions characteristic of Me ₃ -zeatin. Samples of permethylated zeatin (A, 100 ng and B, 5 ng) and of permethylated wheat (1 µl C and D) were injected	286

ABSTRACT

Cytokinin-like activity and soluble sugar and starch levels were monitored during wheat grain (*Triticum aestivum* L.) and potato tuber (*Solanum* spp) development. Cytokinin-like activity was resolved on Sephadex LH-20 eluted with 35 or 20% ethanol and estimated in kinetin equivalents from the soybean callus bioassay.

The cytokinin-like activity increased in tubers larger than 5 - 7.5 mm diameter and reached a maximum in tubers 15 - 20 mm diameter. The amount per tuber was greatest in the largest size category analysed (40 - 50 mm diameter). The amount of sugar and starch per tuber also increased after tuber formation. There was a positive correlation between the highest concentration of cytokinin-like activity and the reported period of intense cell division.

The cytokinin-like activity per ^{plant} pistil and per ^{plant} unit weight increased between ear emergence and pollination. On a per grain basis the activity increased to a high level 14 days after ear emergence (four days post-anthesis) but subsequently decreased to an undetectable level by 21 days. On a per unit weight basis the concentration was high but fluctuated between 10 and 14 days after ear emergence before decreasing. The most polar components of the activity were the *O*-glucosides of zeatin and zeatin riboside. These showed a rapid increase between ear emergence and anthesis but subsequently decreased, whereas zeatin increased most rapidly following pollination and reached a maximum four days post-anthesis. Zeatin riboside remained at a relatively low level at all stages of development. The highest level of cytokinin-like activity correlated with the reported onset of normal cell divisions in the endosperm. The amount of activity remained low as sugar and starch levels in the grain increased.

Both zeatin and zeatin riboside were positively identified by GCMS (MIM) in extract of wheat grains.

CHAPTER I

INTRODUCTION

The cytokinins have been implicated in the growth and development of plants since the liquid endosperm of the coconut (coconut milk) was first used as a source of unidentified growth factors to stimulate growth of excised tissues. Van Overbeek *et al* (1941) used coconut milk to supplement culture media in order to stimulate the *in vitro* growth of young embryos of *Datura*. Ball (1946) noted that if the medium was supplemented with coconut milk, callus composed almost purely of parenchyma cells grew on the cut surface of stem segments taken subadjacent to the apex of *Tropaeolum majus* L seedlings. Caplin and Steward (1948) noted that the factor from coconut milk which promoted the growth of carrot cultures was heat stable and also stable to prolonged autoclaving. Since the activity decreased on dialysis, the active component(s) was considered to be of small molecular size. The "growth factor" was detected in the watery endosperm of the coconut at all stages of development and while some activity was shown by parts of the immature embryo, none was shown by the solid endosperm (Steward and Caplin, 1952). In contrast, Mauney *et al* (1952) suggested that most of the activity was derived from the coconut meat (the solid endosperm).

It was soon noted that the "growth factor" was not confined to coconut tissues. Steward and Caplin (1952) showed that extracts from *Zea mays* in the milk stage, from the gelatinous content of immature fruits of *Juglans regia* (walnut) and also from the young gametophyte of *Ginkgo biloba* could all stimulate growth in the carrot assay.

The level of growth-stimulating activity in developing *Zea mays* was monitored by Steward and Caplin (1952). Water extracts of *Zea mays* showed an increase in activity from a low level prior to pollination to a high level two weeks after pollination. This was followed by a decline to a low level of activity eight weeks after pollination. Extracts from immature wheat grains, barley and oat ovaries showed less activity but Steward and Caplin concluded that since development in these cereals was so rapid following fertilisation, an embryo and its accompanying endosperm

reached in a few days a stage of development comparable to that which *Zea mays* reached after a considerably longer period of time. The authors suggested more frequent sampling would be required to detect high levels of activity from wheat grains.

The condition which Steward and Caplin (1952) considered was common to all plants extracted and which was conducive to the accumulation of the growth factor was the relatively delayed growth of the structure to be nourished (*eg* the embryo) and the relative precocious development of the nutritive material (*eg* the endosperm) at the expense of the nucellus.

Mauney *et al* (1952), aware that inhibitory substances may be masking growth stimulatory activity in plant extracts, tested a range of purification procedures and showed, for example, that yeast extract yielded an active fraction whereas previously the crude extract had been shown to be inactive (Steward *et al*, 1952). Extracts of barley seed at the milky ripe stage, extract of Jack Pine seed and an extract of vascular tissue of tobacco were all found to duplicate the stimulatory effect of coconut meat extract on the growth of carrot callus (Mauney *et al*, 1952). If an auxin, indol-3yl acetic acid (IAA) was added to the medium in addition to a highly purified extract of coconut meat, the growth of carrot callus was increased considerably. This synergistic interaction between an auxin and the active component(s) of coconut meat extract had been noted previously by Caplin and Steward (1948) between IAA and coconut milk, and by Steward and Caplin (1951) between 2,4-dichlorophenoxy-acetic acid (2,4-D) and coconut milk.

Clear confirmation that a specific cell division inducing factor existed, and which was not an auxin, was provided by the cytological studies of Jablonski and Skoog (1954). Coconut milk, malt or water extracts from vascular tissues were shown to promote cell division in tobacco pith tissue, whereas IAA alone promoted growth solely by cell enlargement but not by cell division even though an increase in nuclear material occurred in the expanding cells.

In 1955a, Miller *et al* isolated a compound exhibiting the same physiological activity and requirements as the unidentified coconut milk factor. The compound was isolated from herring sperm DNA and identified as 6-furfurylamino-purine. The structural assignment was verified by synthesis of an authentic sample (Miller *et al*, 1955b). A four-year old preparation of herring sperm was found to be active in the tobacco "wound" callus assay although a fresh preparation was not. However, when water slurries of fresh DNA were autoclaved they became extremely active.

DNA from calf thymus behaved similarly (Miller *et al*, 1955a). The trivial name "kinetin" was proposed for 6-furfurylaminopurine which was found to be capable of stimulating cell divisions in tobacco callus, but only in the presence of an auxin (Miller *et al*, 1955a).

Hall and de Ropp (1955) concluded from their experiments that kinetin was an artifact formed from natural components of DNA. Scopes *et al* (1976) reinvestigated the experiments on kinetin formation from DNA constituents and concluded that kinetin was formed as a result of dehydration and migration of a deoxyribose moiety from the 9-position of adenine to the N⁶-position resulting in the formation of a 6-substituted aminopurine.

Das *et al* (1956) performed a detailed cytological study on the effects of kinetin and IAA on the processes of mitosis and cytokinesis in tobacco pith tissues cultured *in vitro*. Regardless of whether or not kinetin was added to the medium, no mitoses occurred in tissue cultured without IAA. IAA alone induced some mitoses but very little cell division whereas the combination of kinetin and IAA not only increased the frequency of mitoses but caused them to be followed by cytokinesis.

Das *et al* (1956) supported the concept that in plants, growth by cell division was regulated by a proper balance between endogenous growth factors and suggested the term "kinin" as a generic name for all substances which promoted cell division in a manner similar to that of kinetin.

It was soon shown that kinetin could elicit responses from plant tissues in addition to cell division. Many of these responses have been used subsequently to assay for kinetin-like compounds (*eg* Miller, 1963; Letham, 1967; Letham, 1978). Miller (1956, 1958) and Scott and Liverman (1956) found similarities in the effects of kinetin and red light on certain plant responses. Both kinetin and red light were shown to promote leaf expansion. However, while the promotive effect of red light could be reversed by far-red light, that of kinetin or 6-benzylaminopurine could not be reversed by a similar exposure to far-red light (Scott and Liverman, 1956). Miller (1956) also showed that red light and kinetin both caused a reduction in the elongation of pea stem internodes and promoted lettuce seed germination (Miller, 1956, 1958). In addition, Hillman (1957) showed that kinetin could replace red light in promoting dark growth of *Lemna minor* L. Gorton *et al* (1957) showed that kinetin was active in promoting bud formation in a moss (*Tortella caespitosa*) while Richmond and Lang (1957) showed that kinetin delayed chlorophyll loss, reduced or prevented accelerated protein loss and extended the life

span of detached *Xanthium pennsylvanicum* leaves. Moreover, cell division was not observed. Mothes *et al.* (1959) noted the ability of kinetin to mobilise soluble nitrogen compounds towards areas of exogenous kinetin application. The leaf cells were not dividing.

Letham (1958) showed that both immature and mature tissues excised from apples were stimulated to grow when the medium was supplemented with coconut milk and 2,4-D. Using mature tissue, Letham showed that coconut milk could be replaced effectively by an aqueous extract of immature maize seeds or kinetin. In sterile culture Letham and Bollard (1961) showed that excision of apple seeds, which were known to yield active extracts, rendered the growth of apple fruitlet explants more dependent on the exogenous supply of a stimulant of cell division. The authors suggested that the developing fruit may depend, in part, on the developing seeds for a supply of such stimulants. However, Letham and Bollard (1961) showed that apple fruitlets still contained a cell division-promoting factor at a time when cell division had probably ceased and suggested that all cell division was not solely regulated by the presence or absence of such stimulants.

A correlation between "kinin" levels and the frequency of cell division activity in developing fruit was suggested by Goldacre and Bottomley (1959). A high level of "kinin" activity was shown in apple fruitlets at the stage of cell division: a much lower level was observed at a later stage.

Letham and Bollard (1961) further detected cell division stimulants in several more mature fruitlets (*eg* plum, peach, pear) and showed the plum factor to be more similar to coconut milk than to kinetin in its inability to satisfy the non-photosynthetic red light requirement of *Spirodela oligorrhiza* which is analogous to that of *Lemna minor* (Hillman, 1957). At a similar time, Miller (1961) showed that the factor promoting cell division in extracts from immature kernels of *Zea mays* was chemically distinct from kinetin. Water or alcohol extracts of *Zea mays* could substitute very effectively for kinetin in the growth of soybean (*Glycine max*) callus tissue. Miller (1961) applied *Zea mays* extract to a Dowex ion exchange column prepared in the acidic form. Some of the activity was not retained by the column, while a portion was retained and subsequently released by 6N ammonium hydroxide (NH₄OH). Miller suggested that the factor retained by Dowex (H⁺ form) might be a substituted purine. Further investigations led to the suggestions that the factor was an adenine derivative with substitution at the 6-amino group while all the other nitrogens in the purine ring were unsubstituted, that the maize

factor may differ from kinetin only in the nature of the substituent attached to the amino group and that this substituent was unsaturated with probably at least one hydroxyl component (Miller, 1962).

High concentrations of the maize factor were found in both kernels and cobs during the onset and development of reproductive organs (Witham and Miller, 1963). As the kernels matured the concentration of the factor decreased sharply (as previously noted by Steward and Caplin, 1952). However, unfertilised ears also contained high concentrations of the factor.

Letham (1963) suggested the use of the term "cytokinin" as a generic name for all substances which promote cell division since the term "kinin" was already in use with reference to factors causing muscle contraction.

Further correlations with cell division were suggested. In agreement with Goldacre and Bottomley (1959), Letham (1963) found that cell division-promoting activity in apple fruits was high during the period of cell division and declined considerably about the time cell division ceased; activity in plum extracts reached a maximum at the onset of cell division and declined subsequently. Bottomley *et al* (1963) detected cytokinin activity in apples, quince, pear, plum and tomato. A more detailed analysis of the activity in apple and tomato fruits showed that the distribution of activity within these fruits followed the intensity of cell division in the component tissues. However, they noted that the extracts were unpurified so that differences in assayed activity may have been due to factors other than differences in the concentration of cytokinins. Subsequently, Zwar *et al* (1963) resolved the cell division activity in apple extracts into five independent zones. The activity in coconut milk was resolved into four zones which matched four of the zones detected in the apple extracts by the tobacco stem pith culture bioassay. Zwar and Skoog (1963) found that while some of the active material extracted from pea seedlings was retained by Dowex 50 (H⁺ form) and could be eluted from the column with ammonia or 4N HCl, a portion of the active material was not retained by the resin. Differential retention of active compounds was found also by Miller (1961) when purifying extracts of *Zea mays* grains. Zwar and Skoog (1963) suggested that this retention could be used to distinguish at least two groups of cell division-promoting substances.

Nine years after the identification of kinetin, one of the active components in *Zea mays* kernels was isolated, crystallised and characterised by Letham *et al* (1964). The compound was termed zeatin. As suggested

earlier by Miller (1962) zeatin was a 6-monosubstituted purine with an unsaturated side chain containing a hydroxyl group: 6-(4-hydroxy-3-methyl-but-*trans*-2-enyl) aminopurine. Shaw and Wilson (1964) had synthesized the *trans*-isomer of zeatin which allowed Letham *et al* (1964) the unambiguous assignment of the *trans*-configuration for the geometrical isomers of zeatin. Zeatin was later synthesized by an alternative route (Cebalo and Letham, 1967; Letham *et al*, 1969) and a full account of the evidence used to determine the structure of zeatin was presented by Letham *et al* (1964).

Letham (1966b) purified and tentatively identified zeatin riboside by comparison of the UV spectra and chromatographic behaviour on paper with synthetic zeatin riboside. Letham (1966c) tentatively identified a third cytokinin as 9- β -D-ribofuranosylzeatin-5'-phosphate. Although its activity was less than that of zeatin in the carrot root tissue assay, it appeared to contribute more to the total cytokinin activity of sweet corn extracts than either zeatin or zeatin riboside although zeatin appeared to account for most of the activity in plum fruitlets (Letham, 1966a). Letham and Miller (1965) collaborated to establish unequivocally that they were isolating the same compound from *Zea mays* kernels, *ie* zeatin.

Miller (1967) also detected three and possibly a fourth cytokinin in *Zea mays*. In agreement with Letham's findings, zeatin, zeatin riboside and a phosphate derivative containing ribose were tentatively identified. Both qualitative and quantitative changes in the cytokinin components of maize kernels were monitored during maturation (Miller, 1967). Little cytokinin-like activity was detected in maize kernels three days after pollination but by the sixth day both zeatin and zeatin riboside were detected. However, by the eleventh day the activity was highest in the nucleotide region. Calculated on either a per kernel or per unit fresh weight basis, the cytokinin-like activity had fallen to a low level by 21 days after pollination, leaving mature grains with little or no activity. This was similar to the overall pattern determined previously by Steward and Caplin (1952) and Witham and Miller (1963).

Witham and Miller (1965) investigated whether their maize factor, which was identical to zeatin, behaved similarly to kinetin. Zeatin was found to substitute very effectively for kinetin in delaying the senescence of detached leaves (Richmond and Lang, 1957), and in stimulating lateral shoot growth of whole pea seedlings (Sach and Thimann, 1964; Wickson and Thimann, 1958). Zeatin also could replace kinetin in stimulating dark germination of Grand Rapids lettuce seeds which are also sensitive to red light (Miller, 1956, 1958). Far-red irradiation reversed the

effect of red light but did not inhibit germination when either kinetin or the maize factor was present. Both zeatin and kinetin inhibited pea stem elongation in the presence of auxin (Miller, 1956). The maize factor was found to be more effective than kinetin at low concentrations in the soybean callus test introduced by Miller (1961, 1962, 1963). Further differences were apparent in the tobacco pith test where kinetin alone tended to stimulate some growth, the maize factor alone was ineffective. A combination of kinetin and IAA induced growth which was mainly due to cell division whereas zeatin and IAA stimulated both cell division and cell enlargement (Witham and Miller, 1965).

Witham and Miller (1965) suggested that the maize factor may function in the accumulation of nutrients at various loci in the intact plant. Consequently, when present at a high level in a localised part of the plant as found in *Zea mays* grains after fertilisation, zeatin might enhance the preferential transport of nutrients from the vegetative region of the plant to the developing reproductive structures.

By the mid-1960's it was apparent that the cell division promoting activity detected in extracts of a number of tissues was not a single discrete compound but rather that a complex of compounds existed. The activity of zeatin had been shown to be similar, but not identical, to kinetin (Witham and Miller, 1965) and responses to zeatin in addition to cell division had been recorded (Witham and Miller, 1965). Qualitative as well as quantitative changes of cytokinins with plant development had been noted (Miller, 1967) and zeatin had been shown to interact synergistically with another naturally occurring plant growth regulator, the auxin, IAA (Witham and Miller, 1965).

To be classified as a cytokinin, a compound must "in the presence of optimal auxin, induce cell division in tobacco pith and similar tissue cultures (*eg* soybean callus, carrot secondary phloem); in its other activities a cytokinin also resembles kinetin, the first known cytokinin" (Letham, 1978). The definition of a cytokinin is therefore more a physiological one (Horgan, 1978a) based on the ability of a compound to evoke a certain response in plant tissues under defined culture conditions rather than one in which classification is in terms of chemical structure. The above definition (Letham, 1978) recognises the fact that cytokinins do elicit responses in plant tissue in addition to cell division.

Following the identification of zeatin (Letham *et al.*, 1964, 1967), it became evident that the plant cytokinins were a group of structurally related compounds. So far all the naturally occurring cytokinins isolated have been shown to be N⁶-substituted adenines (van Staden and

Davey, 1979) in accordance with the premise by Skoog and Armstrong (1970) that "the structural requirements for high order cytokinin activity include an intact adenine moiety with an N⁶-substituent of moderate molecular length".

Coconut milk has been shown to contain zeatin riboside which was characterised unequivocally by Letham (1974), zeatin which was identified by mass spectrometry by van Staden and Drewes (1975) and a zeatin glucoside (van Staden, 1976a). Van Staden suggested that the glycon was glucose and that the glucoside may be attached at position 3 of the purine ring. This assignment has been queried by Wang *et al* (1977) who suggested that the reported activity of the glucoside was indicative of the glucoside moiety being attached to the isopentenyl side chain, while Letham (1978) suggested that a characteristic ion (*m/z* 160) for zeatin was absent from the spectrum reported by van Staden (1976a). The isolation of 1,3-diphenylurea from coconut milk proved to be an artifact of the extraction process (Shantz and Steward, 1955).

Cytokinin-like activity has been detected in a wide range of plant material by bioassay methods (see detailed reviews by Letham, 1978; van Staden and Davey, 1979). Letham (1978) discusses the sensitivity and assay times of 16 bioassays which are based on the ability of cytokinins to elicit a variety of responses depending on the type of tissue used. Cytokinin bioassays will be discussed in Chapter 2.

Cytokinins occur in plant tissues not only as "free forms" soluble for example in 80% ethanol, but as forms bound to transfer RNA (see reviews by Burrows, 1975; Horgan, 1978; Laloue, 1978; Letham, 1978). The unequivocal identification of free (as distinct from tRNA-bound) endogenous cytokinins are relatively few. Letham (1978) describes and lists all unequivocally identified cytokinins known at the time of publication. Since then further cytokinins have been characterised, several indirectly as a result of metabolic studies.

Parker *et al* (1972) and Parker and Letham (1973) partially characterised a metabolite of zeatin as the 7-glucoside. The compound, assigned the trivial name "raphanatin" was a stable metabolite of [³H]-zeatin which was supplied to de-rooted radish seedlings. The analogous 7-glucoside of 6-benzylaminopurine (6-Bap) was formed when [³H]-6-BAP was similarly supplied through the transpiration stream to de-rooted radish seedlings (Parker *et al*, 1973). In this report, the 9-glucoside was also detected and unequivocally identified as 6-benzylamino-9-β-D-glucopyranosylpurine. The 7- and 9-glucosides were also detected as metabolites of exogenously supplied [³H]-6-BAP to excised immature radish

cotyledons. In addition, a third highly active but minor metabolite (Metabolite C) was detected but not characterised (Wilson *et al*, 1974). The complete structural assignment of raphanatin was reported as the 7- β -D-glucopyranoside of zeatin. The same configuration was assigned to the 7-glucoside of 6-BAP (Cowley *et al*, 1975; Duke *et al*, 1975; Cowley *et al*, 1978). Both assignments had to be made with reference to unambiguously synthesized compounds because of the minute quantities of metabolites isolated (Cowley *et al*, 1978).

Metabolite C from de-rooted radish seedlings was isolated and identified as the 6-benzyl-amino-3- β -D-glucopyranosylpurine (Cowley *et al*, 1975; Parker *et al*, 1975; Letham *et al*, 1975). This was the first report of a compound isolated from plant tissues in which the glycosidic linkage was at position 3 of the purine ring.

When [^3H]-zeatin was supplied to roots of intact *Zea mays* seedlings, to de-rooted seedlings and to a culture of embryonic tissue, the 9-glucoside was detected as a prominent metabolite while the 7-glucoside was of minor significance (Parker *et al*, 1973; Parker and Letham, 1974). The ring form of the sugar and configuration of the glycosidic linkage were confirmed as glucopyranose and β respectively by comparison with synthetic compounds (Cowley *et al*, 1978).

In a preliminary account of experiments in which [^3H]-zeatin was fed through the transpiration stream to de-rooted lupin seedlings, MacLeod *et al* (1975) detected 7- and 9-glycosides of zeatin, but the principal metabolites were two new compounds. One was identified unequivocally by Parker *et al* (1978) and Duke *et al* (1978) as L- β -[6,4-hydroxy-3-methyl-but-*trans*-2-enylamino-purin-9-yl] alanine and assigned the trivial name lupinic acid by MacLeod *et al* (1975). This compound is regarded as the first from a natural source in which an amino acid is conjugated to a purine ring nitrogen atom. The identification of the second compound was confirmed as O- β -D-glucopyranosyl zeatin (Parker *et al*, 1975). The O-glucoside was markedly more active in the radish cotyledon assay than either the 9-glucoside or lupinic acid.

In equivalent experiments with [^3H]-6-BAP and de-rooted *Phaseolus vulgaris* plants L- β -[6-benzylaminopurin-9-yl] alanine, *ie* the 9-alanine conjugate of 6-BAP, was identified as the principal metabolite. Tissues of different ages were investigated and in all tissues the alanine conjugate was detected but the 3-, 7- and 9-glucosides of 6-BAP did not contribute appreciably to the radioactivity of the bean extract (Letham *et al*, 1979).

Letham *et al* (1977) noted the presence of the 4-glucosyloxy moiety

on the isopentenyl side chain of several metabolites of exogenously supplied zeatin, or zeatin riboside in mature leaves of two poplar species. In this and a subsequent paper (Duke *et al*, 1979) the structures of the following compounds were characterised:

- O*- β -D-glucopyranosyl zeatin (OGZ),
- O*- β -D-glucopyranosyl-9- β -D-ribofuranosyl zeatin (OGZR),
- O*- β -D-glycopyranosyl dihydro zeatin (OGDZ), and
- O*- β -D-glucopyranosyl-9- β -D-ribofuranosyl dihydro zeatin (OGDZR).

The compounds were characterised by comparison with unambiguously synthesized compounds. The presence of *cis*-zeatin-*O*-glucoside was attributed to the presence of *cis*-zeatin in the commercial sample of zeatin.

It became apparent that exogenously supplied zeatin and zeatin riboside could be metabolised to a variety of other cytokinins including glucosides, some of which had not been identified as endogenous cytokinins. With the ability to synthesize these metabolites as well as their deuterated derivatives, Summons *et al* (1977) successfully introduced the technique of combined gas chromatography-mass spectrometry (GCMS) using deuterium-labelled internal standards to assist in identifying and to quantify endogenous phytohormones. Raphanatin (7- β -D-glucopyranosyl zeatin) was subsequently identified as an endogenous cytokinin in radish seed (Summons *et al*, 1977).

In addition to quantifying zeatin riboside and zeatin Summons *et al* (1979a) reported the first identification and quantification of zeatin-9- β -D-glucopyranosyl from immature grains of *Zea mays*. Scott *et al* (1980b) subsequently identified this cytokinin in crown gall tissue.

After the *O*-glucosides were identified as metabolites of exogenously supplied [³H]-zeatin or [³H]-zeatin riboside (Parker *et al*, 1975; Letham *et al*, 1977), Morris (1977) identified OGZ and OGZR from *Vinacea rosea* crown gall tissue. Wang *et al* (1977) identified OGDZ in extracts from decapitated *Phaseolus vulgaris* plants. The dihydro derivative of zeatin riboside was identified as a minor component in leaves of the same plant by Wang and Horgan (1978).

Using penta-deuterium labelled internal standards Summons (1979b) accurately determined the amounts of OGZ, OGDZ, OGZR and OGDZR from pod walls and seeds of lupin. ZR was only detected in the seeds. DZR was identified in both pod walls and seed by GCMS. In addition, the other cytokinins quantified were unequivocally identified by complete mass spectral analysis.

All of the metabolites identified by Letham and co-workers following exogenous application of [³H]-zeatin have now been identified

unequivocally as endogenous cytokinins. This includes a recent report of the identification of lupinic acid in lupin pods and of dihydrolupinic acid in *Lupinus luteus* root nodules by Summons *et al* (1981). However, the 3-glucoside of zeatin has not been reported as either a metabolite of exogenously fed zeatin nor as an endogenous cytokinin in contrast to its detection as a metabolite of [³H]-6-BAP (Cowley *et al*, 1975; Parker *et al*, 1975; Letham *et al*, 1975).

Conventional GCMS and comparison with an authentic compound enabled Watanabe *et al* (1978b) to identify isopentenyladenosine (iPA). They claim this to be the first unequivocal evidence of iPA in intact higher plants, *ie* the young shoots of hop plants (*Humulus lupulus* L). Previously iPA had been detected as a free nucleoside in an autonomous strain of tobacco tissue (Dyson and Hall, 1972). Letham (1978) suggested that identification of iPA from cotton ovules was achieved by Shindy and Smith (1975) but although gas chromatography retention times and quantification of the trimethylsilyl-derivatives of iPA were reported, the identity of iPA was not authenticated by their combined GCMS system.

Hashizume *et al* (1979) independently developed the technique of quantifying endogenous cytokinins by mass spectroscopy and stable isotope dilution. Measurable quantities of zeatin riboside and N⁶-isopentenyl-2-methylthioadenosine (msiPA) were detected in extracts of cabbage hearts. Although a full mass spectral analysis was not presented, this was the first indication of the presence of msiPA as a free endogenous cytokinin. Previously it had been found only as a constituent of the tRNA of plants and bacteria (Letham, 1978).

Recently Chaves das Neves and Pais (1980) reported the presence in *Zantedeschaea aethiopica* of the unusual cytokinin *o*-hydroxy-benzyladenosine originally identified from *Populus robusta* leaves by Horgan *et al* (1973). In addition Chaves das Neves and Pais (1980) identified 6-(*o*-hydroxybenzylamino)-2-methylthio-9-β-D-glucofuranosyl purine in the same fruit.

The identification of cytokinins with the *cis*-configuration of the isopentenyl side chain was, until recently, confined to cytokinins bound to tRNA species (*eg* Horgan, 1978; Letham, 1978), and to those produced by bacterial cultures of *Cornyebacterium fascians* (*eg* Scarbrough *et al*, 1973) and *Agrobacterium tumefaciens* (*eg* Chapman *et al*, 1976).

The first report of *cis*-zeatin riboside as a free endogenous cytokinin in plant tissues was by Dauphin *et al* (1977, 1979). The amount of *cis*-zeatin riboside present was smaller than that of zeatin riboside (Dauphin *et al*, 1979), whereas Mauk and Langille (1978) assigned

the *cis* configuration to all the zeatin riboside isolated from potato tissue and identified by GCMS. The *cis* configuration was assigned on the basis of HPLC retention times. Further, Watanabe *et al* (1978c) reported the occurrence of *cis*-zeatin riboside as a free nucleoside in cones of the hop plant and have subsequently reported the presence of not only *cis*- and *trans*-zeatin riboside but also *cis*- and *trans*-zeatin in the tops of the hop plant. This is the first report of the presence of free *cis*-zeatin in plant tissues. Yokota *et al* (1981) reported the presence of both *cis*- and *trans*-zeatin riboside in extracts of *Dilochos lablab* seeds. Hashizume *et al* (1978) reported the presence of *cis*-zeatin riboside in the tops of tobacco plants while McCloskey *et al* (1979), employing the stable isotope dilution technique, detected approximately equal amounts of *cis*- and *trans*-zeatin riboside in bamboo shoots.

The presence of *cis*-zeatin riboside as a free endogenous cytokinin has been reported by four independent laboratories and *cis*-zeatin by one laboratory. It is likely, therefore, that both cytokinins are part of the compliment of free endogenous cytokinins.

There are only two reports of the identification of cytokinins in potato tissues. The presence of *cis*-zeatin riboside was reported in extract from above-ground tissues of the potato plant (Mauk and Langille, 1978) and Arteca *et al* (1980) provided unequivocal evidence by HPLC and GCMS for the presence of zeatin and zeatin riboside in purified potato leaf extracts.

However, much of the early literature can be interpreted now as indicating the presence of endogenous cytokinins in potato tissues and isolated potato tuber tissue has been shown by numerous workers to be responsive to and able to metabolise applied cytokinins (*eg* Steward and Caplin, 1951; Chapman, 1955; Shantz *et al*, 1955; Okazawa, 1968; Deleuze *et al*, 1972; Fox *et al*, 1973; MacLeod *et al*, 1976; Letham *et al*, 1979). In retrospect it is apparent that the response of potato tuber tissue to cytokinin was observed by Haberlandt (1913; cited by Das *et al*, 1956) in experiments in which a substance derived from the phloem (and now presumed to be cytokinin) was shown to diffuse through a thin film of agar and induce cell division in the parenchymatous tissue of potato tuber. He also demonstrated that an application of crushed cells promoted cell division (Haberlandt, 1914, 1921, cited by Das *et al*, 1956).

Cytokinin-like activity was extracted from potato sprouts grown in darkness. Lingappa (1957) found 2% juice of such sprouts could replace coconut milk in inducing undifferentiated callus growth in potato tissue

on a basal media supplemented with NAA and adenine.

There are innumerable examples of the detection of "cytokinin-like" activity from potato tissues following the bioassay of potato extracts. Cytokinin-like activity has been detected from potato tuber tissue (*eg* Okazawa, 1969, 1970; Koda and Okazawa, 1977; Engelbrecht and Bielińska-Czarnecka, 1972; Antis and Northcote, 1975; van Staden, 1976c). Langille and Forsline (1974) and Forsline and Langille (1975) reported the presence of cytokinin-like activity in both above- and below-ground portions of potato plants. Van Staden and Dimalla (1976, 1977a) extracted potato material exhibiting the "little potato" disorder and showed that parent tubers, internodal sprouts, stolon tips and little potatoes all contained cytokinin-like activity as did various tissues removed from potato plants grown in water culture under differing nitrogen regimes (Sattelmacher and Marschner, 1978a,b,c).

The cytokinin-like activity has been resolved into components exhibiting chromatographic properties similar to zeatin ribotide, zeatin glucoside, zeatin riboside, zeatin, isopentenyladenine, isopentenyladenosine and possibly the ribotide of isopentenyladenosine (*eg* van Staden, 1976c; van Staden and Dimalla, 1977a; Koda and Okazawa, 1977).

A number of independent groups have suggested that the cytokinins are closely involved in the tuberisation process. Courduroux (1966, cited by Palmer and Smith, 1970) suggested that the cytokinins may be the stimulus initiating tuberisation. Furthermore, Mauk and Langille (1978) considered that zeatin riboside might indeed be the "tuber forming stimulus" suggested by Courduroux (1966). Palmer and Smith (1969a) suggested that the cytokinins may be mobilising metabolites to the loci of tuber formation by creating a metabolic sink while Palmer and Smith (1969b, 1970) and Smith and Palmer (1970) considered that tuber formation stimulated *in vitro* by kinetin was at least partially a consequence of the stimulating effect of kinetin on starch synthesizing enzymes.

There are three schools of thought concerning tuber initiation. One implicates the movement of a specific tuberisation stimulus to the site of tuber formation. A second concerns the nutrient balance within the whole plant, tuber formation resulting from the accumulation of substrates in the stolon apices. The third school implicates hormonal interactions, tuber initiation occurring only when a certain critical balance is attained.

Garner and Allard (1923) considered tuberisation to be one of the most outstanding features of photoperiodism (a term suggested to them by

a Mr O.F. Cooke and which was described as the response of the plant to the relative length of the day and night). Zimmerman and Hitchcock (1936) attempted to localise the mechanism which regulated tuberisation in plants. Working with *Helianthus tuberosus* the authors suggested that the growing stem tips rather than the entire plant regulated rhizome and tuber development and that the regulators were probably "chemical agents of a hormone-like nature manufactured in the stem tips and sent to other parts of the plant where they exerted a controlling influence on development". Hamner and Long (1939), also working with *H. tuberosus* suggested, in contrast to Zimmerman and Hitchcock that the stem tip was not the locus of the photoperiodic perception but that it was the leaves (even single leaves) which were responsive to short day lengths. Grafting experiments, however, did support Zimmerman and Hitchcock's suggestion that tuberisation may be controlled by a substance with hormone-like properties.

Ito and Kato (1951) conducted experiments with potato sprouts and found that tubers formed at the base of an inverted sprout although the upright controls did not tuberise within the 14 days of the experiment. The authors suggested that tuberisation was due to the downward translocation of a certain substance and that tuber growth occurred under conditions of decreased auxin supply, the "assumptive substance" being intimately related with auxin and possibly being a precursor. Mes and Menge (1954) however, noted that in tissue culture tubers sometimes formed at the tips of upright stems which was not in agreement with the hypothesis that the "stimulus" was translocated in a downward direction.

It was Gregory (1956) who showed that the inducing stimulus controlling tuber formation in cv Kennebec was also a graft transmissible factor. Gregory suggested that plants grown under conditions favourable to tuber formation be considered to be in the induced state while plants grown under conditions leading to complete absence of tubers be considered to be in the non-induced state. Gregory found that cuttings from apical segments of plants which had been grown under inducing conditions tuberised more rapidly than those from sub-apical segments, whereas cuttings from non-induced plants maintained under non-inducing conditions did not tuberise. In tuberising cuttings the basal axillary bud of the cutting first developed a tuber, leading Gregory to suggest that the movement of the stimulus was predominantly basipetal. The induced state appeared to persist for some time even under non-inducing conditions and, although it was not of a permanent nature in whole plants, induction appeared irreversible in cuttings.

In sterile culture, the presence of sucrose was required before induced axillary buds formed tubers, but sucrose did not stimulate tuber formation in non-induced buds and these subsequently grew out as shoots. However, if the induced buds were then transferred onto fresh media the tubers did not resume growth suggesting to Gregory that some factor other than sugars and inorganic nutrients became depleted in the stem piece.

Chapman (1958) suggested that a continuous supply of the tuber-inducing stimulus was necessary to prevent the tubers developing stolons and reverting to a vegetative condition. Chapman (1955) noted that nodes taken from induced stems and grown in sterile culture would form tubers which later grew out forming a vegetative shoot. This was interpreted as indicative of the loss of the tuber-inducing stimulus.

Madec (1963), in an important experiment which does not appear to have been repeated, injected sap from leaves and stems of induced plants of the cultivar Bintje into non-induced plants of the cultivar Ackersegen in the absence of the mother tuber. Tuberisation was hastened by one month compared to the controls which were non-induced plants injected with non-induced sap. Chapman (1958) had earlier failed to stimulate tuber formation at non-induced nodes by incorporation of extract from induced tubers into the culture medium. Madec found that the inducing substance was not inactivated by boiling for five minutes but it was also suggested that the results did not prove that the substance was absent from the sap but simply that it may be present in a concentration too low to initiate tuber formation.

Madec suggested that there were two phenomena involved: the first required the presence of a substance, or a complex of several substances, perhaps of hormonal nature which could be synthesized under certain environmental conditions by the foliage and by the mother tuber of the potato, and the second involved the enlargement of the tuber which required a supply of less specific metabolites, primarily carbohydrates. Enlargement then continued as long as induction was sustained (*eg* Chapman, 1958; Gregory, 1956).

Further experiments supporting the concept of a positive stimulus to tuber formation are reviewed by Madec (1963), Cutter (1978) and Moorby (1978). More recently Chailakhyan *et al* (1980) investigated the nature of the photoperiodic stimulus of tuber formation by conducting experiments with intergeneric graftings of tobacco onto *S. andigena* potato plants. It was noted that control graftings of potato onto potato maintained under short days (SD) developed tubers while those

maintained under long days (LD) did not. Tobacco grafted onto potato stock and maintained under SD induced the stock to form tubers while those plants maintained under LD did not. The authors concluded that a photoperiodic stimulus capable of inducing tuber formation in potato stocks arose in the leaves of the tobacco plant under SD.

Ewing and Wareing (1978) presented further work supporting the existence of a "tuberisation stimulus". Initially they selected seedlings with differing critical photoperiods (CPP). A two-nodal cutting was taken from a clone with a very long critical photoperiod (greater than 20 hours) and similarly from a clone with a CPP less than 13 hours. All plants from which cuttings were excised had been grown under 20 hour photoperiods. The lower leaf of each cutting was excised and reciprocal grafts made between the two clones. The grafted cuttings were then kept under a 20-hour photoperiod. When the leaf (scion) was from the long CPP clone, tubers formed at the underground bud of the stock. The reciprocal graft produced no tubers, showing that the long CPP is a property of the leaf, not of the receptor bud. Whatever its origin, Ewing and Wareing (1978) considered that the stimulus is present in all mature leaves of an induced plant. Ewing (1976) considered that the basis for long critical photoperiods resided in the ability of the leaf to make sufficient "tuberisation stimulus" under LD to induce tubers, not in the ability of the receptor bud or stolon tip to respond to a low level of stimulus. He considered also that there was no evidence that the tuberisation stimulus moved towards gravity or that the expression of the stimulus was directional.

A tendency for inducing conditions to shift growth from above-ground to below-ground buds could be observed earlier than tuberisation itself. If tubers did not form, often the remaining buds grew out as leafy shoots or stolons (Ewing and Wareing, 1978), that is they were not sufficiently induced to form tubers (Ewing, 1976; Ewing, 1981). A tendency for the stimulus to move most strongly to the underground bud most distant from a leaf was noted (Kahn and Ewing, unpublished; cited by Ewing, 1981).

The more recent work cited above supports the early literature which indicated that a "tuberisation stimulus" does exist and which is transmitted from its site of synthesis to its site of action.

Ewing (1981) reported that regardless of the critical photoperiod, shortening the daylength at least to as little as 12 hours increased the intensity of the induction and suggested that the reduced daylength raised the apparent level of the tuberisation

stimulus.

Ewing (1981) considered that the interactions between daylength and temperature and the ability of different varieties to tuberise under different environmental conditions could be explained by the ability of a specific plant to form sufficient tuberisation stimulus under the prevailing conditions. However, Ewing (1981) emphasised that the tuberisation stimulus was not necessarily a single chemical compound and suggested that it is quite possible that the concentrations of several different substances vary in concert to produce the effects attributed to the presence or absence of the stimulus. Indeed it has been shown by Menzel and others (see Menzel, 1980, and references cited therein) that it is possible to modify the responses to photoperiod and temperature by the application of growth regulators. Menzel (1980) suggested that both environmental influences on tuberisation are mediated through hormonal control in which gibberellins and inhibitors play a part.

However, the hypothesis of a hormonally based "stimulus" is not universally accepted. Protagonists of the nutrient theory of tuberisation draw on the effect of the general environmental conditions which promote or delay tuberisation to explain tuber initiation as a result of an excess of carbohydrate over and above that required for growth and respiration.

Wellensiek (1929) noted that aerial tuber formation occurred after ringing or injury of the stem, after removal of young tubers developing in the soil and in plants infested by *Rhizoctonia solani*. He considered that in all cases the transport of food substances to the subterranean parts of the plants was disturbed. This led to an increase in concentration of food substances in the aerial portion of the plant thereby inducing tuber formation. In agreement with Vöchtung (1900, cited by Wellensiek, 1929), Wellensiek considered that all different types of tuber formation, either normal or abnormal, were induced by an increased concentration of food substances. In contrast to Oortwijn Botjes (cited by Wellensiek, 1929), Wellensiek concluded that a relative deficiency of a nitrogenous compound could not account for all instances of tuber formation. Wellensiek (1929), referring to De Vries (1878), Girard (1886-88) and Clark (1921), suggested that the early development of the haulm monopolised the products from photosynthesis. After the stem and leaves had reached a certain stage of development their further growth was checked. Assimilated substances then moved downwards through the stem where they accumulated and gave rise to the formation of tubers.

Slater (1963) considered that tuber initiation occurred earliest in those plants in which a surplus of assimilate occurred. This surplus was the trigger for tuberisation. Bushnell (1928, cited by Driver and Hawkes, 1943) considered that the rate of tuber growth was a function of the available carbohydrate. He considered that decreased yields at high temperatures were as a consequence of a reduction in carbohydrate available for translocation. The reduction was as a result of increased respiration at the higher temperature.

Werner (1934) considered tuber formation was dependent on the carbon/nitrogen ratio within the plant. He suggested that when nitrogen could not be assimilated due to either SD, low temperature or an inadequate supply of inorganic nitrogen, carbohydrate accumulated beyond the needs of the plant and tubers formed. Van Schreven (1956) noted that premature tuberisation of sprouts in darkness was correlated with an increased ratio of soluble carbohydrates to soluble nitrogenous compounds in the mother tubers and the sprouts.

In contrast to Bushnell (1928), Borah *et al* (1960) and Borah and Milthorpe (1963) considered that more assimilate was available at 25°C and more of it was used in growth but that at 25°C a higher proportion of assimilate was diverted to the haulm. These workers considered that tuberisation was promoted by conditions which led to a high concentration of soluble carbohydrate at the sites of tuber production. It was suggested that a high concentration of carbohydrate at the stolon tips could be expected to occur in plants under high radiation and in plants subjected to low night temperatures, both being conditions which promote tuberisation (*eg* Borah and Milthorpe, 1963; Slater, 1968; Frier, 1977).

Borah *et al* (1960) suggested that the "substrate" stimulus had a greater effect than the SD photoperiodic stimulus in determining tuber initiation.

Burt (1964) presented evidence showing increased concentrations of sugars in leaves and stolon tips prior to tuberisation. Plants were grown at a 20°C day/15°C night temperature under a 16 hour photoperiod for three weeks. At week four plants were exposed to low temperatures. After a 3°C exposure high soluble sugar levels occurred during that week and tuberisation occurred by the end of the fifth week. At this stage the level of soluble sugars had decreased again. After an exposure to 9°C an increase in soluble sugar was noted during that week. However, tuberisation did not occur until the end of week seven, this time concurrent with a second increase in soluble sugar level. The control plants tuberised by the eighth week, an increase in

soluble sugars being noted on the seventh. Burt (1964) considered that changes in soluble sugar levels and tuberisation were sufficiently well correlated to suggest that tuber initiation was associated with a high level of soluble sugar in the stolon tips.

Slater (1968) noted that tuber initiation was promoted by lowering the temperature of the whole plant or underground parts of the plant only, and delayed when these were at an elevated temperature. Slater considered that the lowered temperature would allow soluble sugars to accumulate at the stolon tip and thus trigger tuberisation.

Okazawa (1967) reviewed the work performed by Tagawa and Okazawa (1947-1953) concerning the metabolism of various forms of carbohydrate and nitrogenous compounds in developing tubers. Reducing sugars accumulated in the stolon tips briefly just prior to visible swelling of the tips and, simultaneously, there was a high rate of respiration. The subsequent decrease in sugar level was accompanied by the accumulation of starch and a decline in respiratory activity. Okazawa (1973) referred to a similar temporary accumulation of soluble sugar in the stolon tips prior to sprout tuber formation. However, he considered that the accumulation of sugar in the stolon tips before tuberisation was a consequence of induction and not the cause.

In vitro culture of potato stem segments by Okazawa (1955) showed the necessity for a high concentration of a sugar in the medium in order to stimulate tuber formation. Sucrose, maltose, glucose and fructose all gave similar results, acceleration of the rate of tuberisation being proportional to the increase in sucrose concentration. Tuber formation did not occur if the sugar concentration was below 6% (w/v). Tuber formation in induced stem segments was proportional to the increasing sugar concentration in the medium (Okazawa, 1967). No tuber formation occurred, however, irrespective of the sugar content of the medium, if apical tips obtained immediately after sprouting were cultured *in vitro*. Okazawa considered tuber formation *in vitro* depended as much on the physiological age of the stem tissue used as on the culture medium.

Mes and Menge (1954) also transferred stem segments into sterile culture. In many cultures no roots developed. Tuber formation was stimulated by a relatively high sucrose concentration and a relatively low (18°C) temperature. If older stem segments were cultured on 5% sucrose in the dark tuber formation occurred after several weeks in culture. However, under the same conditions young stem segments produced actively growing shoots.

Using nodes from potato sprouts Chapman (1955) observed less than

10% tuberisation after 12 weeks in culture under diffuse light at high temperatures whereas Barker (1953) had observed more rapid and more frequent tuberisation under similar conditions. However, Chapman found that if the cultures were transferred into the dark at a lower temperature over 90% of the cultures then tuberised. Varying the nitrogen level of the medium exerted no effect on the rate of tuberisation. The medium contained only 2% sucrose.

Gregory (1956) removed single-node cuttings from induced and non-induced plants. Under sterile conditions he showed that while sucrose was required from the growth of shoots and tubers it was not responsible for the actual tuber induction since cuttings from non-induced plants did not tuberise. If the tuberising cuttings were transferred to a fresh medium the tubers did not resume growth. Gregory inferred from the results that some factor other than sugars and inorganic nutrients became depleted in the stem piece. Lawrence and Barker (1963) also noted that an adequate supply of carbohydrate was insufficient in itself to induce tuber formation. Segments from etiolated sprouts were maintained for several weeks on a low sucrose medium. A subsequent transfer to a high sucrose medium did not result in tubers forming. Lawrence and Barker (1963) suggested that an unidentified factor was required to induce tuber formation and that this factor became diluted or lost during the prolonged period of culture on low carbohydrate media. They considered the factor to be present in sprouts of stored potatoes and in induced stems of growing potatoes.

From the above results it was shown that consistent tuberisation could be obtained *in vitro* if induced plant material was used. A high level of carbohydrate was required but its effect was secondary to tuber initiation. A decreased level of nitrogen did not increase tuberisation (Chapman, 1955).

Ewing (1981), however, suggested that qualitative and quantitative changes in carbohydrates, along with hormonal changes, may constitute an important part of the tuberisation stimulus.

Goodwin (1978) considers, that taken broadly, storage organ initiation (tubers, corms, bulbs *etc*) is associated with high cytokinin and low gibberellin levels and/or with application of cytokinins or dwarfing agents. Cutter (1978), Moorby (1978) and Ewing (1981) all cite references to the effect that gibberellins reduce tuberisation and that when plants are strongly induced to tuberise their levels of gibberellin-like compounds decrease concomitantly. Noteworthy, however, is the report by Moorby (1978) that the gibberellin level in the stolon tips

is still high after the initial tuber formation as can be observed also from Okazawa's (1967) results. Menzel (1981), however, suggested that high levels of gibberellins in stolon tips inhibited tuberisation.

The possibility that the cytokinins may be involved in the tuberisation process was first advanced by Courduroux (1966; cited by Palmer and Smith, 1970). He suggested that the elusive "tuberisation stimulus" may be akin to the cytokinins.

Palmer and Smith (1969a) claimed to have demonstrated a requirement for cytokinins in the tuberisation of sub-cultured stolons *in vitro*. Etiolated sprouts from potato tubers of the cultivar Norgold Russet were sterilised and grown in culture in darkness. After two weeks the axillary buds had elongated into stolons, and it was those stolons which were then transferred onto fresh media. The media contained 6% sucrose with a variable amount of cytokinin. After 30 days the cultures devoid of cytokinin showed no signs of tuber initiation while those supplied with optimum kinetin concentrations had all tuberised. It was noted that starch accumulation preceded visible signs of tuber formation. Palmer and Smith (1969a) suggested that the cytokinins may be serving to mobilise metabolites to the loci of tuber formation by creating a metabolic sink. The promotion of cell division by cytokinin activity was also suggested. Further work indicated that an adequate supply of carbohydrate was essential for kinetin-induced tuberisation: increasing the concentration of sucrose had no effect if kinetin was not supplied to the medium (Palmer and Smith, 1970). These results lend support to the suggestions that some factor other than sugars and mineral nutrients is required to induce and maintain tuberisation (*eg* Gregory, 1955; Lawrence and Barker, 1963; Ewing, 1981).

Smith and Palmer (1970) showed that more labelled kinetin accumulated in the apical 0.5 cm than in the middle subsection of the stolon cultured *in vitro*. Palmer and Smith (1970) had shown that the cultured segments required a minimum of three days exposure to kinetin containing medium in order to ensure tuberisation. Smith and Palmer (1970) suggested that tuber formation only occurred after a critical amount of kinetin had accumulated at the stolon tip. However, when the accumulated radioactivity extracted as cytokinin was chromatographed, the R_f of the cytokinin activity was less than that for kinetin. There was no major difference between cytokinin extracted from developing tubers and that extracted from stolons prior to tuber initiation with regard to the distribution of radioactivity (Smith and Palmer, 1970). However, Obata-Sasamoto and Suzuki (1979b) suggested that the kinetin metabolite

may have no cytokinin activity.

It has been shown that full induction of whole plants often requires more than a short exposure to inducing conditions (*eg* Chapman, 1958; Hammes, 1972, cited by Hammes and Nel, 1975). Palmer and Smith (1970) have indicated a similar requirement for the cytokinin stimulation of tuberisation *in vitro*. Low temperatures could not replace the requirement for cytokinin by sub-cultured stolons (Palmer and Smith, 1970).

Van Staden and Dimalla (1976) claimed that, although the occurrence of endogenous cytokinins had been reported by Engelbrecht and Bielińska-Czarnecka (1972) and by Antis and Northcote (1975), their presence during tuberisation *in vivo* had not been demonstrated. However, Okazawa (1969) had not only detected cytokinin-like activity in dormant potatoes, but had extracted cytokinins from field grown potatoes of the variety cv Irish Cobler (Okazawa, 1970). The field grown potatoes were sampled periodically over a time span of almost eight weeks. A low level of cytokinin was noted at the first harvest which was about 10 days after the stolon tips had begun to swell. A rapid increase in cytokinin activity then occurred during the next two weeks. This activity subsequently dropped rapidly to a low level and finally disappeared at maturity.

Calculated on a per tuber basis, however, the peak cytokinin level was maintained during the season but dropped at the final harvest. The rapid increase in cytokinin activity occurred just prior to a rapid increase in the fresh weight of the tubers. Okazawa (1970) suggested that there were two peaks of activity, tentatively assigning the activity of the major peak to zeatin. Okazawa (1970) suggested that the cytokinin activity may be important as a cell division-inducing factor. Tagawa and Okazawa (1949, 1952, cited by Okazawa, 1970) had shown that the beginning of starch accumulation in the newly formed daughter tubers was coincident with the onset of a rapid decline in the starch content of the mother tuber. These changes coincided with the rapid increase in cytokinin activity. Okazawa (1970) further suggested that the cytokinins may be serving as "mobilising agents" stimulating the translocation of reserve starch from the mother to the daughter tubers.

Koda and Okazawa (1977) reported the presence of at least six cytokinins in potato tubers harvested one month before full maturity. This was assumed to coincide with the peak of cytokinin-like activity detected by Okazawa (1970). Identifications of zeatin and isopentenyl-

adenine and their respective ribosides were based on chromatographic mobilities with reference to authentic standards. The presence of zeatin glucoside was suggested solely on the basis of R_f . The ribotides of zeatin and isopentenyladenine were tentatively identified after phosphatase treatment and rechromatography. The unexpected presence of nucleotide-like compounds in the butan-1-ol phase after partitioning against water at pH 8 was noted by the authors. The dominant cytokinin was considered to be zeatin riboside.

Antis and Northcote (1975) noted a qualitative change in the cytokinin content of tubers depending on their stage of maturity although different cultivars were extracted. The R_f of the cytokinin-like activity of the cultivar extracted "at the very beginning of their season" was correlated with zeatin while that of the second cultivar obtained "at the end of their season" and which were prone to bud was correlated with zeatin riboside.

If the cytokinins are intimately involved with tuber initiation, exposure of plants to inductive photoperiod and temperature might lead to qualitative and/or quantitative changes in the cytokinin activity within the plant. The influence of temperature and photoperiod on the cytokinin pools of *S. tuberosum* cv Katahdin was investigated by Langille and Forsline (1974). Sprouts were removed from the mother tubers prior to transplanting. Plants under inducing conditions (26°C day/12°C night; 8 hour photoperiod) contained significantly more cytokinin than those under non-inducing conditions (30°C day and night; 16 hour photoperiod). The total cytokinin-like activity reached a maximum in above-ground tissue four days after transfer into inducing conditions and in below-ground tissue (presumably both roots and stolons) the cytokinin-like activity peaked after six days. Non-induced tissues showed small but insignificant changes. Tuber formation occurred after eight to ten days in inducing conditions (Langille and Forsline, 1974). Four zones of cytokinin-like activity were reported in an experiment run under similar conditions (Forsline and Langille, 1975). The total cytokinin activity was again higher in tissue under inducing conditions, but the increase in activity was attributable to only two of the four cytokinins isolated.

Mauk and Langille (1978) reported the identification of zeatin riboside extracted from above-ground tissue after four days in inducing conditions. Using HPLC the *cis* configuration was assigned to zeatin riboside. Identical mass spectra of sample and standard confirmed the identification of zeatin riboside. This cytokinin was reported as the major cytokinin present in the cultivar Katahdin and was at an increased

level in induced tissues. Mauk and Langille (1978) suggested that it may account for most of the cytokinin activity detected in the earlier study. However, a closer examination of the earlier report (Forsline and Langille, 1975) indicated that the component with an R_f -value similar to that expected of zeatin riboside in the solvent system employed was at no stage significantly greater than either the more or less polar components detected.

Langille and Forsline (1974) and Forsline and Langille (1975) detected the peak of cytokinin-like activity in induced above-ground tissues two days prior to the peak in induced below-ground tissues. However, Mauk and Langille (1978) found that in plants exposed to inducing conditions for four days, zeatin riboside peaked simultaneously in both above-ground and below-ground portions of the plant. Tuberculosis was observed after eight days. Mauk and Langille (1978) considered that closer harvest times might have revealed a movement of cytokinins from tops to below-ground tissues similar to that observed previously. They speculated that the elevation in cytokinin levels within the plant marked the change from a non-induced to an induced state. Mauk and Langille considered zeatin riboside to be intimately involved in the tuberisation process and speculated that it might actually be the "tuber forming" stimulus suggested by Courduroux (1966; cited by Palmer and Smith, 1970).

Using the sub-culturing technique of Palmer and Smith (1969a), Mauk and Langille showed that zeatin riboside could promote tuberisation of non-induced potato rhizomes *in vitro*.

In a complimentary *in vitro* study Forsline and Langille (1976) investigated the effect of kinetin on tuberisation of apical, medial and basal nodal segments taken from plants grown under either inducing (low night temperature and SD) or non-inducing (high night temperature and LD) conditions. As noted by Gregory (1956) and Chapman (1958) nodal stem segments from induced plants tuberised more readily than nodal segments from non-induced plants. In agreement with Palmer and Smith (1969a, 1970) the addition of kinetin to the culture medium increased the percentage of tuberisation of non-induced segments, but the addition of kinetin did not increase the percentage tuberisation of induced segments. Forsline and Langille (1976) claimed that the addition of kinetin to the medium eliminated the effect of induction. Induced apical nodal segments tuberised more readily than those from non-induced plants or those from medial or basal segments, but the addition of kinetin to the medium eliminated this difference (Forsline and Langille,

1976). Chapman (1958) and Okazawa and Chapman (1962) considered that the tuberisation stimulus was synthesized largely at active above-ground growing points. The increased tuberisation of induced nodal segments detected by Forsline and Langille supports this idea. The elongation of axillary buds was greater in non-induced segments but the presence of kinetin in the medium reduced the percentage elongation in non-induced segments to that of induced segments. Forsline and Langille (1976) found that neither the percentage tuberisation nor elongation of nodal stem segments cultured *in vitro* was significantly influenced by the number of days plants were grown under inducing conditions. Gregory (1955) suggested that once induced, stem segments tended to remain induced.

Mingo-Castel *et al* (1976b) distinguished between the use of sprout sections and the use of sub-cultured stolons first described by Palmer and Smith (1969a). More tuberisation occurred in the sprout sections than in the sub-cultured stolons without kinetin present in the medium. Mingo-Castel *et al* (1976b) referred to this difference as the endogenous tuberisation potential. Addition of kinetin to the medium increased tuberisation in both sprout sections and sub-cultured stolons.

Van Staden (1976c) extracted cytokinin-like activity from potato tubers stored for eight months at 5°C. Sprouts which had formed on the tubers were included in the extract. Based on co-chromatography zeatin, zeatin riboside and isopentenyladenine were reported to be present. Most of the activity was associated with zeatin riboside. Many of the subsequent investigations carried out by van Staden and co-workers have used stored material.

In order to obtain newly formed tubers van Staden and Dimalla (1976) stored *S. tuberosum* cv BP-1 tubers at 5°C in the dark for nine months. Under these conditions the potatoes aged physiologically and formed tubers prematurely, a disorder known as "little potato" (van Staden and Dimalla, 1976). Material to be extracted was divided into "little potatoes", etiolated sprouts and parent tubers. Cytokinin activity was highest in the little potatoes and lowest in the parent tubers. A more detailed examination revealed the presence of cytokinins reported to co-chromatograph with zeatin, zeatin riboside and *O*- β -D-glucopyranosylzeatin (zeatin-*O*-glucoside). Zeatin riboside appeared to be the major cytokinin in all the tissues examined. Apical halves of tubers appeared to contain more cytokinin-like activity than the whole tubers per unit weight. The total cytokinin content decreased with the age of the tissue: stolon tips and little tubers weighing less than 0.3 g contained higher levels of cytokinins than little potatoes

weighing more than 3 g, although the highest total level of cytokinin occurred in the internodal sprout tissue between the parent tuber and the bulking little potatoes. Zeatin-*O*-glucoside was present in the parent tubers and stolon tips and in very high levels in the internodal sprouts (van Staden and Dimalla, 1977a).

Van Staden extended his investigations on tuberisation to include the effects of ethylene. He found that the formation of "little potatoes" was completely inhibited if the mother tubers were treated with high concentrations of Ethrel (2-chloroethylphosphonic acid, CEPA). Instead, sprouts were formed (Dimalla and van Staden, 1977).

Catchpole and Hillman (1969) reported that both ethylene and Ethrel inhibited extension growth of sprouts and associated stolons grown in the dark. Root development was inhibited and the subapical regions of stolons, stems and axillary buds became swollen. The subapical swellings on the stolons were morphologically and anatomically similar to normal tubers except that little or no starch was present. Direct application of Ethrel to stolon tips led to rapid swelling but again no starch was present (Catchpole and Hillman, 1969).

Ethrel sprays on whole plants can affect rhizome production and tuber number and yield but the effect appears to depend on the time of application, the concentration of Ethrel used, and the time of harvest (*eg* Singh, 1970; Langille, 1972; Bodlaender, 1972). Garcia-Torres and Gomez-Campo (1972) supplied Ethrel to the soil surrounding greenhouse grown plants. If Ethrel was applied to the soil at a concentration of 100 or 500 ppm just prior to tuber formation, an increase in the number of tubers per plant was noted. If Ethrel was applied to the soil just after tuber initiation the promotory effect was not as obvious. The highest concentration of Ethrel (500 ppm) reduced the total weight of tubers per plant at least in the early stages of development. Garcia-Torres and Gomez-Campo (1973), working with etiolated sprouts cultured *in vitro*, showed that tuberisation was advanced and increased when Ethrel was applied at a dose of 5×10^{-5} M. Lower doses were less effective. After three months, treated stolons were shorter and thicker and the root system was stunted. The retarding effect on 10^{-8} M GA₃ on tuberisation could be reversed by 10^{-5} and 5×10^{-5} M Ethrel concentrations but not the effect of 10^{-6} M GA₃.

Palmer and Barker (1973) using etiolated sprout segments *in vitro* reported that ethylene caused a general swelling of the entire stolon but not the localised swelling associated with tuber formation. The lateral buds emerging from the stem segments developed the diageotropic

growth habit characteristic of whole plant stolons. Controls and kinetin-treated explants maintained an upright growth habit. Kinetin-treated segments exhibited localised apical swellings with high starch contents. Little starch was detectable in CEPA-treated cultures. Reducing sugars were higher in CEPA-treated and kinetin-treated cultures compared to control cultures. A subsequent decrease in the reducing sugar content of kinetin-treated cultures coincided with the stage of rapid starch synthesis preceding visible tuber formation. Reducing sugar levels remained high in CEPA-treated cultures but were not associated with tuberisation.

Mingo-Castel *et al* (1974), using sub-cultured stolons (Palmer and Smith, 1969a), showed that ethylene (added to the flasks as the gas) had an inhibitory effect on tuberisation when it reduced the endogenous tuberisation potential from 20% to about 9%. In addition ethylene dramatically decreased the kinetin-induced tuberisation from 100% to 11%. "Hybrid-type" swellings were observed as a result of competition between kinetin and C_2H_4 (Mingo-Castel *et al*, 1976a). These showed a spherical swelling at the base characteristic of normal tuberisation, but the swelling was contained in the upper part by a thickened stolon terminated by a tightly closed hook, both characteristics being typical of C_2H_4 treatment.

Etiolated sprouts cultured *in vitro* have a higher tuberisation potential than sub-cultured stolons (Mingo-Castel *et al*, 1976b). In the presence of kinetin a 30% increase in tuberisation was noted. Mingo-Castel *et al* (1976a) found that a concentration of $5 \mu l l^{-1}$ ethylene inhibited the kinetin-promoted tuberisation.

Dimalla and van Staden (1977) and van Staden and Dimalla (1977c) found that the total endogenous cytokinin content did not differ appreciably between sprout forming mother tubers following treatment with a high concentration of Ethrel compared to untreated mother tubers with associated "little potatoes". However, the cytokinin-like activity of Ethrel-stimulated sprouts was lower than that of the "little potatoes" while the cytokinin activity of the Ethrel-treated mother tubers was slightly higher. No attempt was made to detect zeatin glucoside (Dimalla and van Staden, 1977). The sprouts which developed as a result of the Ethrel treatment had a higher gibberellin content than the control "little potatoes". Langille (1969) treated tubers exhibiting the "little potato" disorder with various growth regulators. CEPA alone had no effect but in combination with GA_3 vigorous top growth was produced.

Ultrastructural studies by Dimalla and van Staden (1977) and

van Staden and Dimalla (1977c) led them to suggest that while some starch was present in Ethrel-treated tissues, cell-division in the procambial region was inhibited.

When tubers predisposed to the "little potato" disorder were treated with a low concentration of Ethrel, tuberisation was incomplete (van Staden and Dimalla, 1977c). The interference resulted in the formation of morphologically incomplete tubers, referred to as "hybrid swellings" (Mingo-Castel *et al*, 1976a). Hybrid swellings contained less cytokinin than "little potatoes". In the control "little potatoes" the peak co-eluting with zeatin riboside was responsible for most of the cytokinin activity. In the "hybrid swellings" most of the cytokinin activity co-chromatographed with zeatin-glucoside (van Staden and Dimalla, 1977c).

Dimalla *et al* (1977) compared the endogenous hormone levels of sprouts (obtained from one-month old tubers maintained in the dark at room temperature for three weeks) and stolons (collected from tubers stored in the dark at 8°C for 10 months) prior to their forming "little potatoes". The cytokinin levels of the sprouts and stolons were found to be similar while the auxin level of the stolons was twice that of the sprouts. The gibberellin content of the sprouts was higher than that of the stolons whereas the opposite was true for the inhibitor content. Both the free and bound inhibitors were reported to co-chromatograph with ABA (Dimalla *et al*, 1977).

Palmer and Smith (1969b) investigated the interaction between ABA and kinetin on tuberisation of stolons obtained from etiolated sprout segments. In the presence of kinetin, increasing concentrations of abscisic acid (ABA) decreased or inhibited stolon elongation and tuber initiation and decreased the size of any tubers initiated. When stolons were incubated on a medium containing kinetin and later transferred to one containing ABA with or without kinetin, the inhibitory effect of ABA decreased appreciably as the time of incubation on kinetin increased, *ie* ABA was shown to be capable of inhibiting kinetin-induced tuber initiation only during the early stages of incubation (Palmer and Smith, 1969).

Carbon dioxide (CO₂) has also been noted to have stimulatory effects on tuberisation (Paterson, 1970). Significantly more tubers were formed on plants whose root systems had been exposed to CO₂. High levels of CO₂ in the internal atmosphere of potato tubers were accompanied by physiologically active levels of ethylene (Paterson, 1975). When all stolons were removed from six weeks old plants and the root

systems fumigated with over 50% CO₂ for 12 hours, tuber numbers were significantly increased but so also was the development of vegetative stolons four weeks after treatment.

Mingo-Castel *et al* (1974), experimenting with sub-cultured potato stolons, showed that CO₂ also could promote tuberisation *in vitro*. The stimulating effect of a range of CO₂ concentrations (1.25% to 20%) was the same (Mingo-Castel *et al*, 1976a). The CO₂-promoted tubers appeared morphologically similar to the control tubers. An antagonism between ethylene and CO₂ was noted, C₂H₄ decreasing the observed CO₂ promotion of tuberisation in sub-cultured stolons. In the presence of kinetin, which promoted 100% tuberisation, CO₂ alone slightly decreased this level, while ethylene alone reduced tuberisation to below the "endogenous potential" level. CO₂ counteracted the effect of ethylene more successfully in the presence than the absence of kinetin (Mingo-Castel *et al*, 1974).

Mingo-Castel *et al* (1976a) further investigated the effect of CO₂ on tuberisation, by culturing sprout sections *in vitro* whose endogenous tuberisation potential was 83% after 30 days in culture. In the total absence of CO₂ and C₂H₄ tuberisation was just over 50% and this was increased 25% in the presence of CO₂ and decreased by 34% in the presence of C₂H₄. In contrast to its effect on *in vitro* sub-cultured stolons, CO₂ could not even partially overcome the inhibition by ethylene in cultured sprout sections. Three to five days exposure to CO₂ was all that was required to promote tuberisation to the same level as 30 days constant exposure. Mingo-Castel *et al* (1974, 1976a) concluded that if CO₂ was involved then sufficient was present in the open control and addition of gaseous CO₂ did not increase the percent tuberisation. However, the absence of CO₂ was detrimental.

Arteca *et al* (1979, 1980) applied CO₂ to the root zones of potato plants grown in water culture. Four days after the CO₂ enrichment of the root zones, Arteca *et al* (1980) detected a decrease in the zeatin content of the root tissues with respect to the control at day 0. After six days the decrease was still apparent. In the leaves there was no significant change in zeatin. Zeatin riboside, however, not only decreased significantly in the roots but there was a corresponding increase in the leaves. Up to six days the roots and leaves showed reciprocal trends. HPLC was employed to separate various plant growth regulators from the potato. A comparison of the mass spectra obtained following GCMS of the sample and corresponding authentic standard established zeatin riboside and zeatin as components of potato leaves. IAA levels

were found to be still increasing in the leaves after six days while those in the roots were constant after a slight increase at two days. ABA levels were virtually the same as the controls except for a small peak shortly after CO₂ treatment.

Arteca *et al* (1980) concluded that CO₂ applied to the root zone modified the hormone levels within the plant. They suggested that the changes may influence the increase in dry matter content while the initial changes may be responsible for the changes in the tuberisation response noted by Arteca *et al* (1979).

Stalknecht (1972) found that coumarin could stimulate tuberisation of sub-cultured stolons *in vitro*. It was also shown that coumarin-stimulated tuberisation occurred earlier and the tubers formed were larger than kinetin-stimulated tuberisation. Stalknecht and Farnsworth (1979) subsequently reported that the relative level of nitrogen and carbohydrate in the medium can influence the affect plant growth regulators may have on stimulating tuber formation *in vitro*. The authors suggested that for critical *in vitro* studies on tuberisation there must be a proper balance between the nitrogen stimulated growth of the axillary shoot and an adequate supply of carbohydrate, in addition to the presence of a tuberisation stimulus such as coumarin.

The influence of nitrogen nutrition on tuber initiation and growth of field grown plants is summarised by Harris (1978). High nitrogen levels in the soil generally tend to delay the apparent date of tuber initiation (*eg* Ivins and Bremner, 1965).

Krauss and Marschner (1971) demonstrated that uninterrupted root growth was an important factor in determining the time of tuber initiation. Plants provided with a constant nitrogen supply (in the form of nitrate) and grown in containers large enough not to limit root growth did not initiate tubers. If a smaller vessel was used under conditions of comparable nitrogen supply, tuber initiation occurred after 45 days of growth. In the large vessel withdrawal of nitrogen promoted tuber initiation.

Application of kinetin or gibberellic acid to the stolon tips reduced the growth rate of stolons but neither growth regulator promoted tuber initiation. Abscisic acid had no effect on the growth of intact stolons (Krauss and Marschner, 1971).

Krauss and Marschner (1976) showed that a continuous supply of ammonia had the same effect as nitrate, completely inhibiting tuber initiation in two *S. tuberosum* cultivars grown in water culture. In *S. andigena* plants grown in water culture under short day conditions

a continuous nitrogen supply delayed but did not prevent tuber initiation.

Marschner and co-workers subsequently examined the effect of nitrogen on tuber initiation and the endogenous cytokinin levels in *S. tuberosum* potato plants grown in water culture.

Sattelmacher and Marschner (1978b) investigated the changes in the endogenous cytokinin level in roots, shoots and exudate of potato plants during the period of tuberisation induced by an interruption in the nitrogen supply. Plants were grown from "physiologically old" tubers to obtain more rapid and uniform tuberisation. They found that prior to tuberisation the ratio of the cytokinin activity in the roots and shoots was in favour of the roots. Upon nitrogen withdrawal there was an increase in cytokinin activity in both roots and shoots but with the onset of tuber growth the ratio changed in favour of the shoots. The increase in shoots, however, was attributed to a shift in the cytokinin activity from the water-soluble fraction in favour of the butanol-soluble fraction. On resupplying nitrogen to the medium "regrowth" of tubers occurred and the cytokinin ratio returned in favour of the roots. Sattelmacher and Marschner (1978a) showed that when physiologically young parent tubers were used, tuberisation was not induced by nitrogen withdrawal but an increase in the cytokinin activity of the roots was still observed. Sattelmacher and Marschner used the combined results to suggest that tuberisation in the potato plants was not related to specific changes in the cytokinin activity of the roots. In addition, the steep increase in the cytokinin activity in the shoot during nitrogen withdrawal and during the time tuberisation took place, did not occur in plants grown from "physiologically young" tubers when nitrogen was withdrawn (Sattelmacher and Marschner, 1978a). However, the aqueous phase was discarded in the latter experiment. It was suggested that the increase in cytokinin activity was not the result of nitrogen withdrawal but was casually connected with tuberisation which was induced by the withdrawal of nitrogen. The increase in cytokinin activity may be related to the increased photosynthetic activity in the leaf ("source") in order to meet the increased assimilate requirement of the growing tuber "sink" (Sattelmacher and Marschner, 1978b).

In further water culture experiments the changes in cytokinin activity in stolon tips and newly formed tubers were studied (Sattelmacher and Marschner, 1978c). Only the butanol-soluble cytokinins were investigated (*ie* those remaining after partitioning of the aqueous extracts against butan-1-ol at pH 7). Tuberisation was again induced by withdrawing nitrogen from the nutrient solution. Coincident with the increase in the

number of induced stolons and newly formed tubers, a temporary and steep increase in the cytokinin activity per unit fresh weight occurred, followed by a decrease. However, a dilution effect was apparent as the cytokinin activity per organ continued to increase over the duration of the experiment. Resupplying nitrogen at a low concentration did not cause regrowth but led to an increase in tuber weight combined with a steep increase in the cytokinin activity per tuber. A shift in the cytokinin spectrum was also noted upon tuberisation although this shift was reversible if regrowth of the tubers was induced by resupplying a high concentration of nitrogen. The peak of cytokinin activity in tubers 4 to 6 mm diameter was attributed to zeatin riboside activity. Sattelmacher and Marschner (1978c) claim that the cytokinins play an important role in tuberisation although they do not consider them to be directly responsible for the onset of tuberisation.

Tizio and Biaiñ (1973) queried the claim made by Palmer and Smith (1969a, b) that the cytokinins may be intimately involved with the process of tuberisation and that the tuber-forming stimulus may be related to the naturally occurring cytokinins (Smith and Palmer, 1970).

Tizio and Biaiñ (1973) cultured pieces of elongating tuber buds each with two to three axillary buds in an *in vitro* system. At concentrations of kinetin, 6-benzylaminopurine and NAA which stimulated "rhizogenesis" but which did not affect the normal growth of roots *in vitro*, tuber formation was delayed. At higher concentrations which partially or completely inhibited root formation and normal growth, tuber formation occurred 12 to 14 days before the controls. Tuber formation was also stimulated by CCC and in this situation root growth was apparent, although details were not given.

Stimulation of tuber formation by CCC occurred without concomitant increase in cytokinin activity (Tizio and Biaiñ, 1973) whereas the induction of tuber formation by NAA and the growth of tubers was coincident with a high level of cytokinin activity. Tizio and Biaiñ claim, from the above results, that the cytokinins may not be considered the specific factor controlling tuberisation. Tizio and Biaiñ (1973) considered that the reported stimulation of tuber formation by kinetin (*eg* Palmer and Smith, 1969a) was a direct consequence of the inhibition of root growth caused by the high cytokinin concentration in the medium. Tizio and Biaiñ (1973) consider that the roots synthesize a factor which delays tuberisation and that the intensity of *in vitro* tuberisation is directly correlated with the intensity of root growth inhibition. However, Mingo-Castel *et al* (1974) found no correlation between rooting

and tuberisation in experiments in which interactions among CO₂, ethylene and kinetin on tuber formation were being observed.

It appears that changes in cytokinin-like activity may be correlated with the change in the plant from a non-induced condition to an induced condition. Langille and co-workers noted a temporary increase in shoots following the transfer of plants into inductive conditions. The increase occurred either simultaneously with (Mauk and Langille, 1978) or prior to (Langille and Forsline, 1974), an increase in the cytokinin content of below-ground portions of the plant in advance of tuberisation. However, transfer of plants into short day conditions has been associated with an increased cytokinin level in plants other than *Solanum* species (see references cited by Letham, 1978, Table 4.5). A causal relationship between changes in cytokinin levels and induction or tuber formation must be regarded with caution especially in view of the transient nature of the elevated cytokinin levels.

Decreased cytokinin levels are usually noted in plants subjected to nitrogen deficiency (*eg* Wagner and Michael, 1969 and 1971; Göring and Mardanov, 1976; Salama and Wareing, 1979; Horgan and Wareing, 1980). However, Sattelmacher and Marschner (1978b) noted a temporary increase in the butan-1-ol-soluble cytokinins in the shoots of potato plants following nitrogen withdrawal and during the associated tuber initiation. Sattelmacher and Marschner (1978c) claim that the cytokinins play an important role in tuberisation although they do not consider them to be directly responsible for the onset of tuberisation, whereas Mauk and Langille (1978) consider zeatin riboside to be the "tuberisation stimulus".

Dimalla and van Staden (1977) noted that ethylene treatment tended to inhibit procambial cell divisions and the treatment also affected the endogenous cytokinin complement of the treated stolons (van Staden and Dimalla, 1977c). These observations are interesting in view of the fact that cell division begins very early in the developing tuber (*eg* Reeve *et al*, 1969; Cutter, 1978) and occurs rapidly until the tuber reaches a diameter of about 20 mm (*eg* Reeve *et al*, 1973a; for a more detailed discussion see pp 173-176).

Changes in the activity of cytokinins in developing stolons and tubers have not been followed in carefully defined size categories or in what could be described as normal tubers growing under reasonably normal conditions. Changes in the cytokinin content of stolons and small tubers was noted by van Staden and co-workers in potatoes suffering from a

physiological disorder and by Sattelmacher and Marschner (1978c) in tubers produced on plants under conditions of severe nitrogen deficiency. Langille's group considered only the above-ground and below-ground portions of the potato plant. The sizes of tubers obtained from field grown potatoes and extracted by Okazawa (1970) were not reported. However, he suggested that the cytokinin levels increased some time after tuber initiation was first observed.

The implication from the reports by Dimalla and van Staden (1977) and van Staden and Dimalla (1977c) is that the cytokinins may regulate cell division in the developing tuber. This would be in accordance with the "role" ascribed to the cytokinins in many developing tissues. Much of the early literature indicates that cytokinin levels are high during periods of intensive cell division (see reviews by Letham, 1978 and Goodwin, 1978). More recently, Davey and van Staden (1977b) demonstrated that the levels of cytokinin rose during development of white lupin fruits and fell when the fruits reached maturity. In seed extracts of white lupin high levels of cytokinin activity were present during the early stages of seed growth and decreased to a low level in the mature seed (Davey and van Staden, 1978b). The authors suggested that cytokinins from the liquid endosperm may stimulate both cell division and expansion of the developing embryo.

The observations made by Davey and van Staden on *Lupinus albus* plants were confirmed and extended by Summons *et al* (1981) using *Lupinus luteus* plants. However, instead of using bioassay methods for quantification, Summons *et al* (1981) used deuterium-labelled internal standards and GCMS (multiple ion monitoring mode). The levels of cytokinins were reported to be considerably higher in developing seed than in seed approaching maturity. The dominant cytokinins in the former were zeatin riboside, DZR and OGDZR. OGDZR was the dominant cytokinin in seed approaching maturity.

Watanabe *et al* (1981) attributed the rapid growth of the fertilised hop cone to the presence of zeatin riboside which accumulated mostly in the seed.

Lorenzi *et al* (1978) noted a change in the cytokinin types according to the stage of development of seeds of *Phaseolus coccineus* L. At early stages of seed development the authors suggested that the cytokinin activity was present in the biologically very active less polar types, whereas at intermediate and later stages it was present in more polar types of less biological activity. The authors suggested that the suspensor supplies specific forms of cytokinin to the growing embryo

until it acquires autonomy.

In agreement with cytokinin analyses performed by bioassay methods (Monselise *et al*, 1978; Davey and van Staden, 1978; and references cited in Letham, 1978) Weiler (1980), employing the technique of radio-immunoassay, noted that there was a gradual decrease in the zeatin content of tomato fruit during development. The highest levels of zeatin were found in the youngest fruits at a stage in which fruit growth was mainly by cell division and the most pronounced decrease in zeatin levels occurred at a time when fruit growth became increasingly due to cell enlargement.

Hopping *et al* (1979) noted that the increase in zeatin could be positively correlated with increasing pericarp cell divisions during development of sour cherry (*Prunus cerasus* L), but there was no marked reduction in the level of zeatin per fruit during the period when cell division ceased. However, results calculated on a per unit weight basis were not presented so the concentration of cytokinin present could not be determined.

Rodgers (1981) agreed with the premise of Sandstedt (1971) that the cytokinins extracted from cotton fruit during early development were involved in cell division. The attainment of the maximum concentration of cytokinins in the seed coincided with intense meristematic activity in the cellular tissues.

Even in developing pea root nodules Syono *et al* (1976) noted that the cytokinin-like activity was highest in the meristematic zones and that the cytokinin-like activity declined with age as did the mitotic index. The authors suggested that the cytokinins influence nodule morphogenesis by regulating the mitotic activity of the nodule meristem.

However, there are a number of references in the early literature to the apparent ability of the cytokinins to mobilise metabolites towards areas of high cytokinin concentration (*eg* Mothes *et al*, 1959; Mothes and Engelbrecht, 1961; Pozsár and Király, 1966) and this has been shown more recently by Turvey and Patrick (1979). A similar "role" has been given to the cytokinins as a possible explanation for the high levels noted in developing seeds or fruit (*eg* Witham and Miller, 1965; Davey and van Staden, 1979; Rodgers, 1981).

It has been noted also that the treatment of plant tissues with cytokinins causes effects which resemble the symptoms of infection with obligate parasites, for example, the production of "green islands" (see references cited by Greene, 1980).

Király *et al* (1967) and Szarvas and Pozsár (1979) reported that

the cytokinin activity was higher in rust infected leaves than in non-infected leaves of Pinto beans and broad beans and suggested that the accumulation of nutrients and the altered pattern of phloem transport following infection were due to the accumulation of cytokinin-like substances in diseased tissues.

In addition, the presence of cytokinins in infected tissues has been implicated in the mobilisation of nutrients to the site of mycorrhizal infection (*eg* Crafts and Miller, 1974), to root nodules (*eg* Henson and Wheeler, 1977) and to sites of larval infection in leaves (Engelbrecht, 1971).

Wang (1961) suggested that the "green island" effect was the result of pigment retention. Richmond and Lang (1957) first reported the senescence delaying property of kinetin, and this effect has been noted frequently (*eg* Osborne and McCalla, 1961; Shaw and Srivastava, 1964; Witham and Miller, 1965). Henson and Wheeler (1977) also commented on the reported influence the cytokinins have on the formation of chlorophyll, chloroplasts and chloroplastic enzymes. Wang (1961) noted the accumulation of starch at rust infection sites and suggested this was the result of *de novo* synthesis from $^{14}\text{CO}_2$ at the infection site and was not due to an enhanced translocation of photosynthates from non-infected leaf areas followed by starch synthesis at the infection site.

More recently, Turvey and Patrick (1979) noted that kinetin was found to promote the movement of both ^{32}P - and ^{14}C -labelled assimilate to the site of hormone action.

Berridge and Ralph (1971) suggested that kinetin did not affect the total CO_2 incorporation into leaf discs of Chinese cabbage floated on a kinetin solution for 24 hours. They further concluded that kinetin can mobilise starch reserves and consequently increase the flow of sugars required for the synthesis of lipids and structural materials in floated leaf discs.

Okazawa (1970) suggested that the cytokinins may be serving as "mobilising agents", stimulating the translocation of reserve starch from the mother to the daughter tubers while Palmer and Smith (1969a) suggested that the cytokinins may be mobilising metabolites to the loci of tuber formation by creating a metabolic sink. Turvey and Patrick (1979) considered that kinetin acted directly to promote the transport of assimilates whereas Mullins (1970) and Gersani *et al* (1980) considered that metabolites moved to centres of growth because of the increased sink activity stimulated by the presence of the hormone.

Organs other than potato stolons or sprouts, grown in media

containing cytokinin have been reported to accumulate starch (*eg* Tasseron-De Jong and Veldstra, 1971; Vasil, 1973). Palmer and Smith (1969b, 1970), Smith and Palmer (1970) and Mingo-Castel *et al* (1976b) considered that tuber formation stimulated *in vitro* by kinetin was at least partially a consequence of the stimulating effect of kinetin on starch synthesizing enzymes whereas Hawker *et al* (1979) using plants grown in water culture, suggested that the change in activity of starch synthesizing enzymes was probably a consequence of tuberisation and not a causal factor. Obata-Sasamoto and Suzuki (1979a) showed a correlation between increased cytokinin levels and ADPG-pyrophosphorylase and suggested that the continued accumulation of starch in developing tubers was caused by cytokinins stimulating enzymes which were involved in starch biosynthesis.

While in the tuber system it may be difficult to separate the cell division occurring after tuber initiation from starch accumulation and to therefore correlate cytokinin changes with one or other developmental event (*eg* Plaisted, 1957; Reeve *et al*, 1973b), in the wheat grain cell division diminishes at about the time starch is beginning to accumulate rapidly in the endosperm (*eg* Jennings and Morton, 1963b; Brocklehurst *et al*, 1978; Jenner and Rathjen, 1978).

Within the wheat ovule changes occur very rapidly following pollination. Free nuclear divisions occur within a few hours of fertilisation (Bennet *et al*, 1973) and cellularisation of the free nuclear endosperm begins within one to two days of anthesis and is complete within about four days (*eg* Morrison and O'Brien, 1976). Normal cell divisions subsequently occur very rapidly. Although reports differ (see pp 268-269 for a detailed discussion), Brocklehurst *et al* (1978) considered that the most consistent time for cessation of cell division in the endosperm is about 14 days after anthesis in field grown wheat. The environmental conditions prevailing during the rapid cell division phase of grain development are considered important in determining the ultimate size of individual grains (*eg* Wardlaw, 1970; Brocklehurst *et al*, 1978; Jenner, 1979) and the final grain size appears to be closely related to the number of endosperm cells formed after anthesis (*eg* Wardlaw, 1970; Brocklehurst, 1977; Brocklehurst *et al*, 1978; Radley, 1978).

The presence of cytokinin-like activity has been reported in wheat grains both pre- and post-anthesis but the activity remains uncharacterised. Steward and Caplin (1952) used the carrot assay to detect growth stimulating activity in immature wheat grains sampled two weeks after

pollination. They suggested that a higher level of activity may have been detected if the grains had been selected sooner after fertilisation. Wheeler (1972) showed that the peak of cytokinin activity in extracts of grains from whole ears occurred at the end of anthesis. However, the development of spikelets, florets and grains within an ear is not uniform (*eg* Rawson and Evans, 1970; Sofield *et al*, 1977; Bremner and Rawson, 1978) and the cytokinin pattern Wheeler (1972) reported for each harvest must reflect grains at different developmental stages. This feature is further reflected in the report by Wheeler (1976) in which the pattern of cytokinin-like activity was shown in the upper, middle and lower thirds of ears (not grains) during development. Notable was the low level of activity in the middle sections and the relatively high activity in the upper and basal sections seven days after anthesis. This section of the ear was considered to be the most advanced (*eg* Rawson and Evans, 1970). Wheeler (1972) found that most of the cytokinin-like activity was present in exudate before anthesis when the ovules were growing very little and suggested that any cytokinin consumed in the grain could not be replaced and that present at the end of anthesis may regulate the early stages of grain growth through an effect on cell division and consequently on final grain size providing photosynthate is adequate. The cytokinin-like activity was resolved into two components, one of which exhibited properties similar to zeatin, while the majority of the activity which was highly polar in nature was not characterised.

Herzog and Geisler (1977) showed that the total cytokinin content of wheat grains was maximal after peak anthesis. They also noted that the wheat cultivar with a final higher grain weight had a higher endogenous cytokinin level in the grain at all analyses and suggested that, at an early developmental stage, the cytokinin activity has a stimulatory effect on cell division.

Dua and Bhardwaj (1979) indicated that the cytokinin content of whole ears was barely detectable two days post-anthesis and was still increasing eight days after anthesis, the date of the final harvest. Cultivar differences in cytokinin content were reported at six days. The cultivar with the higher final grain weight showed a higher cytokinin activity per ear at six and eight days after anthesis. Bhardwaj and Dua (1975) showed that the cytokinin content of grains from the six fertile basal spikelets collected 28 days after anthesis was greater than that at 14 days after anthesis, whereas Wheeler (1972) indicated a continued decrease in the amount of cytokinins in grains from whole ears at this stage after anthesis, as did Herzog and Geisler (1977) for grains

probably selected from the central six spikelets of the ear.

In view of the role ascribed to the cytokinins in stimulating starch synthesizing enzymes in potato tubers, it is interesting to note that some of the enzyme changes reported by Turner (1969) and Kumar and Singh (1980), while not correlating with the cytokinin changes noted by Wheeler (1972) or Herzog and Geisler (1977), could in fact be correlated with the increase in cytokinin-like activity detected by Bhardwaj and Dua (1975).

Bhardwaj and Dua (1975) suggested that the final grain weight is a function of auxins and/or cytokinins which enhance the mobilisation of translocates and/or improve the storage capacity of the grain. However, it would appear that in order to correlate the cytokinin content of the grain with specific developmental events within the grain, the cytokinin analysis must necessarily be of grains selected from a defined position in the ear.

At the commencement of this project in March 1977, the cytokinin-like activity in wheat grains, and stolons and tubers of potato, had not been characterised. The cytokinin changes observed by Wheeler (1972) in wheat grains during development were not verified by Bhardwaj and Dua (1975). As noted above the changes in wheat grains after pollination occur very rapidly and non-uniformly within the ear. Consequently it was felt essential to investigate this phenomenon, taking care to use grains selected from defined positions within the ear.

The cytokinin pattern established by Okazawa (1970) for field grown potatoes was quantified from the bioassay of chromatograms in which the cytokinin-like activity was distributed across the entire chromatogram. Van Staden and Dimalla (1976) had published data obtained from potatoes suffering from the "little potato" disorder. No data was available from physiologically normal plants to verify Palmer and Smiths' (1970) statement concerning a requirement for an accumulation of cytokinin at the stolon tip prior to tuber formation, although Forsline and Langille (1974) had noted that an increase in the cytokinin compliment of the above-ground portion of the potato plants occurred when they were under inducing conditions. This was followed by an increase in cytokinins in below-ground tissues prior to tuber formation. It was suggested that two of the four cytokinin components separated changed in activity during induction. However, changes in stolon tips were not monitored. No data was available concerning the cytokinin content of tubers undergoing rapid cell division.

The aim of this investigation is to establish the nature of the

cytokinins present in two starch-accumulating systems and to follow changes in their levels during the development of the starch-accumulating structures. The systems used are potato and wheat. In potato cell division begins in the tubers after initiation and at least some meristematic activity is retained during the subsequent development of the tuber and during the period of rapid dry matter accumulation. In wheat, cell division in the grains occurs over an extremely short period compared to the time of dry matter accumulation.

The soluble sugar and starch contents of both wheat and potato will be analysed to provide a basis for correlation with developmental changes reported in the literature and which were beyond the scope of this thesis (*eg* cell division and enzyme activity).

CHAPTER II

CYTOKININ BIOASSAYS

2.1 INTRODUCTION

A diversity of cytokinin bioassays have been proposed (see Letham, 1978, Table 4.3). The soybean (*Glycine max* L) callus bioassay is probably the most popular tissue culture assay (Letham, 1978). It is highly sensitive, detecting kinetin at a concentration of $1 \mu\text{g l}^{-1}$ (Miller, 1963) and zeatin at a concentration of $0.02 \mu\text{g l}^{-1}$ (Peterson and Miller, 1977). Miller (1963) reported a linear response between 4 and 10,000 $\mu\text{g l}^{-1}$ kinetin. Although the soybean callus bioassay is specific to cytokinins, ABA at high concentrations and glutathione both inhibit soybean callus growth (Blumenfeld and Gazit, 1970a; Bergmann and Renneberg, 1975). Moreover, ABA at low concentrations can actually enhance at least kinetin activity in the soybean callus bioassay (Blumenfeld and Gazit, 1970a). A major drawback of this bioassay is the long assay time. However, the majority of the more rapid assays lack specificity and are usually non-sterile (see Letham, 1978).

Manos and Goldthwaite (1976) reported the use of soybean hypocotyl segments in a bioassay exhibiting what they found to be several advantages over the conventional soybean callus bioassay. Soybeans were grown to the size needed for the assay, thus eliminating the need to maintain callus cultures and the variability among the explants was reported to be significantly less than the variability between callus transplants. Two-fold concentration differences were easily distinguished. The bioassay could take as little as nine days compared to the 21 to 28 days normally required for the soybean callus assay (Miller, 1968). Manos and Goldthwaite considered the specificity of the bioassay was comparable to that of the soybean callus assay.

It was decided to investigate the soybean hypocotyl bioassay and to compare it with a newly established callus culture.

2.2 MATERIALS AND METHODS

2.2.1 Initiation of soybean callus clones

The original soybean callus clones were established from three soybean cultivars provided by DSIR, Lincoln (Kitamasume, Okuhara and Toyosuzu). The methods used were based on Miller (1963, 1968). Analar grade chemicals were used where possible as was distilled water.

Soybean seed was surface sterilised in 50% (v/v) commercial "Janola" (1.3% sodium hyperchlorite) for four minutes and washed five times in sterile distilled water. 50 ml basal medium (Basal Medium A, Appendix 1A) were added to a series of 100 ml Erhlemeyer flasks and the flasks autoclaved. The medium was mixed thoroughly and left to solidify at room temperature. One seed was planted in each flask, and the flasks incubated at 28⁰C in the dark. After germination each cotyledon was cut into four pieces, and all four pieces were placed on the autoclaved and solidified medium which had been supplemented with α -naphthalene acetic acid (NAA; 10^{-5} M) and kinetin (10^{-6} M) prior to autoclaving. The supplemented medium is subsequently referred to as Transfer Medium A (Appendix 1B).

After 18 days pieces of callus were removed from the cotyledons and placed on fresh Transfer Medium A (Transfer 1). Two clones were established from "Kitamasume" (referred to as Kit A and B), one from "Okuhara" (Oku) and one from "Toyosuzu" (Toy). Callus from each clone was also transferred onto Basal Medium A supplemented with 10^{-5} M NAA but devoid of kinetin.

The next eight transfers were carried out at approximately three week intervals by which time the response to kinetin had begun to diminish and a series of transfers was initiated at seven to 10 day intervals. Care was taken during all transfers to use friable callus, thus avoiding the hard central core, and to select callus pieces in which root tissues were not differentiating, at least after the first transfer.

The specific clone, the number of transfers that clone had been passed through and the age of the callus used as determined from its last transfer, are all indicated with the results of individual experiments. Unless otherwise stated the pieces of callus used in experiments weighed ca 5 to 7 mg each, four pieces of callus were placed in each flask and usually there were three flasks per treatment. At the cessation of all bioassays the total fresh weight of the callus in each flask was measured.

A series of experiments were run to test the different callus clones under differing conditions.

2.2.1.1 It was noticed that with extended periods of incubation the agar medium showed signs of shrinkage. The effect of different strengths of agar on callus growth was determined by supplementing Medium B (Appendix 1C) with 0.65, 0.80, 0.95 and 1.10% (w/v) Difco-Bacto agar. In a second experiment the medium was supplemented with 0.8 and 1.0% agar.

2.2.1.2 The sensitivity of the different callus clones to an extended range of kinetin concentrations was tested. Kinetin standards were prepared by dissolving 5 mg kinetin in a minimal quantity of redistilled ethanol prior to adding the solution to 100 ml of vigorously stirred, warm distilled water to give a final concentration of 50 mg l^{-1} . The solution was diluted to 5 and 0.5 mg l^{-1} and 0.1 to 1.0 ml of the appropriate solution was added to 50 ml of Medium B to give a final kinetin concentration ranging from 1 to $1,000 \text{ } \mu\text{g l}^{-1}$.

2.2.1.3 A similar concentration series to that described above was set up but using, in addition to kinetin, both zeatin and zeatin riboside.

2.2.1.4 A time course experiment was set up using Kit A to establish the optimum bioassay time. The bioassays were stopped at 14, 21, 28 and 35 days after initiation.

2.2.1.5 To determine whether the response of the callus to low cytokinin concentrations could be increased, by using larger pieces of callus at the initiation of each bioassay, callus pieces weighing 7 mg and 20 mg were used as transplants. In an additional experiment callus was transferred onto kinetin-free medium one week prior to the commencement of the bioassay.

2.2.2 Maintenance of a soybean callus clone obtained from Plant Diseases Division, DSIR, Auckland

Soybean callus was obtained from E. Hewett on 3 July 1979. The callus was derived originally from a clone maintained at Aberystwyth, Wales. One line of callus had been maintained on kinetin and a second on 6-benzylaminopurine. The subsequent maintenance of the clones was as described previously at 28°C in a dark incubator but with an increased humidity obtained by placing a tray of water in front of the fan in the incubator. In addition, double-glass distilled water was used to prepare all media. Previously commercial grade sucrose had been used in all media. This was substituted with Pronalysis grade (May and Baker)

sucrose for all stock culture media although this was too expensive to use routinely in the media prepared for large experiments.

Callus was maintained on media supplemented with either 6-benzylaminopurine or kinetin. Both kinetin and 6-benzylaminopurine were dissolved in 2N NaOH prior to the addition of water when making standard solutions. Kinetin or 6-benzylaminopurine was added to Transfer Medium 1 at the rate of $250 \mu\text{g l}^{-1}$ (Appendix 1D).

Transfers of callus for the maintenance of stock cultures were made when the callus was beginning to exhibit exponential growth (10 to 15 days from transfer; B. Fletcher, pers comm), 12 days being used routinely. Callus was used in bioassays 18 days or less after transfer (B. Fletcher, pers comm), 18 days being used routinely.

2.2.3 Bioassays of plant extracts and estimation of cytokinin-like activity

2.2.3.1 The callus derived from cultivar Kitamasume, clone A (clone Kit A) was used in the majority of bioassays of mature tuber material (section 4.2.1), and of material derived from the Potato Field Trial (section 4.2.3) and Wheat Field Trial (section 6.2.1). The clone of callus obtained from DSIR (Auckland) and subsequently maintained on 6-benzylaminopurine (clone Bap) was used in the Plant Physiology Division Potato experiment 1 (section 4.2.4) while that maintained on kinetin (clone Kin) was used in the second wheat field trial (section 6.2.2).

Bioassays of plant extracts resolved either by paper or column chromatography were established following a modification of the procedure of Miller (1961). Difco-Bacto agar (0.4 g) and 50 ml Medium B (Appendix 1C) were added to each 100 ml Erlenmeyer flask containing segments of paper chromatograms or dried column eluant. The material dried on chromatograms or on the base of the flask was dissolved during autoclaving at 104 kPa for 20 minutes. The contents of each flask were mixed thoroughly prior to solidification at room temperature. Four pieces of callus were transplanted onto the medium in each flask as described previously. Control paper chromatograms were bioassayed as was the void volume of the column eluant. Each bioassay was stopped after 25 days and a note made of any blackened callus.

2.2.3.2 Construction of confidence limits To determine if the callus growth was stimulated by the presence of cytokinin-like activity or was due to random variability within the callus itself, 95 and 99% confidence limits were constructed. To obtain limits for the bioassay data on paper chromatograms all data from control chromatograms were

used. However, fewer values were available following column chromatography so values from both the bioassay of the void volume and from flasks containing Medium B were combined, n being the number of values available for the construction of the limits. The limits constructed represent the maximum estimates possible, no refinements of data such as the removal of variability between samples or between zones on paper chromatograms were applied. The limits were not regarded as an absolute criterion for significance and were used as a guide only, due regard being given to the repeatability of peaks between samples and to the R_f value or elution volume of authentic cytokinins.

2.2.3.3 Construction of standard curves A series of flasks containing kinetin standards was set up at the same time as each bioassay. Each kinetin concentration was prepared at least in triplicate and usually in quadruplicate. The standards were prepared as described in section 2.2.1.2. The kinetin, however, was initially dissolved in either redistilled ethanol when standard curves were set up using clone Kit A, or in 2N NaOH when callus from clone Bap or Kin was used.

Regression lines were constructed from the callus response to increasing concentrations of kinetin. The upper and lower limits of the regression line were determined subjectively and these limits were then refined, if necessary, until a significant proportion of the total variation in the callus growth on different cytokinin concentrations was accounted for by linear regression. The equation of each regression line is presented with the results of the corresponding bioassay.

In some instances, however, although peaks may lie well above the 99% confidence limits, the weight of the callus measured may still have lain below the lower limit of the relevant regression line (*eg* Fig 4.26). In these cases the amount of cytokinin-like activity was estimated by direct comparison with the amount of callus growth in the kinetin standards and a minimum estimate presented.

From the regression line or the standards, the concentration of cytokinin-like activity was determined in kinetin equivalents. A kinetin equivalent (KE) is defined as the "amount of kinetin required to elicit the same callus response as an active fraction in a particular assay" (Purse *et al*, 1976).

The variability in the cytokinin-like activity among any three samples is presented as a standard error of the mean (SE).

2.2.4 Soybean hypocotyl bioassay

The soybean hypocotyl bioassay was set up essentially as described

by Manos and Goldthwaite (1976).

2.2.4.1 Germination of soybeans Washed vermiculite was added to test tubes (25 x 150 mm) to a height of 40 mm. To each test tube 11 ml of one-fifth concentration of Hoaglund A nutrient solution was added (Appendix 2). The tubes were covered with aluminium foil, autoclaved for 20 minutes (104 kPa) and allowed to cool. Soybeans were surface sterilised in 50% (v/v) commercial "Janola" (1.3% sodium hyperchlorite) for four minutes with occasional stirring and then rinsed five times in 50 ml of sterile distilled water. One soybean was embedded in the vermiculite in each test tube. The tubes were placed in a dark incubator at 28°C for four to six days when the hypocotyl length was approximately 100 mm.

2.2.4.2 Hypocotyl bioassays The medium used was prepared at 1.11x concentration (Appendix 3). Difco-Bacto agar was added separately to each container to give a final concentration of 0.8% (w/v). Small glass jars (capacity 50 ml) were filled with an appropriate amount of medium and growth regulator solution (see below), covered, autoclaved and allowed to cool. Hypocotyls were removed from the seeds and, under sterile conditions, cut into 1 mm segments. Segments from separate hypocotyls were randomised and then placed on the agar. The containers were placed in a dark incubator at 28°C for five to 15 days. At the end of the incubation period the segments were removed from the containers and the fresh weights recorded.

2.2.4.3 Bioassay 1 4.5 ml medium and 0.5 ml growth regulator solution (10^{-5} M NAA in combination with 0, 10^{-7} and 10^{-5} M kinetin) were added to each glass container. Each treatment was prepared in triplicate. Eight-day old hypocotyls were cut from soybeans of the cultivar Kitamasume. Five segments were used per replicate and the containers were incubated for nine days.

2.2.4.4 Bioassay 2 Medium (9.0 ml) and growth regulator solution (1.0 ml) were added to each container as described for Bioassay 1. Six-day old hypocotyls were used from Kitamasume soybeans. Six segments of hypocotyl were used per replicate and nine replicates were prepared per treatment. Three replicates per treatment were removed at intervals of five, nine and fifteen days.

2.2.4.5 Bioassay 3 A total of 10 or 20 ml of medium and growth regulator solution was added to each container. Six day-old hypocotyls were

removed from Kitamasume soybeans. Six segments of hypocotyl were used per treatment and each treatment was replicated four times. The bioassay was terminated after 10 days incubation.

2.2.4.6 Bioassay 4 In addition to NAA and kinetin, combinations of NAA and isopentenyladenine (iP) were prepared. A total of 20 ml medium and growth regulator solution was added to each jar and the top was covered with foil rather than a loosely applied bakelite lid. All containers were placed in plastic boxes and surrounded with paper towelling which was kept moist for the duration of the bioassay. Three cultivars of soybeans were germinated. Both "Caloria" and "Geiso" produced usable hypocotyls but "Amsoy '71" produced only one usable hypocotyl from eight seeds within eight days. "Caloria" and "Geiso" were used in the bioassays five or six days after planting. Each treatment was prepared in triplicate and six segments were transplanted per replicate. Bioassays were terminated after 10 days.

2.2.4.7 Bioassay 5 A total of 18 ml of medium supplemented with 10^{-5} M NAA was added to the glass containers and 2 ml of kinetin solution were then added to give final kinetin concentrations ranging from 0 to 10^{-9} M. Each treatment was prepared in triplicate. Hypocotyl segments from Caloria were used four days after planting. Three segments were added per replicate. The segments from Geiso were used six days after planting and five segments were added per container. Both bioassays were terminated after 10 days.

2.3 RESULTS

2.3.1 Initiation of soybean callus clones

Soybeans of three cultivars (Kitamasume, Okuharu and Toyosuzu) were used to establish callus clones. Three days after planting onto sterile basal medium the seeds had germinated. Within three days of the transfer of cotyledon segments onto medium supplemented with NAA and kinetin, callus began to form on the cut edges of a number of the segments and within seven days roots had begun to form. The first transfer of callus was performed after 18 days, care being taken to avoid transferring parent cotyledon material and roots.

Prior to the second transfer, 18 days after the first, all pieces of callus from which roots had differentiated were discarded and only callus showing no root formation was used for transfer. Callus maintained

for 18 days on Basal Medium A supplemented only with $10^{-5}M$ NAA was soft and watery. Okuhara callus maintained on Transfer Medium 1 was tinged brown and had grown less after 18 days in culture than had the Kitamasume clones which had shown prolific growth and were creamy white in appearance. Callus from cultivar Toyosuzu showed the least prolific growth.

The third transfer was performed 21 days after the second. At this stage, some root differentiation was still apparent, but those formed were relatively short and sparse. The Kitamasume callus clones were the largest and were creamy in appearance. Kitamasume callus passed once on kinetin-free medium showed little growth and appeared brown as was callus passed twice on kinetin-free medium. Okuhara callus was white and friable and although growth was slightly less prolific than Kitamasume callus, the callus had a smaller, hard central core. Okuhara callus passed twice on medium devoid of kinetin had grown more than the comparable Kitamasume callus but was very small relative to the callus grown on medium supplemented with kinetin. Okuhara callus passed only once onto a kinetin-free medium showed more growth than that passed twice, but was brown and was still very much smaller than the callus grown on Transfer Medium 1. The Toyosuzu callus maintained on kinetin-free medium appeared brown and showed no growth while callus maintained on Transfer Medium 1 showed less growth than either of the other two cultivars.

The effect of agar concentration on callus growth was investigated. Only the two more prolific callus clones were used and callus 18 days post-transfer 2 was used to provide the explants. Higher callus fresh weights were generally obtained from callus grown on medium with a 0.65% (w/v) agar content but this was attributed to the higher water content of the callus. Okuhara callus grown on the 1.10% (w/v) agar medium generally produced lower total callus weights than the callus on media with either 0.80% or 0.95% agar. This difference was not apparent with the Kitamasume variety. Most noticeable, however, was the differing response of two varieties to the kinetin concentration (Fig 2.1). On all concentrations of agar, Kitamasume clone A showed a detectable response to kinetin at $1 \mu g l^{-1}$ whereas Okuhara callus showed no response to kinetin at this concentration. Okuhara callus, however, showed greater growth on $1,000 \mu g l^{-1}$ kinetin.

The fourth transfer was performed 20 days after transfer 3. Kitamasume B and Okhura calluses were more friable than that of

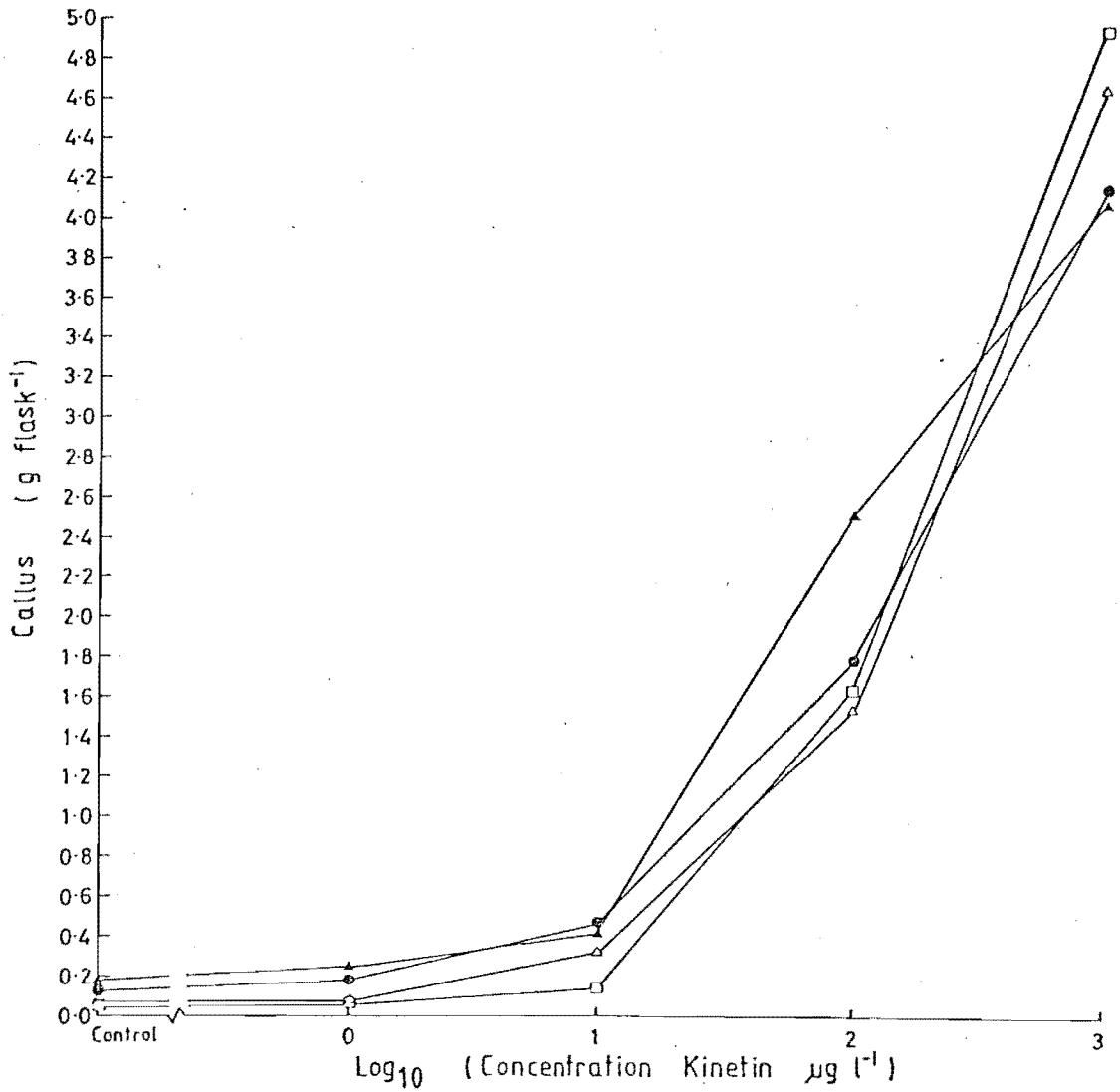


FIGURE 2.1

Response of callus cultured from soybean cultivars Kitamasume (clone A) and Okuhara to increasing kinetin concentrations and two agar concentrations (0.8% and 0.95%).

Key

Callus Clone	Agar concentration (%)	
	0.8	0.95
Kit A (2) ^b	▲	●
Oku (2) ^b	△	□

Control: no cytokinin supplement

Callus: 15 days post-transfer

Length of bioassay: 21 days

^b Number in parentheses indicates number of subcultures.

Kitamasume A while the Toyosuzu clone continued to produce friable but very small pieces of callus following transfer. After four transfers no further root differentiation was apparent in any clone, even in callus maintained for eight weeks.

The remaining third-transfer callus was tested for response to cytokinin concentration in addition to an effect of agar concentration. The two agar concentrations (0.8% and 1.0% (w/v)) did not have consistent effects on final callus yield and the results are not presented. However, significant differences were noted in the response of the different clones to kinetin concentration (Fig 2.2). Kitamasume B callus showed a substantial amount of growth on media without kinetin or with low kinetin concentrations and exhibited a short linear response range. The apparent excessive growth on low kinetin concentrations was due to the response of only some pieces of callus. Small segments of these pieces were transferred to media without kinetin. Most segments showed little growth and were shrivelled and brown within seven days although one segment grew quite substantially.

In contrast to the previous results (Fig 2.1) Kitamasume A showed more callus growth than clone Okuhara at all kinetin concentrations, although the linearity of the response was disturbed at kinetin concentrations of 25 and 50 $\mu\text{g l}^{-1}$ (Fig 2.2). The Okuhara clone showed a greater prevalence for pieces not "striking" on high kinetin concentrations as reflected in the large SE. Both clones showed little growth on media devoid of cytokinin.

Callus from the fourth Kitamasume A transfer was used to determine the optimum time for stopping the bioassay (Fig 2.3). Callus showed considerable growth between 14 and 21 days in culture but an even larger growth increment between 21 and 28 days in culture. The rate of growth, however, decreased between 28 and 35 days in culture (Fig 2.3). By 35 days shrinkage of agar was noticeable in the flasks.

The sensitivity of the Kitamasume A clone to kinetin decreased with time (Fig 2.4). By April 1978, 12 months after establishment of the callus cultures, the Okuhara clone displayed extremely poor and variable response to kinetin. While Kitamasume B showed an increasing growth response to increasing concentrations of kinetin, callus growth at any one concentration was more variable and significantly less than that of clone A. The Toyosuzu clone had shown the most rapid loss of vigour and was discarded within 12 months. A series of rapid transfers of seven to 10 days intervals slowed the deterioration of the Kitamasume

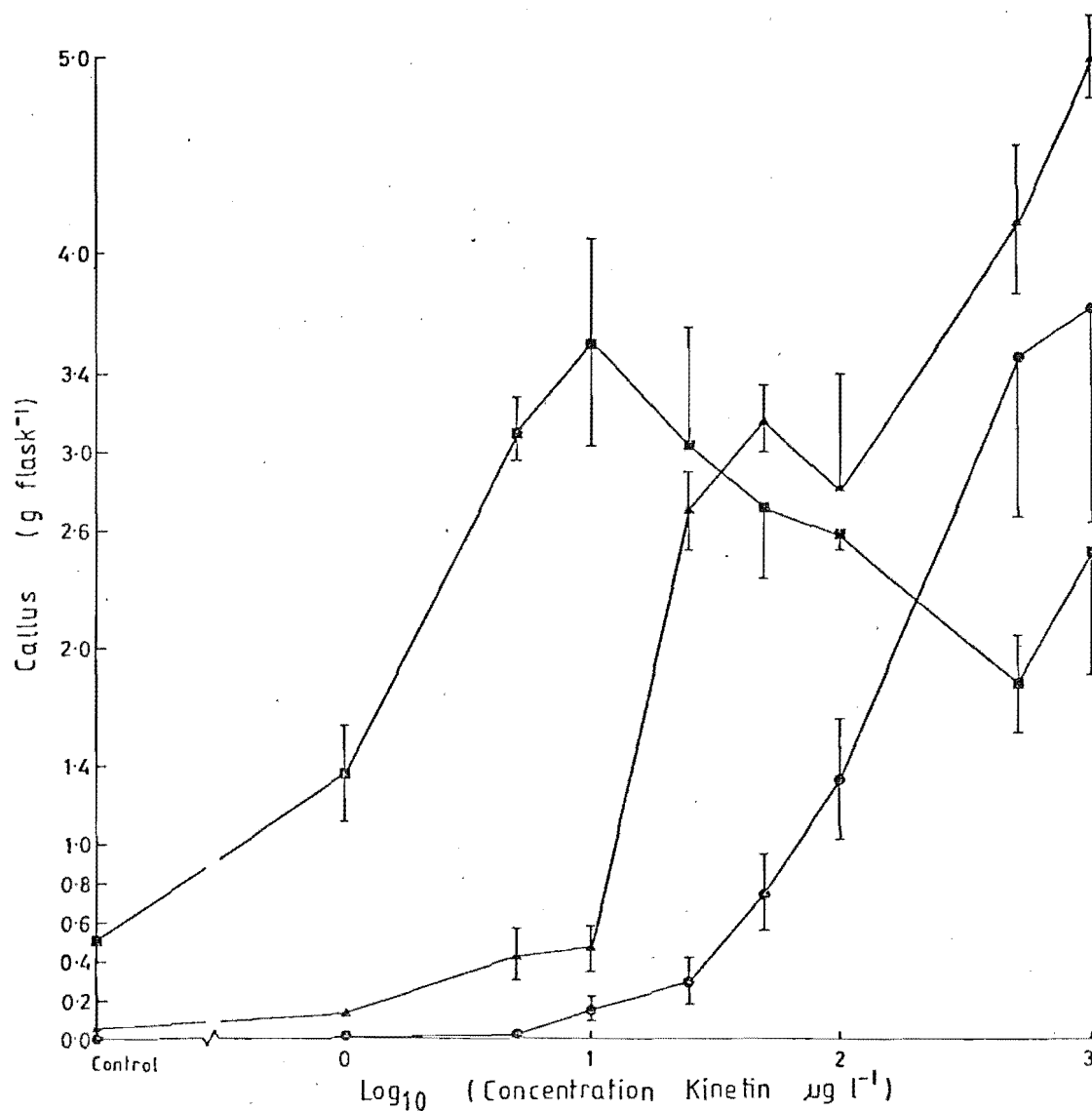


FIGURE 2.2

Response of callus cultured from soybean cultivars Kitamasume (A and B) and Okuhara to increasing kinetin concentrations.

Key	Callus clone
▲	Kit A (3)
■	Kit B (3)
●	Oku (3)

Agar concentration: 0.8%

Control: no cytokinin supplement.

Callus: 22 days post-transfer

Length of bioassay: 21 days.

Once or twice the standard error of the mean is presented as vertical bars above and/or below the line.

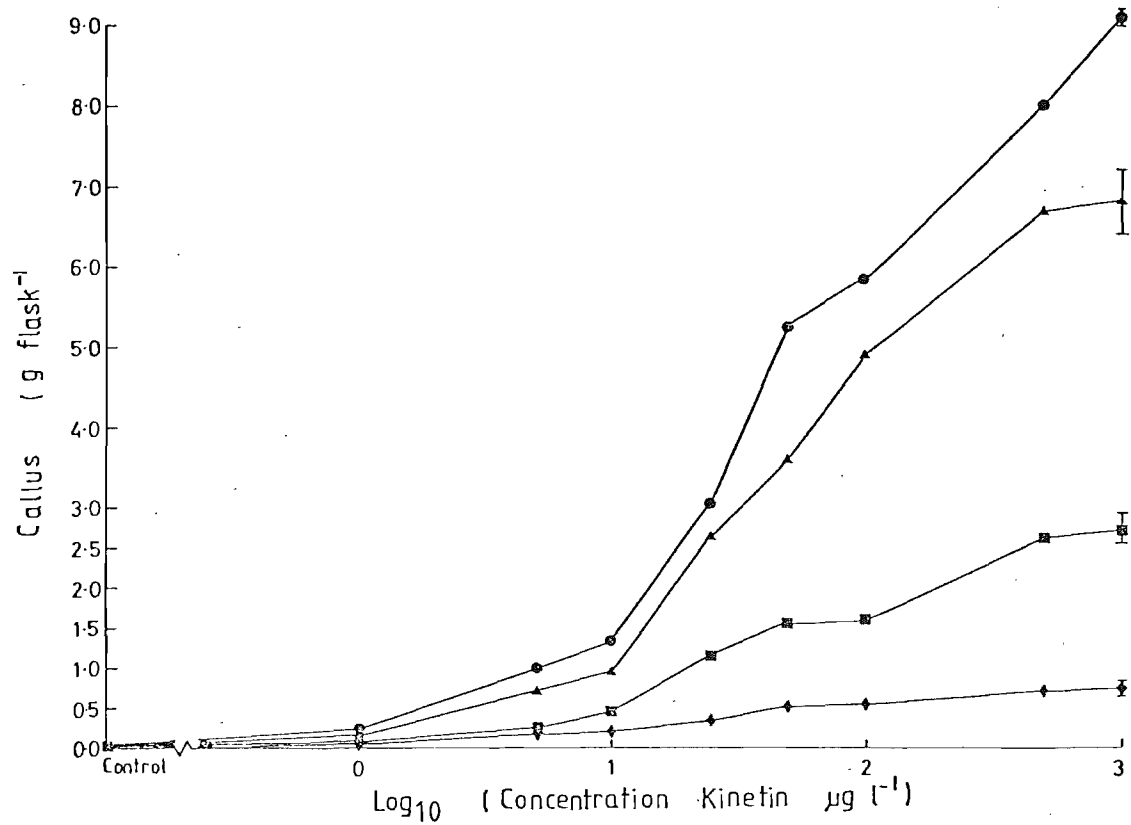


FIGURE 2.3

Soybean callus bioassays terminated after 14 (◆—◆), 21 (■—■), 28 (▲—▲) and 35 (●—●) days.

Control: No cytokinin supplement.

Callus clone: Kit A (4) 31 days post-transfer.

Once or twice the standard error of the mean is presented as vertical bars above and/or below the line.

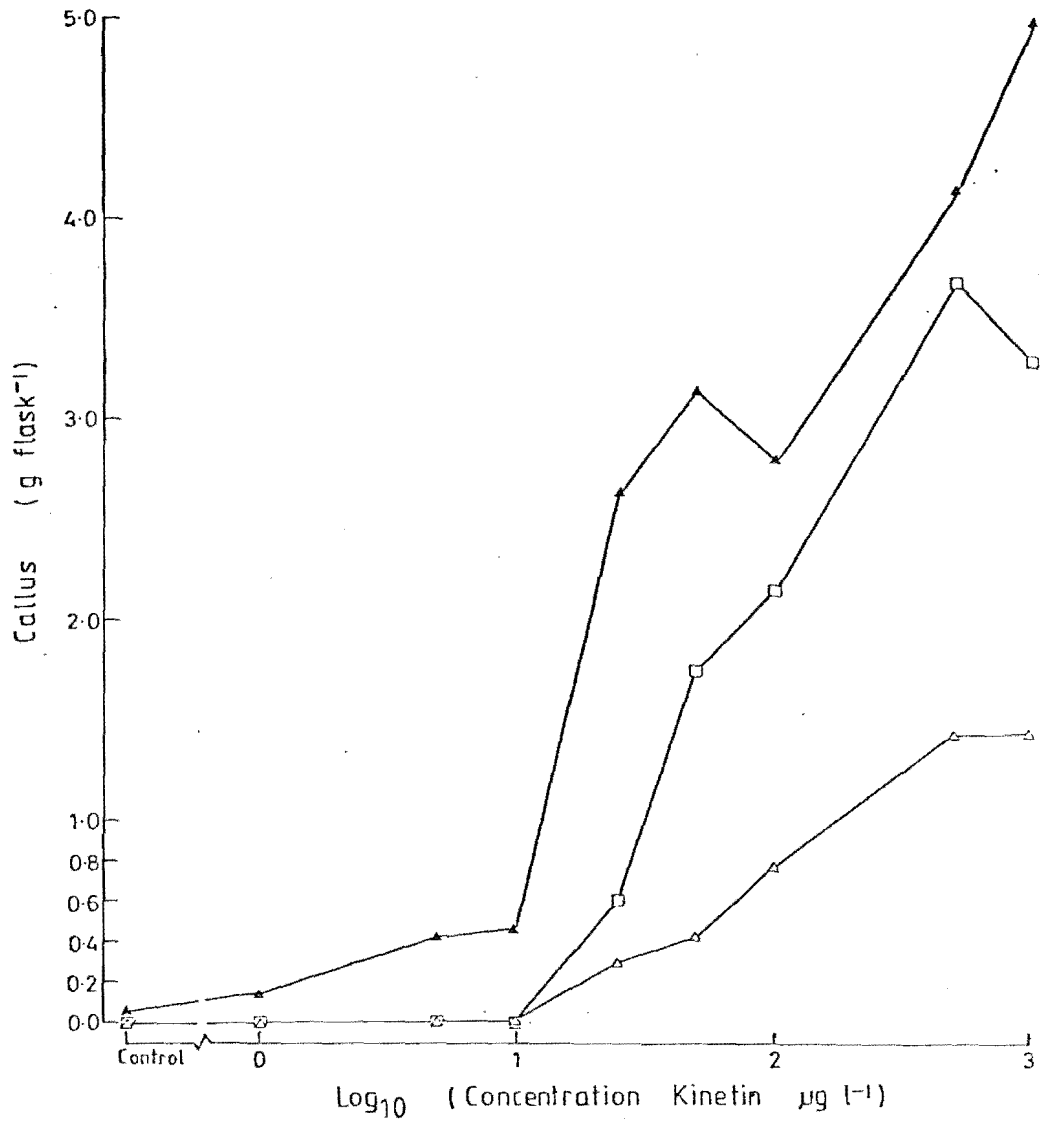


FIGURE 2.4

Comparison of soybean callus bioassays set up with callus passed through different numbers of sub-cultures.

Key

	Clone	Days post-transfer	Length of bioassay (days)
▲	Kit A (3)	22	21
□	Kit A (8)	17	25
△	Kit A (24)	18	25

Control: No cytokinin supplement.

clone A but response to low cytokinin concentrations remained poor.

Larger callus explants (ca 20 mg) were less responsive to increasing cytokinin concentrations than the routine explants (ca 7 mg) while pre-incubation for one week on medium without cytokinin reduced the sensitivity of the callus to cytokinin (Fig 2.5).

Within 19 months of establishing the callus clones, Kitamasume A was showing distinct browning after three to four weeks in culture even on media containing high concentrations of cytokinin.

2.3.2 Maintenance of callus clone obtained from DSIR

The maintenance of callus obtained from DSIR (Auckland) was continued on 6-benzylaminopurine (clone Bap) or kinetin (Clone Kin). However, callus maintained on 6-benzylaminopurine showed a greater response to lower concentrations of kinetin than did callus maintained on kinetin (Figs 2.6 and 2.7). Callus used 18 days post-transfer exhibited greater sensitivity compared to callus used 36 days post-transfer (Fig 2.6).

The callus clone maintained on 6-benzylaminopurine (clone Bap) was originally sensitive to $1 \mu\text{g kinetin l}^{-1}$ but sensitivity decreased and then stabilised at about 3 to $4 \mu\text{g kinetin l}^{-1}$.

Sample standard curves with fitted regression lines are presented in Figure 2.7 for callus clones Kit A, Bap and Kin. The lower and upper limits of the regression line for Kit A callus were 25 and $100 \mu\text{g l}^{-1}$ respectively while those for Bap and Kin callus were 2 to 4 and $500 \mu\text{g l}^{-1}$ respectively (until just before the clone became habituated on cytokinin - see below).

Callus clone Bap was more sensitive to zeatin than to kinetin, especially at low cytokinin concentrations, and slightly more sensitive to zeatin riboside than to kinetin (Fig 2.8).

After 15 months under Canterbury conditions the callus became habituated on cytokinin and for a period of several months was able to be maintained on Transfer Medium B without a cytokinin supplement. Callus maintained on medium devoid of cytokinin, however, did deteriorate in vigour with time, although within this period, the apparent cytokinin-autotroph was used to detect compounds inhibitory to callus growth (Ng, Cole, Jameson and McWha, in Press). Following the termination of the project, transfers of callus were less frequent and at this stage the callus reverted to being dependent on an exogenous supply of cytokinin. A second line of callus obtained from DSIR, Auckland (June 16, 1980) and

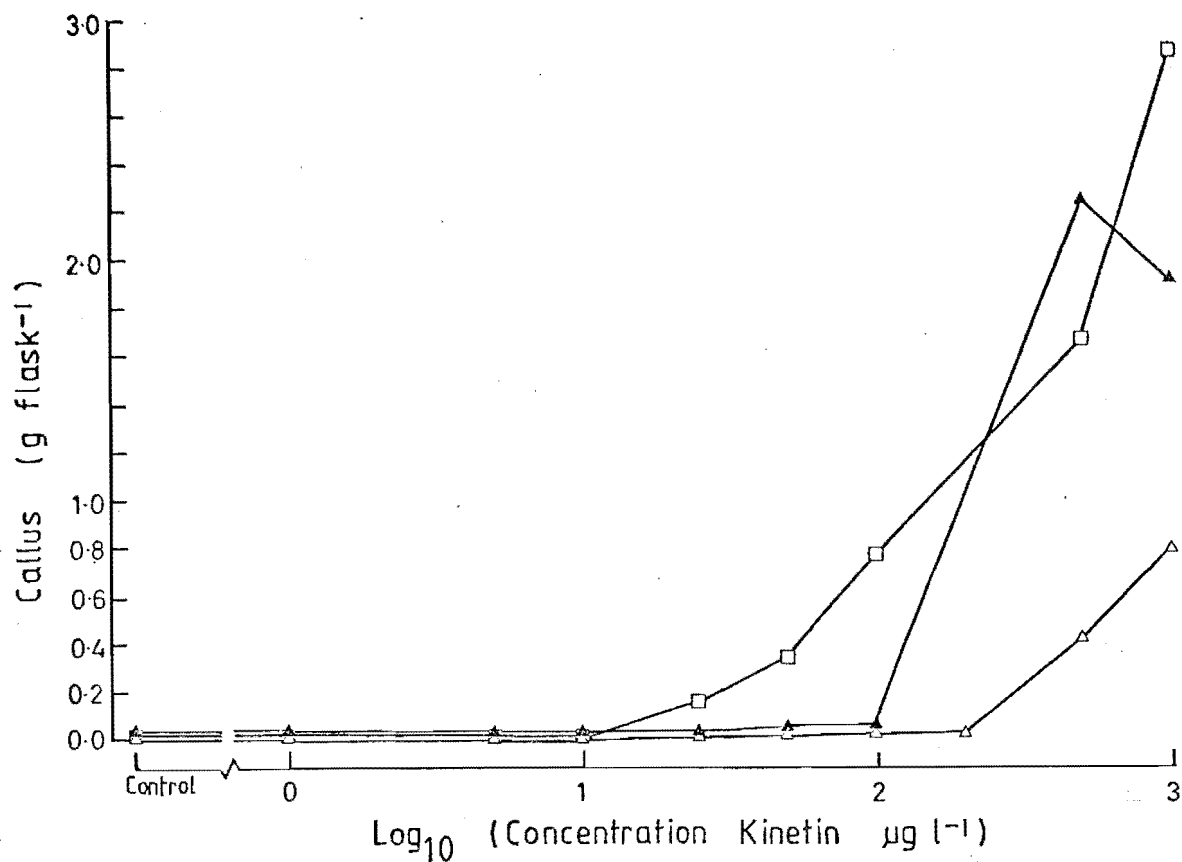


FIGURE 2.5

Comparison of soybean callus bioassays set up with (A) 7 mg transplants (□—□), (B) 20 mg transplants (▲—▲) and (C) callus maintained seven days on medium devoid of cytokinin prior to starting the bioassay (△—△)

Control: no cytokinin supplement

Callus clone: Kit A (43) (A and B)
Kit A (42) (C)

Length of bioassay: 26 days

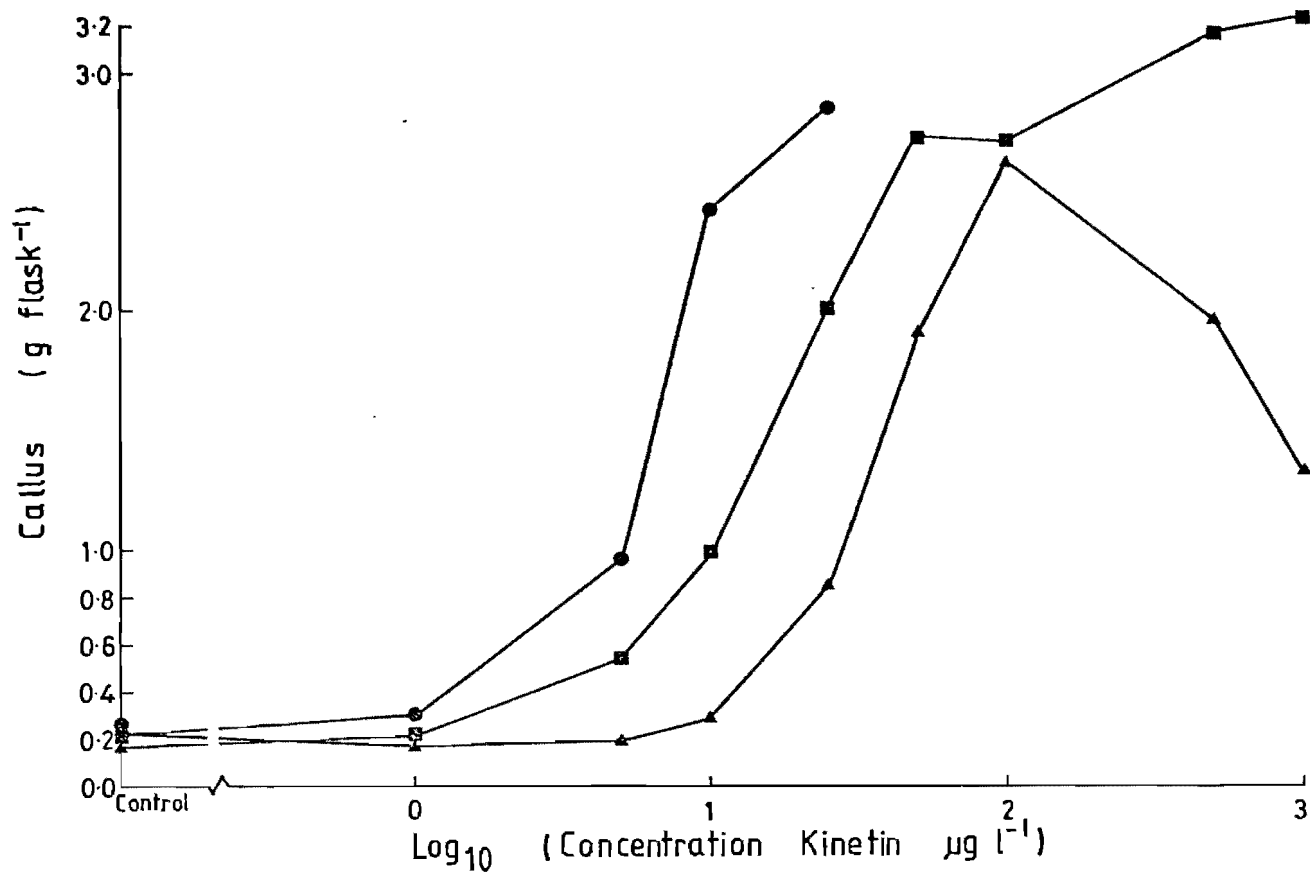


FIGURE 2.6

Comparison of soybean callus bioassays using callus at different ages post-transfer and maintained on either kinetin or 6-benzylaminopurine. Callus clones obtained from DSIR (Auckland)

Key

- Kin (2)^a 18 days post-transfer
- ▲—▲ Kin (1)^a 26 days post-transfer
- Bap (2)^b 18 days post-transfer

Control: no cytokinin supplement

Length of bioassay: 25 days

^a maintained on kinetin

^b maintained for two sub-cultures on 6-benzylaminopurine

FIGURE 2.7

Regression lines calculated from the growth of callus on kinetin standards. The regression lines A, B and C correspond to the bioassays presented in Figures 6.3, 6.15 and 4.29 respectively

	<u>Callus</u>	<u>Regression line</u>
A	Kit A (54)	$Y = 2.09X - 2.78$
B	Kin (13)	$Y = 1.46X - 1.05$
C	Bap (13)	$Y = 0.87X - 0.03$

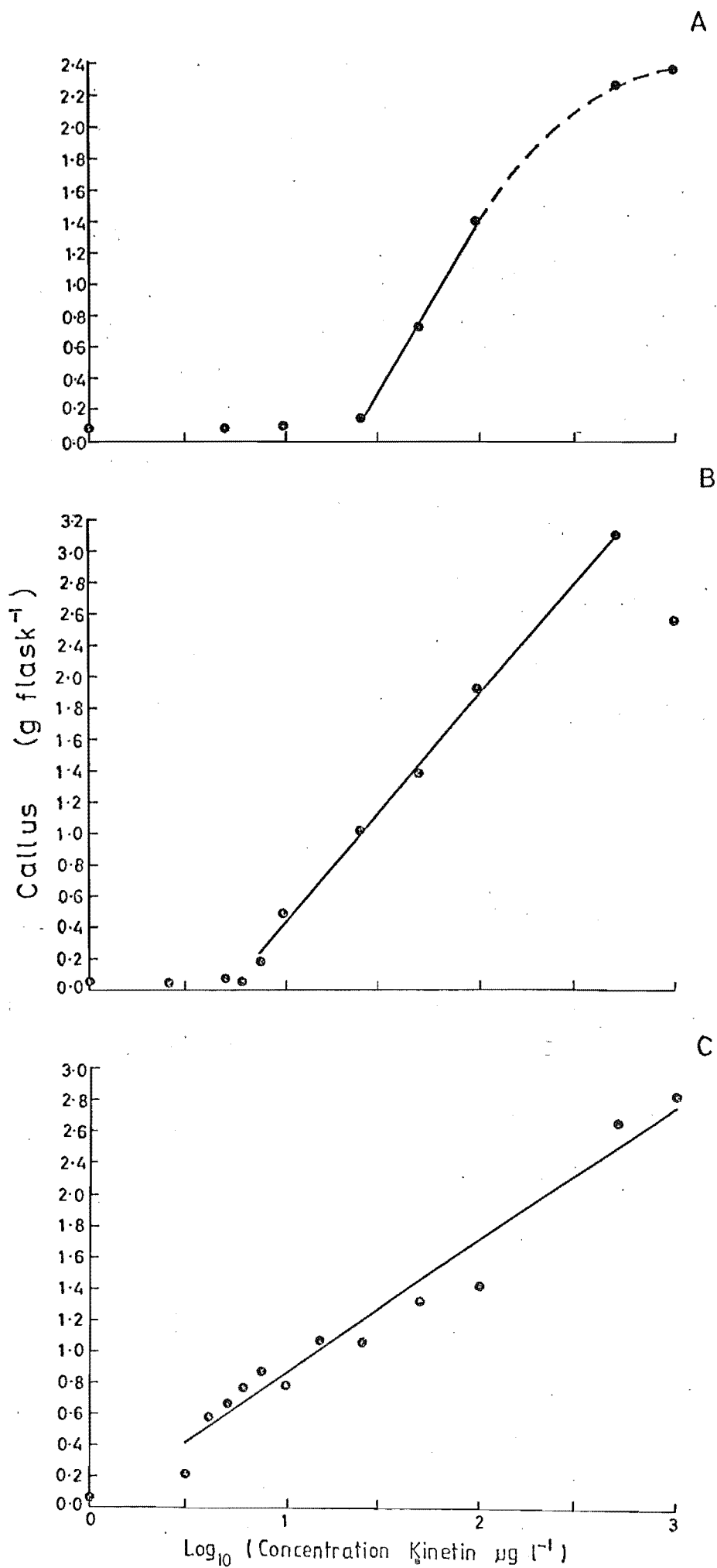


FIGURE 2.7

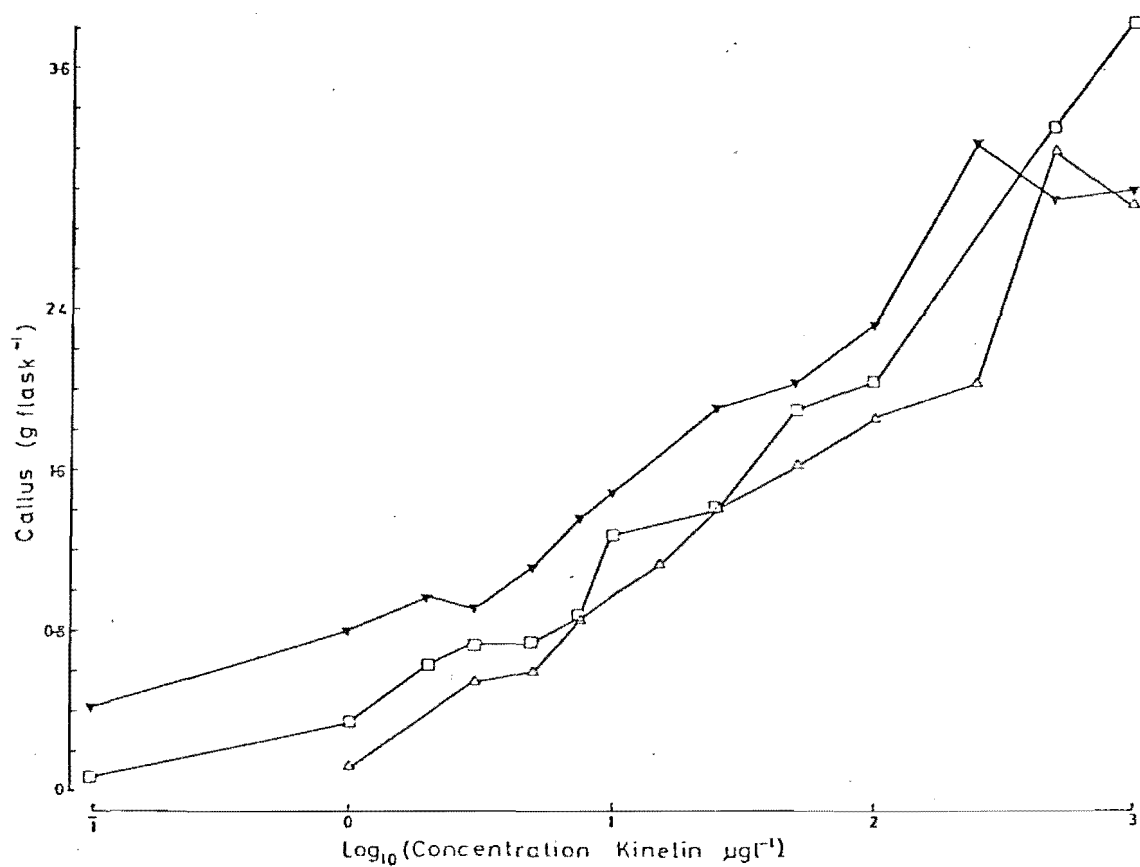


FIGURE 2.8

Response of callus clone Bap to different concentrations of zeatin (▼—▼), zeatin riboside (■—■) and kinetin (△—△).

Length of bioassay: 25 days

Callus: Bap (29) 18 days post-transfer

cultured for four months at Canterbury also exhibited habituation on cytokinin at exactly the same time.

2.3.3 Soybean hypocotyl bioassay

Hypocotyls obtained from soybeans, cultivar Kitamasume, showed little response to NAA or kinetin, alone or in combination. However, an increased bioassay time from nine to 15 days led to a decrease in the fresh weight of the segments. Consequently, in subsequent experiments care was taken to keep the humidity at a high level. An increased quantity of medium was also added to each container.

Since the cultivar Kitamasume was in limited supply, three additional cultivars of soybean were obtained. The use of Amsoy '71 was discontinued because of inconsistent germination and the variable thickness of the hypocotyl. The cultivars Caloria and Geiso produced more uniform hypocotyls although those from cv Geiso grew slightly slower than those from cv Caloria.

A response from both Geiso and Caloria was apparent to both kinetin and isopentenyladenine (Table 2.1). A small positive but consistent response to NAA was also noted. Hypocotyls from cv Geiso used at six days grew slightly more on medium without cytokinin than those used at five days, but grew less at the higher kinetin and isopentenyladenine concentrations (Table 2.1).

An increased response to higher concentrations of kinetin and isopentenyladenine was exhibited by both soybean varieties but the response to isopentenyladenine was not as large as that reported by Manos and Goldthwaite (1976).

Within replicates the response to increasing kinetin concentrations was quite variable (Fig 2.9). Also, the final hypocotyl weights were substantially less than in the previous experiment and an increase in response was noted only between 10^{-7} M and 10^{-6} M kinetin. A similar pattern of response was noted for cv Geiso although the final segment sizes were smaller (data not presented).

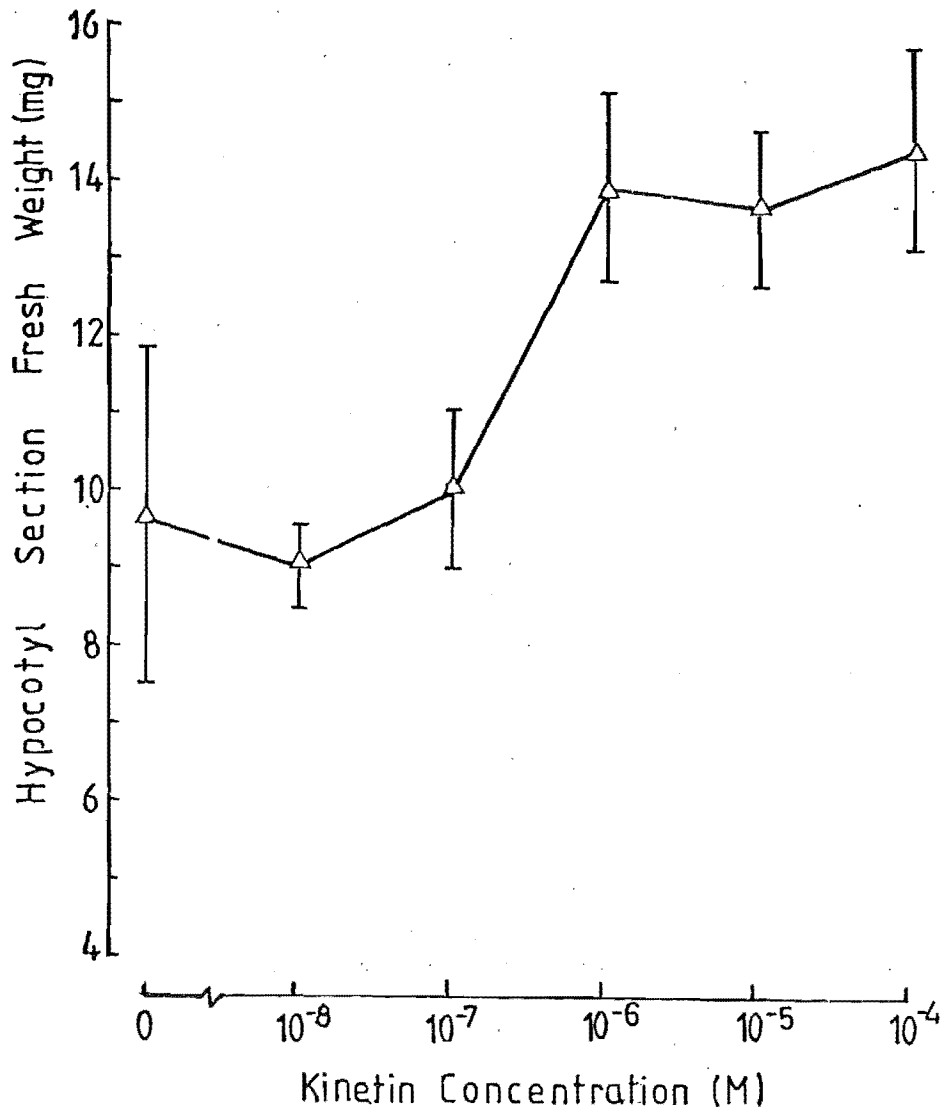


FIGURE 2.9

Soybean hypocotyl bioassay. Response of hypocotyl segments from soybean cv Caloria to different kinetin concentrations

Twice the standard error of the mean is presented as vertical bars

TABLE 2.1

Response of hypocotyl segments to different cytokinins. The bioassay was stopped after 10 days and the average weight (mg) of 18 hypocotyl segments is presented (3 replicates; 6 segments per replicate). NAA (10^{-5} M) was present in all media except the water control

Cultivar	Growth Regulator	Weight (mg) per hypocotyl segment	
		A ^a	B ^b
Geiso	H ₂ O control	8.3	10.7
	10^{-5} M NAA	9.9	14.3
	10^{-7} M iP	15.7	19.3
	10^{-5} M iP	26.0	22.4
	10^{-7} M kinetin	14.1	16.5
	10^{-5} M kinetin	20.8	19.7
Caloria	H ₂ O control		5.8
	10^{-5} M NAA		6.16
	10^{-7} M iP		17.3
	10^{-5} M iP		21.9
	10^{-7} M kinetin		17.6
	10^{-5} M kinetin		27.7

^a Five day old hypocotyls used as transplants

^b Six day old hypocotyls used as transplants

2.4 DISCUSSION

The very wide linear response to kinetin reported for the soybean callus (Miller, 1963) was rarely observed. A more restricted linear response to kinetin was observed and is also apparent in the work of Engelbrecht (1971). A restricted positive response is reported by Newton *et al* (1980) but in contrast to Newton *et al* (1980) the majority of standard curves exhibited linear portions.

Although a response to kinetin and isopentenyladenine was apparent when hypocotyl sections were used as bioassay material, the actual growth increments obtained with increasing cytokinin concentration were considered too small and the response too variable to be useful. The soybean callus was selected for routine use throughout the project.

The conditions under which the soybean callus was grown and the purity of the water and chemicals used in the media appeared to be

critical features for the maintenance of highly sensitive and vigorous callus clones. However, it was also obvious that the cultivar of soybean was critically important to the establishment of a callus clone which was both sensitive to low cytokinin concentrations and which exhibited a wide linear response range (*eg* Figs 2.2, 2.7). Even the two clones established from the same cultivar of soybean exhibited quite different characteristics (Fig 2.2). The maintenance of stock callus on 6-benzylaminopurine appeared to lead to increased responsiveness to low kinetin concentrations (Fig 2.6) and the age of the callus (post-transfer) used to set up a bioassay was also critical (Fig 2.6).

Although in general the limits of the regression lines for each of the callus clones were reasonably consistent, the variation in the upper and lower limits and changes in the slope of the regression lines meant that it was imperative to run a series of standards with each bioassay which is in contrast to the procedure reported by Mauk and Langille (1978). Variable baseline level and sensitivity of callus is also apparent in the literature (*eg* Purse *et al*, 1976; Salama and Wareing, 1979; van Staden, 1976c; van Staden *et al*, 1981).

The Bap clone maintained on 6-benzylaminopurine became habituated on cytokinin and was passed through several transfers on medium devoid of cytokinin. Cytokinin-autotrophy is a notable feature of tobacco callus (see references cited in Letham, 1978, and in Meins and Lutz, 1979) but appears to be less common with soybean callus tissues. Miura and Miller (1969) noted that a clone of soybean callus became habituated on cytokinin after six years of subculturing. No simple reason can be put forward to explain the relatively sudden change in the characteristics of the callus, especially since a clone subcultured at Canterbury for only four months exhibited habituation on cytokinin at the same time as did the clone maintained at Canterbury for 15 months. Meins and Lutz (1979) considered that cytokinin habituation is a result of epigenetic changes rather than genetic mutation.

The nature of the soybean callus bioassay is such that a rigorous statistical analysis of the results is not possible. This problem was noted by Salama and Wareing (1979) and, as also suggested by Hewett and Wareing (1973a,b), is a consequence of the large number of fractions obtained not enabling sufficient replication to be performed during any one bioassay. Up to sixty fractions may be collected from one sample of extract resolved by column chromatography and up to fifty flasks of standards may be required. The choice was either to run a series of

samples selected from different developmental stages with only one replicate for each stage as done by Hewett and Wareing (1973a,b) or to analyse three samples of the same developmental stage. Because of the large number of stages expected to be analysed and the possible variable callus response the latter procedure was selected.

To determine if the callus growth was a response to cytokinin-like activity, confidence limits were constructed. The interval was constructed so as to exclude as far as possible those peaks extending beyond the 99% limit which were due to random callus growth. This procedure meant that some callus growth caused by the presence of cytokinin might be considered non-significant. However, the consistent presence of peaks on the same R_f zone or elution volume and/or the presence of peaks in the elution volume of known cytokinins was given due consideration.

The variability among the three samples presented as standard error (SE) does not incorporate the variability associated with the regression line from which the total values were estimated. To obtain an estimation of the variability about each standard curve a confidence band can be constructed. However, when the individual fraction values are to be determined from the standard curve and added together the confidence values so obtained cannot be summed, so an indication of the variability within each of the three samples cannot be provided.

One of the major drawbacks of the soybean callus bioassay (and of probably all cytokinin bioassays) is that the callus will respond differentially to the different cytokinins as noted with callus clone Bap (Fig 2.8). The difference in response appears to be a feature of the callus clone employed. Engelbrecht (1971) considered zeatin to be about 10 times as effective as kinetin and zeatin riboside in promoting soybean callus growth whereas van Staden and Papaphilippou (1977) showed that zeatin and zeatin riboside promoted the growth of their soybean callus clone to a similar extent.

Van Staden and Papaphilippou (1977) suggested that at low concentrations zeatin and zeatin riboside promoted callus growth to a greater extent than OGZ which is in agreement with the contention by Peterson and Miller (1977) that the threshold detection level for zeatin was less than for OGZ or OGZR. However, van Staden and Papaphilippou (1977) showed that OGZ was less toxic at higher concentrations than either zeatin or zeatin riboside. Wang *et al* (1977) showed that the response of callus to OGDZ was greater than to zeatin which was greater than to kinetin. However, only a narrow concentration range was tested.

Henson (1978) considered that zeatin and dihydrozeatin exhibited similar degrees of biological activity in the soybean callus bioassay while it has been reported that iP is less active than zeatin in the same bioassay (Miller, 1968).

In view of the differing responses reported to the different cytokinins it is apparent that to draw any sort of conclusion concerning differential stimulation of callus, each of the naturally occurring cytokinins should be tested under the specific culture conditions and with the callus clone in routine use. Unfortunately the vast majority of naturally occurring cytokinins are not available commercially.

In the subsequent analyses of wheat and potato extracts for cytokinin-like activity the following assumptions were made.

1. That any peaks of activity extending beyond the 99% confidence limit have a high probability of representing cytokinin-like activity. Peaks lying between the 95 and 99% confidence limits, or on R_f zones or elution volumes characteristic of authentic cytokinins, may represent cytokinin-like activity. Other peaks are probably the result of random fluctuations in callus growth.
2. That for the purpose of obtaining an estimate of the total cytokinin-like activity a non-significant response is regarded as representing a zero concentration of cytokinin as opposed to being reported more accurately as less than a minimum detectable level.
3. That the slope of the regression line prepared using kinetin is representative of the slopes of hypothetical regression lines for all the other endogenous cytokinins, implying, for example, that the relative levels of zeatin, zeatin riboside and their side chain glucosides are the same at all concentrations when estimated in kinetin equivalents.
4. That the nature of the callus used was consistent and did not change in relative sensitivity towards the different cytokinins throughout the analysis of any particular set of plant material.
5. That the amounts of the different cytokinin components reported in kinetin equivalents can indicate trends in cytokinin-like activity occurring during development.

However, at no time can the amounts of cytokinin-like activity reported in kinetin equivalents be considered as estimates of the absolute amounts of the different cytokinins present in potato or wheat tissue.

CHAPTER III

CHROMATOGRAPHY SYSTEMS

3.1 INTRODUCTION

Chromatographic procedures have been used routinely during the purification of cytokinin-containing extracts (see Horgan, 1978). Paper, thin-layer, column and gas chromatography (GC) have all been used extensively, and more recently high performance liquid chromatography was introduced (Carnes *et al.*, 1975). In this project, paper and column chromatography using Sephadex LH-20 (Armstrong *et al.*, 1969) have been used to purify extracts prior to bioassay.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Unless otherwise stated all chemicals used were of Analar (May and Baker) or Pronalys (BDH) grade and all solvents were distilled before use. Water was distilled at least once before use, and double-glass distilled water used with callus clones Bap and Kin (section 2.2.2) Dilutions were on a v/v basis with water and solutions prepared from solid compounds were on a w/v basis with water unless otherwise stated. Kinetin, zeatin, zeatin riboside, isopentenyladenine, 6-benzylamino-purine and α -naphthalene acetic acid were obtained from Sigma Chemical Company.

3.2.2 Paper chromatography

Known quantities of extract were strip-loaded onto Whatman 3 MM chromatography paper in bands 70 mm to 210 mm wide as described for individual experiments. The descending method of chromatography was employed using propan-2-ol-ammonia (SG = 0.91)-water (10:1:1) as solvent (solvent 1). Blank chromatograms, in addition to chromatograms loaded

with extract, were developed for approximately 350 mm before being removed from the solvent and dried in air at room temperature. Chromatograms were then sprayed lightly three times with distilled water and again dried. They were divided into 10 equal R_f strips and each strip cut into small pieces and added to a 100 ml Erhlenmyer flask. In addition to spraying the whole chromatograms with distilled H_2O , ca 2 ml distilled H_2O was added to each flask containing R_f strips of chromatograms from the Potato Field Trial at Lincoln (section 4.2.3) and Wheat Field Trial 1 (section 6.2.1.2). The flasks were dried on a hot plate at $37^{\circ}C$ under a stream of air prior to bioassay.

To determine the relative movement of zeatin and zeatin riboside under the above chromatography conditions 10 μg zeatin, or zeatin riboside, dissolved in 35% ethanol was strip-loaded in a 70 mm band and treated in the manner described above.

3.2.3 Column chromatography

Glass columns, 25 mm x 575 mm (Chromatronix), with adjustable adapters and water jackets were used for all low pressure liquid chromatography. A Techni TE-7 pump was used to draw water through the water jackets of the columns and to maintain the temperature of the water bath at $37^{\circ}C$. When two columns were in use their water jackets were linked in series. The columns were packed to a height of 475 ± 10 mm with Sephadex LH-20 (Pharmacia Fine Chemicals) pre-swollen in either 35 or 20% ethanol. The solvent used was degassed under reduced pressure, although degassing of the actual swollen Sephadex LH-20 prior to packing was not performed until samples from Wheat Field Trial 2 and PPD experiment 1 were being processed.

The solvent flow through the columns was maintained by gravity feed using a constant pressure flask filled with eluant and mounted 0.8 m above the siphon outlet.

The homogeneity of the gel bed was determined by applying 1 ml of Blue Dextran 2000 (Pharmacia Fine Chemicals) dissolved in 35 or 20% ethanol. The Blue Dextran was applied to the base of the column via a three-way valve inlet and eluted by upward flow. The band was monitored using an Isco Model UA-4 absorbance monitor with a Type 4 optical unit and built-in recorder. The UA-4 monitor was set to record percent transmission at a wavelength of 280 nm. The performance of the column was determined by the symmetry and width of the trace. 10 ml fractions were collected by an LKB automatic fraction collector.

3.2.3.1 Extracts of wheat or potato tissue were normally dissolved in 1.5 ml of the appropriate solvent and applied to the base of the column. The sample vial was washed with 2 x 0.5 ml of solvent and the washings were also loaded onto the column. The column was eluted in an ascending manner at a flow rate varying from 35 down to 25 ml h⁻¹ for potato and Wheat Field Trial 1 samples and between 50 and 40 ml h⁻¹ for samples from Wheat Field Trial 2. The 10 ml fractions were combined and transferred to 100 ml Erlenmeyer flasks. Each test tube was rinsed twice with the appropriate solvent and the washings added to the 100 ml flasks. The flasks were dried at 37°C under a stream of air.

Between sample applications the column was back-eluted with 1 L of the appropriate solvent. This eluant was also evaporated to dryness and bioassayed.

Even when upward flow elution is employed Sephadex LH-20 tends to become compressed and the flow rate decreases. Sample loading may also become uneven necessitating repacking of the column. Three samples from any one harvest were chromatographed sequentially and the column repacked, if necessary, only after elution of the third sample.

3.2.3.2 Resolution of cytokinin standards A 1.3 ml sample of kinetin (67 µg) was applied to the column and 10 ml fractions were collected and combined as indicated in Figure 3.2. A 1.0 ml mixture of isopentenyladenine and 6-benzylaminopurine (25 µg respectively) was treated similarly.

In July 1978 authentic zeatin and zeatin riboside were obtained. 1.0 ml samples of zeatin and zeatin riboside (100 µg respectively) were applied to the column. During analysis of the PPD experiment 1 potato extracts and the Wheat Field Trial 2 extracts, zeatin and zeatin riboside standards were applied to the column following each repacking. Elution volumes of zeatin and zeatin riboside for these experiments are indicated by bars drawn on the relevant graphs.

3.3 RESULTS

Both zeatin and zeatin riboside exhibited similar mobilities on paper in solvent 1 (Fig 3.1). Activity was located on the chromatograms between R_f 0.4 and 0.7.

Kinetin, isopentenyladenine and 6-benzylaminopurine eluted from

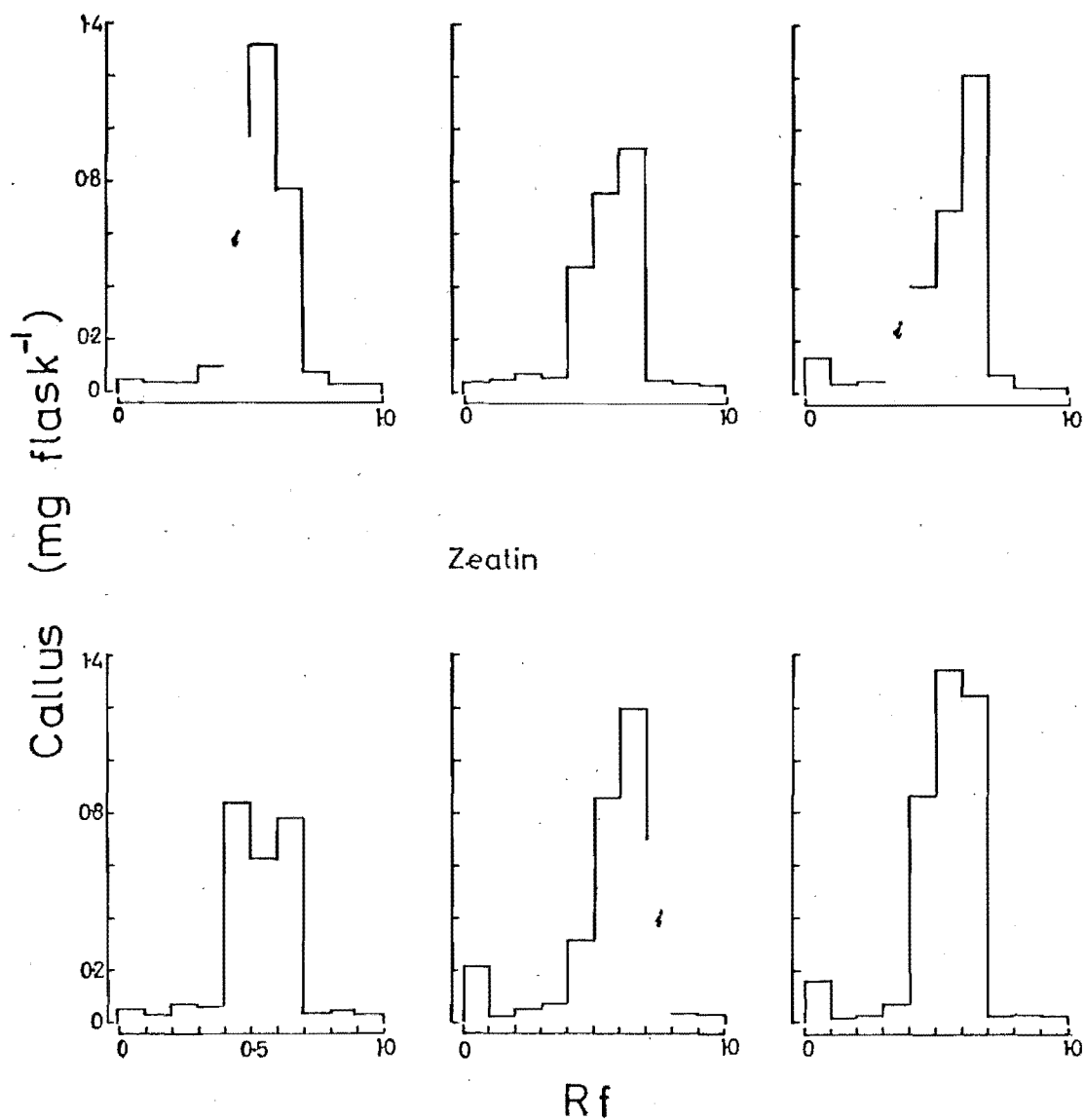


FIGURE 3.1

Mobilites of zeatin riboside and zeatin in propan-2-ol - ammonia - water (10:1:1 v/v) on Whatman 3 MM Chromatography paper . Compounds were detected by using the soybean callus bioassay

Sephadex LH-20 sequentially (Fig 3.2) and were all retained by the gel longer than either zeatin riboside or zeatin (Table 3.1). Zeatin riboside eluted from the column faster than zeatin.

TABLE 3.1

Soybean callus bioassays of authentic zeatin riboside (A) and zeatin (B) following partition chromatography on Sephadex LH-20 using 35% ethanol as eluant. 10 ml fractions were collected and combined as indicated

Fraction Numbers		Elution Volume (ml)	Callus Weight (mg flask ⁻¹)
<u>A</u>	1 - 3	10 - 30	35
Zeatin ribo- side	4 - 6	40 - 60	49
	7 - 9	70 - 90	44
	13 - 15	130 - 150	42
	16 - 18	160 - 180	40
	19 - 21	190 - 210	42
	22 - 24	220 - 240	920
	25 - 27	250 - 270	2418
	28 - 30	280 - 300	331
	31 - 33	310 - 330	24
<u>B</u>	1 - 2	10 - 20	36
Zeatin	3 - 5	30 - 50	121
	24 - 26	240 - 260	128
	27 - 29	270 - 290	114
	30 - 32	300 - 320	1006
	33 - 35	330 - 350	615
	36 - 38	360 - 380	115

The volume of the gel was less when swollen in 20% ethanol compared to 35% ethanol and the flow rate was faster when 20% ethanol was the eluant. UV absorbing compounds first eluted from the column in 90 ml (35% ethanol) or 80 ml (20% ethanol) volumes. For the purpose of establishing a base-line response in the bioassay the first 50 ml of eluant was considered to contain void only.

3.4 DISCUSSION

Initially samples of zeatin and zeatin riboside were unavailable so their approximate elution volumes from the column of Sephadex LH-20

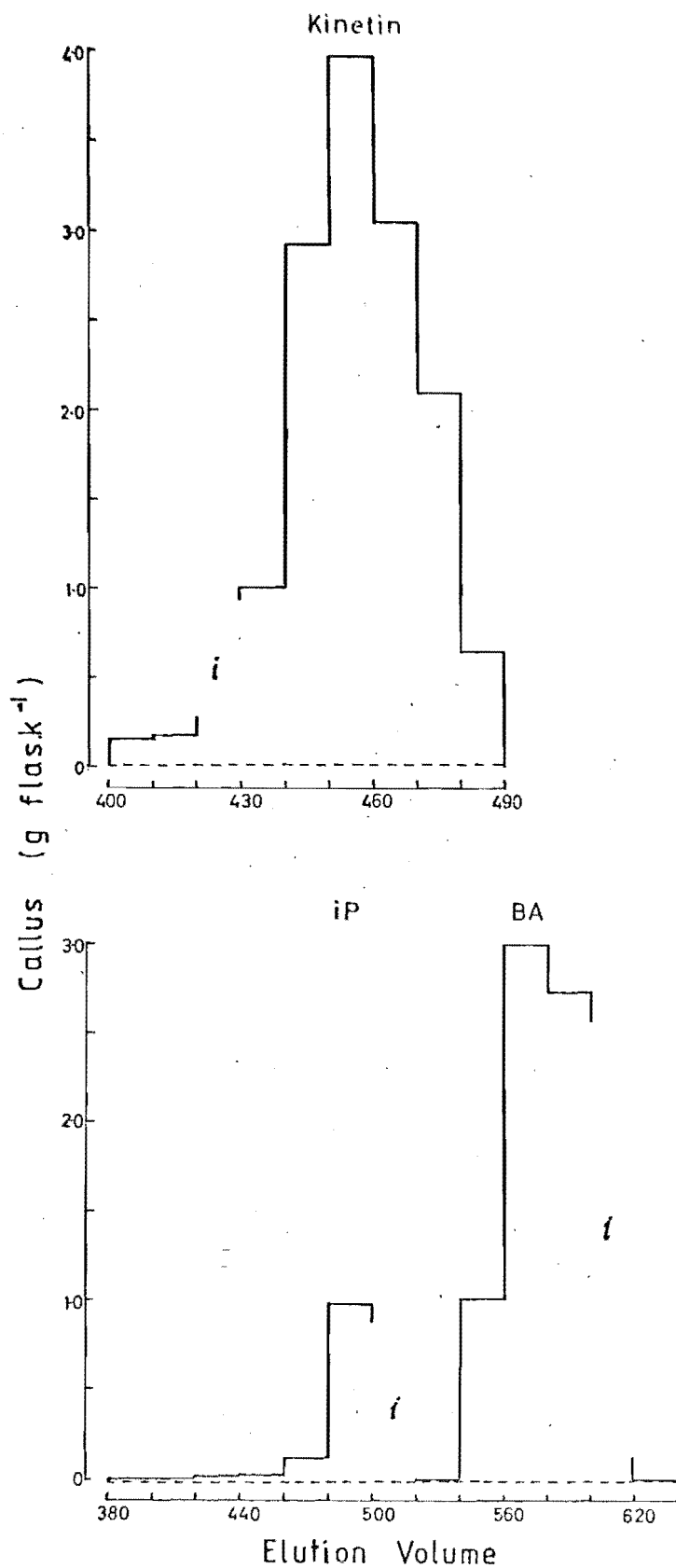


FIGURE 3.2

Elution volumes of kinetin, isopentenyladenine and 6-benzylaminopurine following partition chromatography on Sephadex LH-20 with 35% ethanol as eluant. Compounds were detected by using the soybean callus bioassay

were estimated using kinetin, 6-benzylaminopurine and isopentenyladenine and by reference to Armstrong *et al* (1969). As zone broadening was apparent, fractions were combined and 30 ml volumes were bioassayed in all subsequent experiments unless otherwise stated.

Further discussions of the chromatographic techniques employed are presented in the succeeding chapters.

CHAPTER IV

POTATO

4.1 INTRODUCTION

Following the classification outlined by Hawkes (1978) the potato species used all belong to Series XVIII TUBEROSA Rydb. The cultivated species in the series include *Solanum tuberosum* L. which is subdivided into two subspecies: *tuberosum*, the potato of commerce, and *andigena* (Juz. et Buk.) Hawkes. In the following chapters *S. tuberosum* refers to the subspecies *tuberosum* and to the cultivar Ilam Hardy, while *S. andigena* refers to a subspecies which has been backcrossed to *S. tuberosum* and is referred to as cultivar 165 (Bede, pers comm).

4.2 MATERIALS AND METHODS

4.2.1 Preliminary Experiments with Mature *S. tuberosum* Tubers

Group one seed potatoes (*S. tuberosum* cv Ilam Hardy) were supplied by R G Robinson & CO (Christchurch; 15/4/77). The tubers were subsequently stored at 4°C in the dark.

Tubers were scrubbed and washed in distilled water prior to 100 g of tissue being crushed in a Wareing blender in cold (-20°C) 80% methanol. The extract was transferred to a beaker and extraction continued at 4°C in the dark, the tissue being stirred in a total of 500 ml of solvent. After 24 hours the extract was filtered under reduced pressure through one layer of Whatman 3 MM filter paper and the residue washed with 80% methanol before being resuspended in a further 500 ml cold 80% methanol and extracted as above for another 24 hours. The extract was then filtered through Whatman 3 MM filter paper and the residue washed three times with 60 ml 80% methanol. All filtrates and washes were combined and the extract stored at -20°C prior to further use.

4.2.1.1 (7 June 1977) The extract was evaporated to dryness under reduced pressure at 35°C using a rotary film evaporator. The residue

was redissolved in 80% methanol and the extract subdivided into known fresh weight equivalents. The equivalents of 10 g, 5 g and 2 g fresh weight were strip-loaded onto Whatman 3 MM chromatography paper in 70 mm bands. The chromatograms were developed in the descending manner for 350 mm in solvent 1, propan-2-ol:ammonia:water (10:1:1). Two control chromatograms were also developed. (Refer to Section 3.2.2 for a more detailed description of paper chromatography procedures.)

4.2.1.2 (11 July 1977) A further 100 g mature tuber tissue was extracted by the procedure described above and the methanolic extract subsequently evaporated under reduced pressure at 35⁰C to the aqueous phase. The pH of the solution (200 ml) was adjusted to 7 using 1N NaOH and partitioned four times against equal volumes (200 ml) of water-saturated butan-1-ol over 24 hours. Both aqueous and organic fractions were evaporated to dryness under reduced pressure at 35⁰C. The aqueous-soluble residue was redissolved in 70% methanol and the butanol-soluble residue in butan-1-ol. The equivalents of 10 g and 2 g fresh weight of each residue were strip-loaded onto Whatmann 3 MM chromatography paper in 70 mm wide bands and the chromatograms developed in the descending manner for 350 mm in solvent 1. Each extract was run in duplicate and three control chromatograms were also developed.

4.2.1.3 (30 August 1977) Following extraction of 100 g mature tuber tissue (procedure described above) the extract was evaporated under reduced pressure at 35⁰C to the aqueous phase. The extract was divided in half and one half stored at -20⁰C. The pH of the remaining extract was adjusted to 9 using 1N NaOH and partitioned four times against half-volumes (60 ml) of petroleum ether (BP 40-60⁰C). The first partition was left at 4⁰C for 16 hours and the extract was passed through Whatman 1_S^P phase separation paper on the fourth partition. The petroleum ether phase was evaporated almost to dryness under reduced pressure at 30⁰C before being transferred into a 100 ml Erlenmeyer flask and evaporated to dryness on a hot plate at 37⁰C in a stream of air. The flask was stored at -20⁰C prior to bioassay.

The pH of the aqueous phase was readjusted to 7 with 1N HCl. The extract was partitioned four times against half-volumes (80 ml) of water-saturated butan-1-ol over 24 hours. Whatman 1_S^P phase separation paper was used during the separation of the third partition and the fourth partition was left for 16 hours at 4⁰C. Both aqueous and butanol phases were evaporated to dryness under reduced pressure at 35⁰C. The aqueous-soluble residue was redissolved in 70% methanol and the

organic in butan-1-ol. The equivalents of 10 g and 5 g fresh weight from the organic-soluble residue were strip-loaded onto Whatman 3 MM chromatography paper in 70 mm wide strips, while the 10 g fresh weight equivalent (10 g eq FW) of aqueous-soluble residue was strip-loaded in a 210 mm wide band. The chromatograms were developed in the descending manner in solvent 1.

Duplicate samples, each equivalent to 15 g fresh weight of organic-soluble residue, were evaporated to dryness and redissolved in 1.5 ml 35% ethanol. The samples were filter-centrifuged through a Sinta glass vial of No 3 porosity prior to column chromatography (Section 3.2.3.1).

4.2.1.4 (24 September 1977) Extract from 100 g of mature tuber tissue was evaporated to the aqueous phase and the pH reduced to 2.5 using 2 HCl. The extract was then filtered through Whatman 3 MM filter paper, the residue being retained for bioassay.

A 25 mm x 300 mm column of Dowex 50W-X8 ion exchange resin (dry mesh 50-100) was prepared in the H⁺ form (Appendix 4) and washed with 250 ml 70% methanol prior to application of the extract.

The equivalent of 80 g fresh weight of extract in 768 ml of aqueous solution was applied to the column. Elution of the column with cold solvents (4⁰C) followed the sequence listed below

1. 150 ml 70% methanol
2. 200 ml H₂O
3. 200 ml 2 N NH₄OH (0.91 SG Analar Grade)
4. 500 ml 5 N NH₄OH
6. 300 ml 5 N NH₄OH

Material not retained by the column when the extract was applied or when the column was eluted with 70% methanol or with water, were pooled (Fraction A). The material displaced from the resin by the first two ammonium hydroxide washes was also pooled (Fraction B) while the final 300 ml of 5N NH₄OH was kept separate (Fraction C).

Fraction B was divided in half and each half evaporated to dryness and the residue redissolved in methanol/NH₄OH (2N) (70:30 v/v). In one instances 2 g (in duplicate), 5 g and 10 g fresh weight equivalents were strip-loaded onto Whatman 3 MM chromatography paper in 90 mm wide bands. The loaded chromatograms and three control chromatograms were developed in the descending manner in solvent 1. In the second instance 5 g and 10 g fresh weight equivalents were evaporated to dryness under reduced pressure at 35⁰C, redissolved in 1.5 ml 35% ethanol, filter centrifuged and subjected to resolution by column chromatography (Section 3.2.3.1).

The remaining 20 g eq FW of methanolic tuber extract was evaporated to dryness and the residue redissolved in methanol/NH₄OH (2N) (70:30 v/v). The equivalents of 2 g (in duplicate) and 5 g fresh weight of extract were strip-loaded onto Whatman 3 MM chromatography paper in 90 mm wide bands and subjected to resolution by descending chromatography. A further 10 g eq FW of extract was evaporated to dryness, redissolved in 1.5 ml 35% ethanol and filter centrifuged prior to column chromatography.

4.2.2 Controlled Growth Rooms Experiments (Canterbury)

4.2.2.1 Ilam Hardy "Bolter" plants "Bolters" were selected from a field of Ilam Hardy plants being grown in mid-west Canterbury to produce "seed" potatoes. All "normal" plants in the field had died back but the "bolter" plants still displayed healthy deep green foliage (Dr Bedi, pers comm). Tubers from each of the eleven plants dug on 18/4/77 were bagged separately. On 11/5/77 selected tubers were washed in water containing a small amount of commercial Janola solution (3.6% active sodium hyperchlorite) and rewashed in running tap water. Tubers were placed on damp sterilised sand under a perspex chamber in the dark at about 24°C. Sprouting tubers were planted at a depth of 125 mm in PB8 bags (26/5/77) containing a modified UC Soil Mix II(c) (pers comm, D.J. Woolley; Appendix 5). Plants were placed in a growth room maintained at 20°C and with a 16 hour photoperiod (Appendix 6). 50 mls of a quarter-strength Hoaglund nutrient solution (Appendix 2) was added on alternative days five weeks from planting.

On 27/6/77 five plants were transferred to short day conditions (SD) with an eight hour photoperiod (Appendix 6). On 14 July it was noticed that the lights in the SD growth room were on continuously so when all plants were harvested on 15/8/77, plants in the SD growth room may have been subjected to only four weeks of SD. One plant was transferred from LD to SD on 18/7/77.

Harvesting involved removing the plants from the PB8 bags, washing roots and stolons in running tap water and excising stolon tips (*ie* tip plus 15 mm stolon) and tubers (*ie* tuber plus 10 mm unswollen stolon). Excised plant material was washed in ice-cold distilled water, weighed and placed in 80% methanol (-20°C). 4 g of stolon material and 5 g of tubers were extracted in 100 ml and 125 ml of solvent respectively. Plant material was fragmented after 24 hours using a test tube grinder and the extracts were stirred continuously for a further 48 hours in darkness at 4°C. After 24 hours the extracts were filtered through Whatman 3 MM filter paper and the residues resuspended in equivalent

amounts of solvent for a further 24 hours. The extracts were again filtered through Whatman 3 MM filter paper, and the residues washed with 80% methanol. The respective stolon or tuber filtrates and washes were combined and the methanol removed by evaporation under reduced pressure at 35°C.

The aqueous extracts were adjusted to pH 7 using 0.05 M NaOH and partitioned four times over 24 hours against half volumes (25 ml) of water-saturated butan-1-ol. Aqueous and organic phases were evaporated to dryness and subsequently redissolved in small amounts of 70% methanol and butan-1-ol respectively. The equivalents of 4 g fresh weight were strip-loaded onto Whatman 3 MM chromatography paper in 70 mm bands. The loaded chromatograms and three control chromatograms were developed in a descending manner in solvent 1.

4.2.2.2 *Solanum andigena* cv 165 Experiment 1 (Canterbury) Tubers of *S. andigena* were supplied by Dr Bedi, Crop Research Division, DSIR, Lincoln. The tubers had been harvested in late March from field plots in mid-west Canterbury. When obtained (7/7/77) the tubers were sprouting. Tubers were washed and cut into segments containing at least one developing "eye" and left 24 hours with the cut surface exposed to the air. Seed pieces were planted on 27/7/77, at a depth of 125 mm in PB8 bags containing a prepared soil mix (Appendix 5). A total of eleven seed pieces were planted, one piece per PB8 bag. Plants were maintained under LD conditions (16 hour photoperiod) at 20°C (Appendix 6). After 15 days (six days after shoot emergence) all plants were trimmed to one main stem and staked. Twenty-eight days after planting 50 ml of quarter-strength Hoaglund nutrient solution (Appendix 2) was supplied and subsequently applied regularly. Twenty-eight days after shoot emergence six plants were transferred to SD (eight hour photoperiod) at 20°C (Appendix 6). After 21 SD's (*ie* 49 days after shoot emergence) all plants were removed from the PB8 bags and roots and stolons washed under running tap water. Stolon tips (1.765 g) and tubers (4.710 g) were removed, washed in ice-cold distilled water and placed in 50 ml 80% methanol at -20°C for three hours. Tissue was ground with a mortar and pestle and extraction continued as described in Section 4.2.2.1.

The tuber extract was evaporated to the aqueous phase under reduced pressure at 35°C and subsequently divided into known fresh weight equivalents. The equivalent of 1.8 g fresh weight of tuber tissue was evaporated to dryness under reduced pressure at 35°C. The total stolon extract was similarly evaporated to dryness. The respective residues

were redissolved in 70% methanol (v/v) and evaporated to dryness on a hot plate at 37°C under a stream of air. The residues were solubilised in 1 ml 35% ethanol and filter centrifuged. The retained residue was washed with a further 0.5 ml solvent and the combined filtrate subjected to column chromatography (Section 3.2.3.1).

The volume of the remaining tuber extract (2.9 g eq FW) was increased to 50 ml with distilled water and the pH adjusted to 7 with 0.05 M NaOH. The extract was partitioned over 24 hours against four equal volumes of water-saturated butan-1-ol. Both aqueous and organic fractions were evaporated to dryness under reduced pressure at 35°C and the residues redissolved in 70% methanol and butan-1-ol respectively. The extracts were strip-loaded onto Whatman 3 MM chromatography paper in 90 mm wide bands. The loaded and two control chromatograms were developed for 350 mm by descending chromatography in solvent 1 (Section 3.2.2).

4.2.2.3 *Solanum andigena* cv 165 Experiment 2 (Canterbury)

S. andigena plants grown under prevailing field conditions in mid-west Canterbury were dug on 16/3/78. The tubers were washed and placed on moist towels in a plastic container which was placed in an unlit incubator at 28°C.

Sprouting tubers were removed from the incubator after six days and cut into segments, each segment bearing at least one developing eye, and left for 24 hours with the cut surface exposed to the air. The segments were planted singly at a depth of 125 mm in PB8 bags containing a prepared soil mix (Appendix 5). Thirty-three segments were planted on 23/3/78 and the PB8 bags placed in an unheated glasshouse until 4/4/78 when shoot emergence was first apparent. Plants were then transferred to a growth room maintained at 20°C and with a 16 hour photoperiod. Plants were trimmed to a single main stem and staked nine days after transfer. After 37 LD, 12 plants were transferred to SD (eight hour photoperiod) with 20°C day and night temperatures. All plants were examined for tuberisation 53 days after transfer to the controlled growth room.

4.2.2.4 *Solanum andigena* cv 165 Experiment 3 (Canterbury)

S. andigena plants growing under prevailing field conditions in mid-west Canterbury were dug on 26/3/79. The tubers were stored in the dark at room temperature for two months. Sprouting tubers were washed, cut into segments, and subsequently treated as described in Section 4.2.2.2. Two seed pieces were planted in prepared soil in each PB8 bag on 21/5/79.

Ten PB8 bags were placed in growth room A maintained at 20°C with a 16 hour photoperiod. Lighting was provided initially from fluorescent sources only (Appendix 6). Eight PB8 bags were placed in growth room B under a 16 hour photoperiod at 23°C. Lighting was provided from fluorescent and incandescent sources (Appendix 6). Each plant was trimmed to a single shoot 14 days after sowing (seven days after shoot emergence) and staked. All lateral shoots were removed four weeks after shoot emergence. Incandescent lights were placed in Room A five weeks after shoot emergence. Plants were harvested 35 and 49 days after shoot emergence and examined for the presence or absence of tubers.

4.2.3 Potato Field Trial (Lincoln, Mid-Canterbury)

Sprouting Ilam Hardy tubers (Group 1 seed; C 1693) were planted by hand by DSIR personnel (18/10/77) in ground that had been taken from pasture. Superphosphate had been applied at the rate of 377 kg per hectare prior to sowing. Plots were sown with 10 tubers per plot, 300 mm between tubers and 760 mm between parallel plots. Forty-three of the plots sown were assigned to the experiment. Each of these plots was randomly allocated a number from 1 to 43 inclusive. Plots were subjected to routine grubbing and moulding, and plants were sprayed with herbicide ("Sancur") when 10 cm high. Sprouting "seed" tuber material was analysed for cytokinin-like activity by the procedures described below.

The first harvest (25/11/77) was scheduled 10 days after 50% emergence (15/11/77). Subsequent harvest dates are listed in Table 4.1. Harvesting was carried out in as short a time as possible starting at 9.00 am and being completed normally by 11.00 am. The number of plots dug at each harvest is given in Table 4.1. Plants were dug carefully and wrapped individually in newspaper which was dampened thoroughly with distilled water. All plants from one plot were placed in one large plastic bag which was labelled with the plot number. Plants from each plot were assigned to one of three groups. Each group was subsequently processed independently. On return to the laboratory the bags of plants were placed in a cold room at 4°C and removed to room temperature only as required. Plants were washed carefully, stolon tips and tubers removed, counted and weighed after being grouped in the size categories described in Table 4.1. All plants were processed on the day of harvest.

Selected samples of fresh material were placed in 80% methanol (-20°C) as soon as practical after excision from the whole plant (Table 4.1). The tissue remained in the solvent (10 ml g⁻¹ tissue) at -20°C for approximately 15 hours before being ground with mortar and pestle. After

TABLE 4.1

Potato Field Trial. List of harvest dates, total number of rows harvested and size categories to which excised plant material was allocated

Harvest date	Number of plots harvested	Stolon tips	Size Category											
			All sizes	<7.5	<4	4 < 7.5	Tubers (mm radial diameter)			20 < 30	30 < 50	30 < 40		
							7.5 < 15	<15	7.5 < 20					
25/11/77	9	✓ ^a	✓											
29/11/77	9	✓ ^a	✓											
13/12/77	9	✓		✓				✓	✓					
20/12/77	12	✓			✓ ^a	✓ ^a		✓	✓					
3/1/78	1								✓ ^a	✓		✓		
17/1/78	3 plants							✓		✓	✓			✓ ^a

^a Weighed samples placed in 80% methanol at -20°C after excision (refer to text for details)

a further 24 hours, during which the extract was stirred at 4°C in the dark, the extract was filtered under reduced pressure through Whatman 3 MM filter paper. The extract was washed with a measured quantity of water followed by 80% methanol. This was repeated prior to resuspending the residue in the solvent (10 ml g⁻¹ tissue) and repeating the extraction described above for a further 24 hours. The extract was then filtered through Whatman 3 MM filter paper and the residue washed as described above. All filtrates and washings were combined and treated in one of two ways.

4.2.3.1 The extract was evaporated to the aqueous phase under reduced pressure at 35°C. The volume was increased to 50 ml with distilled water and the pH adjusted to 7 with 0.05 M NaOH. The extract was partitioned four times against an equal volume of water-saturated butan-1-ol at room temperature over 24 hours, the fourth partition being left overnight. Both aqueous and butanol fractions were evaporated to dryness under reduced pressure at 35°C. The residue was redissolved in methanol/NH₄OH (2N) (70:30 v/v), divided into the required fresh weight equivalents and evaporated to small volumes suitable for loading onto paper chromatograms. Small quantities of water-saturated butan-1-ol, in addition to methanol/NH₄OH (2N) (70:30 v/v), were used to ensure complete dissolution of all the residue in the organic fraction. Extracts from both the aqueous and organic fractions equivalent to 5 g and 2 g fresh weight were strip-loaded onto Whatman 3 MM chromatography paper. The 5 g eq FW of aqueous-soluble residue was strip-loaded in a 200 mm wide band and the remaining extracts in 90 mm wide bands. The chromatograms together with controls were developed in the descending manner for 350 mm in solvent 1.

4.2.3.2 The extract was evaporated to dryness under reduced pressure at 35°C and redissolved in methanol/NH₄OH (2N) (70:30 v/v). Measured quantities of extract were then evaporated to dryness on a hot plate at 37°C under a stream of air, redissolved in 1 ml 35% ethanol and filter centrifuged through a Sinta glass vial of No 3 porosity. The residue was washed with a further 0.5 ml solvent. The combined 1.5 ml of extract was applied to a column of Sephadex LH-20 and eluted with 35% ethanol.

4.2.4 Controlled Environment Experiments, Plant Physiology Division DSIR, Palmerston North

4.2.4.1A PPD Experiment One *S. andigena* plants which had been grown under prevailing field conditions in mid-west Canterbury were harvested 16/3/78. Tubers were stored in a potato storage shed at approximately 10°C until 25/7/78, when they were desprouted and placed in a cold room at 4°C. They were re-examined 3/8/78 and any further sprouts removed. On 6 January 1979 tubers were removed from the cold room and placed on damp paper towels within a plastic container. The container was placed in the dark at 27°C until sprout elongation was apparent. Tubers were then sent to Plant Physiology Division (DSIR), Palmerston North, where they were cut into segments (10/1/79), each segment bearing at least one developing eye. After being left for 24 hours with the cut surfaces exposed to the air, the tuber pieces were planted in a modified UC Soil Mix prepared six days in advance (Appendix 5). The pieces were planted at a depth of 125 mm with two pieces planted in each 9L pail. The soil mix was thoroughly moistened and the pails were placed in a heated glasshouse with daylight extension lighting from 0400 to 0800 and 1700 to 2100 hours. Temperature ranged from 15 to 25°C. Pails were checked daily and watered sufficiently to maintain the mix in a damp but well aerated condition.

When emergent shoots were 50 mm tall (25/1/79), plants were trimmed to two main shoots and transferred from the glasshouse to the controlled environment (CE) rooms. The day of transfer is referred to subsequently as Day 0 (25/1/79). Eight pails containing two plants per pail were placed in each CE room making a total of 96 plants per room. Three CE rooms were employed initially. All three rooms were run as LD rooms with a 16 hour photoperiod. The rooms were illuminated with eight hours of high intensity, photosynthetically active radiation and the four hours either side of this period with low intensity photoperiodically active radiation. (Refer to Appendix 7 for technical details.) The day temperature was set at 20°C, the night temperature at 16°C. The temperature changeover, day/night and night/day was two hours in duration. Day temperatures were programmed to be on only during the eight hours of high intensity lighting.

The plants' mineral nutrition requirements were maintained during the experiment by providing, through an automated microtubule system, 2 x 400 ml applications per pail per day of a quarter-strength Hoaglund A solution modified by the use of chelated iron (Appendix 2).

After 18 LD's the plants in one pail were examined for tuberisation. After 20 LD's two plants were examined. Harvest 1 took place after 21 LD's. Harvests 1 to 5 were at daily intervals; subsequent harvests were at increasing intervals until day 84 (Table 4.2). The number of pails examined at each harvest is listed in Table 4.2.

The trolleys in each CE room were randomly assigned a number from 1 to 6 inclusive and each of the eight pails per trolley was similarly assigned a number from 1 to 8 inclusive. At any one harvest three independent samples were collected, numbered pails being allocated to samples A, B and C by a predetermined sequence. At the end of each harvest period the remaining pails were rerandomised on their respective trolleys.

TABLE 4.2

PPD Experiment 1. Harvest schedule detailing the number of pails and plants harvested after a given time in the controlled environment rooms

Day Number	Number of pails	Number of plants
18	1	2
20	1	2
21	18	26
22	19	38
23	22	43
24	17	34
25	18	36
30	15	30
35	12	24
42	12	24
56	5	10
84	5	10

Harvesting entailed washing the roots free of the soil mix and excising the stolon tips and tubers from the plants. The material was then rinsed in ice-cold distilled water before the radial diameter of the tubers was measured and the plant material placed in the categories described below and listed in Table 4.3.

Stolon tips were grouped according to whether they were removed from plants forming tubers (Stolons (A)) or from plants not showing any signs of tuberisation (Stolons (NA)). Tubers of diameter less than 5 mm were divided into three categories. Tubers placed in the category

3 mm to 5 mm diameter were visibly well shaped tubers; those of diameter between 1.5 mm and 3 mm showed a definite sub-apical swelling and unfolding of the stolon hook while the third, less well-defined category (JA) were selected on the basis of "opaqueness" in the sub-apical region, but there was little visible swelling. Larger tubers were grouped as described in Table 4.3. The numbers of stolons or tubers in each category were recorded as were their fresh weights before the material was flash frozen in liquid air.

Only two plants (from one pail) were harvested at any one time and the sequence from cutting to flash freezing was performed in the minimum time possible. All material was stored below 0°C prior to freeze drying and reweighing to obtain dry weights. Subsequently storage of tissue was at room temperature. At selected intervals, root, stem and leaf material was similarly treated.

4.2.4.1B Analysis of tissue for cytokinin-like activity

1. For all size categories the equivalent of 4 g fresh weight (4 g eq FW) of tissue was extracted (Table 4.3). With the larger tuber sizes it would have been necessary to cut tubers and hence work with a small sample population to obtain 4 g eq FW. In these instances, larger samples were ground and 4 g eq FW removed from the total sample (Table 4.3). Three independent samples were extracted for all but the two largest size categories (Table 4.3). In these instances one large sample was ground and three portions of 4 g eq FW removed and extracted.

The 4 g eq FW freeze-dried tissue was ground by hand using a mortar and pestle and added to 50 ml 80% methanol previously stored at -20°C. The extract was left, stirring, in the dark at 4°C for 24 hours and then filtered under reduced pressure through Whatman 3 MM filter paper. The residue was washed with 10 ml 80% methanol followed by 10 ml of distilled water before being resuspended in 40 ml of cold 80% methanol and extracted under the above conditions for a further 24 hours. The extract was again filtered through Whatman 3 MM filter paper and the residue washed with 15 ml 80% methanol and 15 ml distilled water. Filtrates and washes were combined and the extract evaporated to dryness at 35°C under reduced pressure using a rotary film evaporator. The residue was redissolved in methanol/NH₄OH (2N) (70:30 v/v) and evaporated to dryness on a hot plate at 37°C in a stream of air. The residue was redissolved in 2 ml 35% ethanol and centrifuged for 30 minutes at 3,000 g. The supernatant was decanted and the pellet resuspended in 1 ml 35% ethanol and recentrifuged. This procedure was repeated. Both washes

TABLE 4.3

PPD Experiment 1. Harvest schedule listing the categories excised stolon and tuber material were allocated to, the number of stolons or tubers ground prior to extraction and the fresh weight of material extracted.

Size Category	Sample	Number of stolons or tubers	Fresh weight (g)	Equivalent fresh weight extracted
Stolons (NA)	A	197	3.983	3.983
	B	237	4.052	4.052
	C	114	4.002	4.002
Stolons (A)	A	210	4.022	4.022
	B	218	4.010	4.010
	C	190	4.003	4.003
JA	A	225	4.041	4.041
	B	206	4.082	4.082
	C	179	3.993	3.993
1.5 < 3	A	148	3.966	3.966
	B	146	3.992	3.992
	C	155	3.979	3.979
3 < 5	A	65	3.948	3.948
	B	66	4.024	4.024
	C	67	4.015	4.015
5 < 7.5	A	17	3.983	3.983
	B	17	3.922	3.922
	C	23	4.005	4.005
7.5 < 10	A	8	4.030	4.030
	B	7	4.232	4.232
	C	8	4.085	4.085
10 < 15	A	4.5	5.096	4.00
	B	4	4.591	4.00
	C	5	5.104	4.00
15 < 20	A	4	12.895	4.00
	B	3	9.650	4.00
	C	3	8.625	4.00
20 < 25		5	30.449	3 x 4.00 ^a
40 < 50		16	974.40	3 x 4.00 ^a

^a Replicates are not independent

and supernatant were combined and evaporated to dryness on a hot plate at 37°C in a stream of air. The extract was stored at -20°C until required when it was dissolved in 1.5 ml 35% ethanol and applied to a column of Sephadex LH-20 (Section 3.2.3.1).

2. Stolons from tuberising plants and five size categories of tubers were extracted and bioassayed for cytokinin-like activity (Table 4.4). Only one sample, equivalent to 8 g fresh weight, was extracted from each size category (Table 4.4). The extraction procedure was as described in the preceding section. However, the extracts were resolved by column chromatography using 20% ethanol as the eluant.

TABLE 4.4

PPD Experiment 1. Extraction schedule listing the number of stolons or tubers ground prior to extraction, and the fresh weight and dry weight of material extracted

Size Category	Number of Organs	Fresh Weight (g)	Equivalent Dry Weight (g)
Stolons (A) ^a	393	8.0	0.62
Tubers (mm diam)			
1.5 < 5	173	8.2	0.76
5 < 7.5	39	8.0	0.59
7.5 < 10	15.5	8.0	1.04
10 < 15	5.5	8.0	1.17
15 < 20	1.6	8.0	1.37
20 < 25	1.3	8.0	1.18

^a Stolons removed from tuber forming plants

4.2.4.1C Soluble Sugar and Starch Analyses The analysis of potato tissue for soluble sugar and starch content followed the procedure outlined by Haslemore and Roughan (1976). All analyses were performed at Plant Physiology Division, DSIR, Palmerston North under the supervision of Dr R. Haslemore.

Freeze-dried plant material of known weight and tuber number was ground to a fine powder using a mortar and pestle, before small quantities were removed from each sample for analysis (Table 4.5). Analysis of each sample was performed in triplicate unless otherwise indicated.

1. Soluble Sugar Analysis The dried plant material was extracted

TABLE 4.5

PPD Experiment 1: Starch and Soluble Sugar Analyses. Details of the analyses showing the dry weight, fresh weight and number of stolons or tubers ground per sample, and the dry weight extracted per replicate. The volumes to which the starch hydrolysates were diluted and the aliquots removed for incubation with glucose oxidase are also indicated

Size Category ^a	Stolon/ tuber number	Amount of material ground per sample		Dry Weight Extracted (mg)	Volume hydrolysate diluted to (ml)	Aliquot tested for starch (μ l)
		FW (g)	DW (g)			
Stolons (NA)	52	1.242	0.100	50.1	10	250
Stolons (A)	303	7.508	0.680	51.6 53.4 52.3	" " "	100 " "
JA	139	4.422	0.394	51.5 50.9 58.7	" " "	" " "
1.5 mm < 3 mm	196	4.527	0.524	57.4 53.1 50.3	" " "	50 " "
3 mm < 5 mm	98	4.901	0.650	54.2 52.9 53.1	" " "	" " "
5 mm < 7.5 mm	27	3.936	0.698	55.0 51.5 52.5	" " "	250 " "
7.5 mm < 10 mm	8	3.291	0.538	51.4 52.9 50.8	" " "	" " "
10 mm < 15 mm ^b	3	3.565	0.571	56.6 50.8 51.2	" " "	" " "
15 mm < 20 mm	3	10.022	1.890	52.4 52.2 56.8	" " "	" " "
25 mm < 30 mm	3	38.077	6.661	51.9 52.6 53.1	" " "	" " "
35 mm < 40 mm	3	81.689	13.318	58.4 52.8 53.2	" " "	" " "
40 mm < 50 mm	3	165.313	32.453	52.9 52.7 55.1	" " "	" " "
Stolons (NA) (from PPD Experiment 2)	-	-	0.144	40.2 37.5	" "	" "
10 < 15 ^b				100.1 100.7	50 "	100 "

^a See Section 4.2.1.4A for details

^b Aberrant result, extraction repeated

with 10 ml 62.5% methanol for 15 minutes at 55°C using screw-capped culture tubes with Teflon-faced caps. The samples were centrifuged before 4 ml aliquots were transferred to similar tubes each containing 0.1 ml of a neutral saturated aqueous solution of lead acetate. The pellet was retained for subsequent starch analysis. Standards were prepared by diluting 0.5 ml, 1.0 ml and 1.5 ml of the sucrose standard solution (10 mg ml⁻¹ in 65.5% methanol) to 10 ml with 62.5% methanol. Aliquots of 4 ml were then removed from the standards and treated in the same manner as the plant samples to give standards equivalent to 5, 10 and 15% soluble sugars on a dry weight basis respectively. A control of 4 ml 62.5% methanol was also treated similarly. Samples, standards and control were shaken twice with the lead acetate solution over a period of 10 minutes before 5 ml chloroform was added. The solutions were vigorously shaken and centrifuged to aid phase separation. Aliquots of 50 µl were removed from the aqueous phase and added to 0.95 ml water. 1 ml 5% phenol (w/v) was added and the solutions mixed before the addition of 5 ml concentrated H₂SO₄ (SG 1.84). The acid was added by pipette and care was taken to direct the stream of acid directly onto the surface of the liquid to aid mixing as recommended by Dubois *et al* (1956). Samples were left at room temperature for 60 minutes to cool and the absorbances were read at 490 nm.

2. Starch Analysis The pellet of plant material remaining following soluble sugar extraction was resuspended in 4 ml methanol and heated at 100°C for five minutes. Following centrifugation the washings were aspirated and discarded and the process repeated. Starch standards were prepared from a 0.2% solution of soluble starch (BDH) in water. 2 ml starch solution were added to 2 ml water.

The methanol-washed plant residue was suspended in 4 ml water and samples and starch standards were heated for 60 minutes at 100°C to gelatinise the starch. When the solutions had cooled, 2 ml 0.25 M sodium acetate buffer (pH 4.5) was added. A solution of purified amyloglucosidase (Haslemore and Roughan, 1976) in 25 mM sodium citrate (pH 6.0) containing 2 to 3 mg protein ml⁻¹ was then added (0.1 ml) and the solutions incubated for 60 minutes at 55°C. Samples were finally diluted with water to 10 or 50 ml as indicated in Table 4.5 and starch standards to 10 ml. Samples and starch standards were then thoroughly mixed and centrifuged.

Glucose was determined by incubating aliquots of the diluted hydrolysates (Table 4.5) in a final volume of 1 ml water with 2 ml glucose

oxidase reagent (Haslemore and Roughan, 1976) at 37°C for 60 minutes. Glucose standards were prepared from a stock solution of 1 mg ml⁻¹ glucose in water: 25, 50, 75 and 100 µl aliquots were treated as above to give standards equivalent to 25, 50, 75 and 100 µg glucose. A control of water only was also prepared. 5 ml hydrochloric acid (5 M) was then added and the solutions thoroughly mixed. Absorbances were subsequently read at 540 nm.

4.2.4.2 PPD Experiment 2 Tubers from field grown *S. andigena* plants were harvested on 20/2/79 and forwarded to Plant Physiology Division (DSIR), Palmerston North, where they were placed on damp paper towels at approximately 20°C for one week. The procedure followed for cutting and planting of tuber segments was that used in PPD Experiment 1. Seed pieces were planted on 28/2/79 and entry to CE rooms (criteria and pre-treatment as in Experiment 1) was on 23/3/79 referred to as Day 0.

Three CE rooms were employed and maintained under the following conditions:

- (1) 8 hours high intensity lighting plus 8 hours photoperiodic extension lighting; 20°C day and 20°C night temperatures.
- (2) 8 hours high intensity lighting plus 8 hours photoperiodic extension lighting; 20°C day and 16°C night temperatures. After 38 LD's the 8 hours extension lighting was discontinued, *ie* the CE room was changed to SD conditions.
- (3) 16 hours high intensity lighting: 20°C day and 20°C night temperatures.

Day/night temperature changeover, lighting details and mineral nutrient application were as described in Controlled Environment Experiment 1 (Section 4.2.4.1A and Appendix 7).

On Day 22 (13/4/79) eight pails from each of the three rooms were harvested. The harvest schedule is detailed in Table 4.6 and the harvest performed as described in PPD Experiment 1.

TABLE 4.6

PPD Experiment 2. Harvest schedule detailing the number of pails (and plants) harvested after a specified time in each of the Controlled Environment rooms

Day Number	Room No	Number of pails (Number of plants)		
		1	2	3
21		8 (14)	8 (15)	8 (13)
28		-	-	8 (12)
42		8 (16)	8 (14)	-
56		8 (16)	8 (14)	-

4.3 RESULTS

4.3.1 Preliminary Experiments with Mature *S. tuberosum* Cultivar Ilam Hardy Tubers

4.3.1.1 The presence of cytokinin-like activity in a methanolic extract of mature tuber tissue was indicated by a positive bioassay response (Fig 4.1). Peaks of activity lying beyond the 99% confidence limit were considered significant. Cytokinin-like activity was observed in bioassays of 2 g and 5 g fresh weight equivalents of tuber tissue but not of 10 g (Fig 4.1). The observed activity was not confined to a single R_f zone on the paper chromatogram nor was the major peak of activity consistently located at the same R_f . The amount of activity lay between 1 and 10 $\mu\text{g KE l}^{-1}$. A loss of detectable activity when 10 g of extract were bioassayed was also noted in a previous experiment but this bioassay was affected by solvent residue on the chromatograms and the results are not presented. Both chromatograms carrying 10 g eq FW were obviously overloaded when visualised under long wavelength (354 nm) ultraviolet light.

To obtain a consistent bioassay response it was found necessary to spray the paper chromatograms several times with a fine mist of distilled water, allowing the chromatograms to re-dry between sprayings. Failure to do this resulted in the callus browning, though not necessarily dying, within a few days after the commencement of the bioassay. Callus in flasks containing the standard kinetin concentrations showed no discolouration within this time.

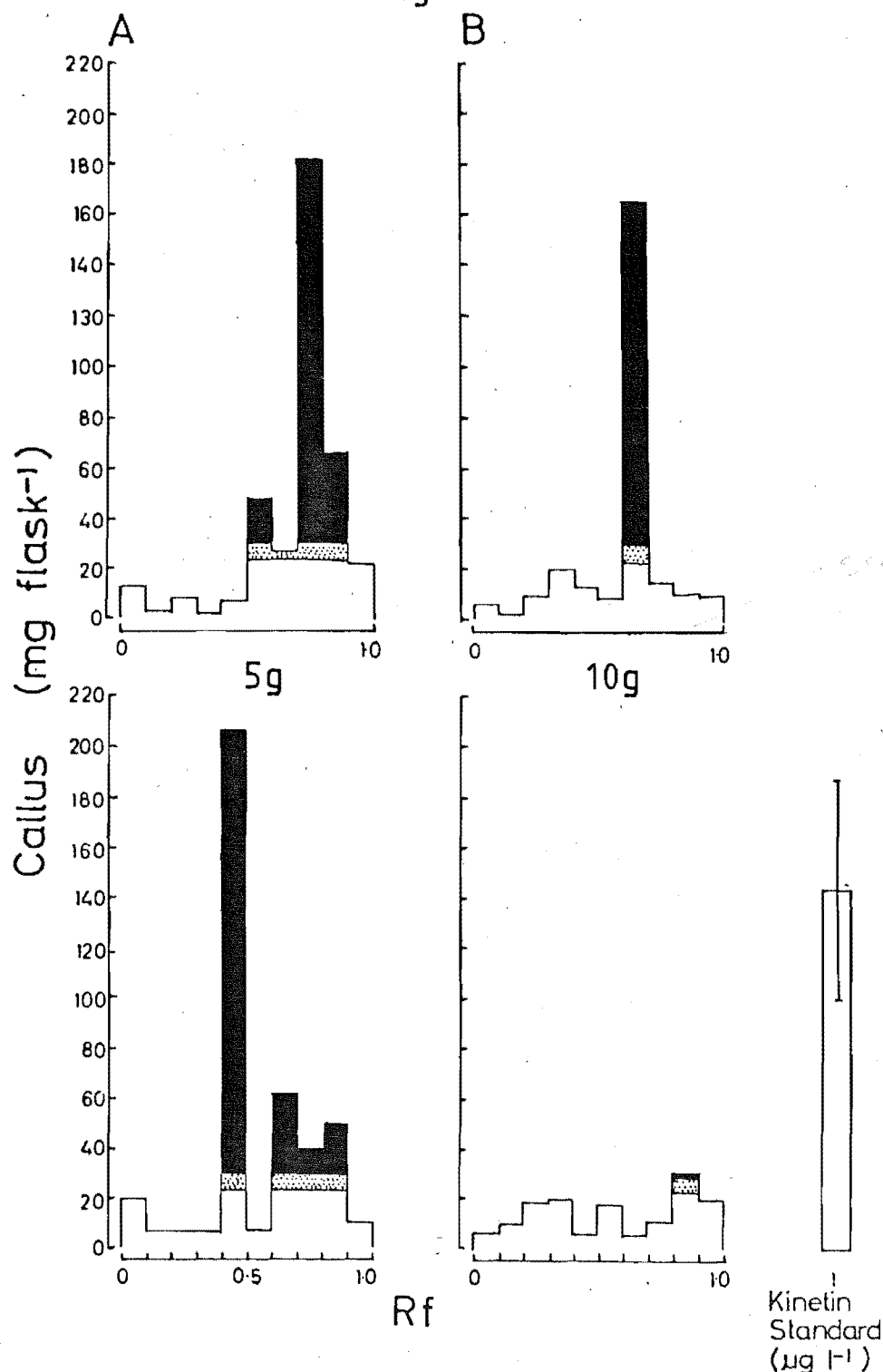


FIGURE 4.1

Soybean callus bioassay of paper chromatograms loaded with methanolic extract of mature tuber tissue

2 g (2 replicates, A and B), 5 g and 10 g fresh weight equivalents were bioassayed.

Solvent: propan-2-ol - ammonia - water (10:1:1 v/v)

Callus: Kit A (4) (4 representing the number of sub-cultures)

Confidence limits: $n = 20$ (n being the number of values the intervals were constructed from)

Stippled area represents values lying between the 95 and 99% confidence limits. Shaded area represents values extending beyond the 99% confidence limit. A kinetin standard is indicated. The vertical bar represents twice the standard error of the mean.

4.3.1.2 When the mature tuber extract was reduced to the aqueous phase and partitioned against butan-1-ol at pH 7, the majority of the cytokinin-like activity was found in the organic phase (Fig 4.2). The sensitivity of the callus was significantly reduced compared to the experiment described above and cytokinin-like activity present in the organic phase from 2 g eq FW of tissue was barely detectable (Fig 4.2). However, a significant response was obtained from the bioassay of 10 g eq FW of tuber tissue. Activity was located on R_f zones of 0.5 to 0.8 in both replicates but in no zone did the activity exceed that obtained from the $10 \mu\text{g l}^{-1}$ kinetin standard (Fig 4.2).

All chromatograms were observed under long wavelength ultraviolet light prior to bioassay. Chromatograms loaded with 10 g eq FW of aqueous-soluble material showed distinct signs of overloading; those with an equivalent amount of the butanol-soluble material appeared only slightly overloaded. The majority of activity, however, was located on that region of the chromatogram to which zeatin and zeatin riboside were shown to migrate under similar chromatography conditions (Fig 3.1).

4.3.1.3 Bioassay of material which partitioned into the petroleum ether phase revealed neither detectable cytokinin-like activity nor callus blackening (Table 4.7). However, when the extract was partitioned against petroleum ether (pH 9) and then butan-1-ol (pH 7) and the organic-soluble material passed through a column of Sephadex LH-20, blackening of the callus in the subsequent bioassay indicated that inhibitory compounds were present in fraction 7 (Table 4.7).

Cytokinin-like activity was detected in fraction 9 in both replicates; callus growth being similar to that observed on the $5 \mu\text{g l}^{-1}$ kinetin standard (Table 4.7). Paper chromatograms were loaded with extract from both butanol and aqueous phases. However, the response of the callus to both control and loaded chromatograms was extremely variable and no conclusions can be drawn from the data (results not presented).

4.3.1.4 An attempt to purify the mature tuber extract by passing the extract through Dowex 50 (H^+ form) cation exchange resin resulted in an apparent loss of cytokinin-like activity (Figs 4.3, 4.4).

Significant cytokinin-like activity was detected following paper chromatography and bioassay of both 2 g and 5 g eq FW of unpurified methanolic extract (Fig 4.3). The chromatogram carrying 5 g eq FW showed slight signs of overloading when visualised under long ultraviolet wavelengths. The most prominent peak of cytokinin activity lay

FIGURE 4.2

Soybean callus bioassay of paper chromatograms loaded with aqueous and butan-1-ol soluble components of mature tuber tissue extract

2 g and 10 g fresh weight equivalents were bioassayed.

Solvent: propan-2-ol-ammonia-water (10:1:1 v/v)

Callus: Kit A (5)

Confidence limits: $n = 30$

Shading and standards as in Figure 4.1

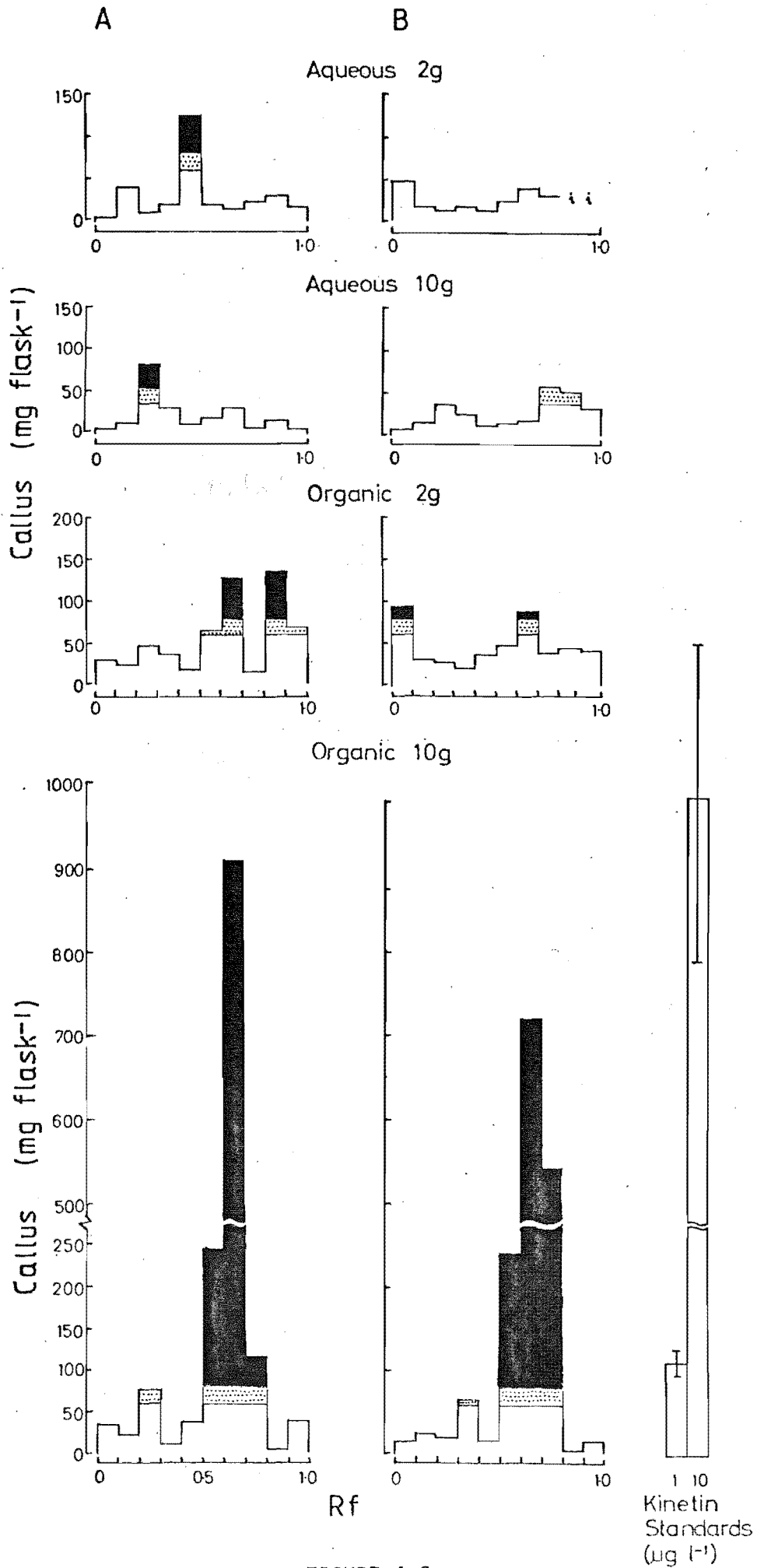


FIGURE 4.2

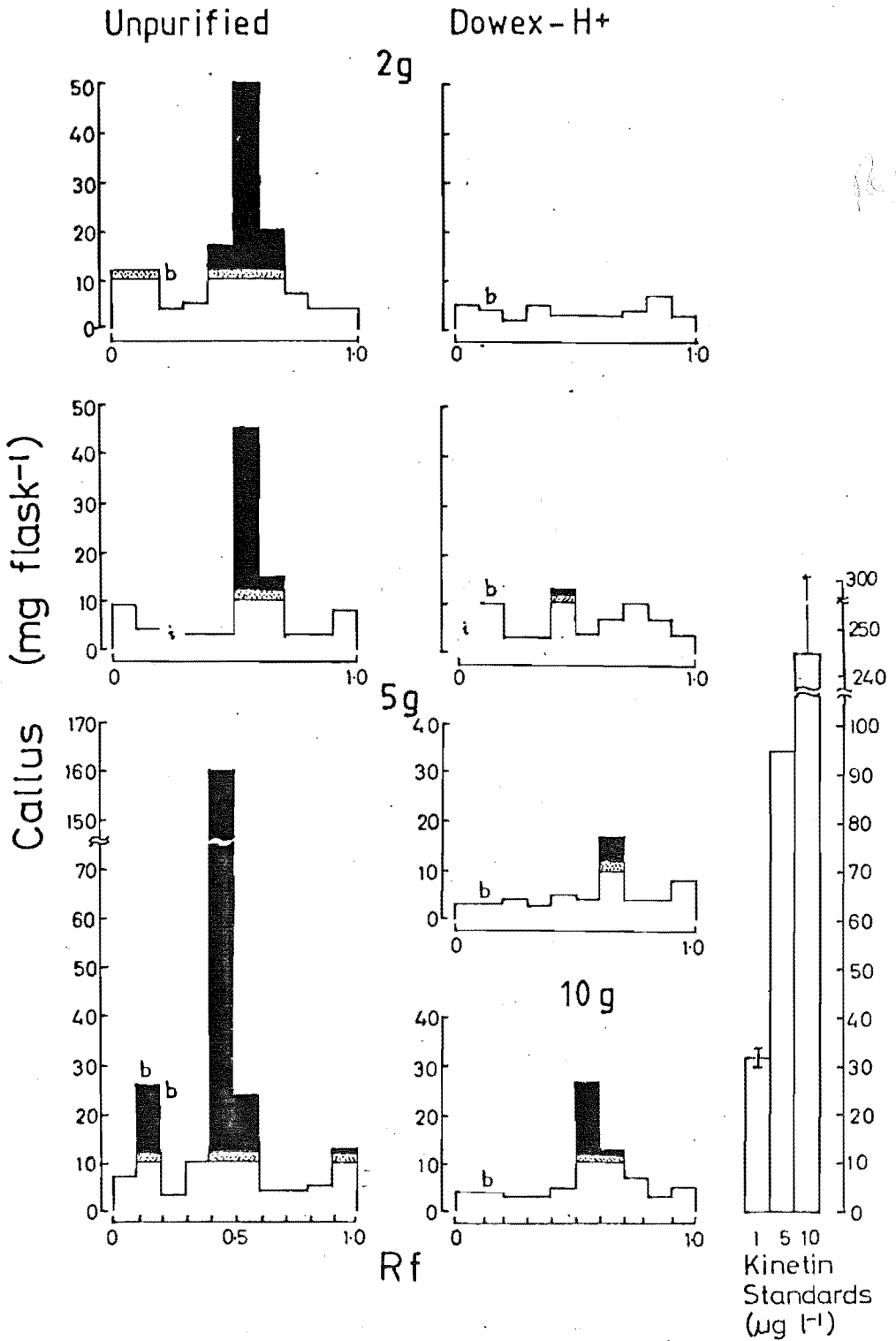


FIGURE 4.3

Soybean callus bioassay of paper chromatograms loaded with methanolic extract and extract partially purified by Dowex 50 (H⁺ form) cation exchange chromatography

- b: callus blackened
- Solvent: propan-2-ol - ammonia - water (10:1:1 v/v)
- Shading as in Figure 4.1

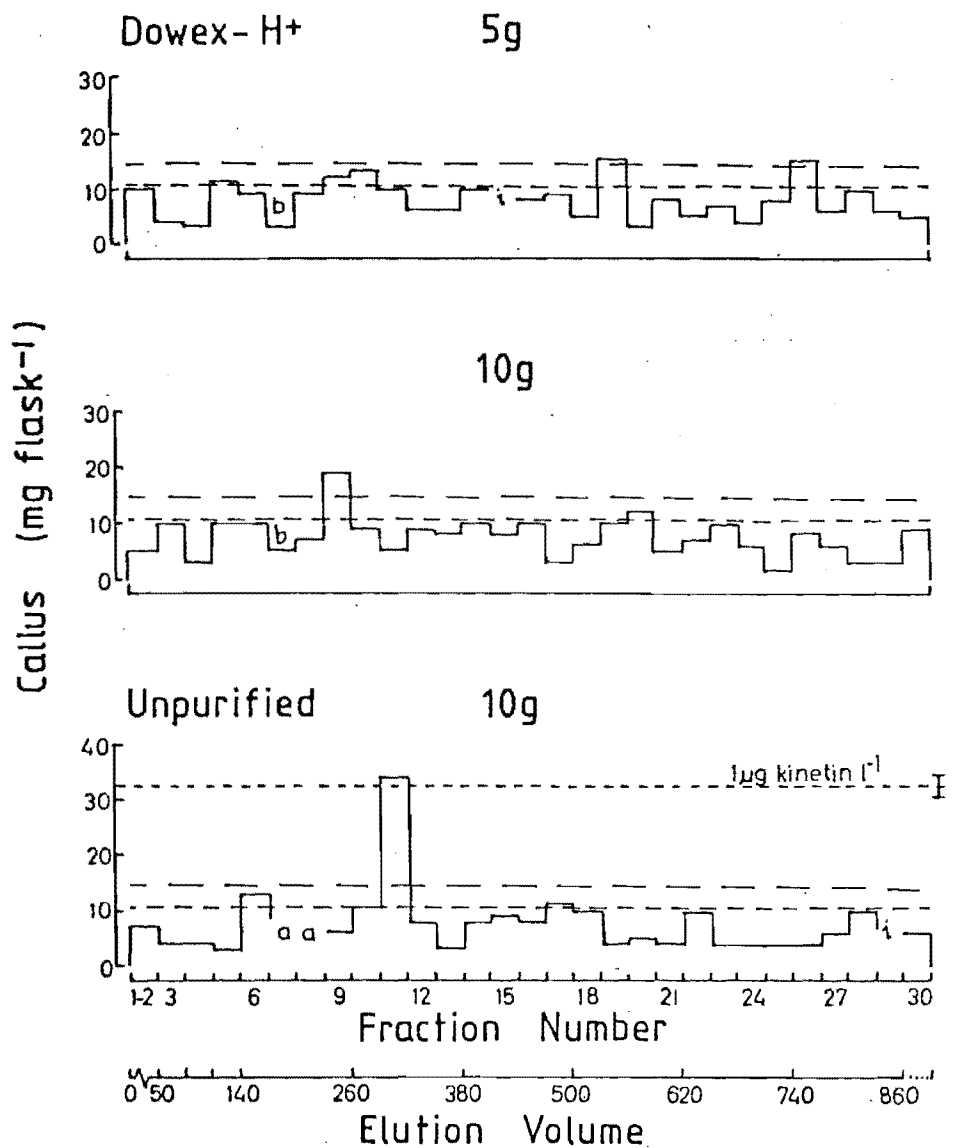


FIGURE 4.4

Soybean callus bioassay of methanolic extract and of extract partially purified by Dowex 50 (H⁺ form) cation exchange chromatography. Both extracts were resolved by partition chromatography on Sephadex LH-20

a: agar medium not set

b: callus blackened

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fractions 1-2 (50 ml) and fraction 30 (40 ml)

Callus: Kit A (9)

Confidence limits: n = 29

99% limit — — —

95% limit - - - - -

Kinetin standards: as shown on the figure and also presented with Figure 4.3

between R_f 0.4 and 0.5 while the major peak of activity on chromatograms to which only 2 g eq FW was applied lay between R_f 0.5 and 0.6. Small peaks of activity were also present at R_f 0.1 to 0.2 and 0.9 to 1.0 on chromatograms carrying the equivalent of 5 g fresh weight. Callus blackening was observed at R_f 0.2 to 0.3.

TABLE 4.7

Soybean callus bioassay of petroleum ether- and butan-1-ol-soluble components of potato tuber extract. The butanol-soluble components were resolved by partition chromatography on Sephadex LH-20

Solvent	Fraction	Colour of Callus	Callus FW ^a (mg flask ⁻¹)
Petroleum ether	-	cream	6
Butan-1-ol:			
Replicate A	6	cream	5
	7	black	3
	8	cream	3
	9	cream	32
	10	cream	3
Replicate B	6	cream	4
	7	black	3
	8	cream	3
	9	cream	31
	10	cream	3

Callus: Kit A (6)

^a Standards:	Kinetin ($\mu\text{g l}^{-1}$)	Callus (mg flask ⁻¹ ISE)
	0	2.7 ± 0.3
	1	13 ± 3
	5	29 ± 5

Little cytokinin-like activity was detected in extract applied to paper chromatograms following Dowex 50 (H^+ form) ion exchange treatment. Callus blackening was noted on all chromatograms between R_f 0.1 to 0.2 and the activity apparent in 10 g eq FW was less than $1 \mu\text{g l}^{-1}$ kinetin equivalent (Fig 4.3).

A similar reduction in detectable activity was also observed following column chromatography and bioassay of extract subjected to Dowex 50 (H^+ form) ion exchange treatment (Fig 4.4). Inhibitory material was again apparent in fraction 7 and low levels of cytokinin-like

activity were present in fractions 9 and 10. The bioassay of extract not subjected to ion-exchange chromatography revealed a higher total level of cytokinin-like activity but the activity was present in fractions 10 and 11 and not in fraction 9. The media containing fractions 7 and 8 respectively did not solidify.

Material not retained by the ion-exchange resin was incorporated into the basal medium. The medium did not solidify following sterilisation and the material was discarded.

4.3.2 Controlled Growth Room Experiments (Canterbury)

4.3.2.1 Ilam Hardy "bolter" plants Tubers were collected from Ilam Hardy "bolter" plants grown in the field to provide a possible source of short day obligate potato plants (Bedi, pers comm). The plants, grown from the selected tubers under controlled growth room conditions (Appendix 6) were tall, with weak stems and small leaves. On the day of harvest plants had been exposed to either 56 long days or 28 long days followed by 28 short days.

All five plants transferred to short days had formed tubers. Tuber sizes ranged from a few mm to 17 mm diameter. Plants remaining in long days had formed longer, more numerous stolons, some of which had reverted to an upright growth habit. Under long day conditions, one "bolter" plant had formed large tubers (17 mm and 25 mm diameter) while two further plants had barely detectable swellings on a few stolon tips. When tested with I/KI solution positive starch tests were observed. These plants were considered to be tuberising, indicating that three of the eight "bolter" plants were capable of forming tubers within 56 LD, *ie* under a 16 hour photoperiod.

Tubers from SD plants and stolons from LD plants were extracted and bioassayed for cytokinin-like activity. Too few stolon tips were present on the SD plants for cytokinin analysis. Following partitioning against butan-1-ol at pH 7, the majority of the cytokinin-like activity present in the tubers excised from SD plants appeared to stay in the aqueous phase and remain on the origin after chromatography in solvent 1 (Fig 4.5). Cytokinin-like activity partitioning into the organic fraction was located at R_f 0 to 0.1, 0.3 to 0.4, 0.5 to 0.6 and 0.8 to 1.0.

Substantially less total cytokinin-like activity was detected in stolons excised from plants maintained under LD conditions (Fig 4.5). No activity was detected in the aqueous fraction, although activity was located at R_f 0.3 to 0.4, 0.5 to 0.6 and 0.8 to 0.9 following chroma-

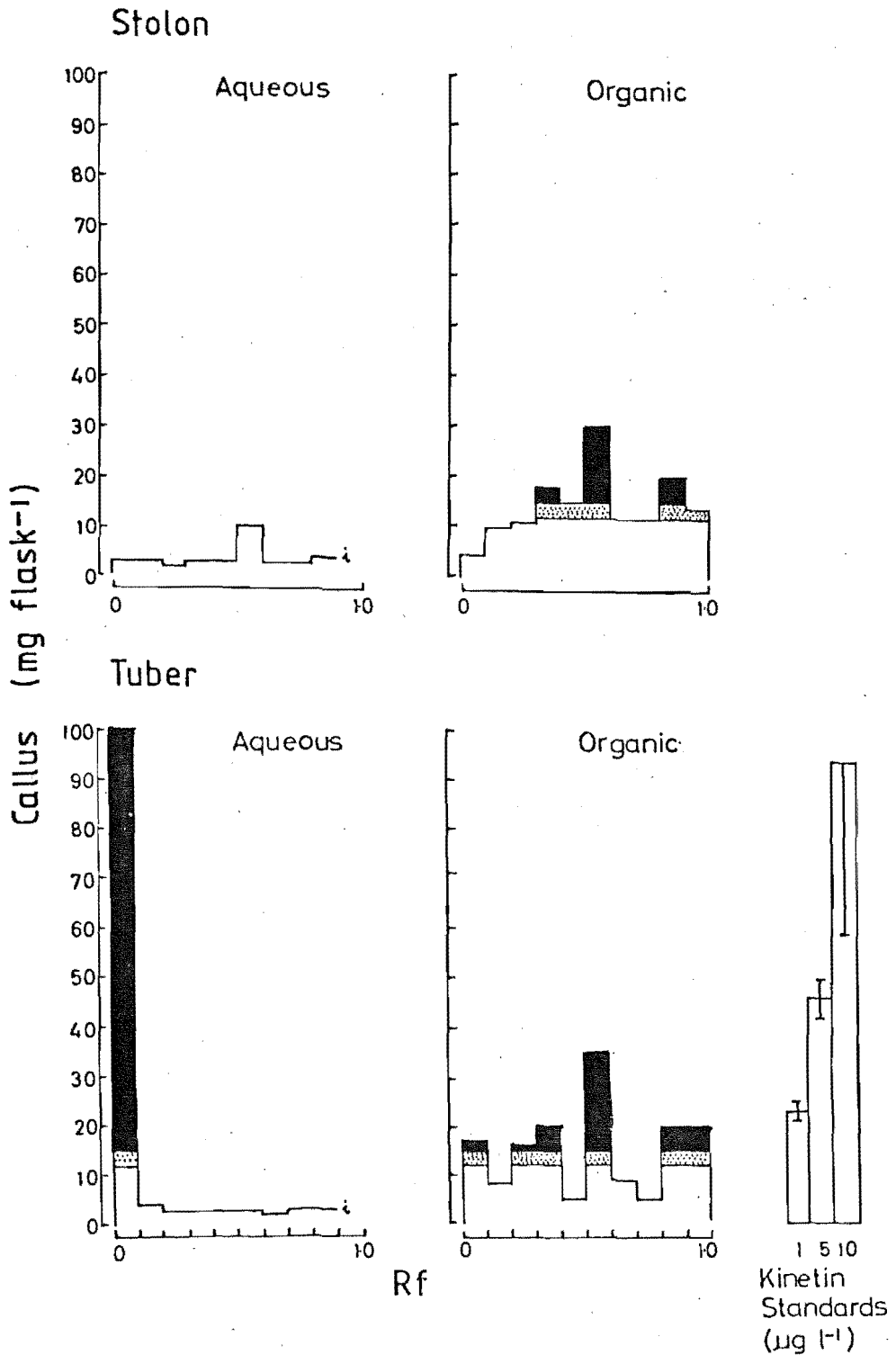


FIGURE 4.5

Soybean callus bioassay of paper chromatograms loaded with extract of Ilam Hardy "bolter" plants. Tubers were removed from plants under SD conditions, stolons from plants under LD conditions. 4 g fresh weight of tissue was extracted and bioassayed

Solvent: propan-2-ol - ammonia - water (10:1:1 v/v)

Callus: Kit A (6)

Confidence limits: n = 28

Shading and standards as in Figure 4.1

tography of butanol-soluble material.

On a fresh weight basis the total cytokinin content of young tubers was greater than that of stolon tips. On a per organ basis the difference would be accentuated but the total number of stolons and tubers extracted was not recorded.

4.3.2.2 *Solanum andigena* cv 165 Experiment 1 (Canterbury)

S. andigena plants were grown under both long and short day conditions to determine whether this variety of potato required SD conditions for induction of tuber formation.

Nine days after planting sprouted tuber segments, shoots had emerged from the soil. Plants were examined 49 days after shoot emergence. Plants maintained under long day conditions showed no signs of starch accumulation while those exposed to 21 short days following transfer from long days, exhibited numerous swellings on the ends of stolons. Selected swellings were tested with I/KI solution and yielded a positive starch test, while the remaining tubers were extracted for cytokinin analysis.

Both qualitative and quantitative differences were observed between extracts from stolons and from small developing tubers (Fig 4.6) following resolution of the unpurified extract by column chromatography. Cytokinin-like activity present in the tuber extract eluted immediately after the void volume, but a major proportion of the activity detected was present in fraction 9. Cytokinin-like activity was barely detectable in the stolon extract although a low level of activity was present in fraction 9. Material collected in fraction 7 from both tuber and stolon extracts caused the callus to turn black during the bioassay.

Both aqueous and organic phases of tuber extract partitioned against butan-1-ol at pH 7 were resolved by paper chromatography and bioassayed (Fig 4.7). Cytokinin-like activity soluble in butanol was located on R_f zones 0.5 to 0.7. The flask containing material present on R_f zones 0.7 to 0.8 became contaminated and was discarded. Cytokinin-like activity had previously been located at this R_f (Fig 4.2) so the total activity present in the butanol phase cannot be estimated. Notable, however, is the lack of detectable activity in the aqueous phase of extract from small tubers (Fig 4.7 cf Fig 4.5).

4.3.3 Potato Field Trial (Lincoln, mid-Canterbury)

A field plot containing 430 plants of *Solanum tuberosum* cv Ilam Hardy was harvested at increasing time intervals from sowing to provide stolons and tuber material at different developmental stages for cyto-

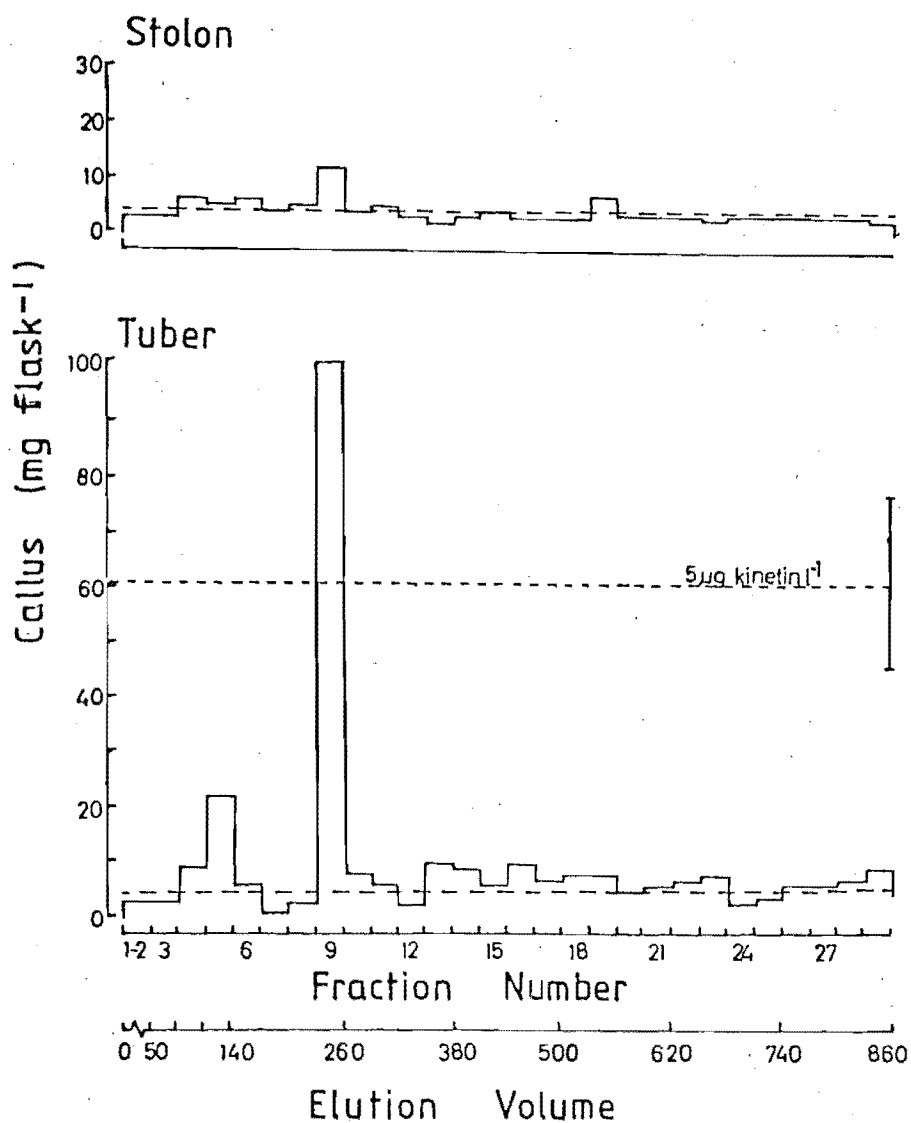


FIGURE 4.6

Soybean callus bioassay of extract of *S. andigena* cv 165. Tubers were removed from plants under SD conditions, stolons from plants under LD conditions. 1.8 g fresh weight of tissues were extracted and resolved by partition chromatography on Sephadex LH-20

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of Fractions 1-2 (50 ml) and fraction 30 (40 ml)

Callus: Kit A (8)

Confidence limits: $n = 20$

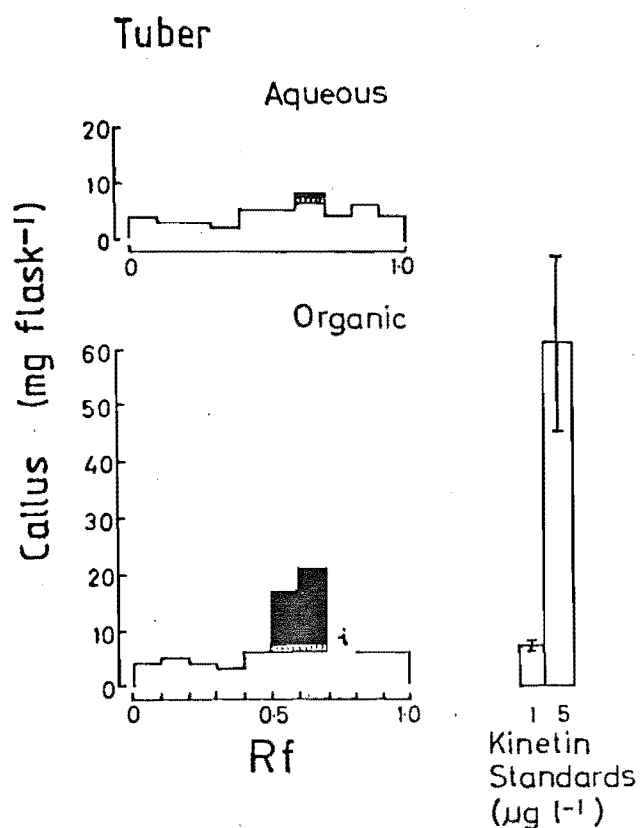


FIGURE 4.7

Soybean callus bioassay of paper chromatograms loaded with aqueous and butan-1-ol-soluble components of extract of *S. andigena* cv 165 tubers. 2.9 g fresh weight equivalent of tissue was extracted and bioassayed.

Solvent: propan-2-ol - ammonia - water (10:1:1 v/v)

Other details as in Figure 4.6 but with shading as in Figure 4.1

kinin analysis.

The emergence of the plants was sporadic initially but within 38 days of planting over 85% of the plants had emerged. The plants were up to 150 mm in height by this time. Stolons were generally short but small tubers (0.03 g to 0.50 g FW) were present on over 40% of the plants. After 56 days from planting a wide range of tuber sizes was evident (0.03 g to 33 g FW) and the majority of plants were forming tubers.

Detailed growth data was not recorded at each harvest (Table 4.1) although the data presented in Figure 4.8 indicates that the tuber fresh weight increased rapidly as the diameter increased. Although the absolute rate of increase was initially slow in the smaller tubers, the relative rate of fresh weight increase was at its greatest in tubers less than 20 mm in diameter.

Stolon and tuber material from field grown plants was analysed for cytokinin-like activity (Section 4.2.3). Extracts from all samples were partitioned against butan-1-ol (pH 7) and resolved by paper chromatography in solvent 1 (Figs 4.9, 4.11, 4.13, 4.14). Selected extracts were also applied to a column of Sephadex LH-20 and eluted with 35% ethanol (Figs 4.10, 4.12, 4.15).

Both the aqueous and butanol-soluble components of the sprouting "seed" tuber extract were resolved by paper chromatography (Fig 4.9) Significant amounts of cytokinin-like activity were detected in the organic fraction. A low level of activity was located at R_f 0.6 to 0.7 on only one of the three chromatograms loaded with 2 g eq FW. However, following the bioassay of 5 g eq FW cytokinin-like activity was located at R_f 0.5 to 0.6 in samples A and B. In sample C the response of the callus to material located between R_f 0.5 to 0.7 lay between the 95 and 99% confidence limits. On comparison with samples A and B, the activity present in sample C appears to be distributed across the chromatogram in a broader band and is therefore considered significant. The cytokinin-like activity detected lay on the region of the chromatogram to which zeatin riboside and zeatin were shown to migrate under similar conditions (Fig 3.1) but represented less than $5 \mu\text{g KE l}^{-1}$. Little if any cytokinin-like activity was detected in the aqueous fraction, but both aqueous and organic fractions contained material, located between R_f 0.1 and 0.2, which was inhibitory to callus growth.

Following resolution by column chromatography and bioassay of 10 g eq FW, it was apparent that the cytokinin content of the "seed" tuber extract was low (Fig 4.10) but that the activity was not

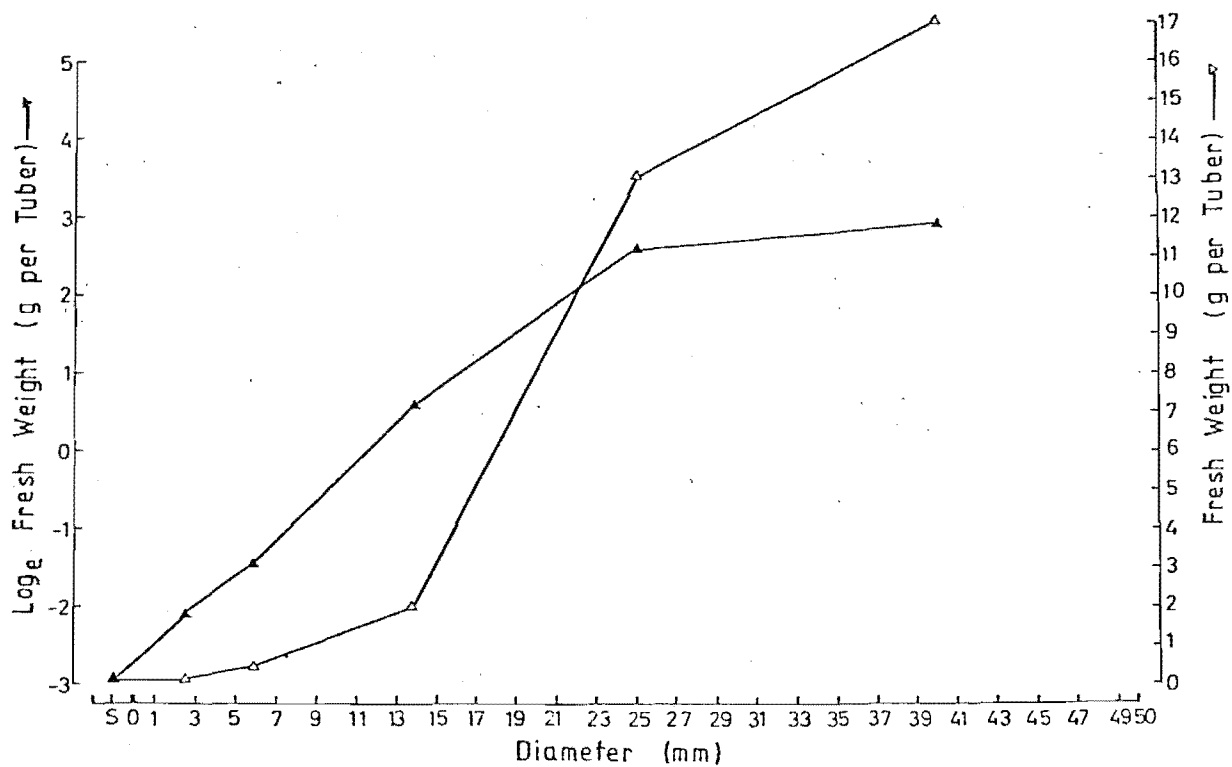


FIGURE 4.8

Potato Field Trial. Fresh weight increase with increasing tuber size. The midpoint is taken for each size category to represent the average tuber weight in that category (eg tubers 7.5 to 20 mm diameter are represented by a point at 13.8 mm)

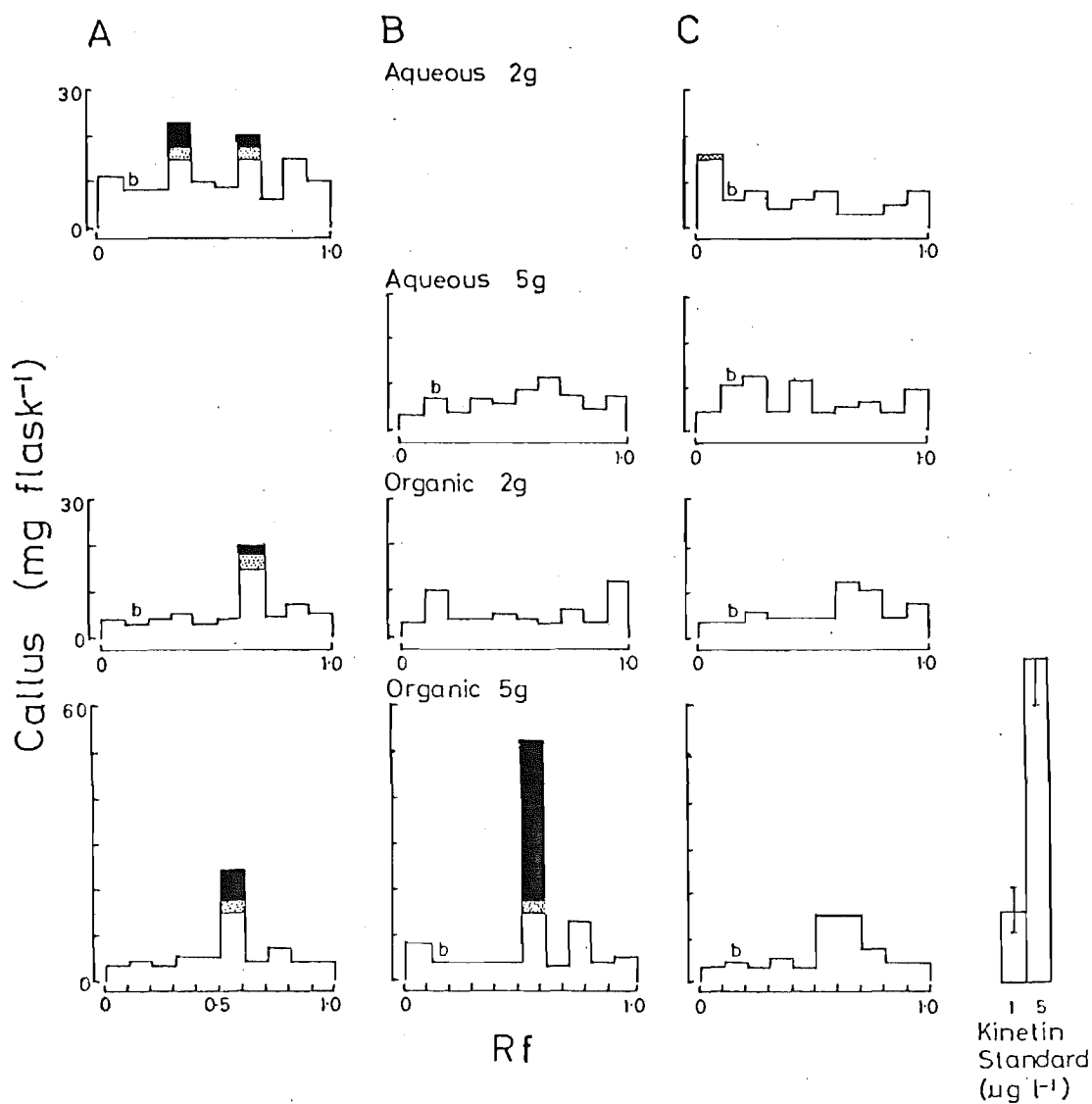


FIGURE 4.9

Potato Field Trial. Soybean callus bioassay of paper chromatograms loaded with extract of sprouting *S. tuberosum* "seed" tubers. Aqueous- and butanol-soluble components of 2 g and 5 g eq FW of three samples (A, B and C) were bioassayed

Solvent: propan-2-ol - ammonia - water (10:1:1 v/v)

Callus: Kit A (11)

Confidence limits: n = 39

Shading and standards as in Figure 4.1

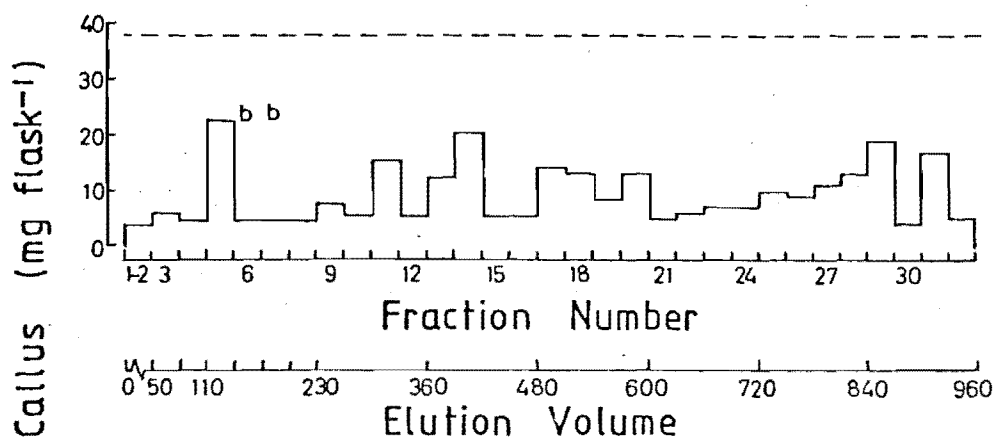


FIGURE 4.10

Potato Field Trial. Soybean callus bioassay of extract of *S. tuberosum* cv Ilam Hardy "seed" tubers. 10 g fresh weight of tissue extracted and resolved by partition chromatography on Sephadex LH-20

b: callus

Fraction volume: 30 ml with the exception of fractions 1-2
(50 ml)

Solvent: 35% ethanol

Confidence limit: n = 6

95% confidence limit shown — — —

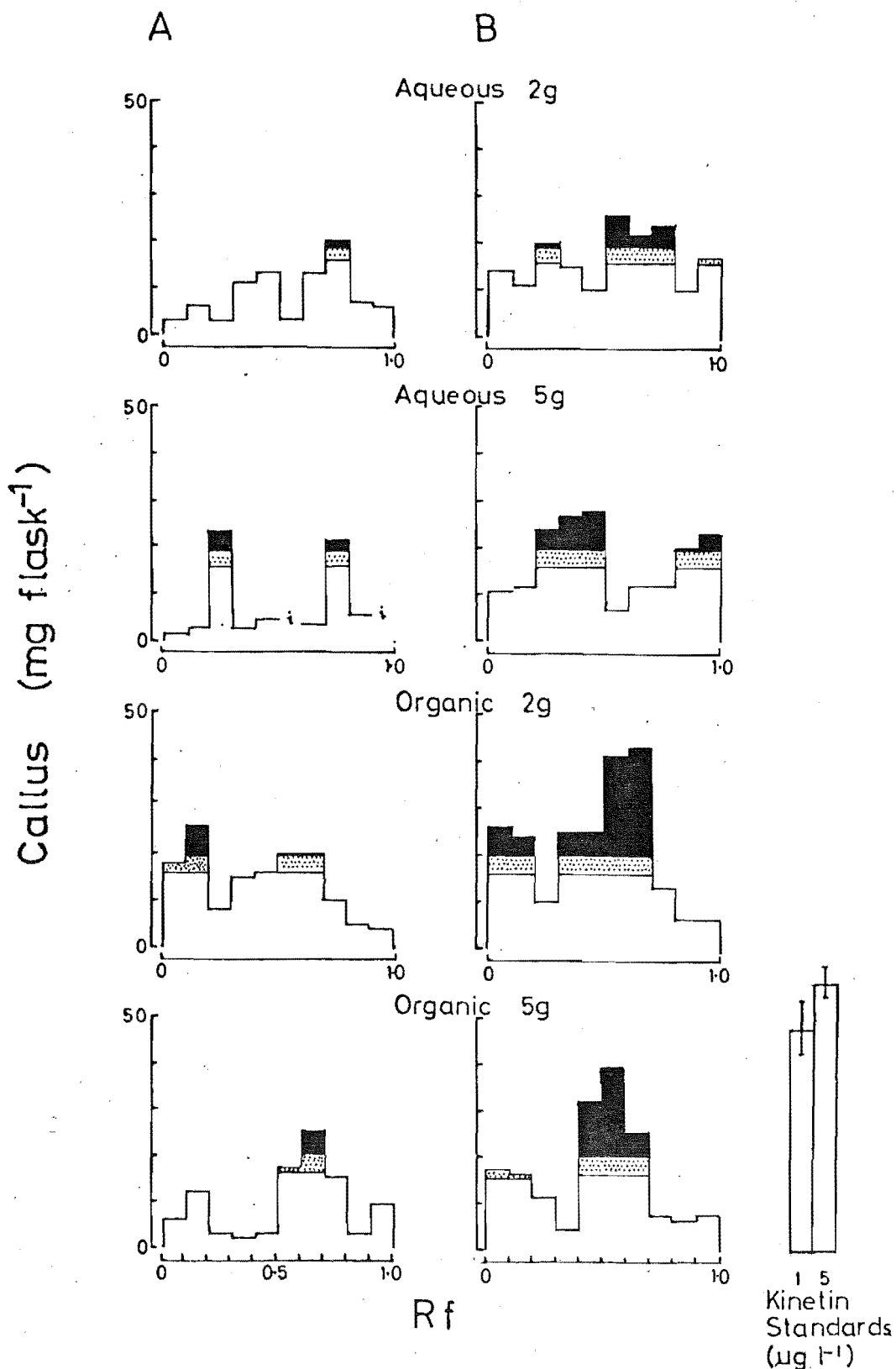


TABLE 4.11

Potato Field Trial. Soybean callus bioassay of paper chromatograms loaded with aqueous- and butan-1-ol-soluble components of stolon tip extracts. Extracts of 7 g fresh weight were subdivided into 2 g and 5 g eq. FW. Two samples (A and B) were bioassayed

Solvent: propan-2-ol - ammonia - water (10:1:1 v/v)

Callus: Kit A (15)

Confidence limits: $n = 39$

Shading and standards as in Figure 4.1

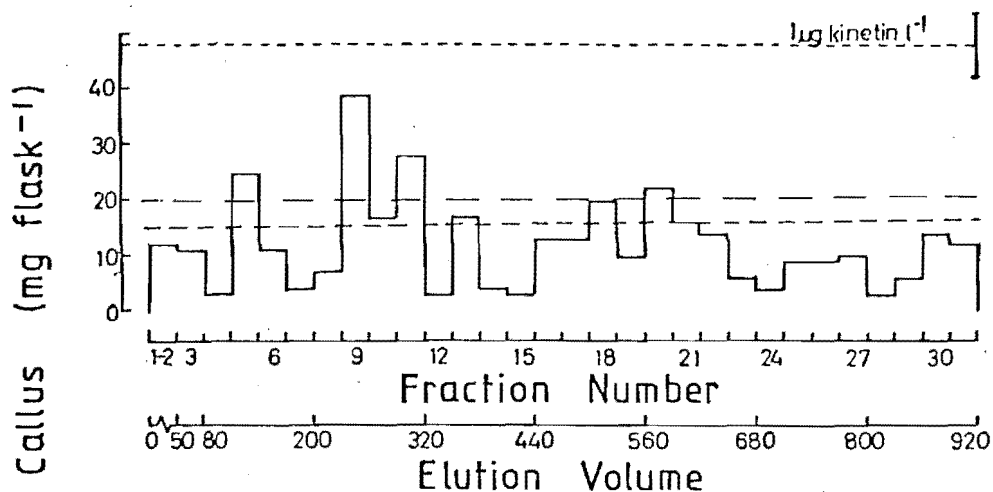


FIGURE 4.12

Potato Field Trial. Soybean callus bioassay of stolon extract. 5 g fresh weight (105 stolon tips) extracted and resolved by partition chromatography on Sephadex LH-20.

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1-2
(50 ml)

Callus clone, confidence limits, and standards as in Figure 4.11

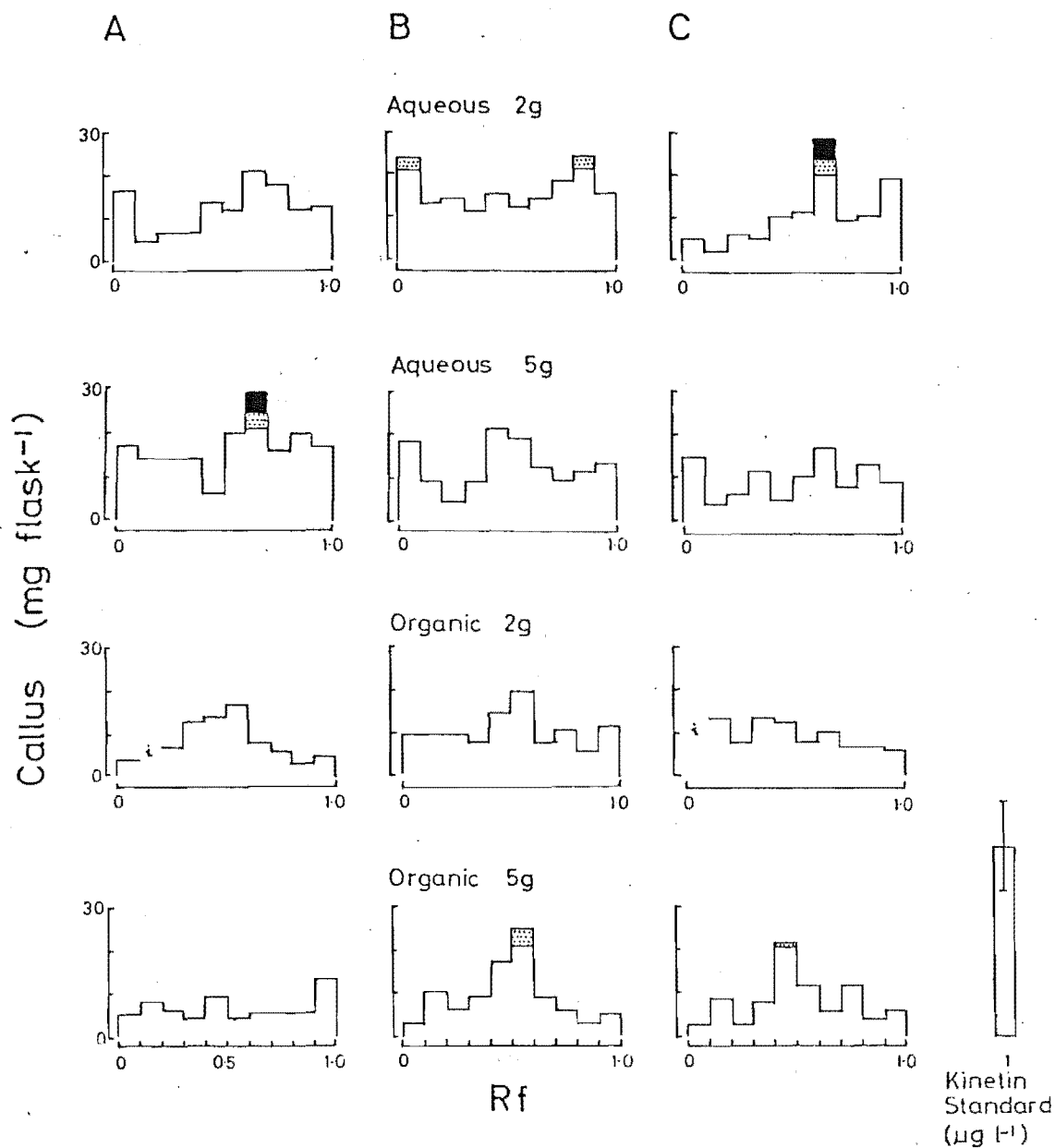


FIGURE 4.13

Potato Field Trial. Soybean callus bioassay of paper chromatograms loaded with aqueous- and butanol-soluble components of extract of tubers less than 4 mm diameter. Extracts of 7 g fresh weight were subdivided into 2 g and 5 g eq FW. Three samples (A, B and C) were bioassayed

i: contaminated and discarded flask

Solvent: propan-2-ol - ammonia - water (10:1:1 v/v)

Callus: Kit A (18)

Confidence limits: $n = 29$

Shading and standards as in Figure 4.1

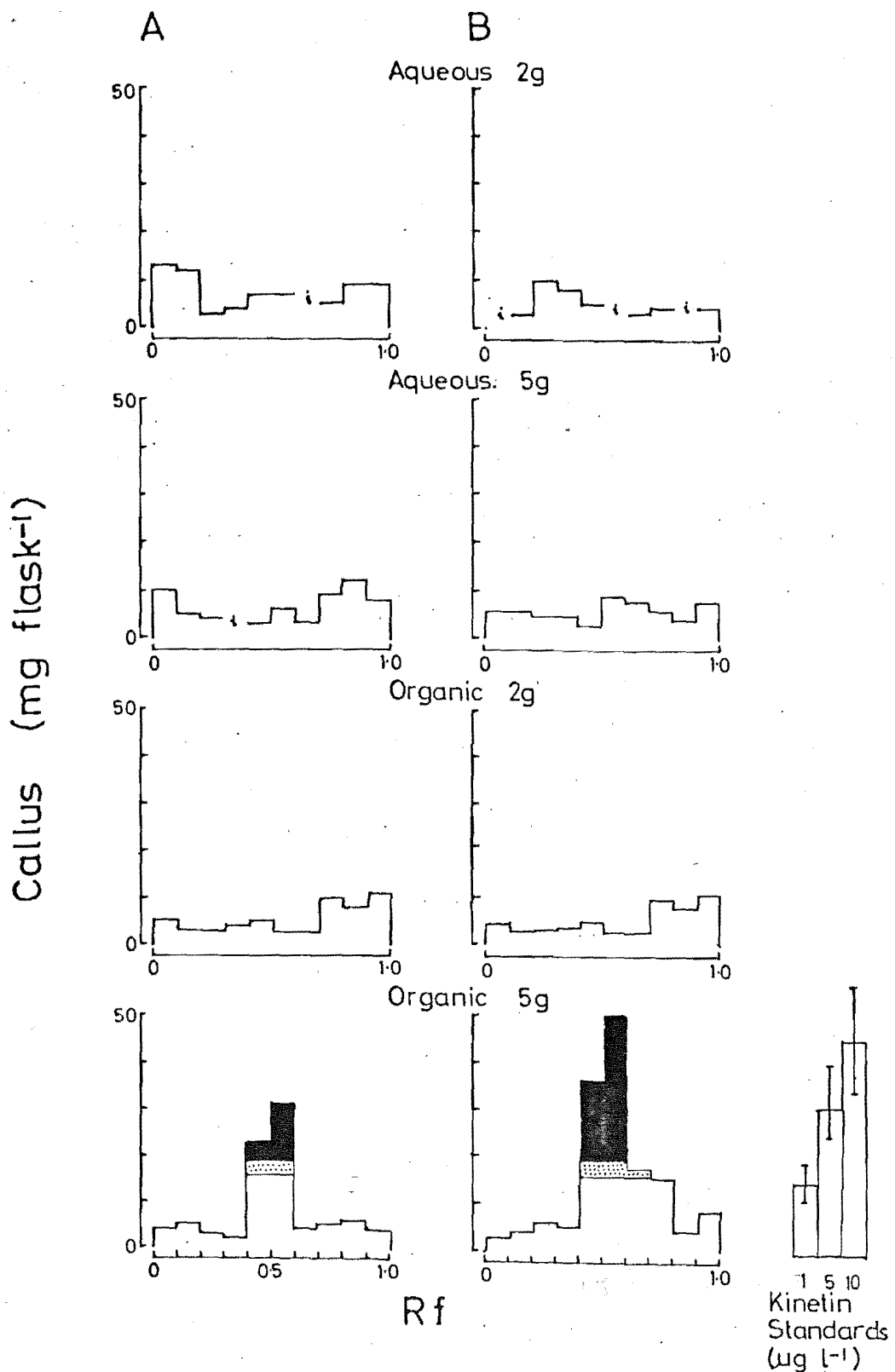


FIGURE 4.14

Potato Field Trial. Soybean callus bioassay of paper chromatograms loaded with aqueous- and butan-1-ol-soluble components of extracts of tubers between 4.0 and 7.5 mm diameter. Extracts of 7 g fresh weight were subdivided into 2 g and 5 g eq FW. Two samples (A and B) were bioassayed

- i: contaminated and discarded flasks
- Solvent: propan-2-ol-ammonia - water (10:1:1 v/v)
- Callus: Kit A (19)
- Confidence limits: 28
- Shading and standards as in Figure 4.1

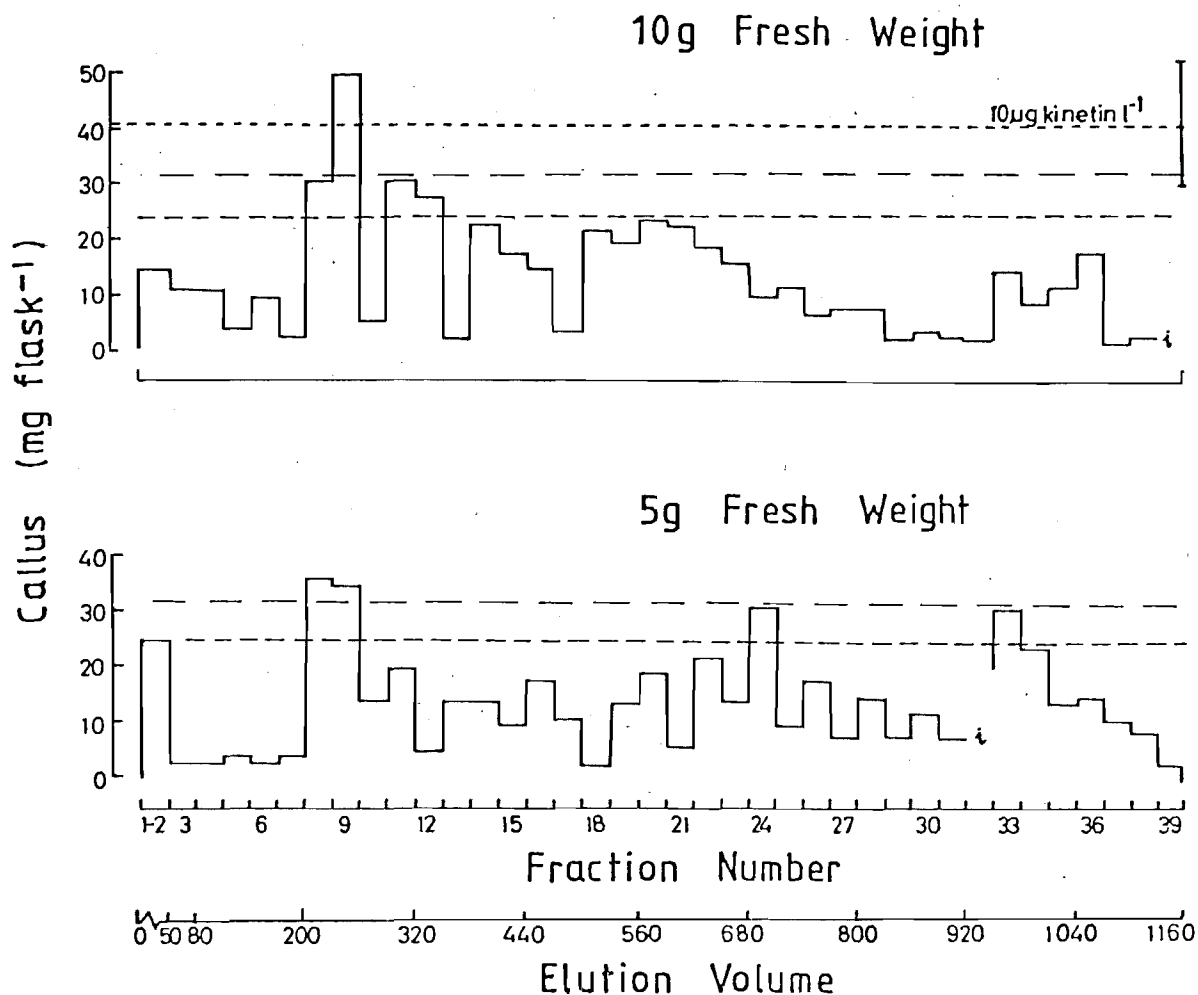


FIGURE 4.15

Potato Field Trial. Soybean callus bioassay of extract of tubers 4 to 7.5 mm diameter. 10 g and 5 g fresh weight (44.3 and 22 tubers respectively) were extracted and resolved by partition chromatography on Sephadex LH-20.

i: contaminated and discarded flask

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fractions 1-2
(50 ml)

Callus: Kit A (19)

Confidence limits: 28

restricted to a single discrete compound. Activity collected in fraction 11 corresponded to the elution volume of zeatin (Table 3.1). However, activity also eluted from the column both faster and slower than zeatin. Inhibitory activity was present in fractions 6 and 7 as determined by blackening of the callus during the bioassay.

Stolons, excised from tuberising and non-tuberising plants at harvest 1 (25/11/77), contained demonstrable cytokinin-like activity (Fig 4.11). The major proportion of the activity moved into the organic phase following partitioning of the stolon extract against butan-1-ol at pH 7. Most of the activity was located on the chromatogram in the region to which zeatin riboside and zeatin were shown to migrate under similar chromatography conditions (*ie* R_f 0.4 to 0.7; Fig 3.1) but some of the cytokinin-like activity remained near the origin. Small amounts of activity were also detected in the aqueous fraction (Fig 4.11). The concentration of cytokinin-like activity in any one flask did not exceed $1 \mu\text{g KE l}^{-1}$. The amount of activity detected in sample 1 was less than in sample 2. Contamination of the extract by a faulty magnetic flea used during the extraction process may have caused this.

Extract partitioned by column chromatography and assayed simultaneously with the paper chromatograms showed cytokinin-like activity associated with different elution volumes (Fig 4.12). The elution volume of activity collected in fractions 9 and 11 corresponded with peak elution volumes of zeatin riboside and zeatin markers respectively (Table 3.1). Cytokinin-like activity was also detected in fractions 18 and 20 to 22. The elution volume of the activity detected in fraction 18 corresponded with the elution volume for isopentenyladenine (Fig 3.2). Cytokinin-like activity collected in fraction 5 and retained by the column longer than isopentenyladenine was also detected in the sprouting "seed" tuber extract (Fig 4.10). Compounds present in fraction 6 caused the callus to darken but not turn black during the bioassay and callus blackening did not occur during the bioassay of the paper chromatograms (Figs 4.12 and 4.11 respectively).

A wide range of tuber sizes was collected at harvest 3 (13/12/77) (Table 4.1) but insufficient tubers of relatively small diameter were available for cytokinin analysis. However, sufficient material was collected at harvest 4 (20/12/77) for cytokinin analysis of tubers less than 4 mm diameter and between 4 mm and 7 mm diameter.

The amount of cytokinin-like activity in tubers less than 4 mm diameter was low (Fig 4.13). Peaks which extended beyond the 99% confidence limit were not repeated consistently within or between

samples although extremely low levels of activity may have partitioned into the organic fraction and be located at R_f 0.4 to 0.6. Cytokinin-like activity soluble in the aqueous phase may also be located at R_f 0 to 0.1 and R_f 0.6 to 0.7 (Fig 4.13).

Cytokinin-like activity was readily detected in extract from tubers of diameter 4 mm to 7.5 mm (Figs 4.14, 4.15). The cytokinin-like activity partitioned into butan-1-ol at pH 7 and was located on the chromatograms at the R_f to which zeatin and zeatin riboside were found to migrate (R_f 0.4 to 0.7, Fig 3.1). Individual peaks of activity lay between 5 and 10 $\mu\text{g KE l}^{-1}$ (Fig 4.14). Cytokinin-like activity was not detected in the aqueous fraction.

Extract partitioned by column chromatography was assayed at the same time as the paper chromatograms described above. Resolution of the cytokinin-like activity into a number of discrete fractions was apparent following chromatography of 10 g eq FW but less so with 5 g eq FW (Fig 4.15). As described previously, three of the four components of the cytokinin-like activity collected in fractions 8 to 22 corresponded to the elution volumes of zeatin riboside, zeatin and isopentyladenine standards respectively.

Activity detected in fraction 5 in both the stolon and sprouting "seed" tuber extract (Figs 4.12 and 4.10 respectively) was not apparent in the extract from tubers 4 to 7.5 mm in diameter although cytokinin-like activity may again be eluting after a substantial retention time. Blackening of callus was not apparent during the bioassay.

Tubers of the size range 7.5 to 20 mm and 30 to 40 mm diameter respectively were extracted and the extracts partitioned and applied to paper chromatograms. However, extreme temperature fluctuations during the greater part of both bioassays may have contributed to otherwise unaccountable random callus responses on both control and loaded chromatograms. The results are not presented.

Although significant cytokinin-like activity was detected in stolons and small tubers, the amount present was very low and lay below the straight line portion of the standard curve which was constructed in kinetin equivalents for each bioassay (Fig 2.7A). However, by comparison between the bioassays of extracts of stolons and tubers (Figs 4.12, 4.15) and the appropriate kinetin standards, it is apparent that tubers of diameter 4 mm to 7.5 mm contained more cytokinin-like activity on both a fresh weight and per organ basis than did stolons (Table 4.8).

TABLE 4.8

Field Trial 1. Comparison of the total cytokinin-like activity of stolons from harvest 1 and tubers of diameter 4 to 7.5 mm from harvest 4. Cytokinin-like activity was estimated in kinetin equivalents (KE)

Organ	Fresh weight extracted (g)	Organ number extracted	Cytokinin-like activity		
			$\mu\text{g KE l}^{-1}$	ng KE g DW^{-1}	ng KE organ^{-1}
Stolon tips	5	105	ca 1	ca 10	ca 0.5
Tubers	10	44.3	20	120	22.1

4.3.4 Controlled Environment Experiments, Plant Physiology Division, DSIR, Palmerston North

4.3.4.1 PPD Experiment 1 Potato plants were grown in controlled environment rooms at PPD to provide a supply of plants at similar development stages. *S. andigena* cv 165 was selected as a variety with a reputed SD requirement for tuber induction (Table 4.17). By controlling the time of tuber induction it was hoped to obtain a population of plants in which tuber development was reasonably uniform. Sufficient plants were grown to provide material for cytokinin analysis as well as for analysis of soluble sugar and starch content. The harvest schedule is outlined in Section 4.2.4.1.

Two plants were examined for tuber formation after 18 days in the controlled environment rooms and a further two after 20 days. None of the plants were forming tubers. However, when 26 plants were harvested after 21 days only 10 of these were not tuberising. The proportion of tuberising plants increased very rapidly (Table 4.9).

The total weight of stolons and tubers per plant was variable at the first four harvests (Table 4.10). An increasing trend in total stolon and tuber weight per plant was noted at all subsequent harvests except at Day 35.

Following the initial tuberisation period the relative rate at which tuber weight per plant increased was very high until Day 30 but less thereafter (Fig 4.16).

The total number of stolons and tubers per plant did not increase substantially following Day 30 with the exception of an apparent transient

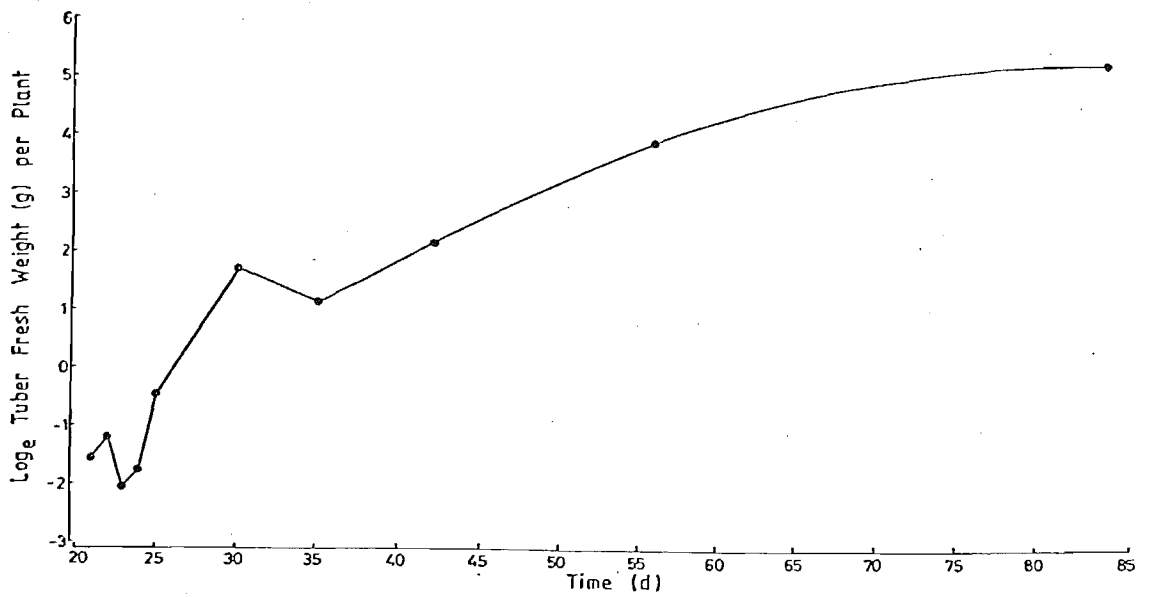


FIGURE 4.16

PPD Experiment 1. Growth analysis of the population of plants sampled between 21 and 84 days after transfer to controlled environment rooms. Relative rate of tuber weight increase as a function of time after transfer.

increase at Day 56 (Table 4.10). The number of tubers per plant was low until Day 25 and was then similar for harvests at Days 30, 35, 42 and 84. An exceptionally high value was again noted at Day 56.

TABLE 4.9

PPD Experiment 1. Percentage tuberisation of *S. andigena* plants under long day conditions in controlled environment rooms

No of days in CE	No of plants harvested	Percentage tuberisation
18	2	0.0
20	2	0.0
21	26	61.5
22	38	65.8
23	43	76.7
24	34	68.0
25	36	90.9
30	30	100.0
35	24	96.0
42	24	100.0
56	10	100.0
84	10	100.0

TABLE 4.10

PPD Experiment 1. The number of plants harvested, total weight and number of stolons and tubers per plant and the total number of tubers per plant is presented for each harvest.

Days after transfer to controlled environment rooms	Number of plants harvested	Stolons and Tubers		Tubers
		Total weight (g) per plant	Total number per plant	Total number per plant
21	26	0.571	4.5	21.3
22	38	0.518	4.1	14.3
23	43	0.307	4.1	14.7
24	34	0.360	5.1	14.7
25	36	1.225	10.8	17.5
30	30	5.730	17.2	20.4
35	24	3.410	16.9	28.6
42	24	9.000	17.8	24.0
56	10	50.82	42.5	45.1
84	10	203.56	24.8	24.8

The number of stolon tips or tubers in a particular size category at each harvest is represented in Figure 4.17 as a proportion of the total number of stolons and tubers collected at that harvest.

The proportion of unswollen stolon tips decreased initially very rapidly but new tips were formed at least up to Day 35. Concurrent with the initial decrease in stolon tips was an increase in small tubers less than 3 mm in diameter. The proportion of the tubers in the smallest size category (JA) showed a pattern similar to that of the stolons but displaced slightly in time, whereas the proportion of tubers 1.5 to 3 mm in diameter remained relatively constant up to Day 84.

Tubers in the sizes categories from 3 mm to 15 mm in diameter constituted an increasing proportion of the total tuber number from Day 24 to Day 30. A decrease then occurred but this was followed by a gradual increase from Day 42 to the final harvest. The proportion of tubers enlarging to sizes greater than 15 mm increased slowly with time (Fig 4.17).

A large proportion of the tubers collected at Day 84 were relatively small. Almost 50% of the tubers were less than 7.5 mm in diameter whereas less than 30% were greater than 20 mm in diameter.

The contribution from non-tuberising stolon tips to the total fresh weight (of stolons plus tubers) decreased very rapidly as tuberisation proceeded, as did the contribution from tubers less than 5 mm in diameter (Fig 4.18). Tubers between 5 and 15 mm in diameter made the most noticeable contribution to tuber fresh weight at Day 25 and thereafter the contribution shifted in favour of increasingly larger tuber sizes. By Day 84 57% of the total fresh weight was due to tubers greater than 40 mm in diameter. Moreover, less than 9% of the total number of tubers contributed to over 50% of the fresh weight after the initial tuberisation period (Table 4.11).

The rate at which both the fresh and dry weight increased as a function of increasing tuber diameter showed a long lag period followed by an exponential increase in weight (Fig 4.19). A rapid relative rate of fresh or dry weight increase did not occur immediately following tuber formation. However, the relative rate was extremely high in tubers 3 mm to 10 mm in diameter but decreased as tubers expanded further.

4.3.4.1A Starch Analysis In expanding tubers much of the dry weight was attributable to starch as was the case even in relatively small tubers (Fig 4.20, Table 4.12).

The relative rate of starch increase per organ was very high as tubers first began to accumulate starch but then decreased to a steady

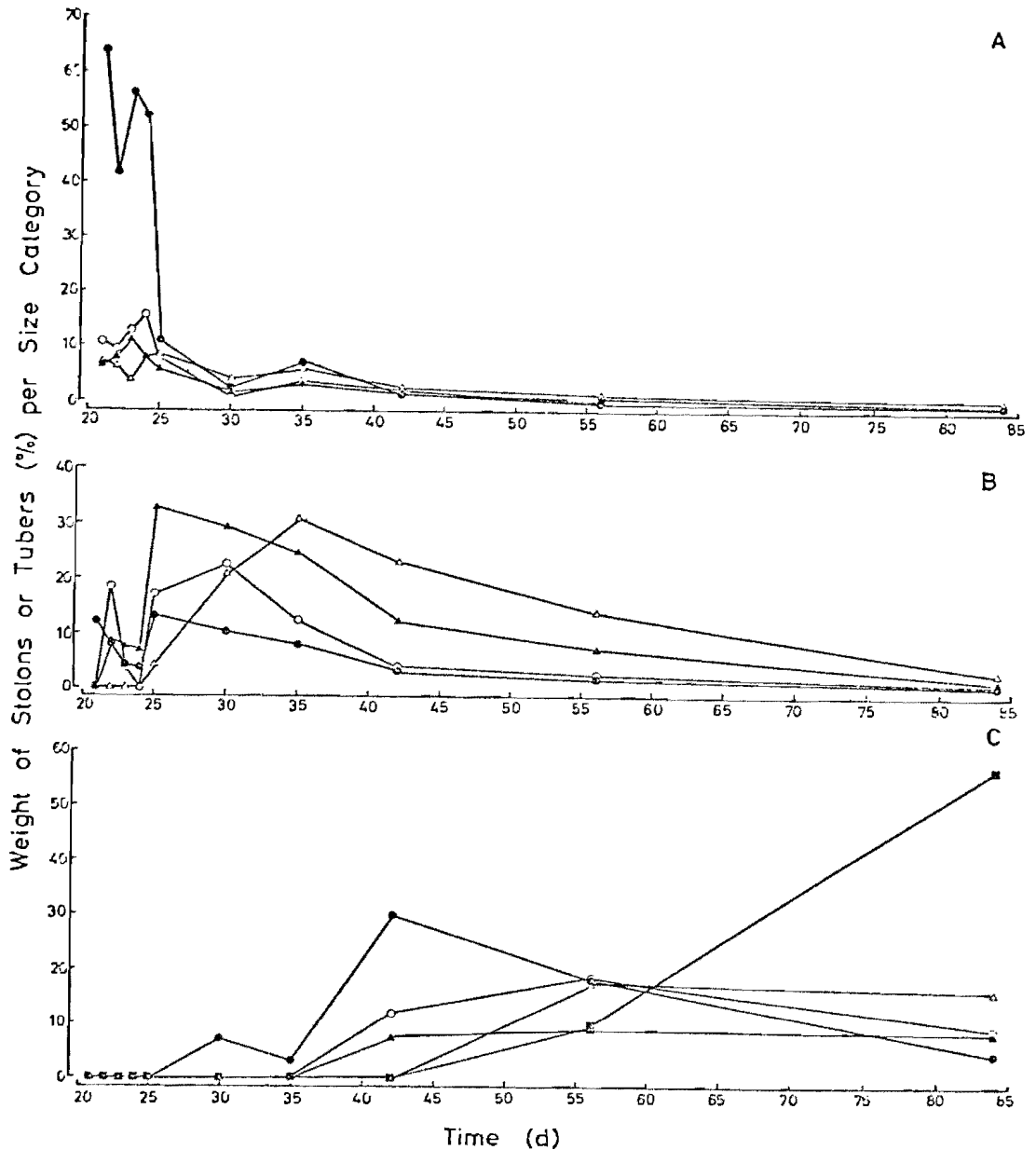


FIGURE 4.17

PPD Experiment 1. Growth analysis of the population of plants sampled between 21 and 84 days after transfer to controlled environment rooms. Each size category of stolons and tubers is presented as a proportion of the total stolon and tuber weight per plant as a function of increasing plant age.

Key



	mm diameter		
	A	B	C
●	stolon tips	5 < 7.5	20 < 25
○	JA	7.5 < 10	25 < 30
▲	1.5 < 3	10 < 15	30 < 35
△	3 < 5	15 < 20	35 < 40
■			40 < 50

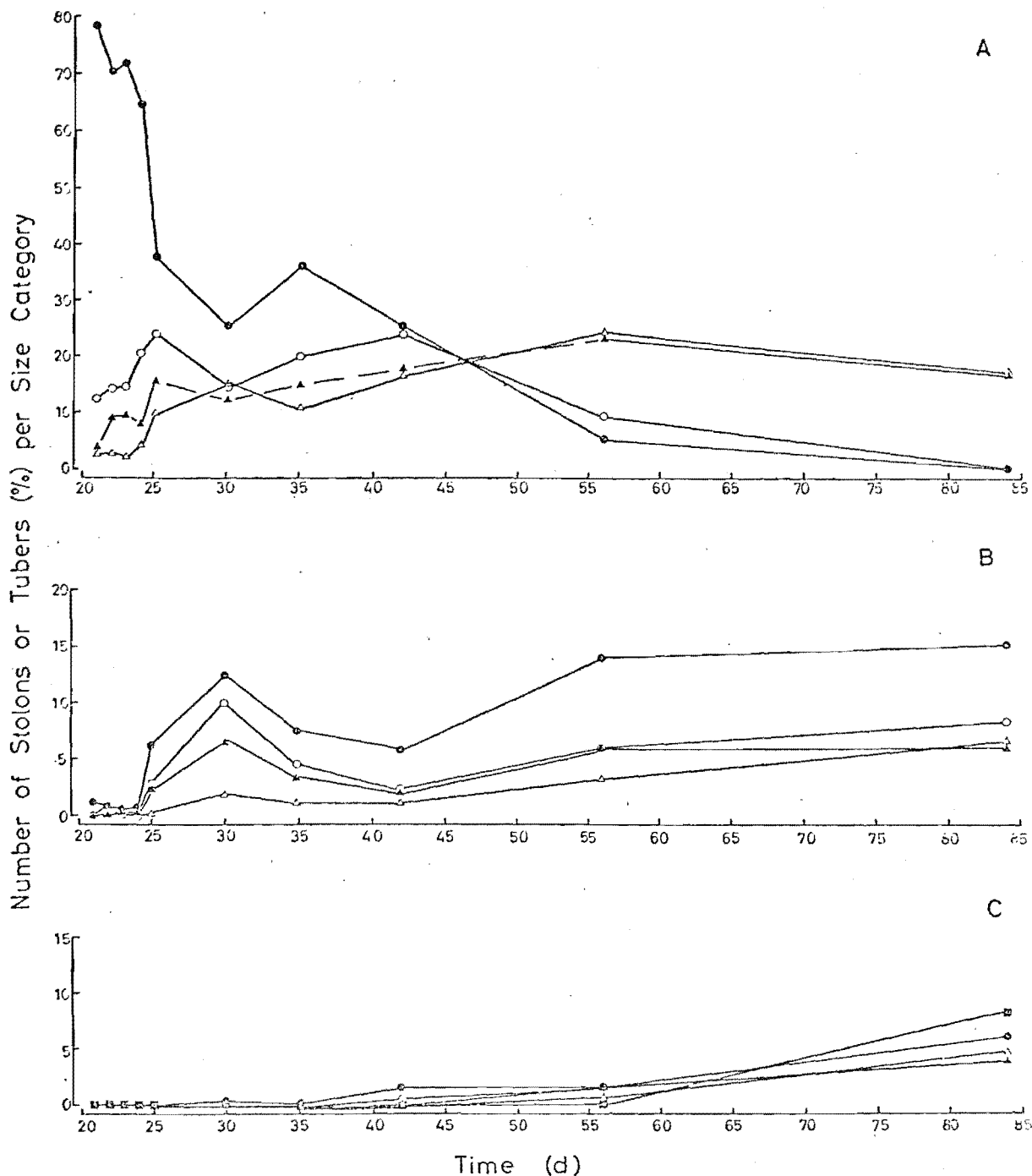


FIGURE 4.18

PPD Experiment 1. Growth analysis of the population of plants sampled between 21 and 84 days after transfer to controlled environment rooms. Each size category of stolons and tubers is presented as a proportion of the total stolon and tuber number per plant as a function of increasing plant age.

Key	mm diameter		
	A	B	C
●	stolon tips	5 < 7.5	20 < 25
○	JA	7.5 < 10	25 < 30
▲	1.5 < 3	10 < 15	30 < 35
△	3 < 5	15 < 20	35 < 40
■			40 < 50

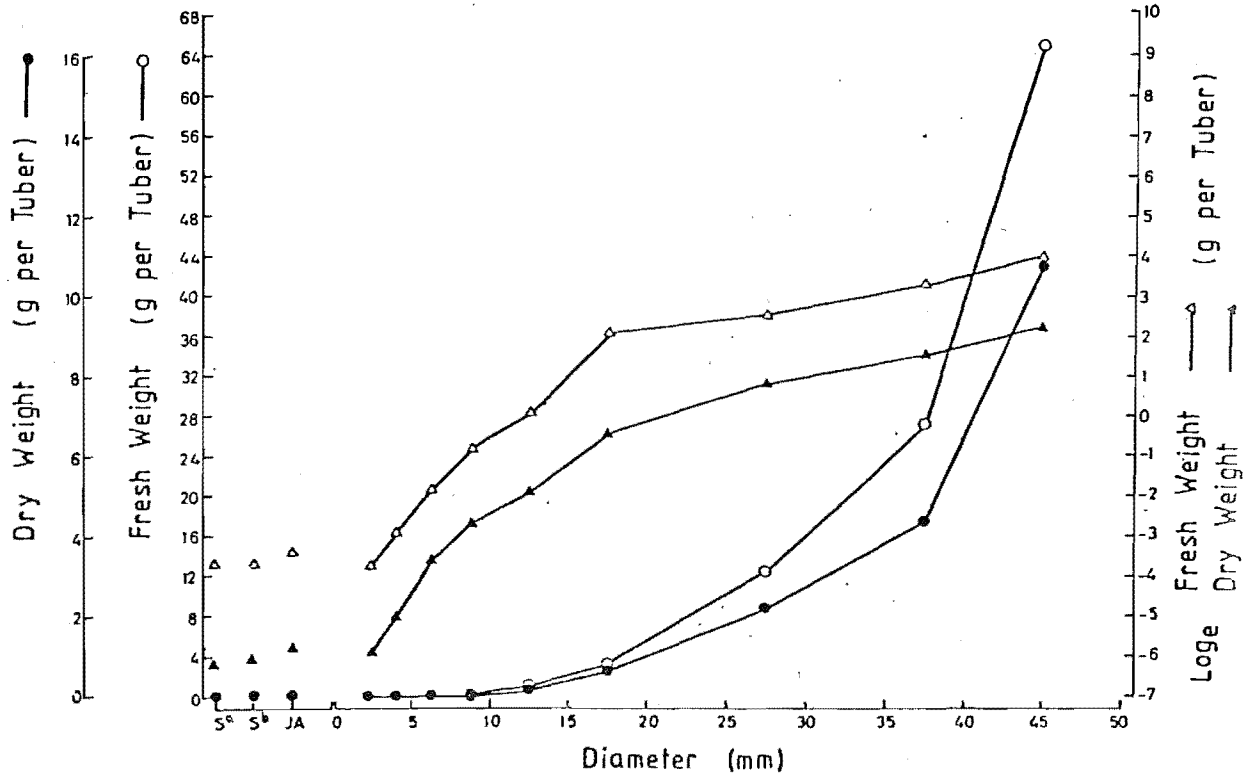


FIGURE 4.19

PPD Experiment 1. Increase in dry and fresh weight as a function of tuber diameter

- S^a: stolons excised from non-tuberising plants
 S^b: stolons excised from tuberising plants
 JA: stolon tips visibly accumulating starch

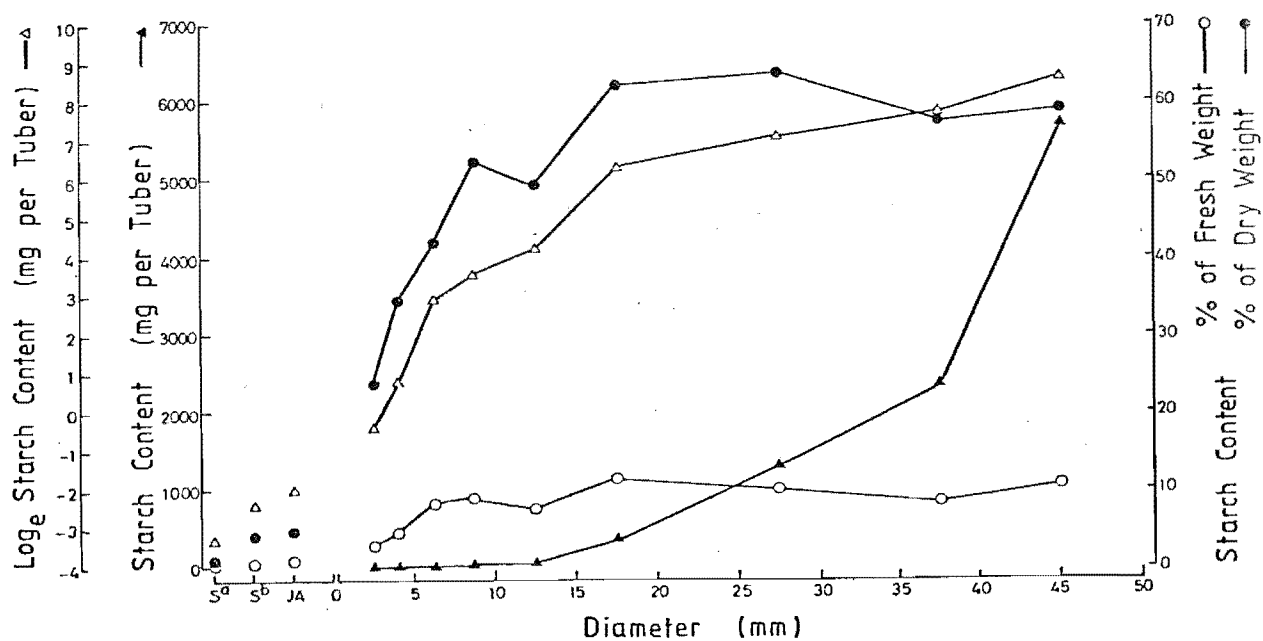


FIGURE 4.20

PPD Experiment 1. Starch content of stolons and tubers as a function of tuber diameter. All analyses were performed in triplicate. The coefficients of variation were less than 6.4% with the exception of the analysis of tubers 15 to 20 mm diameter when it was 11.8%. Refer to Figure 4.19 for further details

exponential rate in tubers greater than 20 mm in diameter (Fig 4.20).

TABLE 4.11

PPD Experiment 1. Range of tuber sizes and the proportion of the total tuber number contributing to approximately 50% of the total fresh weight of tubers

Days after transfer to controlled environment rooms	Tuber diameter (mm)	Proportion of total tuber number (%)	Proportion of total tuber weight (%)
21	JA < 7.5	21.34	36.02
22	JA < 15	28.98	58.01
23	JA < 15	27.86	42.67
24	JA < 15	34.60	47.89
25	7.5 < 20	5.32	54.08
30	10 < 25	8.79	57.64
35	10 < 25	4.66	58.83
42	20 < 35	2.26	50.04
56	25 < 40	3.11	55.13
84	> 40	8.47	56.85

TABLE 4.12

Starch content of stolons and tubers less than 7.5 mm in diameter

Size Category (mm diameter)	µg	Starch	
		% DW	% FW
Stolons (NA)	36.0	1.90	0.15
Stolons (A)	92.4	4.20	0.38
Tubers: JA	132.5	4.73	0.42
1.5 < 3	650	24.10	2.79
3 < 5	2,301	34.87	4.62
5 < 7.5	12,190	47.00	8.34

An increase in the amount of starch per organ was noted at about the time of tuber initiation (Table 4.12, Fig 4.20). In fact stolon tips removed from tuberising plants contained substantially more starch than stolon tips removed from non-tuberising plants. Stolon tips removed from non-tuberising plants after 21 days in PPD Experiment 2 (Table 4.20) contained only 20 μg of starch per stolon tip which represented 0.8% of the total dry weight.

A marked increase in the starch content on both a per organ and percentage dry weight basis occurred as the stolon hook unbent (Table 4.12, Fig 4.20).

Fifty-two percent of the dry weight in tubers between 7.5 and 10 mm diameter was attributable to starch. A decrease in the starch content of tubers 10 to 15 mm diameter was noted. Tubers 15 to 30 mm diameter contained over 60% of their dry weight as starch and this proportion decreased only slightly in the larger size categories analysed.

As a percentage of the fresh weight, starch increased from a low amount in stolons to about 10% in small tubers (Fig 4.20) after which the level remained relatively constant.

4.3.4.1B Soluble Sugar Analysis Over 20% of the dry weight of stolons was attributed to soluble sugars (Table 4.13, Fig 4.21). The total amount of soluble sugar per organ increased until tuber formation was detectable. A sudden decrease in the soluble sugar content on both a percent dry weight and per organ basis was apparent as the stolon hook unfolded (tubers 1.5 to 3 mm diameter). The soluble sugar content measured as a proportion of the dry weight continued to decrease until tubers were up to 5 mm diameter. An increase then occurred but the proportion of soluble sugar decreased to a relatively constant level of less than 2.5% of the dry weight in tubers greater than 20 mm in diameter. Following the initial tuber expansion the soluble sugar content did not exceed 1.3% of the total fresh weight, and in tubers greater than 15 mm diameter it remained at less than 0.5% of the fresh weight. However, the total amount of soluble sugar per tuber increased rapidly until tubers measured 10 to 15 mm diameter. The amount of soluble sugar then increased at a constant rate as tubers expanded further (Table 4.13, Fig 4.21).

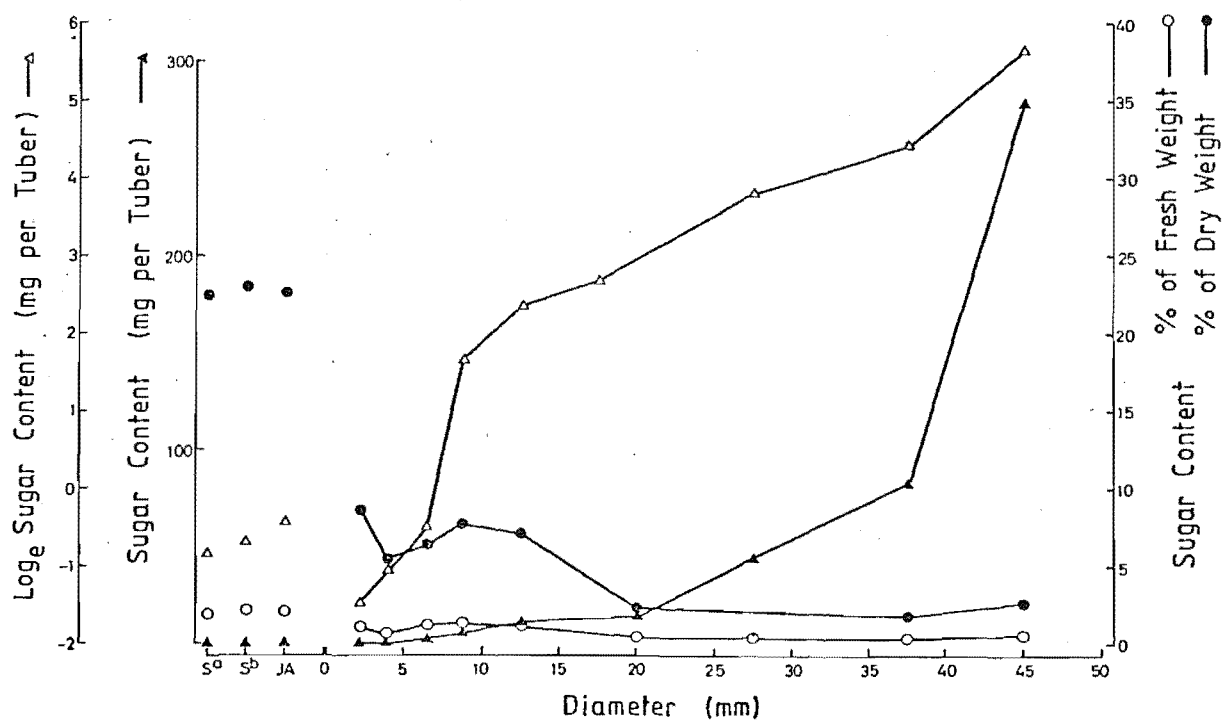


FIGURE 4.21

PPD Experiment 1. Soluble sugar content of stolons and tubers as a function of tuber diameter. All analyses were performed in triplicate and the coefficients of variation for all analyses were less than 6.2%. Refer to Figure 4.19 for further details.

TABLE 4.13

Soluble sugar content of stolons and tubers less than 7.5 mm diameter

Size Category (mm diameter)	Soluble sugars		
	μg	% DW	% FW
Stolon tips (NA)	425.6	22.40	1.78
Stolon tips (A)	506.0	23.00	2.04
Tubers: JA	632.8	22.60	1.99
1.5 < 3	231.0	8.56	1.00
3 < 5	345.0	5.40	0.71
5 < 7.5	1,630	6.36	1.13

4.3.4.1C Analysis of tissue for cytokinin-like activity Stolon tips removed from plants without tubers contained a series of cytokinin-like compounds (Fig 4.22). Cytokinin-like activity eluted from the column in the same volume of eluant as did authentic zeatin, zeatin riboside and isopentenyladenine, respectively. A peak of activity between the elution volume noted for zeatin and isopentenyladenine was also evident and some activity appeared to be retained by the Sephadex LH-20 gel longer than would be expected for isopentenyladenine. No activity promoted callus growth more than $2 \mu\text{g l}^{-1}$ kinetin.

The bioassay of extracts of stolon tips just accumulating starch was detrimentally affected by both low callus sensitivity and infection. Cytokinin activity less than $5 \mu\text{g KE l}^{-1}$ would not have been detected. The bioassay of tubers 1.5 to 3 mm diameter was similarly affected by low callus sensitivity but less so by infection. The results are presented in Figure 4.23. Cytokinin-like activity was detected in

FIGURE 4.22

2PD Experiment 1. Soybean callus bioassay of extracts of stolon tips (4 g eq FW). Extracts were resolved by partition chromatography on Sephadex LH-20. Three samples (A, B and C) were bioassayed.

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml), fraction 39 (sample A; 60 ml), fraction 40 (sample B; 40 ml) and fraction 41 (sample B; 40 ml)

Callus: Bap (5)

Confidence limits: n = 10

99% limit — — —

95% limit - - - - -

Regression line: $Y = 2.51X - 0.70$

Lowest detectable amount of kinetin: $2 \mu\text{g l}^{-1}$ ($819 \pm 62 \text{ mg callus flask}^{-1}$)

Kinetin standard as indicated: $\mu\text{g KE l}^{-1}$

Elution volumes of zeatin riboside (ZR) and zeatin (Z) are indicated (-——-). Solid line represents peak elution volume.

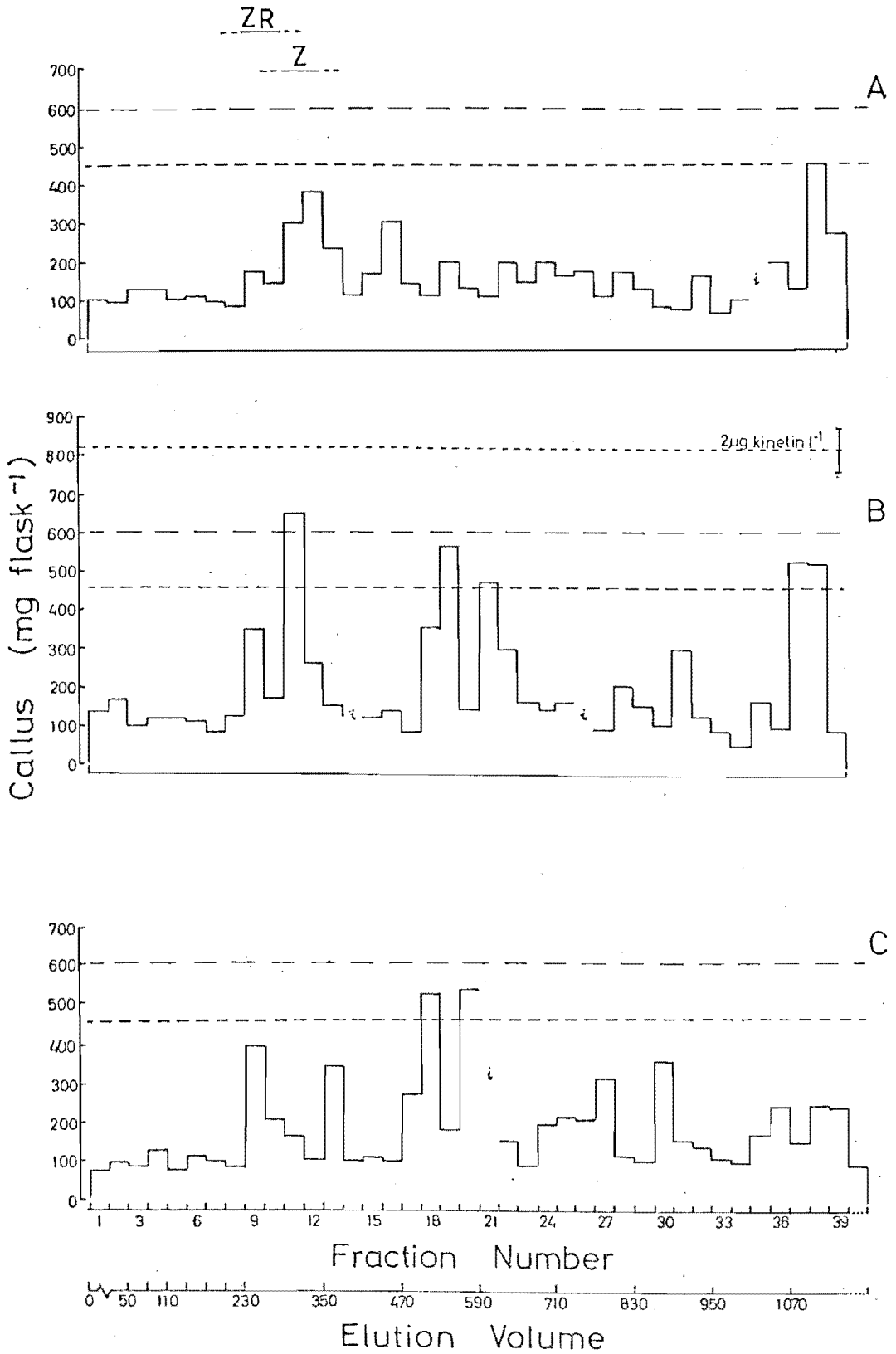


FIGURE 4.22

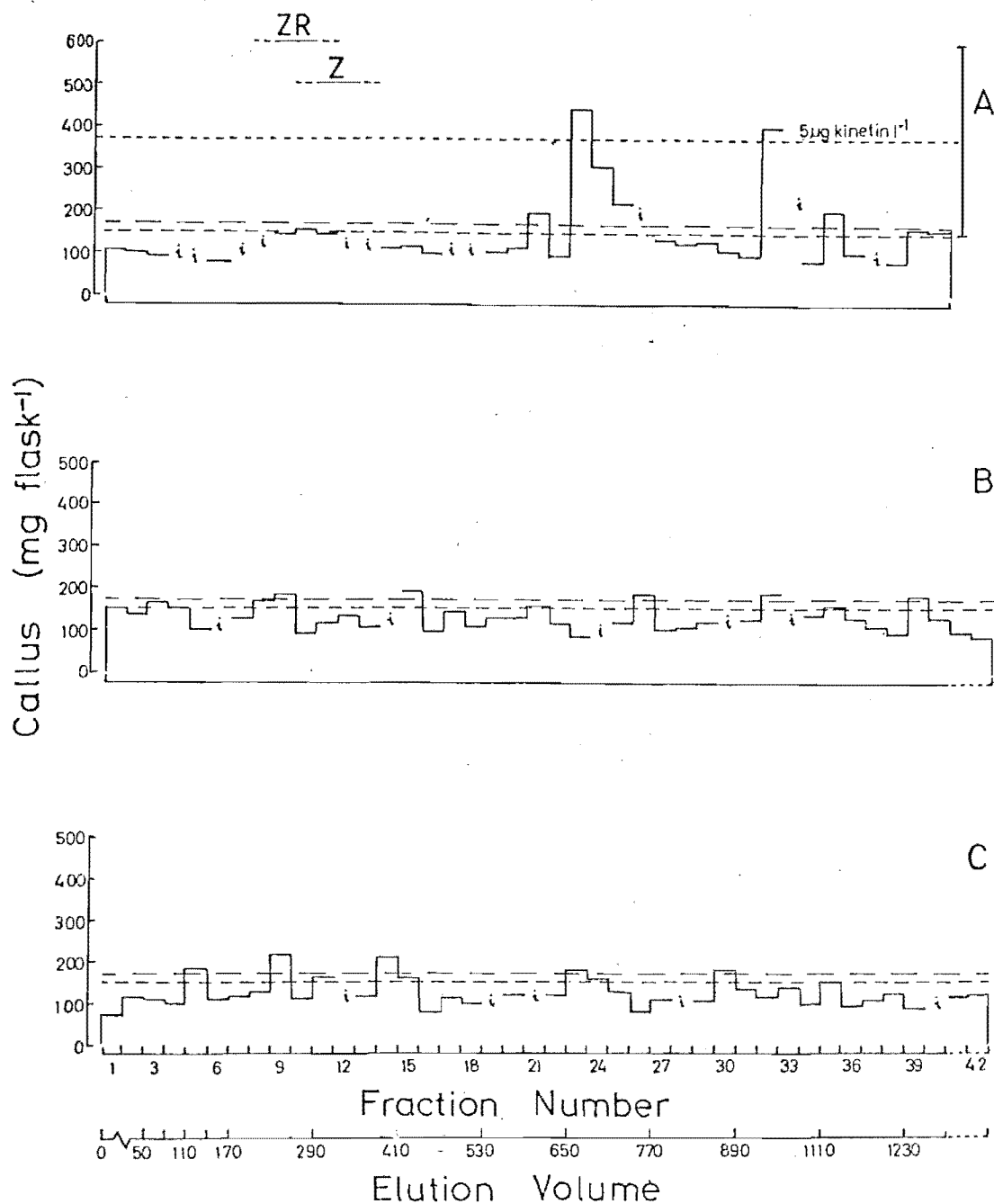


FIGURE 4.23

PPD Experiment 1. Soybean callus bioassay of extracts of tubers 1.5 to 3 mm diameter (4 g eq FW). Extracts were resolved by partition chromatography on Sephadex LH-20

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml), fraction 41 (50 ml) and fraction 42 (50 ml)

Callus: Bap (6)

Confidence limits: $n = 10$

Lowest detectable amount of kinetin: $3 \mu\text{g l}^{-1}$ ($174 \pm 46 \text{ mg callus flask}^{-1}$)

Further details are tabulated with Figure 4.22

fraction 9 in two of the three samples and in fractions 14 and 15 of samples B and C respectively. Significant peaks of activity with longer retention times were detected in sample A. The initial bioassay of samples of tubers 3 mm to 5 mm diameter was contaminated. A single sample was subsequently extracted and bioassayed concurrently with tubers 7.5 to 10 mm diameter. Barely detectable activity was present in the fractions expected to contain zeatin riboside and isopentenyladenine (Fig 4.24). Observations of callus growth in the flasks not infected during the initial bioassay support the above results.

The cytokinin-like activity in tubers 5 to 7.5 mm diameter was resolved into at least three discrete components (Fig 4.25). Two of the components exhibited similar elution volumes to zeatin riboside and zeatin respectively. The third component eluted from the column more rapidly than would be expected of isopentenyladenine. Low amounts of activity exhibiting elution volumes similar to or greater than isopentenyladenine would not have been detected since the remainder of the bioassay was set up with older and less sensitive callus. The amount of cytokinin-like activity in elution volumes corresponding to zeatin and zeatin riboside and isopentenyladenosine was approximately equivalent to $1 \mu\text{g KE l}^{-1}$ each. The total amount of cytokinin-like activity was calculated from the regression line $Y = 1.59X + 0.65$ and by reference to the kinetin standards themselves.

Increased levels of cytokinin-like activity were apparent in at least two of the three samples of tubers 7.5 to 10 mm diameter (Fig 4.26). This increase was due mainly to increased activity with an elution volume similar to that of authentic zeatin riboside. Other peaks of activity were not repeated consistently among the samples.

Cytokinin-like activity collected in fraction 9 was again the most prevalent activity in tubers 10 to 15 mm diameter (Fig 4.27), 15 to 20 mm diameter (Fig 4.28) and 20 to 25 mm diameter (Fig 4.29). Little cytokinin-like activity was detected in extracts of tubers 40 to 50 mm diameter (Fig 4.30). Only traces of cytokinin-like activity were detected after fraction 9 in extract of tubers greater than 10 mm diameter (Figs 4.26, 4.27, 4.28, 4.29, 4.30).

Accurate determination of the cytokinin-like activity could only be made in three of the size categories selected for analysis (Table 4.13). Cytokinin-like activity was detected in the remaining categories

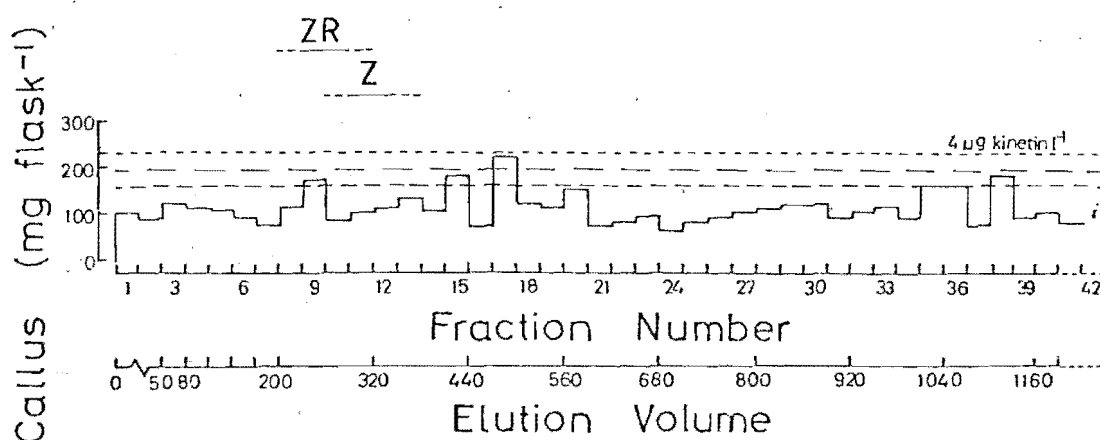


FIGURE 4.24

Soybean callus bioassay of extract of tubers 3 mm to 5 mm diameter (4 g eq FW). Extracts were resolved by partition chromatography on Sephadex LH-20

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml) and fraction 41 (50 ml)

Callus: Bap (7)

Confidence limits: $n = 8$

Regression line: $Y = 1.72X - 0.28$

Lowest detectable amount of kinetin: $4 \mu\text{g l}^{-1}$ ($232 \pm 21 \text{ mg callus flask}^{-1}$)

Further details are tabulated with Figure 4.22

FIGURE 4.25

Soybean callus bioassay of extracts of tubers 5 mm to 7.5 mm diameter (4 g eq FW). Extracts were resolved by partition chromatography on Sephadex LH-20

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml), fraction 41 (50 ml) and fraction 42 (50 ml)

Callus: Bap (3) (fractions 1-17); Bap (2) (fractions 18-42)

Confidence limits: $n = 9$ (fractions 1 - 17 only)

Regression line: $Y = 1.59X + 0.65$ (Bap 3)

Lowest detectable amount of kinetin:

Bap 3; $1 \mu\text{g l}^{-1}$ (836 ± 68 mg callus flask $^{-1}$)

Bap 2; $6 \mu\text{g l}^{-1}$ (249 ± 36.4 mg callus flask $^{-1}$)

Further details are tabulated with Figure 4.22

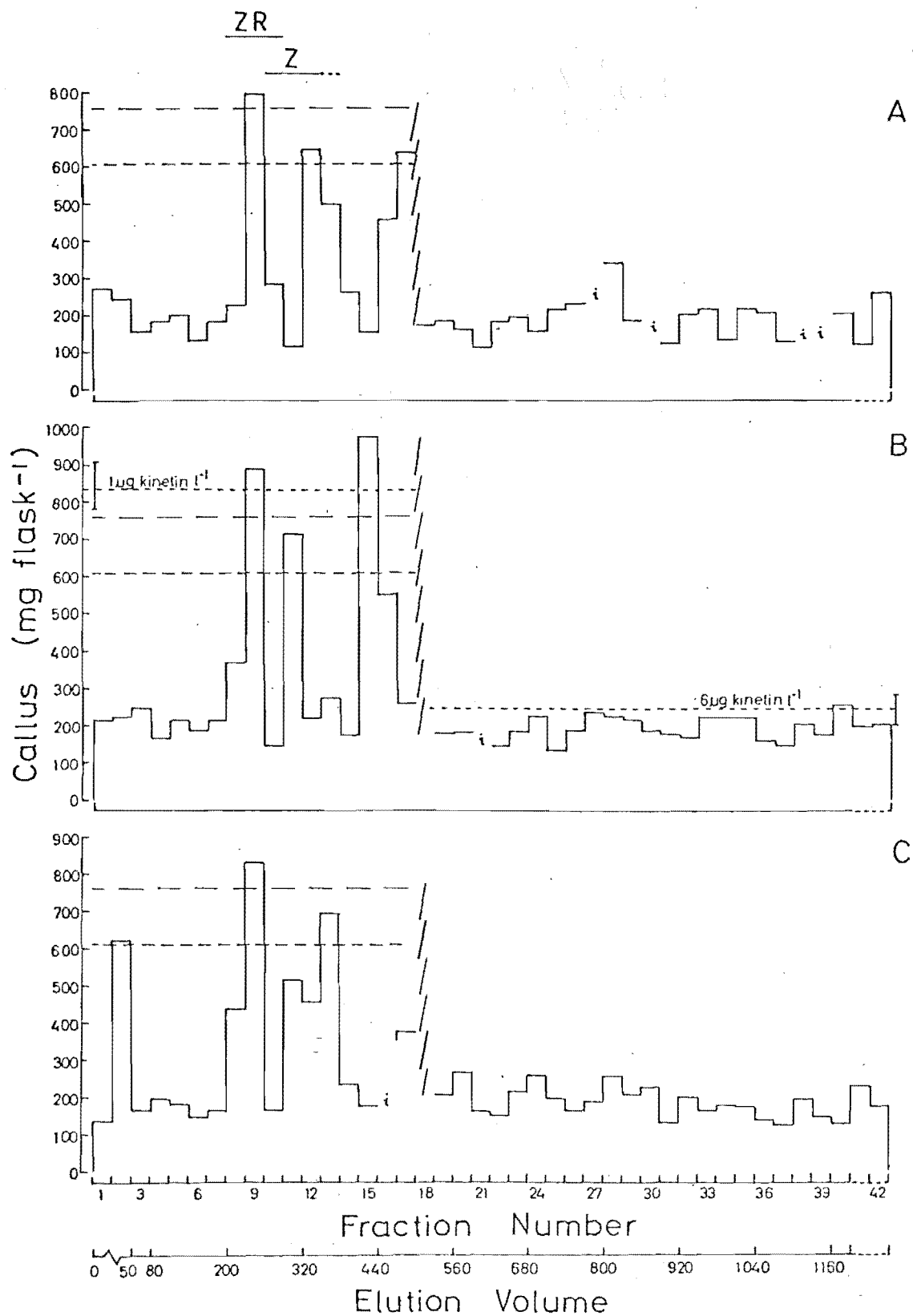


FIGURE 4.25

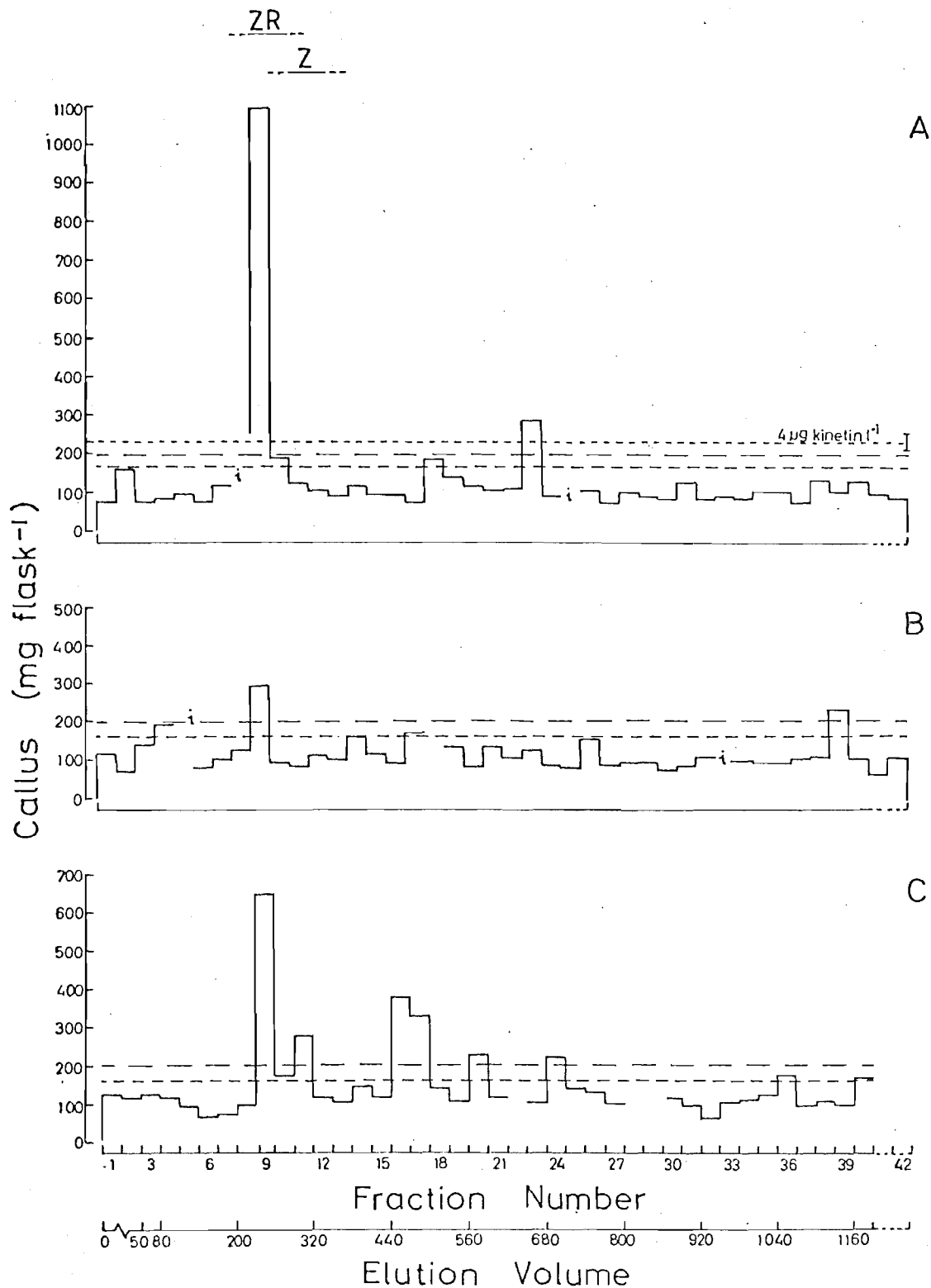


FIGURE 4.26

Soybean callus bioassay of extracts of tubers 7.5 to 10 mm diameter (4 g eq FW). Extracts were resolved by partition chromatography on Sephadex LH-20.

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml), fraction 41 (50 ml) and fraction 42 (50 ml)

Callus: Bap (7)

For further details see Figure 4.24

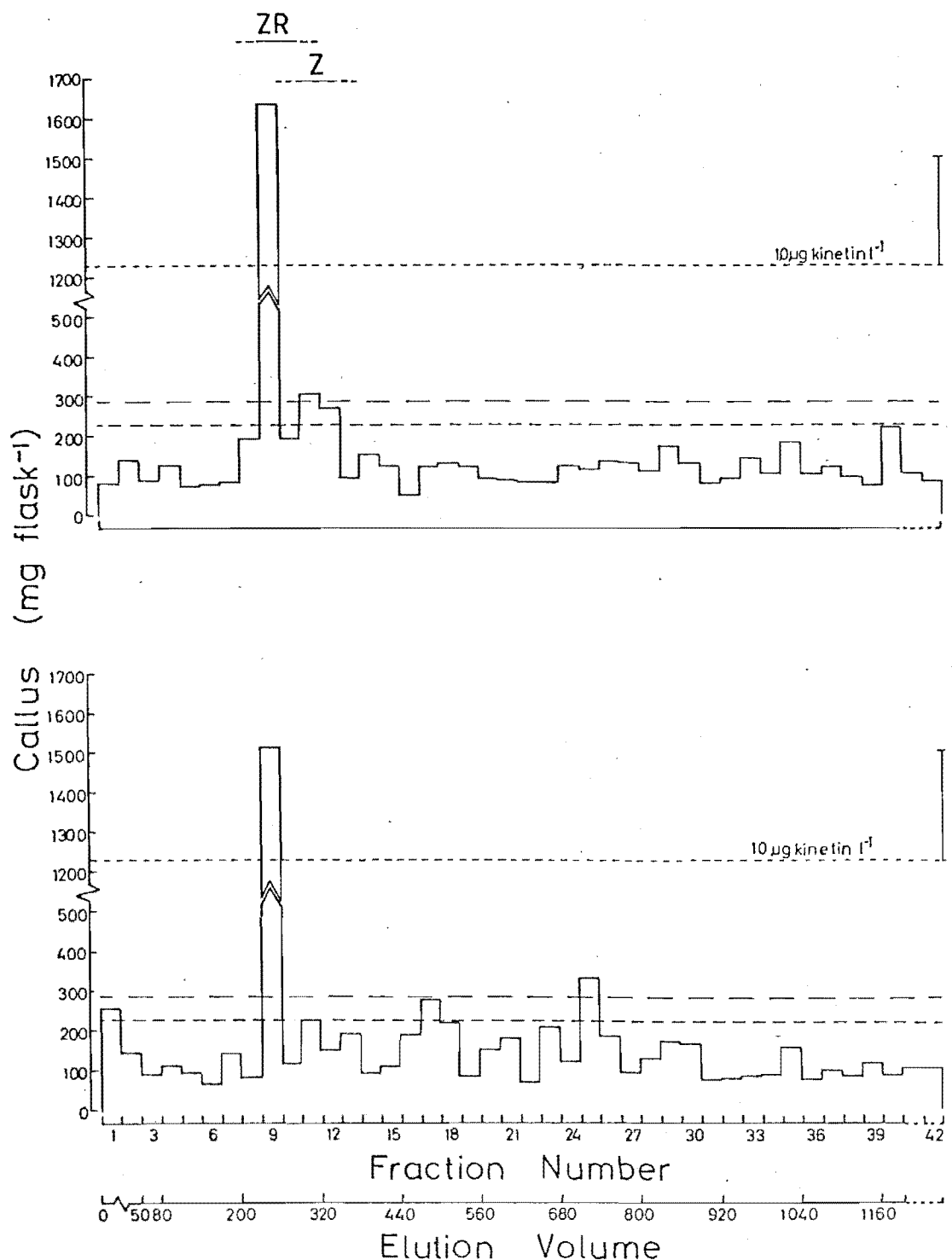


FIGURE 4.27

Soybean callus bioassay of extracts of tubers 10 to 15 mm diameter (4 g eq FW). Extracts were resolved by partition chromatography on Sephadex LH-20

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 41 (50 ml) and fraction 42 (50 ml)

Callus: Bap (9)

Confidence limits: $n = 10$

Regression line: $Y = 1.96X - 0.65$

Lowest detectable amount of kinetin: $4 \mu\text{g l}^{-1}$ ($431 \pm 179 \text{ mg callus}$
 flask^{-1})

Further details are tabulated with Figure 4.22

FIGURE 4.28

Soybean callus bioassay of extracts of tubers 15 to 20 mm diameter (4 g eq FW). Extracts were resolved by partition chromatography on Sephadex LH-20

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 41 (50 ml) and fraction 42 (50 ml)

Callus: Bap (12)

Confidence limits: $n = 10$

Regression line: $Y = 1.10X - 0.31$

Lowest detectable amount of kinetin: $3 \mu\text{g KE l}^{-1}$ ($122.7 \pm 25 \text{ mg callus flask}^{-1}$)

Further details are tabulated with Figure 4.22

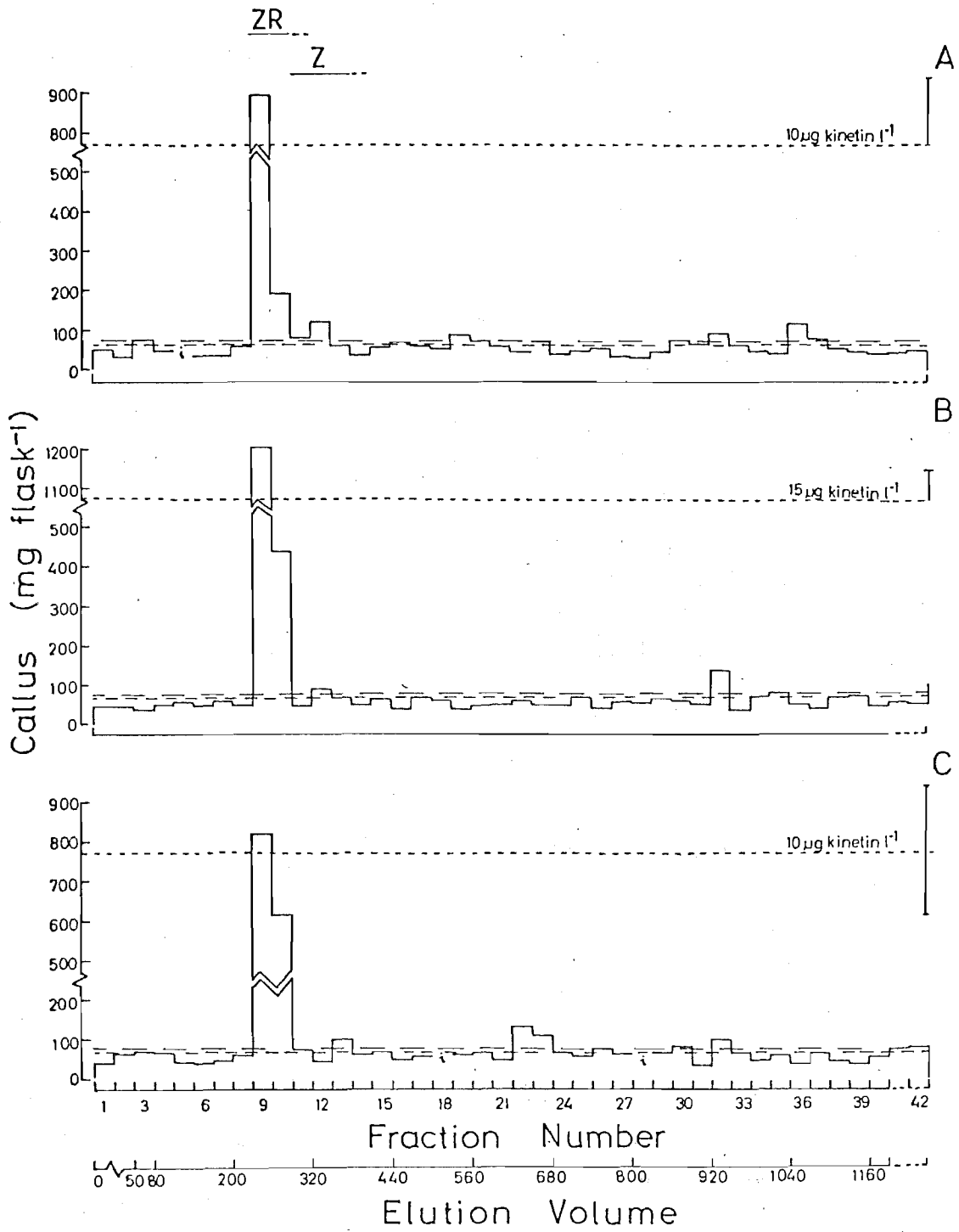


FIGURE 4.28

FIGURE 4.29

Soybean callus bioassay of extracts of tubers 20 to 25 mm diameter (4 g eq FW). Extracts were resolved by partition chromatography on Sephadex LH-20

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 41 (50 ml) and fraction 42 (50 ml)

Callus: Bap (13)

Confidence limits: $n = 10$

Regression line: $Y = 0.84X + 0.02$

Lowest detectable amount of kinetin: $3 \mu\text{g l}^{-1}$ (226 ± 90 mg callus
flask $^{-1}$)

Further details are tabulated with Figure 4.22

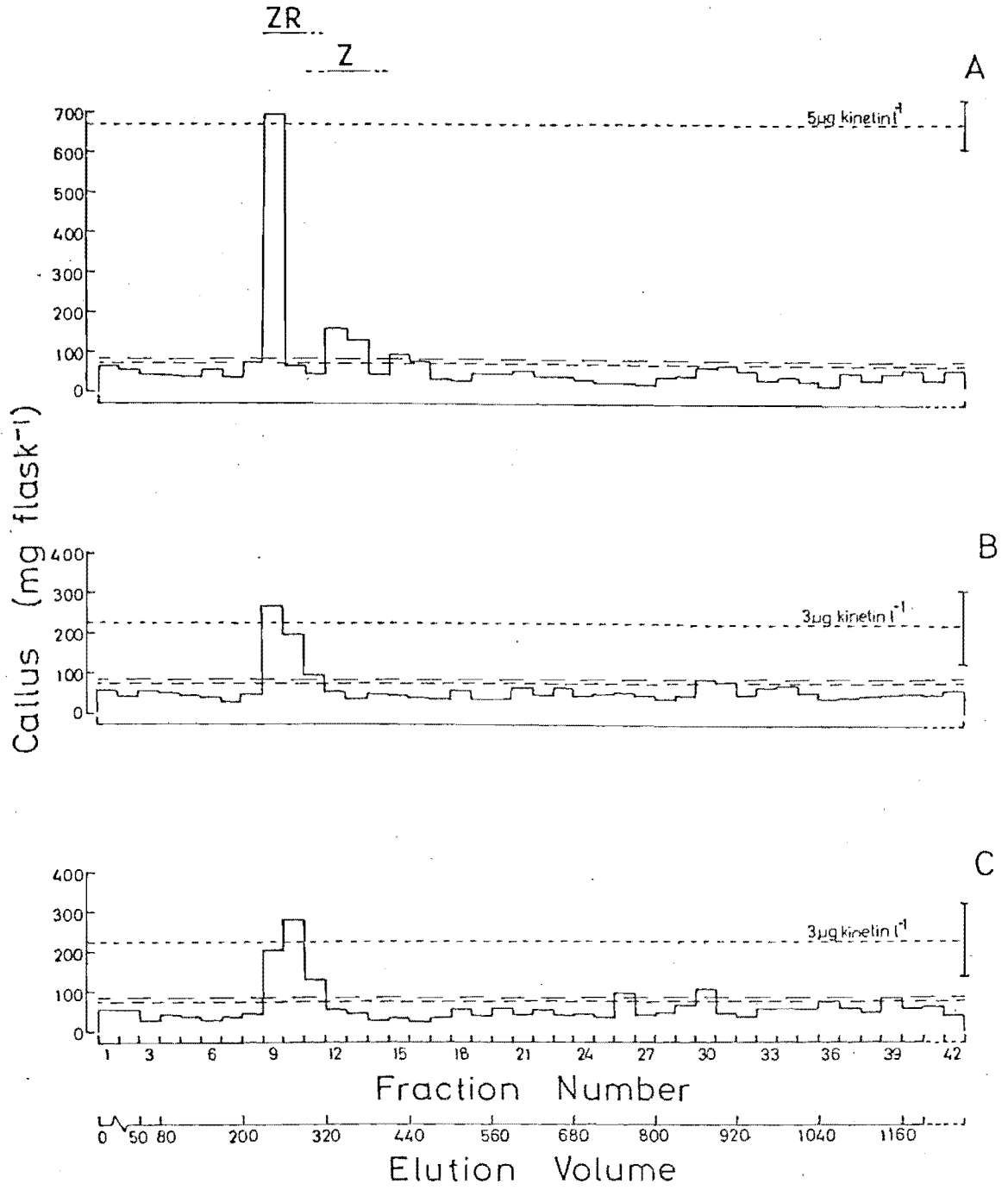


FIGURE 4.29

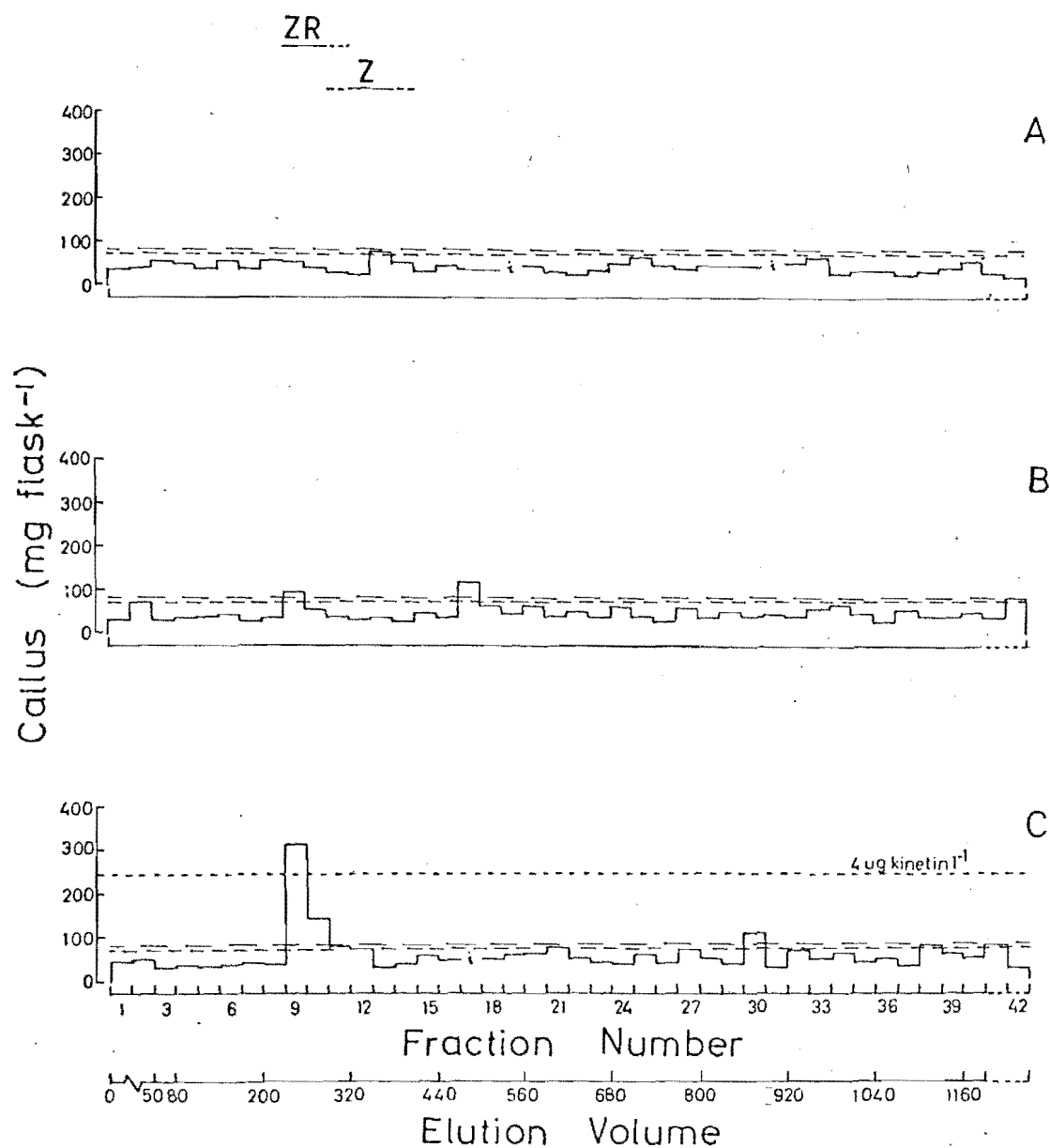


FIGURE 4.30

Soybean callus bioassay of extracts of tubers 40 to 50 mm diameter (4 g eq FW). Extracts were resolved by partition chromatography on Sephadex LH-20.

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 41 (50 ml) and fraction 42 (50 ml)

Callus: Bap (14)

Confidence limits: $n = 10$

Regression line: $Y = 1.27X - 0.61$

Lowest detectable amount of kinetin: $2 \mu\text{g l}^{-1}$ (193 mg callus
flask⁻¹)

Further details are tabulated with Figure 4.22

TABLE 4.14

PPD Experiment 1. Estimation of the cytokinin-like activity present in 4 g fresh weight samples of stolon and tuber material

Size Category Tuber (mm diam)	Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)								Total
		9	10	11	Fractions 12 13		15	17	25	
5 < 7.5	A	1.24			ca 1 ^a			ca 1 ^a		3.24
	B	1.41		1.10			1.60			4.09
	C	1.30				1.06				2.35
7.5 < 10	A	6.32								6.32
	B	ca 4. ^a								ca 4
	C	ca 4. ^a								ca 4
10 < 15	B	14.71		3.10						17.81
	C	12.80					3.02	3.21		18.02
15 < 20	A	12.48								12.48
	B	24.46	4.85							29.31
	C	10.86	7.01							17.87
20 < 25	A	6.28								6.28
	B	ca 6. ^a								ca 6
	C	ca 6. ^a								ca 6
40 < 50	A	nd ^b								
	B	trace								
	C	5.32								5.32

^a Below straight-line portion of standard curve. Activity estimated by reference to the appropriate set of kinetin standards

^b nd - not detectable

and for tubers 7.5 to 10 mm and 20 to 25 mm diameter activity in samples B and C was calculated by direct reference to the kinetin standards (Section 2.2.3.3).

The majority of the cytokinin-like activity in tubers greater than 7.5 mm diameter was detected in the same fractions (9 and 10) as were found to contain samples of authentic zeatin riboside, and it was this activity which changed with increasing tuber size in extracts of 4 g tissue fresh weight. The total cytokinin-like activity is presented in Figure 4.31 on a fresh weight, dry weight and per tuber basis as a function of increasing tuber diameter. The cytokinin-like activity was low in small tubers but a much higher level of activity was present in tubers between 10 and 20 mm diameter. A rapid decrease in the cytokinin-like activity occurred in larger tubers when calculated on a per unit fresh or dry weight basis. However, the overall trend on a per tuber basis was that of an increase.

Extracts of 8 g eq FW of stolon and tuber material were partitioned on a column of Sephadex LH-20 using 20% ethanol as the eluant (Section 4.2.4.1B). All samples were analysed for cytokinin-like activity in the same bioassay (Fig 4.32). The horizontal bars indicate that complete resolution of zeatin riboside and zeatin standards was obtained. Fractions 12 and 13 would be expected to contain zeatin riboside, and fractions 16 and 17 to contain zeatin. Under the same chromatography conditions isopentenyladenine was collected in fractions 27 to 29. These fractions were not collected for the above bioassay.

Cytokinin-like activity was detected in fraction 16 of the stolon extract although no activity was apparent at this elution volume in an extract of tubers 1.5 to 5 mm diameter. An increasing amount of cytokinin-like activity was detected in extracts of the larger tubers up to 15 to 20 mm in diameter, whereas activity in fraction 16 of tubers 20 to 25 mm diameter was barely detectable.

A small amount of cytokinin-like activity was detected in fraction 12 in tubers 5 to 7.5 mm diameter. However, cytokinin-like activity was detected in this fraction in extract of tubers 10 to 15, 15 to 20 and 20 to 25 mm in diameter. Small amounts of cytokinin-like activity may be present in other fractions, for example fractions 8, 9, 20, 23 and 24, although some peaks may simply be the result of a random callus response as exemplified by fractions 1 (tubers 7.5 to 10 mm diameter) and 3 (tubers 20 to 25 mm diameter).

The amount of cytokinin-like activity was determined in kinetin equivalents from the equation of the regression line $Y = 1.35 X - 0.32$ for

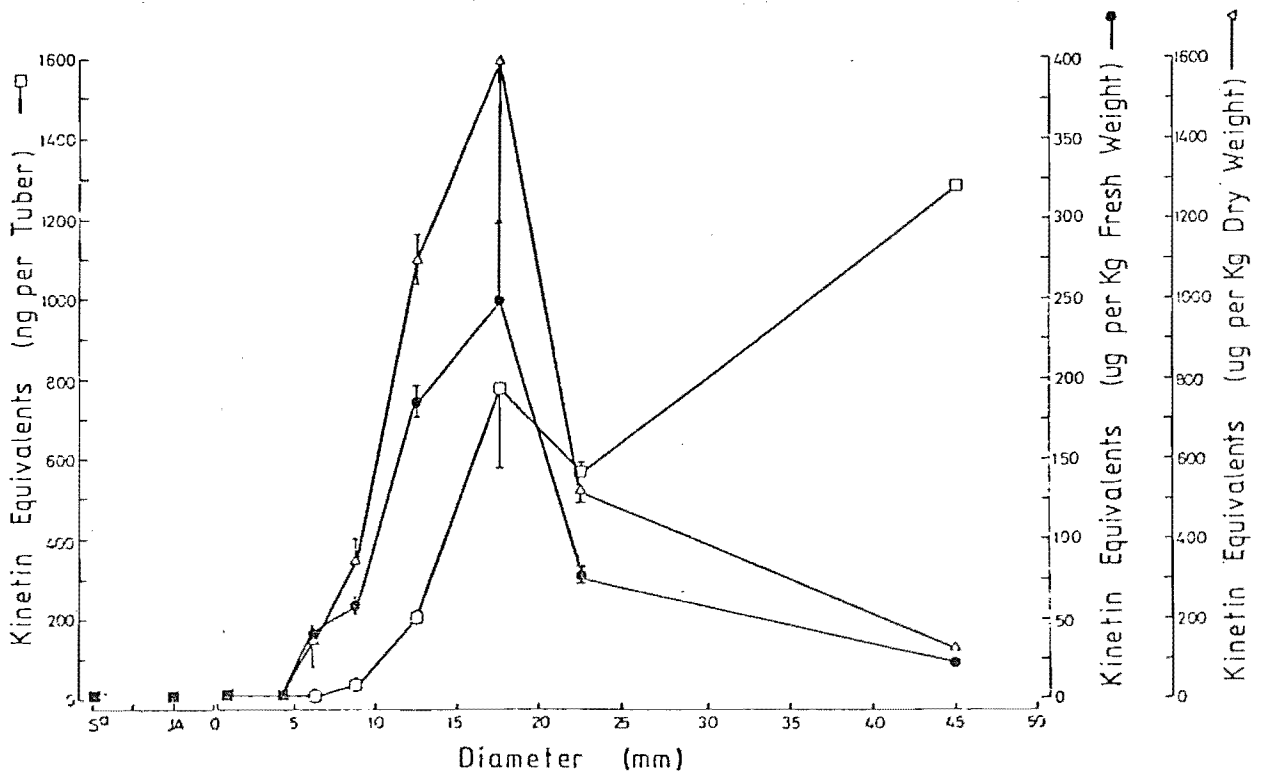


FIGURE 4.31

PPD Experiment 1. Total cytokinin-like activity in potato tubers following the extraction of 4 g eq FW tissue. The data are expressed as kinetin equivalents either on a per tuber or per fresh or dry weight basis.

Vertical bars show one standard error above and/or below the line.

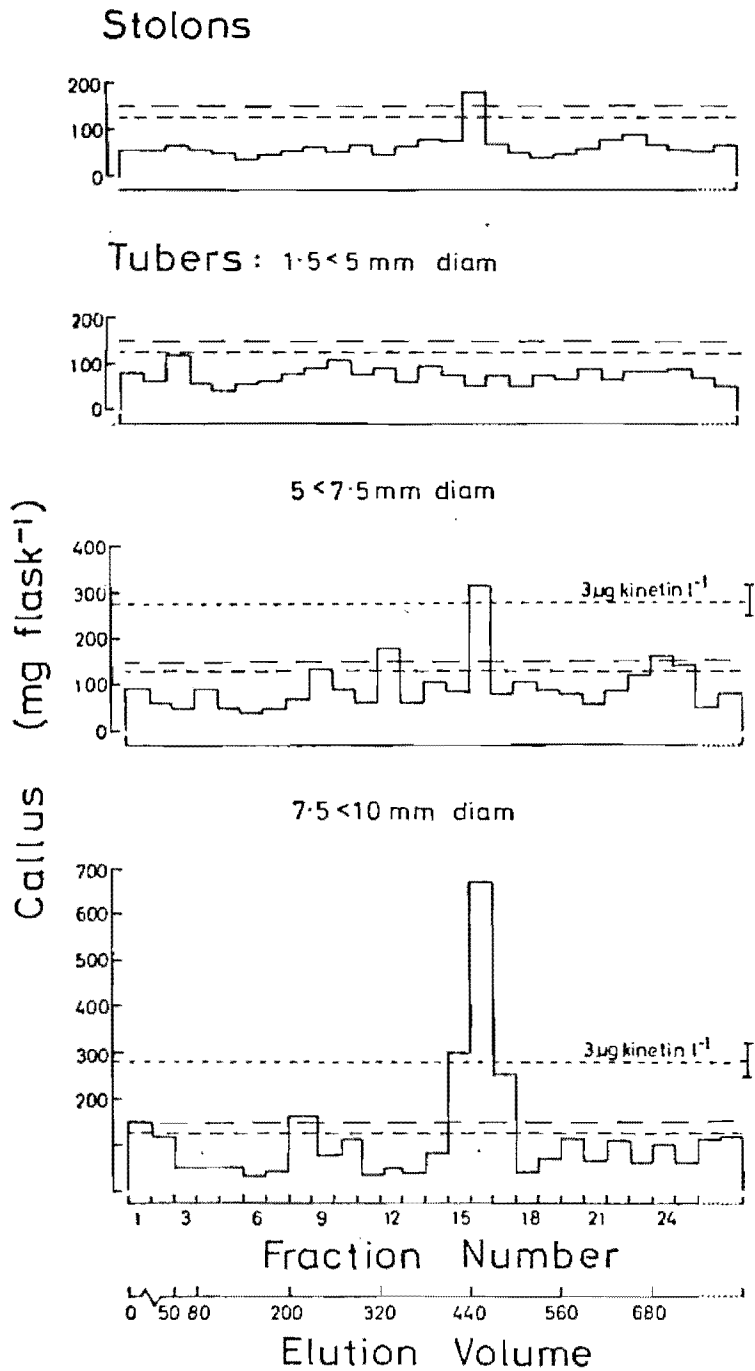
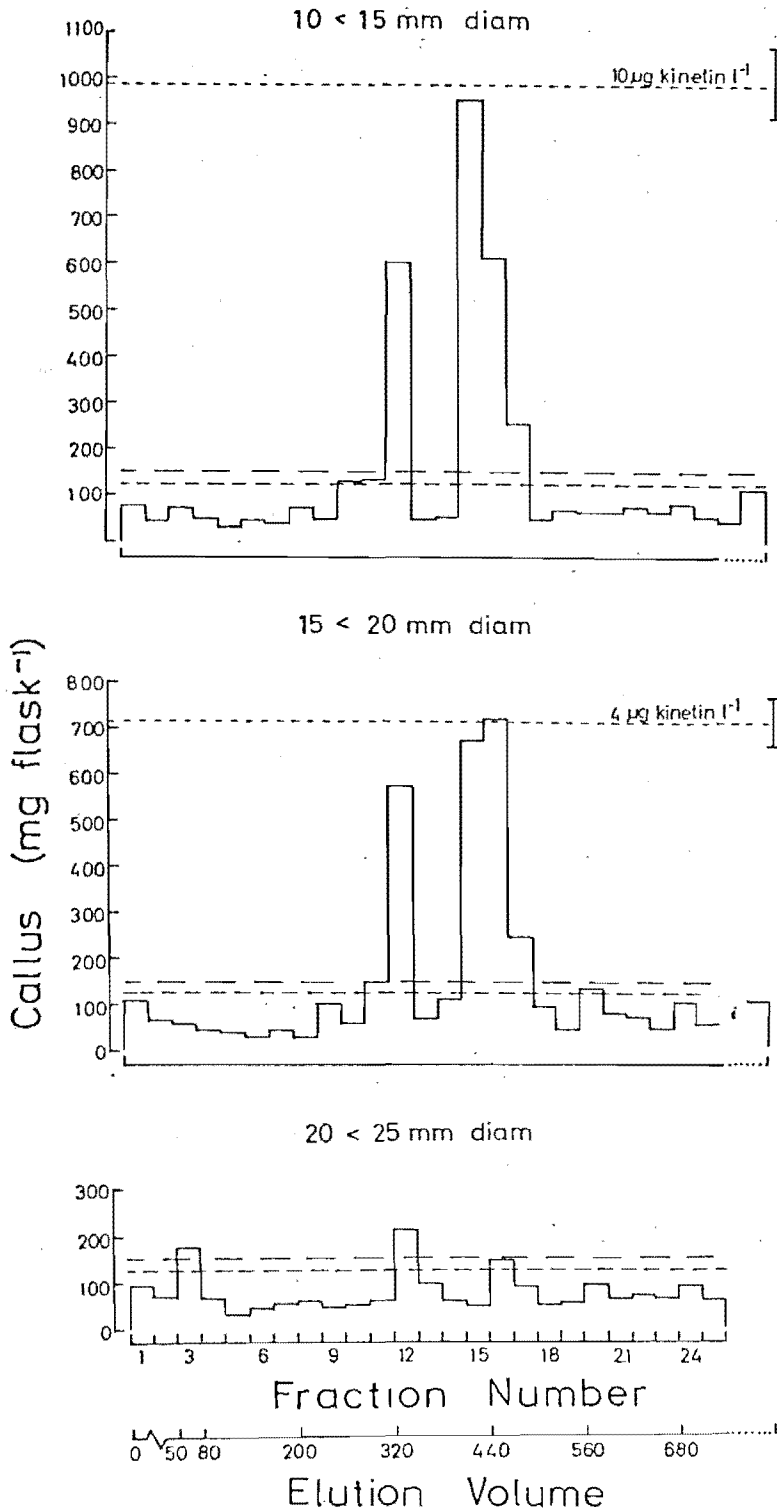


FIGURE 4.32

Soybean callus bioassay of extracts of stolons and tubers of the sizes specified on the figure. 8 g eq FW extracted and resolved by partition chromatography on Sephadex LH-20

Solvent: 20% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 26 (250 ml) and fraction 27 (310 ml)



Callus: Bap (31)

Confidence limit: n = 18

Regression line: $Y = 1.35X - 0.32$

Lowest detectable amount of kinetin: $1 \mu\text{g l}^{-1}$ ($125 \pm 22 \text{ mg callus flask}^{-1}$)

Further details are tabulated with Figure 4.22

those values lying beyond the 99% confidence limit (Table 4.15).

TABLE 4.15

Estimation of the cytokinin-like activity present in 8 g eq FW of stolon and tuber extracts. The activity was resolved by partition chromatography on Sephadex LH-20 eluted with 20% ethanol

Size Category	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)				Total
	8 - 9	Fractions		24	
		11 - 12	15 - 17		
Stolons ^a	nd ^b	nd	2.35	nd	2.35
Tubers (mm diam)					
1.5 < 5	nd	nd	nd	nd	
5 < 7.5	ns ^c	2.35	3.00	2.27	7.62
7.5 < 10	2.28	nd	10.93	nd	13.21
10 < 15	nd	4.82	16.25	nd	21.06
15 < 20	nd	4.55	13.87	nd	18.42
20 < 25	nd	2.46	ns	nd	4.78

^a Stolons from tuber forming plants

^b nd = not detectable

^c ns = not significant

A comparison of the cytokinin-like activity collected in fraction 12 following the bioassay of 8 g eq FW samples (Table 4.15) and fraction 9 following the bioassay of 4 g eq FW samples (Table 4.14) is presented in Table 4.15. Significantly less activity with an elution volume similar to zeatin riboside was detected after the bioassay of 8 g fresh weight than of 4 g.

Figure 4.33 was compiled from the relevant sections of Tables 4.14 and 4.15. The values representing "zeatin riboside-like" activity were obtained after the bioassay of 4 g eq FW tissue (fraction 9; Table 4.14) while those representing "zeatin-like" activity were obtained after the bioassay of 8 g eq FW tissue (fractions 15 to 17; Table 4.15).

A slightly higher level of "zeatin-like" activity may be present in the smaller tubers but there is significantly more "zeatin riboside-like" activity in tubers 15 to 20 mm diameter determined on both a fresh and a dry weight basis (Fig 4.33). The decrease in "zeatin riboside-like" activity in the larger tubers was mirrored by a decrease in the "zeatin-like" activity to a barely detectable level when calculated on a per unit weight basis. The extract of 8 g fresh weight of tubers 40 to 50 mm diameter was not bioassayed so the increase in the

FIGURE 4.33

PPD Experiment 1. Cytokinin-like activity expressed as kinetin equivalents in stolons and potato tubers. The solid symbols represent the cytokinin-like activity extracted from 4 g FW tissue which had an elution volume similar to that of zeatin riboside. The open symbols represent the activity from 8 g FW tissue which had a similar elution volume to that of zeatin. Vertical bars show one standard error above and/or below the lines.

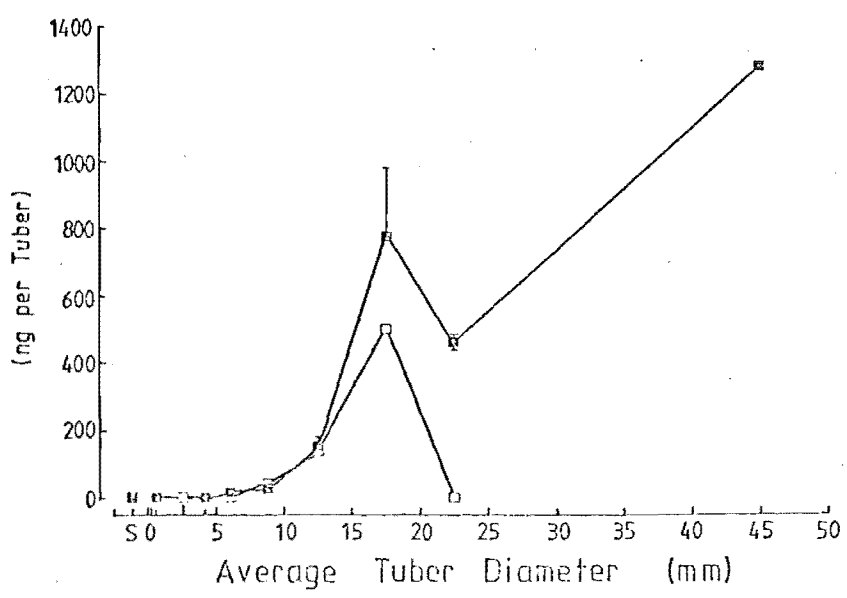
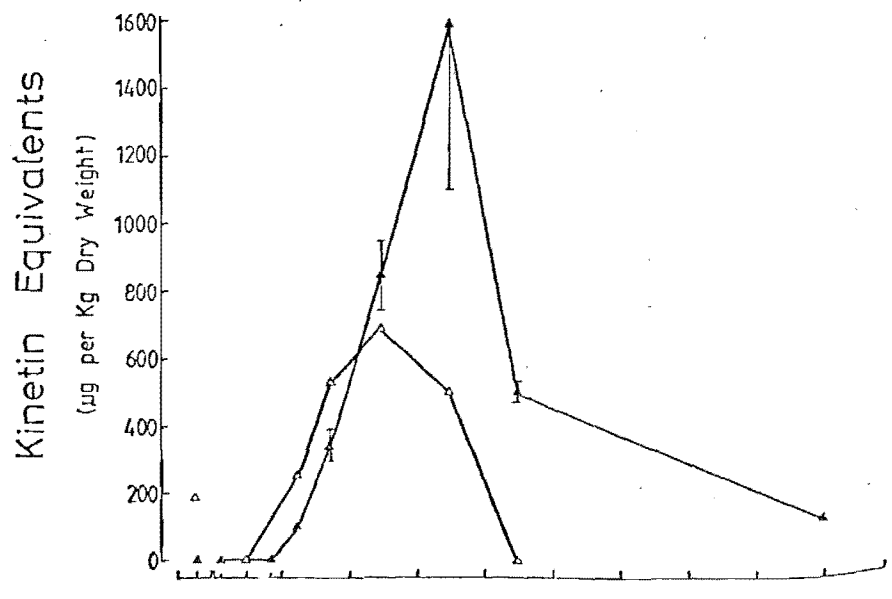
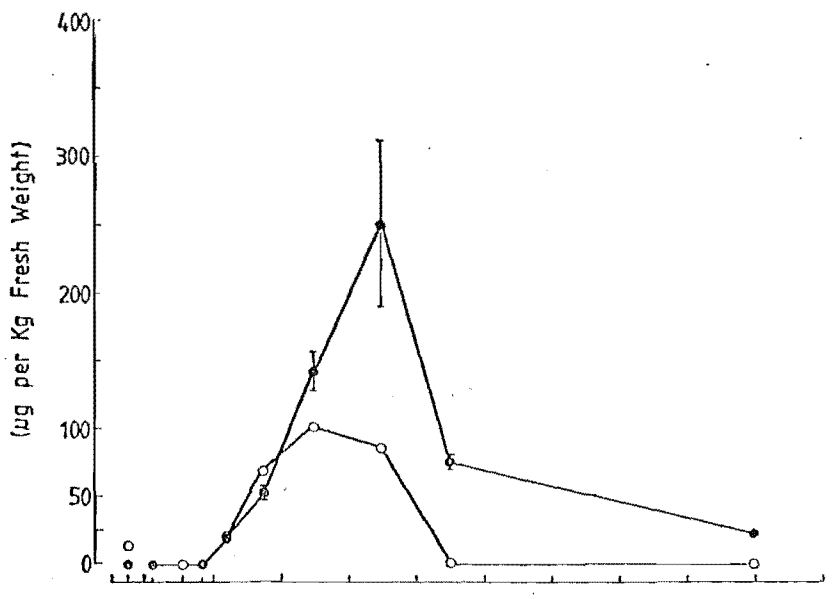


FIGURE 4.33

cytokinin-like activity on a per tuber basis detected in the bioassay of 4 g fresh weight was not confirmed.

TABLE 4.16

PPD Experiment 1. Comparison of the amount of cytokinin-like activity present in fraction 9 and fraction 12 following the extraction of 4 g eq FW and 8 g eq FW respectively of stolons and tubers. The activity was resolved by partition chromatography on Sephadex LH-20 eluted with 35% ethanol (4 g eq FW) and 20% ethanol (8 g eq FW), respectively

Size Category	µg KE per Kg fresh weight	
	Fraction 9	Fraction 12
Stolons	Trace	nd ^a
Tubers (mm diam)		
5 < 7.5	16.5	14.7
7.5 < 10	58.2	nd ^a
10 < 15	142.8	30.1
15 < 20	248.5	28.4
20 < 25	76.2	15.4
40 < 50	22.4	-

nd^a - not detected

A combination of the "zeatin riboside-" and "zeatin-like" activities from the two different sets of experiments is presented in Figure 4.34. A steady increase in the cytokinin-like activity per unit dry weight was apparent as tubers expanded from 5 mm in diameter to 20 mm in diameter. A marked decrease occurred as tubers expanded further.

Tubers 10 to 20 mm in diameter contained more cytokinin per unit weight than the other size categories analysed (Figs 4.31, 4.34). After 25, 30 and 35 days in the controlled environment room, tubers 10 to 20 mm in diameter contributed 37, 50 and 56% respectively to the total fresh weight of stolons and tubers (Fig 4.18, Table 4.17). This represented only 2.5% of the total number of stolons and tubers at Day 25, 8.5% at Day 30 and only 4.5% at Day 35 (Fig 4.17, Table 4.17). By Day 84, 12.5% of the tubers had high cytokinin concentrations, but these tubers contributed only 2.5% to the total fresh weight.

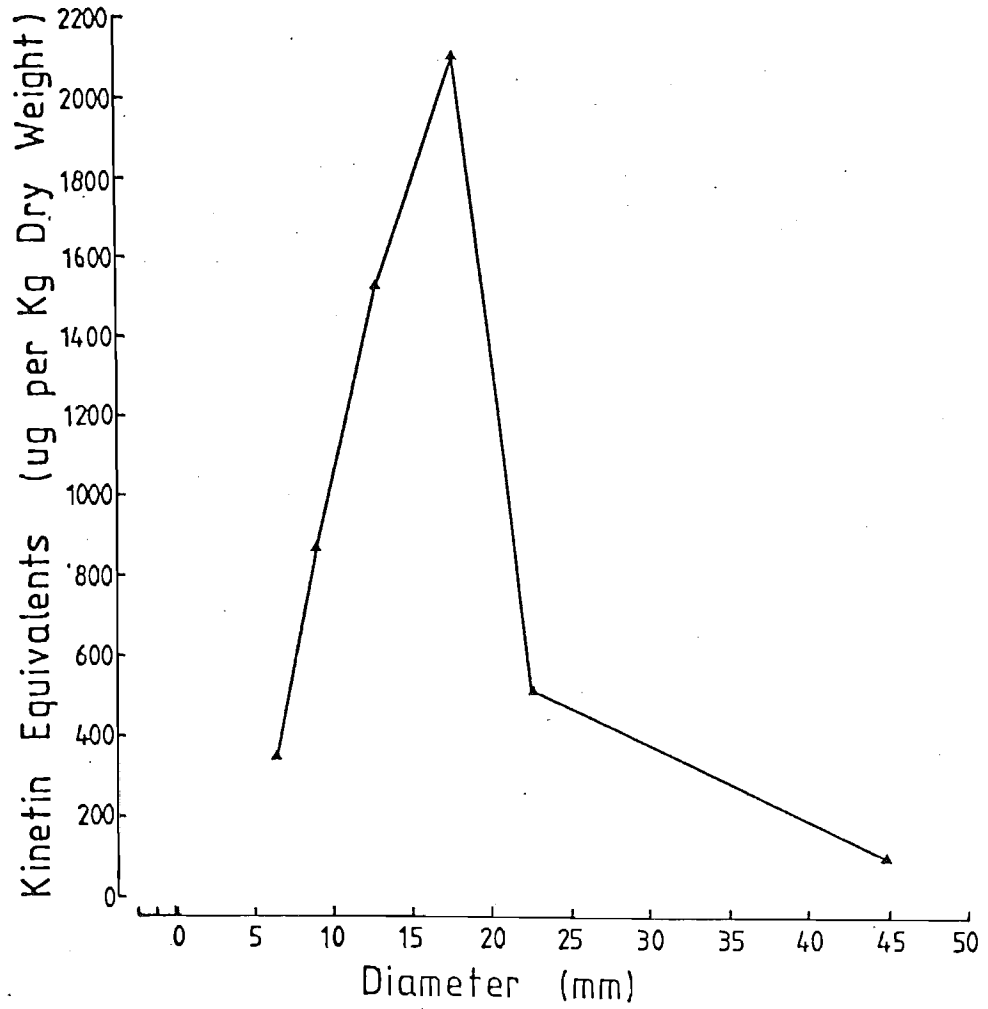


FIGURE 4.34

PPD Experiment 1. Cytokinin-like activity expressed as kinetin equivalent in stolons and potato tubers. The figure represents the total of the two component parts presented in Figure 4.33

TABLE 4.17

PPD Experiment 1. Contribution from *S. andigena* tubers of the size categories 10 to 15 mm and 15 to 20 mm diameter to the total number and weight of stolons and tubers per plant

Days after transfer to controlled environment conditions	Percentage Contribution			
	10 < 15	Tuber Number 15 < 20	Total	Tuber Weight Total
21	0	0	0	0
22	0.1	0	0.1	8.2
23	0.3	0	0.3	7.6
24	0.2	0	0.2	6.7
25	2.3	0.2	2.5	37.0
30	6.6	1.9	8.5	50.3
35	3.4	1.2	4.5	55.7
42	2.1	1.2	3.3	35.4
56	6.0	3.3	9.3	20.5
84	6.1	6.5	12.5	3.7

4.3.4.2 Observations on the effects of different growth conditions on the tuberisation response of *S. andigena* cv 165 The response of *S. andigena* plants grown under long day conditions in the controlled growth rooms at the University of Canterbury differed from those observed in the controlled environment rooms at Plant Physiology Division (PPD), DSIR, Palmerston North.

Plants grown under LD conditions at Canterbury in 1977 and 1978 were slow to tuberise but tuber initiation was promoted by SD conditions (Table 4.18).

TABLE 4.18

Response of *S. andigena* to long day and short day conditions

Location and number of experiment	Days from shoot emergence	Length of exposure		Number of plants harvested	Percentage tuberisation
		LD ^a	SD ^b		
Canterbury:					
Experiment 1	49	49	0	5	0
	49	28	21	6	100
Experiment 2	53	53	0	21	9
		37	16	12	100

^a 16 hours photosynthetically active radiation (ca 150 $\mu\text{E m}^{-2}\text{s}^{-1}$)

^b 8 hours photosynthetically active radiation (ca 150 $\mu\text{E m}^{-2}\text{s}^{-1}$)

Many of the plants grown at PPD for experiment 1 tuberised within 21 long days (Table 4.10). Tuber material used in the growth room experiments was obtained in different seasons and subjected to different storage times. A summary of the field and storage details is presented in Table 4.19.

TABLE 4.19

Field harvest dates, storage conditions and length of storage of *S. andigena* tubers used in all controlled environment experiments

Location and number of experiment	Field harvest date	Storage history and sprout appearance	Total storage time
Canterbury:			
Experiment 1	Late March 1977	4 months storage shed (sprouting)	4 months
2	16/3/78	1 week at 28 ⁰ C and high humidity (sprouting)	1 week
3	26/3/79	20 ⁰ C; dark (sprouting)	2 months
PPD:			
Experiment 1	Late March 1978	4 months storage shed; sprouts removed 25/7/78. 5½ months 4 ⁰ C; sprouts removed 3/8/78. Sprouting promoted 28 ⁰ C, high humidity	9½ months
2	21/2/79	1 week 20 ⁰ C, dark. Sprouting not readily achieved	1 week

Details of the day/night temperature regime and percentage tuberisation of plants from different mother tuber material is presented in Table 4.20.

An increased tendency to tuberise under LD conditions had occurred over the three years as shown by the three experiments conducted at Canterbury (Table 4.20). However, the same tendency was also noted if Canterbury experiment 2 is compared to PPD experiment 1. The tubers used in the Canterbury experiment were freshly dug and collected from the field plot, those for PPD experiment 1 were collected from the DSIR

storage sheed (Table 4.19). The tubers were reported to be the same seed lot. Tubers used in PPD experiment 1 had been stored for a substantial period of time and in addition a lowered night temperature was used.

TABLE 4.20

Percentage tuberisation attained by *S. andigena* plants grown from seed tubers with different storage histories

Location and number of experiment	Field harvest date	Length of storage	Days after shoot emergence	Day/night temp (°C)	Number of plants examined	Percentage tuberisation
Canterbury: ^a						
Experiment 1	Late March 1977	4 months	49	20/20	5	0
2	16/3/78	1 week	52	20/20	21	9.0
3	26/3/79	2 months	28 44	20/20	8 12	25.0 16.0
Days after transfer to controlled environment rooms						
PPD: ^b						
Experiment 1	Late March 1978	10 months	21 30	20/16	26 30	61.0 100.0
2	21/2/79	1 week	21 42	20/20	14 16	0 ^c 81.3

^a 16 hours photosynthetically active radiation (ca 150 $\mu\text{E m}^{-2} \text{s}^{-1}$)

^b 8 hours photosynthetically active radiation ($158 \pm 3 \text{ W m}^{-2}$) plus 8 hours photoperiodically active radiation (7 W m^{-2})

^c Stolon tips removed for starch and soluble sugar analysis.

Maintaining equal day and night temperatures did not prevent tuberisation from occurring under LD conditions in PPD experiment 2 (Table 4.21). Over 81% of the plants had tuberised within 42 days. Tuberisation did not occur as early when the seed tubers used were freshly dug although a full comparison is confounded by temperature (Table 4.21).

TABLE 4.21

Growth of *S. andigena* under differing conditions in the CE rooms (PPD). Plants were subjected to different day/night temperature regimes^a

Location and number of experiment	Length of storage	Days after transfer to controlled environment rooms	Temperature (°C)		Number of plants	Percentage tuberisation
			Day	Night		
PPD:						
Experiment 1	10 months	21	20	16	26	61.0
		30				30
Experiment 2	1 week	21	20	16	15	0
		21				20
	42	16	81.3			

^a Photoperiod: 8 hours PAR plus 8 hours low intensity lighting. Refer to Table 4.20 for light intensities.

The intensity and the daily length of exposure to photosynthetically active radiation also differed between experiments 1 and 2 at Canterbury and experiment 1 at PPD (Appendices 6 and 7). The response of *S. andigena* plants to differing amounts of photosynthetically active radiation (PAR) was tested during experiment 2 at PPD (Table 4.22). Tuber formation occurred faster when plants were exposed to 16 hours of high intensity PAR than when exposed to eight hours of PAR and eight hours of low intensity but photoperiodically active radiation per day. This is in direct contrast to the observations made in the two previous years when plants were grown at Canterbury (experiments 1 and 2; Table 4.22) to 16 hours PAR delayed tuber formation rather than hastened it.

Experiment 3 at Canterbury used tuber material which had been stored for two months longer than that used in experiment 2 at PPD (Table 4.19). Plants tuberised readily under 16 hours of PAR (Table 4.23). A higher day and night temperature coupled with a slightly higher light intensity did not inhibit tuber formation under LD conditions in the Canterbury growth rooms during experiment 3 (Fig 4.22).

TABLE 4.22

Response of *S. andigena* plants to differing amounts of photosynthetically active radiation (PAR). Seed tubers were planted within one week of harvest. Day and night temperatures were constant at 20°C.

Location and number of experiment	Exposure to Photosynthetically Active Radiation ^a				Number of plants examined	Percentage tuberisation
	Hours day ⁻¹	Days	Hours day ⁻¹	Days		
Canterbury:						
Experiment 2	16	52	0	0	21	9.0
	16	38	8	14	12	100.0
<hr/>						
Hours of Light Exposure						
	PAR ^a	Low intensity ^a radiation	Days of light exposure		Number of plants examined	Percentage tuberisation
<hr/>						
PPD:						
Experiment 2	8	8	21		14	0
	8	8	42		16	81.3
	16	0	21		13	83.3
	16	0	28		12	83.3

^a Refer to Table 4.20 for light intensity data

TABLE 4.23

A comparison of the response of *S. andigena* plants grown in different controlled growth rooms at Canterbury (experiment 3). Both light intensity and temperature differed between the rooms

Room Number	Temperature (°C)		Light intensity ($\mu\text{E m}^{-2} \text{ s}^{-1}$)	Days from shoot emergence	Number of plants examined	Percentage tuberisation
	Day	Night				
1	20	20	100	28	8	25
				44	12	100
2	23	23	212	28	4	0
				44	8	100

4.4 DISCUSSION

4.4.1 Preliminary Experiments

Mature non-sprouting potato tubers were readily available during the winter and were used initially in trial extractions and bioassays as they were claimed to be a source of cytokinin-containing tissue (eg Okazawa, 1969, 1970; Engelbrecht and Bielińska-Czarnecka, 1972; Antis and Northcote, 1975; Conrad and Köhn, 1975; van Staden and Dimalla, 1978; van Staden and Brown, 1978).

Traditionally the extraction of free cytokinins has involved maceration of the tissue and extraction in aqueous methanol or ethanol (eg Letham, 1978; Miller, 1961, 1975a; van Staden *et al.*, 1972; Horgan, 1978; Davey and van Staden, 1981). Most extractions of free endogenous cytokinins from potato tubers have been performed by this technique (eg van Staden, 1976; van Staden and Dimalla, 1976, 1977a; Dimalla and van Staden, 1977; Koda and Okazawa, 1977; Sattelmacher and Marschner, 1978c; Obata-Sasamoto and Suzuki, 1979a; Wurr *et al.*, 1980; and references cited above). Langille and Forsline (1974), Forsline and Langille (1975) and Mauk and Langille (1978) used the same extraction procedure to obtain cytokinin-like activity from potato plants divided into above-ground and below-ground portions.

Different research groups appear to require quite different amounts of equivalent fresh weight of potato tissue in bioassays before cytokinin-like activity can be detected.

Antis and Northcote (1975) indicated that activity inhibitory to

the growth of callus was present in mature tuber tissue. The inhibitory activity moved into the organic phase following partitioning of the extract against butan-1-ol at unknown pH and prevented the growth of callus during the bioassay of the crude butan-1-ol fraction. Resolution of the extract on paper chromatograms, however, revealed cytokinin-like activity somewhat inconsistently. The amount of tissue assayed was not reported.

Cytokinin-like activity was detected in extracts of the apices of potato tubers by Engelbrecht and Bielińska-Czarnecka (1972). The extract had been partially purified by cation exchange chromatography and between 2 g and 13.5 g fresh weight equivalents were bioassayed without further resolution by chromatography and cytokinin-like activity detected.

Van Staden and co-workers, however, bioassayed tuber, stolon and sprout extracts of 25 g fresh weight equivalents (Dimalla and van Staden, 1977; Dimalla *et al.*, 1977), 50 g fresh weight equivalents (van Staden and Dimalla, 1976, 1978; van Staden and Brown, 1979) and 100 g fresh weight equivalents (van Staden, 1976c). The cytokinin-like activity was detected following cation exchange, paper and/or column chromatography. Okazawa (1970) also used large quantities of tissues which were partially purified by cation exchange chromatography and the cytokinin-like activity detected following the bioassay of paper chromatograms. Wurr *et al.* (1980) detected cytokinin-like activity which appeared to be less than 1 µg of 6-benzylaminopurine equivalents per litre following paper chromatography and the assay of 75 g fresh weight equivalents of butan-1-ol-soluble material obtained from small developing tubers. However, Obata-Sasamoto and Suzuki (1979a) detected cytokinin-like activity in small tubers following the bioassay of approximately 4 g fresh weight equivalents. Using even smaller amounts of tissue Langille and Forsline (1974) and Forsline and Langille (1975) detected cytokinin-like activity in 1 g fresh weight samples purified by a petroleum ether partition and resolved by paper chromatography. This tissue, however, had simply been divided into the above-ground and below-ground portions of potato plants.

Van Staden (1976c) applied 100 g eq FW of tuber and sprout tissue to a column of Sephadex LH-20. The extract was subsequently eluted with 35% ethanol and the bioassay of fractions from the column indicated the presence of cytokinin-like activity. However, no activity was detected in the fractions expected to contain zeatin riboside. When the compounds collected in these specific fractions were resolved on paper chromatograms developed in solvent 1, it was apparent that cytokinin-like activity had been masked by the co-elution from the column of compounds

inhibitory to callus growth.

In subsequent work van Staden and co-workers applied extracts to paper chromatograms which were then developed in solvent 1. R_f zones 0.2 to 0.9 were subsequently eluted and rechromatographed on a column of Sephadex LH-20 prior to bioassay (van Staden and Dimalla, 1976, 1977a, 1978; van Staden and Brown, 1979). This procedure led to the detection of cytokinin-like activity which, according to van Staden and Dimalla (1976) "co-eluted with *O*- β -D-glucopyranosylzeatin". Notable, also, is the apparent increase in activity of the total "zeatin/zeatin riboside" fraction (van Staden and Dimalla, 1976).

In further work van Staden and Dimalla (1977c) again noted that no cytokinin-like activity was detected on paper chromatograms at the location of zeatin glucoside and suggested that "this does not exclude the possibility of glucosylated cytokinins being present, as impurities may inhibit the growth of soybean callus in the R_f -strips concerned (R_f 0.20 to 0.50)".

Compounds inhibitory to callus growth are present in a wide range of potato tissues. They occur in both sprouting and non-sprouting tubers (van Staden, 1976c; van Staden and Dimalla, 1978) as well as in the parent tubers, internodal sprouts, stolon tips and tubers obtained from material developing the "little potato" disorder (van Staden and Dimalla, 1976) and in "hybrid swellings" caused by Ethrel treatment (van Staden and Dimalla, 1977c). It would appear that the callus-inhibiting material which co-eluted with zeatin glucoside on paper chromatograms developed in solvent 1 was being loaded onto the Sephadex LH-20 column along with cytokinin-like compounds eluted from paper chromatograms. Its elution volume may, at least partially, coincide with that of zeatin riboside (van Staden, 1976c).

The presence of material which masked cytokinin-like activity was extracted from mature tubers of the cultivar Ilam Hardy (Fig 4.1). However, this inhibitory effect was eliminated by dilution (Fig 4.1). A reduction in the masking effect of the inhibitor occurred following a butanol partition since cytokinin-like activity was detected in the bioassay of 10 g eq FW of butanol-soluble material (Fig 4.2) and not in the bioassay of 10 g eq FW of crude methanolic extract (Fig 4.1). As callus inhibitory material partitioned into butan-1-ol (Table 4.7, Fig 4.3) (Antis and Northcote, 1975), an explanation of the apparent discrepancy in the results may lie in the poor resolution of the chromatogram loaded with 10 g eq FW of crude methanolic extract and the subsequent co-elution of cytokinin-like activity and inhibitory compounds (Fig 4.1).

Compounds inhibitory to callus growth were detected on paper chromatograms (eg Figs 4.4, 4.5) at R_f -values similar to those reported for zeatin-*O*-glucoside (R_f 0.2 to 0.4; eg van Staden and Dimalla, 1977a). Cytokinin-like activity was occasionally detected on R_f zones 0.2 to 0.4 in extracts of mature tuber tissues. Distinct inhibition of callus growth (as detected by the blackening of the callus) was not observed consistently during the bioassay of potato extracts further supporting the suggestion that compounds both promotory and inhibitory to callus growth may be co-chromatographing.

Callus inhibitory activity was reported by Blumenfeld and Gazit (1969) to be present in methanolic extracts of avocado fruits, but to partition completely into petroleum ether at pH 9.0. A petroleum ether partition (pH 9.0) was employed by Langille and Forsline (1974), Forsline and Langille (1975) and Mauk and Langille (1978). However, the callus inhibitory activity detected by van Staden and co-workers appears to be less mobile on paper in solvent 1 than the inhibitory activity reported by Blumenfeld and Gazit (1969).

The inhibitory activity extracted from mature tubers did not partition into petroleum ether (Table 4.8). Emulsion formation was also a significant problem and at a low pH Dekhuijzen and Gevers (1975) found that the emulsion between petroleum ether and water contained a significant amount of cytokinin activity. Van Staden *et al* (1972) detected "callus-stimulating activity" in the organic phase following a petroleum ether partition but considered that the activity was not due to a purine derivative. However, van Staden (1973a) considered that some cytokinin-like activity (possibly zeatin) may partition into petroleum ether at pH 9.0, a factor which must be considered with regard to Langille and co-workers' results. The use of a petroleum ether partition was not further investigated.

On the basis of partition coefficients determined with pure compounds (Letham, 1974b) the cytokinin bases and nucleosides in plant extracts are expected to partition into butan-1-ol at pH 7.0 whereas cytokinin nucleotides are expected to remain in the aqueous phase. The partition coefficients determined by Purse (1974; cited by Horgan, 1978) indicate a similar distribution of bases, nucleosides and nucleotides during partitioning against butan-1-ol at pH 8, although Letham (1978) comments that the diverse compounds in crude plant extracts could modify the partition behaviour of cytokinins.

The majority of the cytokinin-like activity detected in mature tuber tissue was present in the organic phase following partitioning of

the extract against butan-1-ol at pH 7 (Fig 4.2). A large proportion of the cytokinin-like activity present in small tubers obtained from "Bolter" plants (Fig 4.5) was detected in the aqueous fraction. This level of cytokinin-like activity in the aqueous fraction was not subsequently observed and the result is regarded with caution. Cytokinin-like activity which migrated to R_f 0.6 to 0.7 in solvent 1 was present in the aqueous fraction of tuber tissue (Fig 4.7) and possibly at R_f 0.5 to 0.6 in the aqueous fraction from stolon tissue (Fig 4.5). Water-soluble cytokinins were also detected in extracts from material collected during the field trial. This activity will be discussed in more detail below.

If the aqueous phase has been bioassayed following partitioning of plant extracts against butan-1-ol at pH 7.0 or 8.0, water-soluble cytokinin-like activity has often been detected. In early work van Staden and co-workers appeared to consider that the only water-soluble cytokinins were the cytokinin nucleotides (*eg* van Staden *et al.*, 1972; van Staden, 1973b,c). However, more recently van Staden and Davey (1976) suggested that a loss of up to 50% of the activity which "co-eluted" with zeatin occurred when an extract of xylem sap was partitioned against butan-1-ol at pH 7. Menary and Van Staden (1976) partitioned an extract from tomato sap against butan-1-ol (pH 8) and suggested that the activity located at R_f 0.6 to 0.8 in the aqueous fraction was a result of incomplete partitioning. Sattelmacher and Marschner (1978b) detected water-soluble cytokinins in extracts of potato shoots following purification of the extract by cation exchange chromatography and partitioning against butan-1-ol at pH 7. However, they considered that the substances detected in the organic and aqueous phases were not identical even though they have similar R_f -values as further partitioning against butan-1-ol did not remove the activity from the aqueous phase. A change in favour of butan-1-ol-soluble cytokinins occurred on induction (Sattelmacher and Marschner, 1978b).

Letham (1974a) suggested that coconut milk contained cytokinins which were not extracted from aqueous solution by butan-1-ol. Moreover, not all the water-soluble cytokinins were converted to butanol-soluble forms following treatment by alkaline phosphatase and of the 40% solubilised in this manner only a little of the activity was attributed to zeatin riboside. Further, Hopping *et al.* (1979) found that there was cytokinin-like activity in the aqueous fraction of sour cherry extract which was not degraded to zeatin riboside by alkaline phosphatase.

Henson (1978) and Stuchbury *et al* (1979) suggested that the side chain glucosides of zeatin and zeatin riboside were soluble in butan-1-ol although no indication was given of the efficiency of the extraction. Significantly, the partition coefficients of the *O*- β -D-glucosides between organic solvents and water do not appear to be in the literature.

However, it would appear that not all the cytokinin-like activity present in the aqueous phase can be attributed to either incomplete partitioning, to the cytokinin nucleotides or to the *O*- β -D-glucosides. For example, Taylor *et al* (1974) noted that some of the cytokinin-like activity from both exudate and fruits of cotton was highly polar in nature. Although some of this activity could be attributed to ribonucleotide-like activity (Sandstedt, 1974) the rest which had remained in the aqueous phase following partitioning against butan-1-ol was actually retained by cation exchange resin but not by anion exchange resin.

In addition, Dekhuijzen (1980) suggested that there was a novel cytokinin in the water-soluble fraction of club root extract which was retained by anion exchange resin and which yielded free zeatin and glucose-6-phosphate after treatment with β -glucosidase. He suggested that the compound was a glucose-6-phosphate derivative of zeatin.

It would appear that there are endogenous cytokinins yet to be unequivocally identified, and it would be of interest to identify the water-soluble cytokinins in potato tissues.

Problems such as incomplete partitioning between organic and aqueous solvents, residual solvent on paper chromatograms and variable callus growth as well as overloading of chromatograms, were overcome to some extent by using column chromatography. Column chromatography using Sephadex LH-20 gel has been employed almost routinely for the resolution of cytokinin-like compounds in plant extracts since its introduction by Armstrong *et al* (1969) (for references see Carnes *et al*, 1975; Brenner, 1981). The Sephadex LH-20 column eluted with 35% ethanol operates by reverse phase partition chromatography (Anonymous, Sephadex LH-20, Chromatography in Organic Solvents, Pharmacia Fine Chemicals, Svegea, AB) and therefore, in general, the more polar compounds are expected to elute more rapidly than the less polar ones (Purse *et al*, 1976).

The extraction technique utilising aqueous methanol at 4°C does not inactivate certain non-specific phosphatase enzymes (Bialeski, 1964) and consequently the enzymatic degradation of cytokinin nucleotides may occur during extraction (*eg* Letham, 1966a; Parker *et al*, 1972; Letham, 1978; Horgan, 1978; Brenner, 1981).

The presence of cytokinin nucleotides in potato tubers was suggested by Koda and Okazawa (1977) on the basis of a change in mobility of callus-stimulating activity following alkaline phosphatase treatment and rechromatography (*eg* Miller, 1965; Vonk and Davelaar, 1981).

Under the chromatography conditions employed in this investigation, cytokinin nucleotide activity is expected to be located near the origin of paper chromatograms developed in solvent 1 (*eg* Koda and Okazawa, 1978; Smith and van Staden, 1979) and to elute from a Sephadex LH-20 column soon after the void volume (*eg* Smith and van Staden, 1979; Dekhuijzen, 1980).

Cytokinin-like activity obtained from non-sprouting mature tuber tissues by extraction in 80% methanol at 4°C was not detected on either paper chromatograms or in column eluate in the R_f zone or column fraction expected to contain cytokinin nucleotide activity, although small amounts of water-soluble cytokinin-like activity were occasionally detected as discussed previously. However, when plant material was left in 80% methanol at -20°C for up to 24 hours before the tissue was homogenised, cytokinin-like activity with chromatographic properties similar to cytokinin nucleotides was detected (Figs 4.5, 4.6, 4.12, 4.13).

A large proportion of the cytokinin-like activity present in small tubers obtained from "Bolter" plants was detected in the aqueous fraction and located on the chromatogram developed in solvent 1 at R_f 0.0 - 0.1 (Fig 4.5). However, this result was not confirmed in any subsequent experiment.

Small amounts of cytokinin-like activity exhibiting nucleotide-like properties were detected in the stolon tips, and very small tubers from field grown material (Figs 4.11, 4.12, 4.13). Similar activity was not detected, however, in tubers 4 mm to 7.5 mm in diameter (Fig 4.15).

The results could be interpreted as a shift in cytokinin activity in favour of less polar cytokinins following tuberisation, although Horgan (1978) suggested that immersion in cold (0°C) 80% methanol was only effective in inhibiting phosphatase activity in soft tissues such as callus. Consequently the lack of detection of cytokinin-like activity exhibiting the chromatographic properties of cytokinin nucleotides may be a result of increasing tuber size rather than the stage of development.

The conclusion that cytokinin-nucleotide(s) were present in the potato tissues examined is based on chromatographic mobility and the fact that the activity was only detected when tissues were immersed in 80% methanol at -20°C for several hours prior to maceration (with the exception of the sprouting "seed" tuber, Fig 4.10). However, the

extraction procedure was such that the relative amounts of cytokinin nucleotide, free base or riboside could not be assessed (Horgan, 1978). Cytokinin-like activity with nucleotide-like chromatographic properties was detected in non-induced and induced tissues of the above-ground and below-ground portions of potato plants (Forline and Langille, 1975) and also in potato tubers extracted two weeks after harvest, although similar activity was not detected in tubers stored for 120 days at 5°C (van Staden and Brown, 1978).

Immersing tissue in a solvent mix of methanol-chloroform-formic acid (7N)-water (12:5:3) at -20°C was suggested by Bielecki (1964) to inhibit most phosphatase action. However, maceration of tissue is performed after inactivation of the phosphatase enzymes (Horgan, 1978). Unfortunately, differential penetration would occur as tubers increased in size, and for this reason and because the major experiment was performed at PPD with the extractions at Canterbury, Bielecki's solvent cocktail was not used during the extraction of fresh potato tissues.

Cation exchange resins were introduced by Miller (1961) as a partial purification procedure for extracts from *Zea mays*. The initial purification of crude plant extracts by cation exchange chromatography is still currently employed (eg Horgan, 1978; Letham, 1978; Brenner, 1981).

Dowex 50 ion exchange resin in the acid (H^+) form was used by Okazawa (1970), Koda and Okazawa (1977) and Borys and Jeske (1978) to clean up potato extracts. Engelbrecht and Bielińska-Czarnecka (1972) used a cation exchange resin "Wofatit KPS" while Zerolit 225.52 cation exchange was used by Sattelmacher and Marschner (1978a, b, c). In all papers following the initial publication (van Staden, 1976c), van Staden and co-workers passed extract from potato tissues through Dowex 50 (H^+ form).

During this investigation it was found that there was a substantial reduction in detectable cytokinin-like activity after extract of mature tuber tissue was subjected to cation exchange chromatography (Figs 4.3, 4.4) in which Dowex 50 (H^+ form) was used and the retained material eluted with 2N and 5N ammonium hydroxide (Hewett and Wareing, 1973a).

It was also noted that material inhibitory to callus growth was initially retained by the resin and subsequently released by NH_4OH (Figs 4.3, 4.4). Van Staden and co-workers (van Staden, 1976c; van Staden and Dimalla, 1977a, 1978) showed that compounds masking cytokinin-like activity on paper chromatograms developed in solvent 1 were not

retained by Dowex 50 (H^+ form) cation exchange resin.

A change in the cytokinin complement of extracts subjected to cation exchange chromatography was commented on by Kefford (1963). Miller (1965, 1967, 1968), Tegley *et al* (1971) and Dekhuijzen and Gevers (1975) suggested that hydrolysis of zeatin riboside to zeatin may occur during cation exchange chromatography using Dowex 50 (H^+ form).

Both Miller (1965, 1967) and Letham (1973) showed that zeatin riboside may be hydrolysed by heating with a sulphonic acid resin. Vreman and Corse (1975) suggested that the major forces binding zeatin to the polystyrene resin (Dowex 50, H^+ form) were not ionic, and that 1N NH_4OH eluted only about 70% of the applied zeatin from the column. Sattelmacher and Marschner (1978a) suggested the recovery rate for kinetin was about 60% when tested in their extraction and purification procedure which incorporated the use of a strong cation exchange resin and a butan-1-ol partition (pH 7).

Van Onckelen and Verbeek (1972) claimed to have achieved 100% recoveries of zeatin and zeatin riboside respectively from Dowex 50 (H^+ form) eluted with 3N NH_4OH . Vreman and Corse (1975), however, suggested these values were overestimates due to the methods used to determine the recovery.

Challice (1975), referring to preliminary results, suggested that a range of cytokinin standards added to leaf extracts, were not recovered when the extracts were purified by procedures which involved the use of strong cation exchange resins in either H^+ or NH_4^+ forms, whereas van Staden (1976) considered that there was neither hydrolysis of cytokinin glucosides nor loss during cation exchange treatment using Dowex 50 (H^+ form) and continues to use this strong cation exchange resin routinely (*eg* Forsyth and van Staden, 1981; van Staden and Davey, 1981; van Staden *et al*, 1981).

To prevent the possible degradation of cytokinins arising from the production of heat during elution steps (*eg* Miller, 1965; Letham, 1973) Dyson and Hall (1972) maintained the temperature of the resin below $5^{\circ}C$ during neutralisation as did Watanabe *et al* (1981). This precaution was not taken by Tegley *et al* (1971) or Dekhuijzen and Gevers (1975).

Elution of retained cytokinins from Dowex 50 (H^+ form) with ethanolic-ammonium hydroxide led to reports of almost complete recovery of applied sample (Miller, 1974) of zeatin (ca 85%) (Vreman and Corse, 1975), and 94.9% recovery of $[8-^{14}C]$ *cis*-zeatin riboside (Watanabe *et al*, 1981).

Cytokinin nucleotides were reported not to be retained by cation exchange resins (*eg* Miller, 1965, 1967; van Staden and Dimalla, 1980). These compounds are expected to be retained by anion exchange resin (*eg* Miller, 1965; Letham, 1973; Horgan, 1978; van Staden and Dimalla, 1980). However, it appears that cytokinin-like compounds susceptible to degradation by alkaline phosphatase may be, at least partially, retained by cation exchange resin (*eg* Koda and Okazawa, 1977; Smith and van Staden, 1979). Moreover, van Staden and Dimalla (1980) showed that isopentenyladenosine and isopentenyladenine were incompletely retained on Dowex 50 (H^+ form) and possibly retained in a more quantitative manner by the anion exchange resin, Dowex 1. It may be interpreted from their data that relatively more isopentenyladenosine was retained by Dowex 50 (H^+ form) than isopentenyladenine.

It is obvious from the literature cited and the results reported in this thesis that the use of strong cation ion exchange resins is problematical. Van Staden and co-workers have used Dowex 50 (H^+ form) cation exchange resin to partially purify extracts of potato tissues (see references cited, p 146) apparently without taking precautions against heat accumulation during neutralisation of the resin. Neither were retained cytokinins eluted with ethanolic-ammonium hydroxide. Both the relative and total levels of cytokinins reported by van Staden and co-workers must be interpreted with caution.

In preference to sulphonic acid resins such as Dowex 50, Letham (1978) and Horgan (1978) recommend the use of cellulose phosphate columns prepared in NH_4^+ form. The purification of potato samples on cellulose phosphate columns was not investigated although Dowex 50 (H^+ form) was not further used.

4.4.2 Plant Physiology Division Experiment One

It was apparent from the studies performed under controlled growth room conditions and from the field trial that there was a pronounced difference in the cytokinin content of stolons and small tubers (Fig 4.6, Table 4.9). To monitor cytokinin activity during the early stages of tuber development required a large number of plants to provide sufficient material for analysis, and plants grown under controlled conditions appeared more suitable. The time lag between harvesting the plants and removal of stolons and tubers could be reduced to a minimum and the harsh field conditions (caused by the prevalent hot north-west wind) avoided. These conditions may have led to the observed non-uniform stolon and tuber formation.

In order to minimise losses during the cytokinin analysis, purification and clean-up procedures were kept to a minimum (section 4.2.4.1C). Small tissue weights were analysed initially in order to dilute out the masking effect of inhibitory compounds (Fig 4.1) (van Staden, 1976c). Cytokinin nucleotides are not expected to be isolated quantitatively by the extraction procedure used (see p 151).

Cytokinin-like activity was detected in all the material tested following the bioassay of 4 g fresh weight equivalent of tissue. In an attempt to verify the pattern shown in Figure 4.31 which was compiled from a series of different bioassays and to try and confirm the presence of "zeatin-like" activity in developing tubers (*eg* Figs 4.25, 4.27A, 4.28A, B and C), a larger tissue fresh weight was analysed at the risk of increasing inhibitor interference (van Staden, 1976c). The results of the bioassays of 8 g eq FW (Fig 4.33), although showing the same total trend with respect to tuber size as obtained from the bioassays of 4 g eq FW (Fig 4.31), showed a marked reduction in the amount of activity with chromatographic properties similar to zeatin riboside (Table 4.15). The marked reduction in detectable zeatin riboside-like activity is explained on the basis of co-elution of inhibitory material (van Staden, 1976c). Zeatin-like activity was consistently detected in the analysis of samples of 8 g eq FW. A comparison of the relevant bioassays indicates that the amount of zeatin-like activity detected in the second analysis would be barely, if at all, detectable during the first series of bioassays (Fig 4.14 cf Figs 4.22 to 4.30 inclusive).

The cytokinin-like activity in the stolon tips was resolved into at least four distinct fractions (Fig 4.22). Activity eluted from the columns in the same volumes as did zeatin riboside, zeatin and iP markers. However, cytokinin-like activity was also detected in an elution volume between that determined for zeatin and for isopentenyladenine suggesting that this activity had a retention time similar to isopentenyladenosine (iPA) (*eg* Armstrong *et al*, 1969; van Staden, 1976c; Koda and Okazawa, 1977). The presence of cytokinin-like activity with retention times on Sephadex LH-20 (35% ethanol) similar to iPA and iP has not been reported previously in stolon tips. The complement of cytokinin-like activity in the stolon tips of *S. andigena* grown under controlled environment conditions was essentially similar to that of stolon tips obtained from field grown plants of *S. tuberosum* except for the lack of activity in fraction 5 (Fig 4.22 cf 4.12) which would be expected to contain cytokinin nucleotide activity. Although the

absence of activity in fraction 12 (Fig 4.32) can be explained on the basis of phosphatase activity there is only a vague indication of activity with a retention time longer than zeatin. Band broadening may occur for compounds with long retention times on Sephadex LH-20 eluted with 20% ethanol and may account for the lack of detection of compounds with retention times longer than zeatin (Armstrong *et al*, 1969). However, an iP marker was collected in fractions 27 to 29 and as not all these fractions were collected and bioassayed owing to the limitation placed on the total number of flasks able to be analysed in any one bioassay, iP and compounds retained for longer times would not have been detected.

Although a high, variable base-line may account for some of the apparent peaks of cytokinin-like activity observed in extract of 4 g of stolon tips and tubers, some activity appeared to be retained by Sephadex LH-20 for a substantial period of time (*eg* Figs 4.22, 4.23, 4.26, 4.27). Similarly retained activity has been reported by Hewett and Wareing (1973) and, in poplar leaves, corresponds to 6-(*o*-hydroxybenzylamino)-9- β -D-ribofuranosylpurine. 2-Methylthioisopentenyl-adenosine (msiPA) has been shown to have a very long retention time on Sephadex LH-20 (35% ethanol) (Vreman *et al*, 1978) and to be present as a free cytokinin in extracts of cabbage (*eg* Hashisume *et al*, 1979). Moreover, Sattelmacher and Marschner (1978a) showed that a compound present in potato tissues was retained for a substantially longer period of time on a column of polyvinylpyrrolidone than was iP.

It is probable then that the response shown by the callus was to cytokinin-like activity extracted from stolons and tubers of *S. andigena* and *S. tuberosum* (Fig 4.15) and retained by Sephadex LH-20 (35% ethanol) for long periods of time.

The amount of cytokinin-like activity detected in fractions 9 to 21 of stolon extracts (Fig 4.22) could not be estimated in kinetin equivalents since the only activity which exceeded the 99% confidence limit lay well below the straight-line portion of the standard curve. The relative amounts of the four cytokinins detected by bioassay in fractions 9 to 21 was not consistent and, in fact, small relative changes cannot be verified solely by bioassay techniques.

Van Staden and Dimalla (1976) reported that stolon tips collected from potatoes affected by the "little potato" disorder contained substantially more activity "co-eluting" with zeatin riboside than "co-eluted" with zeatin or "zeatin glucoside". Moreover, Mauk and Langille (1978) considered that zeatin riboside levels lay anywhere

between five and 100 times greater than any other cytokinin detected in non-induced or induced *S. tuberosum* cv Katahdin tissues. However, the direct comparison between non-induced and induced "below-ground tissue" and stolon tips from potatoes exhibiting the "little potato" disorder is difficult to make.

In contrast, Sattelmacher and Marschner (1978c) suggested that the main activity in non-induced stolon tips of plants grown in water culture was not zeatin riboside, but made no assumptions as to its identity. Sattelmacher and Marschner (1978c) reported a change in the relative levels of the two bands of cytokinin-like activity following induction of the potato plants by removal of nitrogen from the nutrient medium. The amount of activity with chromatographic properties similar to zeatin riboside increased relative to the less mobile cytokinin-like activity although the absolute difference in activity was probably quite small (Sattelmacher and Marschner, 1978c).

The cytokinin analysis reported in Figure 4.22 was performed on stolon tips removed from non-tuberising plants. Although Sattelmacher and Marschner (1978c) used the presence of a swelling on the subapical region of at least one stolon as an indicator of induction, tuberisation occurred so rapidly during this experiment (Table 4.9) that the assumption that all the plants not bearing stolons with sub-apical swellings were not induced does not appear to be justified. However, as will be discussed in more detail subsequently, stolon tips removed from non-tuber forming plants had substantially less starch per tip than stolon tips removed from plants with tubers (Table 4.11) and only slightly more than detected in stolon tips removed from non-induced plants during PPD Experiment 2. Therefore, on the basis of starch levels, the suggestion that the stolon tips extracted were from non-induced plants, appears justified.

Callus of low sensitivity was used in the bioassay of extract of tubers 1.5 to 3 and 3 to 5 mm diameter. Cytokinin-like activity less than $5 \mu\text{g KE l}^{-1}$ (Fig 4.23) or less than $4 \mu\text{g KE l}^{-1}$ (Fig 4.24) would not be easily detected. The relative increase in zeatin riboside-like activity, and decrease in more polar activity just after induction reported by Sattelmacher and Marschner (1978c) may not have been detected in the above bioassays but it is not apparent in extract of tubers between 5 and 7.5 mm diameter (Fig 4.25). Extracts of tubers from this size category with an average fresh weight of 0.4 g were resolved into at least three discrete groups of cytokinin-like activity. The response of the callus to cytokinin-like activity present in the fraction

expected to contain zeatin (fraction 11) is only slightly less than that in fraction 9 which is expected to contain zeatin riboside. This pattern is repeated in tubers between 4 and 7.5 mm diameter obtained from field grown plants (Fig 4.15). The presence of cytokinin-like activity in fractions expected to contain iPA and iP was noted in extract from the field grown plants. Callus of different ages was used in the bioassays of tubers between 5 and 7.5 mm diameter grown under controlled conditions and a decrease in the sensitivity of the callus meant that small amounts of activity with chromatographic properties similar to iP would not have been detected.

The presence of cytokinin-like activity with a similar elution volume to zeatin was confirmed in extract of tubers 5 to 7.5 mm diameter following the bioassay of 8 g eq FW (Fig 4.32). Only a small amount of activity was detected in fraction 12, expected to contain zeatin riboside. Activity with a long retention time was also collected in fractions 24 and 25 and may reflect the presence of activity similar to iPA. Subsequent extractions of wheat grain indicated that the *O*- β -D-glucosides of zeatin and zeatin riboside are eluted from Sephadex LH-20 with 20% ethanol faster than zeatin riboside (Fig 6.15). Cytokinin-like activity detected in extracts of tubers in fraction 9 may be indicative of the presence of a small amount of side-chain glucoside activity in developing tubers. Similar activity was found by van Staden and Dimalla (1977a) in potato tubers developing the little tuber disorder.

An increase in the cytokinin-like activity collected in fraction 9 relative to the total cytokinin-like activity was apparent in tubers between 7.5 and 10 mm diameter with an average fresh weight of 0.4 g (Fig 4.26). The distinct peak of activity in sample A was estimated in kinetin equivalents from the standard curve but the corresponding peaks in samples B and C lay below the straight line portion of the standard curve. However, as the weight of callus obtained was greater than that obtained from the bioassay of $4 \mu\text{g}$ kinetin l^{-1} , a minimum value of $4 \mu\text{g}$ KE l^{-1} was assigned to the cytokinin-like activity present in samples B and C (Table 4.13, Fig 4.31). In sample C small peaks of activity were detected which had elution volumes corresponding to those expected from zeatin, iPA and iP (Fig 4.26).

The presence of activity with chromatographic properties similar to zeatin was confirmed following the bioassay of 8 g eq FW of tubers 7.5 to 10 mm diameter although no activity was detected in fraction 12 which is again explained on the basis of co-elution of material inhibitory to callus growth (Fig 4.32). The presence of activity with chrom-

atographic properties similar to iPA or iP was not confirmed although activity in fraction 8 may represent side-chain glucoside activity.

The size category 7.5 to 10 mm in diameter would probably include the largest size category sampled by van Staden and Dimalla (1976) when "little potatoes" weighing less than 0.3 g per tuber were extracted. Considerably more cytokinin-like activity was detected in the fraction which "co-eluted" with zeatin riboside or with OGZ respectively. By comparison there would appear to be slightly more zeatin-like activity than zeatin riboside-like activity in tubers of *S. andigena* with an average fresh weight of 0.4 g.

A substantial amount of cytokinin-like activity was detected in the fraction expected to contain zeatin riboside in samples of tubers 10 to 15 mm and 15 to 20 mm in diameter in extract of both 4 g and 8 g eq FW (Figs 4.27, 4.28, 4.32). The presence of zeatin-like activity was confirmed but a relatively lesser amount of ZR-like activity was detected following the bioassay of 8 g eq FW (Table 4.15). Little glucoside-like activity was detected.

The larger tubers, with an average fresh weight of 3.3 g, contained the highest concentrations of cytokinin-like activity per unit weight of tissue (Figs 4.31, 4.33, 4.34). Van Staden and Dimalla (1976) noted a decrease in the amount of cytokinin-like activity which "co-eluted" with zeatin riboside in "little potatoes" weighing more than 3.0 g compared to those weighing less than 0.3 g. However, the size range of the larger tubers extracted was not given (van Staden and Dimalla, 1976). A decrease in the amount of cytokinin-like activity in fraction 9 was observed in the extract from the largest tubers analysed (Figs 4.29 and 4.30) and this feature may have influenced the result reported by van Staden and Dimalla (1976).

Wurr *et al* (1980) considered that there was more total cytokinin-like activity in "little potatoes" than in normal tubers in samples which included tubers up to 40 mm diameter. However, the results could have been biased by a dilution effect depending on the size of tubers selected for each sample.

Although monitoring of relatively small changes in the pattern of cytokinin-like activity in stolons and tubers less than 5 mm diameter was not possible in these experiments, there was an obvious increase in the cytokinin-like activity which had elution volumes similar to zeatin riboside and to zeatin in expanding tubers up to 20 mm diameter (Fig 4.33).

Van Staden and Dimalla (1976) reported that the cytokinin

content of stolons was greater than that of little potatoes on a per unit weight basis. This feature was not observed in any of the comparisons between stolons and developing tubers reported in this thesis. Developing tubers contained substantially more cytokinin-like activity than stolons obtained from "Bolter" plants grown under controlled conditions at Canterbury (Fig 4.5), from *S. andigena* cv 165 plants grown under similar controlled conditions (Fig 4.6), *S. tuberosum* cv Ilam Hardy plants grown under field conditions (Table 4.9) and from *S. andigena* cv 165 plants grown in controlled environment rooms at PPD (eg Figs 4.22, 4.25, 4.26, 4.32). Sattelmacher and Marschner (1978c) and Obata-Sasamoto and Suzuki (1979a) also reported that there was less cytokinin-like activity in stolons than developing tubers on a per unit fresh weight basis.

A comparison between the transient increase in zeatin riboside activity prior to tuberisation in below-ground tissue (Mauk and Langille, 1978) and the high level of activity detected in stolon tips by van Staden and Dimalla (1976) would be extremely tenuous. The activity van Staden and Dimalla recorded could more readily be explained as a feature of the "little potato" disorder.

According to Sattelmacher and Marschner (1978c) an increase in cytokinin-like activity following induction occurred at an early developmental stage and was at its highest level in tubers 4 to 6 mm in diameter. Tubers 4 to 6 mm in diameter had a total cytokinin content of $ca\ 130 \pm 20\ ng\ g\ FW^{-1}$ while induced stolons had $ca\ 80 \pm 20\ ng\ g\ FW^{-1}$. Therefore the increase in the total cytokinin content was not substantial. In this thesis the cytokinin content of tubers 5 to 7.5 mm diameter was greater than that of stolons but the peak of cytokinin-like activity occurred in substantially larger tubers (Figs 4.31, 4.34). The slight decrease in total cytokinin-like activity measured by Sattelmacher and Marschner (1978c) on a per unit FW basis in enlarging tubers may be a consequence of an overall decrease in the cytokinin level within the plant due to the absence of nitrogen from the culture medium (see references cited, p.33).

Okazawa (1970) showed that a pronounced peak of cytokinin-like activity occurred about 11 days after tuber formation was first detected in field grown potatoes. Presumably the samples included a broad range of tuber sizes. The peak of cytokinin-like activity detected by Okazawa (1970) occurred just prior to the rapid increase in fresh weight of the tubers which is in agreement with the results reported in this thesis (Figs 4.19, 4.34).

Koda and Okazawa (1977) collected a bulk sample of tubers from field grown material purportedly at the same developmental stage as the tubers containing maximal cytokinin-like activity as previously determined by Okazawa (1970). Following several rechromatography steps it was apparent that there was more cytokinin-like activity with chromatographic properties similar to zeatin riboside than to zeatin. Activity with an elution volume similar to iPA was detected although no activity was detected with an elution volume similar to iP. The presence of small amounts of cytokinin nucleotides and possible glucoside were also reported. If Koda and Okazawa (1977) have selected tubers containing peak cytokinin levels then the relative amounts of the cytokinin compounds in *S. tuberosum* cv Irish Cobbler were slightly different from those detected in *S. andigena* cv 165 tubers between 10 and 20 mm diameter (Figs 4.27, 4.28, 4.33) when only a trace of activity was detected at the elution volume of iPA (eg Fig 4.27c). Since some precaution was taken against hydrolysis of zeatin riboside during cation exchange chromatography, the lower relative level of zeatin riboside-like activity may be due to co-elution of inhibitory material (van Staden, 1976) and is shown to some extent by Koda and Okazawa (1977) themselves, during rechromatography of different fractions.

Obata-Sasamoto and Suzuki (1979a) suggested the highest level of cytokinin-like activity was in the largest tuber size extracted (35 mm diameter). However, they did not extract tubers of any size between 5 and 25 mm diameter although they suggested that there was more cytokinin-like activity on a per unit weight basis in tubers of 35 mm diameter than of 25 mm diameter. The cytokinin-like activity measured was distributed across all 10 R_f zones following chromatography in butan-1-ol-acetic acid-water (4:1:1).

The size categories selected by Obata-Sasamoto and Suzuki (1979a) did not include those sizes in which the cytokinin-like activity was maximal in *S. andigena* (Figs 4.31, 4.34). However, neither was a comparison of the cytokinin content of tubers 25 and 35 mm diameter performed in this study, although an overall decrease in the cytokinin-like activity on a per unit weight basis was noted in extracts of tubers 20 to 25 mm diameter and tubers 40 to 50 mm diameter (Figs 4.31, 4.33).

The time span of tuberisation has been reported by Liz-Kaczyńska and Listowski (1977) to range from 8.8 to 16 days in pot experiments under different environmental conditions. Krijthe (1955) suggested that the initial development of tubers in box experiments was complete within two to three weeks while Ivins and Bremner (1963-5) suggested

that the majority of tubers on each plant were initiated in the field within a period of two weeks. Moorby (1967) also suggested that all the tubers appeared to be initiated over a very short period.

Under controlled environment conditions (PPD), tuberisation of *S. andigena* occurred very rapidly. Although 61.5% of the plants were found to be forming tubers by Day 21 (Table 4.8), the small proportion of the larger tuber size categories at Days 21 to 24 (Figs 4.17, 4.18) led to the conclusion that the first tuber formation must have occurred just prior to Day 21. All plants examined on Day 30 were tuberising. *S. andigena* plants therefore, appeared to tuberise at least as rapidly and uniformly as *S. tuberosum* is reported to do under field conditions.

The marked contribution from tubers greater than 7.5 mm diameter to the total tuber weight on Days 21 and 22 (Fig 4.18) was a reflection of the relatively few larger tubers found on the plant at those harvests (Fig 4.17).

Following the initial tuberisation period it has been suggested that tuber numbers then remain relatively constant (Krijthe, 1955; Ivins and Bremner, 1963-5; Bremner and Taha, 1966; Moorby, 1967). With the exception of Day 56 this appeared to be the case for *S. andigena* (Table 4.10). The transient high number recorded at Day 56 may be a reflection of the small sample size available.

The majority of stolons formed at least very small tubers (Fig 4.17). Liz-Kaczyńska and Listowski (1977) noted that 89% of the stolon tips tuberised in one experiment although this number was obviously affected by growing conditions. Krijthe (1955) noted that tubers could form at the tip of every stolon regardless of position on the stem or order and that many of the very small tubers were located on the tips of second order stolons at the end of the experimental growth period, an observation also made during this study.

Stolons and tubers less than 5 mm in diameter contributed 33% to the total weight at Day 25 but only 0.2% at Day 84 (Fig 4.18). However, this latter value represented 32% of the total stolon and tuber number (Fig 4.17). It is apparent that after the initial tuberisation period relatively few of the tubers formed developed into larger tubers (Fig 4.17) but those few contributed substantially to the observed total fresh weight (Fig 4.18). Less than 9% of the total number of tubers contributed to over 50% of the fresh weight after the initial tuberisation period (Table 4.11).

In field grown potatoes it has been noted that many tubers do not progress beyond the initial stage (*eg* Krijthe, 1955; Bremner and

Taha, 1966). Borah and Milthorpe (1963) showed that immediately following tuber initiation the relative growth rate was high but soon fell to a slower constant value. Moorby (1970) suggested that after a short exponential phase the rate of tuber growth was linear over most of the growing season. In PPD experiment 1, tuber growth per plant was still in an exponential phase by Day 84 although the relative growth rate was substantially less by this time than during the initial phase of tuber growth (Fig 4.16).

It is suggested that the growth analyses for *S. andigena* grown under CE conditions show that the growth features observed were essentially similar to those observed by other workers in both pot and field experiments.

Stolons and tubers were removed and combined in samples regardless of the node of origin of the stolon. This practice was of practical necessity in order to obtain sufficient plant material for cytokinin analysis but it was also made obligatory because the individual stolons occupied a very compact zone at the base of each stem as was also noted by Wurr (1977) when he grew potatoes in containers. However the node of origin of stolons may have a subsequent bearing on the relative growth rate of individual tubers (*eg* Gray, 1973). Moreover, although Krijthe (1955) suggested that the larger tuber grows most rapidly, both Moorby (1967, 1968, 1978) and Gray and Smith (1973) have suggested that the largest tubers were not necessarily growing faster than the smaller tubers. Moorby (1967) suggested that only a few tubers on a plant grew rapidly at any one time. Periods of tuber expansion were in fact interrupted by periods when tubers grew slowly if at all (Wurr, 1977). In PPD experiment 1 the peak noted at Day 30 in the proportion of tubers with diameters ranging from 3 to 15 mm was followed later in time by a slower increase in the relative proportions of these size categories (Fig 4.17). This could be indicative of a fast "turn-over" of the more rapidly developing tubers and the subsequent

appearance of either non-developing or slower bulking tubers. Moorby (1975) in fact suggested that some tubers started to grow immediately after initiation and, while others may be initiated later, they rarely grow to an appreciable size. Borah and Milthorpe (1963) also noted that tubers of very small size appeared to be present at all times and suggested that tubers were actually being initiated and resorbed. These features were not able to be accommodated when collecting stolons and tubers for analysis, although in future experiments consideration should be given to collecting and analysing tubers which are rapidly or slowly bulking. It would be useful to determine whether the variability in the cytokinin content noted among the samples of tubers 15 to 20 mm diameter (Table 4.13) could be accounted for on the basis of an unconscious selection of slower or faster growing tubers in any one of the three samples analysed.

If the tubers analysed were a true representation of the population of stolons and tubers at a particular developmental stage then over a period from Days 25 to 42, over 35% of the total stolon and tuber fresh weight had a relatively high concentration of cytokinin-like activity, although this represented a relatively small proportion of the total number of stolon tips and tubers (Table 4.17).

In order to be able to relate more closely the growth and developmental stages of *S. andigena* tubers to the growth of tubers reported in the literature, an analysis of both the soluble sugar and starch contents of both stolons and tubers was performed (section 4.2.4.1C).

The soluble sugars were extracted into 62.5% methanol (Haslemore and Roughan, 1976). This procedure, however, not only extracted soluble sugars but lipids and phenolic compounds as well. In the non-specific determination of total soluble sugars the presence of phenolic compounds would markedly interfere with the required specificity of the phenol/sulphuric acid reaction. Lead acetate was added to the solution to precipitate phenolic compounds. A slight loss of soluble sugars was reported to occur at this step (Haslemore and Roughan, 1976). Following the addition of chloroform, lipid materials

partitioned into the organic phase, the precipitated phenolic compounds remained at the chloroform aqueous interface, leaving the aqueous layer containing the carbohydrates free from phenolic and lipid contaminants.

All carbohydrates (including starch) present in the aqueous aliquot react with the phenol/sulphuric acid mixture. The assumption, however, was made that only soluble sugars were present. The contribution of soluble sugars other than sucrose, fructose and glucose was assumed to be negligible in developing tubers as has been found in mature tubers (*eg* Schwimmer *et al*, 1954; Cronin and Smith, 1979).

The phenol/sulphuric acid technique has been used previously to determine the total soluble sugar content in potato tubers (*eg* Ohad *et al*, 1971; Munns and Pearson, 1974; Moorby *et al*, 1975). However, it is important to note that different sugars will give slightly different absorbance readings following the formation of coloured chromophores. According to the standard curves constructed by Dubois *et al* (1956) the amounts of glucose and fructose would be underestimated if sucrose was used as the standard by which to estimate either glucose or fructose.

Mares and Marschner (1981) reported that the sucrose and reducing sugar contents of tuber tissues varied quite considerably within different parts of one tuber and between tubers on the same plant, and it was suggested that this may depend on such factors as tuber growth rate, growth conditions and time of harvest. Hawker *et al* (1979) showed that the amount of sucrose was less than the amount of reducing sugar in fast-growing tubers while the reverse was apparent in slow-growing tubers. Burton and Wilson (1970) noted that the relative amounts of reducing sugars and sucrose changed during development and that relative levels may be associated with the environment. Pressey (1969), however, considered that the predominant sugar in immature potatoes was sucrose although reducing sugar levels were high at early stages of tuber development.

It is evident therefore, that both sucrose and reducing sugars are present during tuber development and so the results presented in

Figure 4.21 and Table 4.13 are necessarily an underestimate of the total soluble sugar content of *S. andigena* tubers. In contrast to a slight underestimation of soluble sugar levels, the procedure employed for the starch analysis should yield an accurate determination of starch levels since care was taken to use a highly purified amyloglucosidase preparation (Haslemore and Roughan, 1976) and the released glucose was detected by a highly specific glucose oxidase reagent. In computing the amount of starch in a sample a correction factor was incorporated to allow for the addition of water during the hydrolysis of starch to glucose.

As discussed in the introduction, one school of workers considers that tuber initiation is associated with those conditions which lead to a high concentration of carbohydrate (Borah and Milthorpe, 1963) or of soluble sugars in the stolon tips (eg Burt, 1964; Slater, 1968).

Burt (1964) purported to show a close association between a high soluble sugar content in the stolon tip and tuber initiation. An increase in the soluble sugar concentration of stolon tips removed from control plants occurred shortly before visible tubers were detected. The soluble sugar concentration of the tubers was not reported. Burt noted that thickenings in the subapical regions of stolons were visible after plants had been exposed to low temperatures (3°C and 9°C) for one week. High soluble sugar concentrations were detected in these swellings. However, he then stated that those plants exposed to 3°C for one week had "visible" tubers present by the end of the following week by which time the soluble sugar concentration had decreased. "Visible" tubers were not detected on stolons until three weeks after the plants had been exposed to 9°C , this time tuberisation occurred concomitantly with an increased concentration of soluble sugar. Clarification of terms used and an indication of tuber diameters may assist in interpreting his data and conclusions.

Lovell and Booth (1967) considered that there was a poor correlation between high soluble sugar levels and tuber initiation. The total sugar content per unit dry weight increased in the stolons of both control and GA_3 -treated plants prior to tuberisation, but although the total sugar content was higher in the GA_3 -treated plants, the untreated plants tuberised earlier.

Burton and Wilson (1970) suggested that the total sugar content of tubers rose to a maximum within about a week of tuber initiation, after which it declined. Burt (1964) suggested that the sugar concentration probably fell soon after tuber initiation. If the total sugar

content is calculated from the sum of sucrose and reducing sugar values reported by Hawker *et al* (1979), it is apparent that the sugar content per unit fresh weight decreased very shortly after tuber formation on plants grown in liquid culture. However, if the data presented by Plaisted (1957) is recalculated, it is found that the proportion of total soluble sugars on a per fresh weight basis decreased only slightly immediately following tuber formation but dropped more substantially as the tubers developed.

The soluble sugar present in stolons and tubers has also been analysed as the component reducing and non-reducing sugars. Okazawa (1973) presented a summary of the results obtained by Tagawa and Okazawa (1949-1955, published in Japanese) and suggested that the reducing sugar level in stolon tips reached a peak just before tubers began to swell but subsequently dropped sharply. Plaisted's (1957) results (recalculated on a per unit weight basis) showed a higher level of reducing sugar than non-reducing sugar in stolon tips and a decrease in reducing sugar immediately following tuber formation. However, the amount of non-reducing sugar showed a transient increase immediately following tuberisation.

The results presented by Lovell and Booth (1967) indicate a decrease in the concentration of non-reducing sugars in stolons prior to tuberisation. Tubers were not analysed for non-reducing sugars. The concentration of reducing sugars (calculated approximately as the difference between the total and non-reducing sugar concentrations) contributed the major proportion of the total sugar level at the time of tuberisation of the control plants. A higher level of reducing sugars was actually present in the stolons of GA_3 -treated plants in which tuberisation was delayed. However, Mares *et al* (1981) suggested that the GA_3 -treated tuber tissue was characterised by a high reducing sugar content. They also noted that stolons growing out from tubers following GA_3 -treatment had high reducing sugar levels and low sucrose levels similar to those of normal stolon tips analysed prior to tuberisation as was indicated also by Hawker *et al* (1979). Hawker *et al* (1979) noted that the amount of sucrose as a proportion of the fresh weight tended to increase with increasing tuber size although there was a noticeable decrease in the reducing sugar content following tuberisation.

Burton and Wilson (1970) suggested that the content of reducing sugars was high in stolon tips just prior to tuber initiation, but fell following initiation. In contrast the sucrose content was low in stolon

tips prior to initiation but increased rapidly to a maximum value during the first one to two weeks following tuber initiation.

When the soluble sugar content of stolons and tubers from *S. andigena* plants was calculated as a proportion of the dry weight, little difference was detected in the sugar content of stolon tips removed from either tuberising or non-tuberising plants or in the stolon tips which were visibly tuberising but in which the hook had not straightened out (Category JA, Table 4.13). However, increasing amounts of starch (Table 4.12) mask the fact that the amount of soluble sugar per stolon tip was increasing and was at a peak level after tuber formation had begun but before the stolon hook unfolded (Table 4.13). A substantial decrease occurred in both the total amount of soluble sugar per tuber and the amount calculated as a proportion of the total dry or fresh weight as the stolon hook unfolded. Plaisted (1957) also detected a high level of soluble sugar when tuber formation was detected as a slight thickening in the subapical region of the stolon. However, the maintenance of this level of total sugar appeared to be the result of a transient increase in non-reducing sugar at the time of tuber formation.

The increase in sucrose following tuber initiation noted by Burton and Wilson (1970) was somewhat less transient but may be related to the size of samples chosen.

It would appear that a decrease in the sugar content of stolon tips and especially a decrease in reducing sugar levels is indicative of tuber formation but conversely a high total, reducing or non-reducing, sugar content in stolon tips may not always be a reliable indicator of imminent tuber formation. More definitive work at the time of tuber initiation and tuber formation is obviously required with close attention being given to the relative levels of reducing and non-reducing sugars before the "cause" of tuberisation can be ascribed to the high levels of soluble sugars in stolon tips.

As tubers of *S. andigena* increased in diameter the total soluble sugar content per tuber increased. The decrease in soluble sugar content as a proportion of dry weight continued until tubers were about 5 mm in diameter. A transient increase then occurred as was apparent also on recalculating Plaisted's results. If the total soluble sugar is expressed as a percentage of the fresh weight the pattern is reflected on a smaller scale as shown also in Hawker *et al's* (1977) results. This decrease may well have been detected by Burton and Wilson (1970) who suggested that sucrose levels were at a maximum per unit fresh weight within one to two weeks following tuber initiation.

Once tubers have reached 15 to 20 mm diameter soluble sugars contribute only 2% to the total dry weight which is a similar proportion to that reported by Burton and Wilson (1970). This proportion remains constant as the tubers continue to increase in size and is substantially less than in the tubers analysed by Plaisted (1957) where the soluble sugars contributed over 10% of the total dry weight until the tubers were between 150 and 200 g fresh weight.

Analysis of the starch content of the stolons and tubers of *S. andigena* showed that there was a substantial increase in the amount of starch present in stolon tips removed from plants with tubers (stolons (A)) compared to those removed from plants which had no tubers (stolons (NA)) (Table 4.12). An even lower concentration of starch was noted in stolon tips removed from plants from a population in which tuberisation was not evident (PPD experiment 2, Table 4.20). The starch content continued to increase as tuberisation became visible (Table 4.12, Fig 4.21).

These results confirm the cytological observations made by Artschwager (1924) who suggested that the cortical cells behind the growing region of the stolon contained starch and that during early tuber expansion starch was present in cortical cells at the time divisions in the pith were occurring prior to the formation of the major starch-storage tissue. Noda and Yamamoto (1950; cited by Okazawa, 1967) detected an accumulation of starch grains in the endodermis tissue of stolon tips immediately after the cessation of stolon elongation as well as the subsequent accumulation of starch grains in tissues, including the cortex and pith, as tuberisation proceeded.

Reeve *et al* (1969) reported that starch granules were abundant in all young parenchyma cells in tubers of diameter 1.5 to 2.0 mm. Cutter (1978) showed that starch grains were present in cells of the stolon tip before the stolon hook had straightened out although she considered that deposition of starch at this early stage of tuber development may be a varietal feature.

The results (Table 4.11) also confirm observations made by Lovell and Booth (1967) who claimed that in 10 non-tuberising plants, almost no starch was detected by iodine staining near to the stolon tips whereas in 10 plants which had obvious but small tubers, starch was frequently detected in a localised area just behind the tip of stolons which themselves showed no external signs of tuberisation. Lovell and Booth (1967) claimed that the onset of starch deposition was a precise indicator of tuber initiation.

The amount of starch detected in stolon tips by Plaisted (1957) (ca 35% DW) was substantially more than that detected in stolon tips obtained from non-tuberising or tuberising plants of *S. andigena* (1.9 and 4.2% DW respectively). The results obtained from *S. andigena* were of the same order of magnitude as those detected by Burt (1964: 3% DW) and by Sowokinos (1976: ca 5% DW). As a percentage of the fresh weight the starch levels of stolon tips from *S. andigena* (0.15 and 0.38%) compare well with those reported by Obata-Sasamoto and Suzuki (1979a: ca 0.25% FW) but not with those of Plaisted (1957: ca 3% FW). Hawker *et al* (1979) reported a starch level of 1% fresh weight in stolon tips of plants grown in liquid culture although the diameter of these stolon tips appears greater than those of pot grown or field grown tubers (section 4.2.4.1 and Obata-Sasamoto and Suzuki, 1979a). In the "just swelling" tips Obata-Sasamoto and Suzuki (1979) reported an increased amount of starch (0.65% FW) which is slightly higher than that detected in *S. andigena* (0.42% FW). In this case the difference may be attributable to the selection of slightly different size categories. However, in a similar developmental stage, 6.5% of the fresh weight was attributable to starch according to Plaisted (1957). It would appear that the data presented by Plaisted (1957) concerning the starch levels in stolons and small tubers is incorrect.

The early developmental stages of *S. andigena* tubers were characterised by a very rapid increase in the concentration of starch which contributed to over 50% of the dry weight in tubers 7.5 to 10 mm in diameter (Fig 4.10). The decrease in the proportion of starch in tubers 10 to 15 mm diameter was not readily explainable except on the basis of a faulty analysis. However, as the weight and size of tubers increased, a high concentration of starch was maintained as starch continued to accumulate in the tubers at an increasing exponential rate. A rapid increase in the concentration of starch (% DW) immediately following tuberisation was reported by Sowokinos (1976). Fifty eight percent of the dry weight was starch in tubers 10 mm diameter and this level was maintained during further tuber expansion.

As a proportion of the fresh weight the starch content increased rapidly following tuberisation and reached a level of 8.3% in tubers between 5 and 7.5 mm diameter, changing only slightly as the tubers further increased in size. A similar accumulation pattern is shown in the starch content (% FW) of tubers obtained from plants grown in water culture (Hawker *et al*, 1979). A starch level of 7.4% of the fresh weight was determined in tubers of 7.3 mm diameter. The concentration

fluctuated slightly as the tubers increased in size. Mares and Marschner (1980) suggested that the starch content of tubers weighing between 15 and 30 g from plants grown in water culture was greater in the basal tissue (8 to 11% FW) than in the apical tissue (7 to 9% FW).

Although the levels of starch determined by Obata-Sasamoto and Suzuki (1979a) for stolons and small tubers are in agreement with those reported in this thesis, the proportion of starch as a function of the fresh weight calculated from the data for larger tubers was very low. A peak starch level of 3.5% FW occurred in tubers of 10 mm diameter. This is in contrast to 8.6% FW and 7.7% FW for *S. andigena* tubers of sizes 7.5 to 10 mm diameter and 10 to 15 mm diameter respectively and does not agree with the starch content of 6.5% fresh weight reported by Hawker *et al* (1979) for tubers of a similar size grown in water culture.

Although the levels of starch are reported to be different in slower and faster accumulating tubers of the same size (Hawker *et al*, 1979), the levels of starch reported by Obata-Sasamoto and Suzuki (1979a) in expanding tubers appear to be very low.

In mature tubers the starch content was reported to be 10 to 25% of the fresh weight, with a maximum of 35% depending on the variety (Cutter, 1978). However, in the analyses cited above mature tubers were not extracted and agreement with the lower figure (10% FW) can only be obtained by extrapolation (Fig 4.20).

The relative rate of starch and soluble sugar accumulation in tubers was at its highest in tubers about 3 mm in diameter but decreased until a more constant relative rate was attained in tubers of 15 mm diameter or greater (Figs 4.20, 4.21). During this period of decreasing relative rate of solute accumulation the cytokinin concentration in the tubers increased to its maximum and subsequently decreased at the time when a constant relative rate of accumulation was established. A similarly poor correlation was observed if either the concentration on a fresh or dry weight basis or the amount of cytokinin-like activity per tuber was related to the (absolute) rate of starch or soluble sugar accumulation (Figs 4.20, 4.21).

Obata-Sasamoto and Suzuki (1979a) suggested that decreasing auxin and increasing cytokinin levels in developing tubers enhanced the activities of starch synthesizing enzymes to support continuing starch deposition. An increased ADPG-pyrophosphorylase activity was correlated with an increase in the cytokinin content of tubers 25 mm and 35 mm diameter.

ADPG-pyrophosphorylase is regarded as an important enzyme in the regulation of starch synthesis (eg Mares *et al*, 1981 and references cited therein). In contrast, starch synthase is not considered to have an important regulatory function while the activity of UDPG-pyrophosphorylase is thought to be related to the requirement for nucleotide sugars (Mares *et al*, 1981).

Obata-Sasamoto and Suzuki (1979a) suggested that ADPG-pyrophosphorylase could be detected only in tubers of diameter 25 mm and 35 mm although a transient increase in activity in tubers 2.5 mm diameter is shown in their data. In contrast Sowokinos (1976) showed that there was a very rapid increase in ADPG-pyrophosphorylase activity following tuber formation. The highest activity was present in tubers 10 and 15 mm in diameter. A rapid decrease in activity occurred as tubers expanded further.

Hawker *et al* (1979), using stolons and tubers from plants grown in water culture, showed that ADPG-pyrophosphorylase did not increase immediately following tuberisation, a feature which would not show in the data presented by Sowokinos (1976). The enzyme level was similar in stolons and tubers 3.5 mm in diameter. However, the activity rose rapidly as tubers increase in diameter from 3.5 mm to 7.3 mm in diameter. The activity remained high as tubers expanded but levelled off rather than decreased to the same extent as observed by Sowokinos (1976).

These data cited above lead to the suggestion that the peak of activity of ADPG-pyrophosphorylase was, inexplicably, not detected by Obata-Sasamoto and Suzuki (1979a). Consequently the correlation drawn between ADPG-pyrophosphorylase and the cytokinin content by Obata-Sasamoto and Suzuki must be considered with caution. However, the increase in ADPG-pyrophosphorylase activity measured by Hawker *et al* (1979) occurred in only slightly smaller tubers than did the first detectable increase in the cytokinin-like activity of *S. andigena* tubers.

The possible relationship between tuber formation, enzyme activity and the cytokinins will be discussed further in relation to *in vitro* studies in Chapter 5.

The literature referring to cytological events at the initial stages of tuber development appears confused. However, the region of the stolon in which tuber formation occurs is not debated. As noted in this study and by Cutter (1978 and references cited therein), tuber formation begins in the subapical region of the stolon but whether the initial radial expansion is caused simply by cell enlargement or by cell division and enlargement is still uncertain. Artschwager (1924)

suggested that successive cell divisions in the region of the pith caused the initial radial expansion although simultaneously with the divisions in the pith cells were changes in the peripheral cortex including both tangential stretching and radial divisions. Subsequently the major areas of cell division occurred in the pericycle and the perimedullary zone (the Markkrona).

Yamamoto and Noda (1947-1951) suggested that both cell enlargement and an increase in cell number contributed to the size increase at early developmental stages. Plaisted (1957) showed that the total cell number per tuber increased immediately after tuber formation while cell expansion in the pith cells was noticeably less than that in the cortical cells.

Reeve *et al* (1969) suggested that the initial radial enlargement of the stolon tips was by cell division at random planes and cell growth in the young pith and pith branches with accompanying cell divisions in the cortex whereas Reeve *et al* (1973b) suggested cell enlargement, in fact, initiated tuberisation. Moreover, Booth (1963) claimed that at the very early stages of tuber development there was little evidence of cell division, except in the cortex, although in slightly older tubers cell division was detectable throughout the pith as well as in the cortex and vascular tissue. Lesham and Clowes (1972) suggested that the initial tuber growth was by expansion of the pith cells accompanied by some cell divisions.

In a preliminary study Cutter (1978) tended to agree with Booth (1963) and suggested that radial expansion in tubers of 1.0 to 1.5 mm diameter was primarily attributable to enlargement of the pith cells, although she considered that cell division probably rapidly followed. Peterson and Barker (1979) endeavoured to resolve the controversy by culturing induced stolons *in vitro*. They claimed that early radial expansion of tubers was due to an increase in cell diameter of both pith and cortical cells.

The more recent studies appear to confirm Booth's observation that the initial radial expansion in tubers is primarily by the enlargement of pith cells. Cell division, however, occurred very soon after tuber formation in field grown potatoes (*eg* Cutter, 1978) whereas in tubers formed *in vitro* cell division was delayed (Peterson and Barker, 1979). In fact, for at least six days after the first radial expansion was observed tuber enlargement did not involve cell division in the cortex or pith but rather cell enlargement in these tissues and some cell division in the vascular cambium and phellogen. Even in tubers up to

6 mm diameter the perimedullary zone contributed little to tuber diameter (cf Reeve *et al*, 1973b). However, as Peterson and Barker (1979) pointed out, 6 mm was the maximum diameter obtained under *in vitro* conditions and it was suggested that limiting factors may be operating. Growth hormones were not supplied exogenously.

The relative contribution of cell division and cell enlargement to further tuber expansion has also been debated. Artschwager (1924) suggested that rapid and continuous cell divisions occurred in the perimedullary zone and that cell division continued until the tuber was mature. Yamamoto and Noda (1947-1951) suggested both cell divisions and cell enlargement contributed to tuber expansion until the end of the swelling period. Plaisted (1957) suggested that the increase in tuber size was more directly related to an increase in cell number than to an increase in cell volume and that cell division, and expansion, continued in tubers at least up to 200 g fresh weight. However, it could be interpreted from his data, that cell division may make a decreasing contribution to tuber enlargement in tubers more than 74 g fresh weight.

Reeve *et al* (1973b) were critical of the conclusions drawn by Plaisted (1957). Reeve *et al* (1969, 1970) and Reeve *et al* (1973a,b) considered that the later stages of tuber growth were mainly by cell enlargement. Reeve *et al* (1973b) reported that many new cells were formed early in tuber growth but that there was an early reduction in the cell division rate and cell divisions were greatly diminished in tubers over 20 mm diameter. While considerable cell enlargement did occur in young tubers (Reeve *et al*, 1973a), it became increasingly dominant in tubers beyond 30 to 45 g fresh weight (Reeve *et al*, 1973b).

By the time tubers were about 4 mm in diameter cell division in tissues contributing to starch-storage parenchyma had been initiated and the tissue was well differentiated in tubers about 10 mm in diameter (Reeve *et al*, 1973b). When tubers were between 20 to 30 mm diameter the starch-storage parenchyma had become well established and the starch granules had begun to reach sizes which were peculiar to the different tissues of the mature tubers, growth by cell division diminished and growth by cell enlargement became increasingly dominant (Reeve *et al*, 1970; Reeve *et al*, 1973b). Reeve *et al* (1970) suggested that in tubers of this size many of the procambial cells retained meristematic characteristics although these were more pronounced towards the bud ends than the stem ends of the young tubers and became less evident as tubers exceed 30 mm diameter.

The phase of rapid cell division suggested by Reeve and co-workers would coincide with the increasing concentrations of cytokinin-like activity detected in developing tubers of *S. andigena* (Figs 4.31, 4.34). It was suggested that cell divisions in tubers from 20 to 30 mm diameter were greatly reduced (eg Reeve *et al.*, 1969). The concentration of cytokinin-like activity was also found to be low in tubers of these diameters. Okazawa (1970) suggested that there was an increasing amount of cytokinin-like activity per tuber until near maturity. Cell divisions are considered to occur in potato tubers until, or at least near to, maturity (eg Plaisted, 1957; Reeve *et al.*, 1970). However, these divisions were suggested to become more localised in the apical region of the potato as it increased in size (Reeve *et al.*, 1970).

Cytokinin-like activity was detectable in two of the three replicate bioassays of extract of *S. andigena* tubers 40 to 50 mm diameter. It is probable that a dilution effect has occurred as it was noted by van Staden and Dimalla (1978) that less cytokinin-like activity was detectable in the internal tissues of mature tubers (one week after harvest) than in the cortex adjacent to apical or lateral buds.

It is most probable that cytokinin-like activity was present in tubers 40 to 50 mm diameter. However, the actual concentration of cytokinin-like activity was very low in extracts of whole tubers 40 to 50 mm diameter. It would be interesting to extract and determine the concentration of the cytokinin-like activity in the regions around the developing buds relative to the rest of the tissue in this size category of tubers as cell divisions are purportedly still occurring in the phloem strands near the developing tuber buds (Reeve, cited by Moorby, 1978).

A closer correlation can be drawn between the amount and concentration of cytokinin-like activity in developing tubers and the frequency of cell divisions as reported by Reeve and co-workers than between either soluble sugar or starch accumulation or the reported activities of ADPG-pyrophosphorylase.

4.4.3 Tuberisation response of *S. andigena* grown under different growth conditions

The objective in growing a population of plants in the controlled environment rooms at Plant Physiology Division (PPD), Palmerston North, was to maintain one set of plants in non-inductive conditions to provide non-tuberising stolon tips for cytokinin, soluble sugar and starch analysis while the remaining plants were to be initiated to form tubers

by transfer into SD conditions. These plants were to provide a source of induced, but non-swollen, stolon tips and a range of tuber sizes. Trial experiments conducted in the growth rooms at Canterbury appeared to indicate that the timing of tuber induction could be controlled by a transfer of plants of *S. andigena* cv 165 from LD to SD conditions (Table 4.18).

However, the unexpectedly rapid tuberisation under LD conditions during PPD experiment 1 (Table 4.9) has meant that a definitive picture of cytokinin, soluble sugar and starch changes in stolon tips before and after tuber initiation has not been obtained.

The conditions under which the plants were grown in the Canterbury growth rooms (Appendix 6) were not replicated exactly in the controlled environment rooms at PPD (Appendix 7) and several reasons could be advanced for the more rapid tuber formation under the PPD conditions. A lowered night temperature was used during PPD experiment 1 and this has often been found to promote tuber formation in *S. tuberosum* cultivars (eg Gregory, 1956; Borah and Milthorpe, 1963; Slater, 1968) although Woolley (pers comm) suggested that the critical night temperature for *S. andigena* was about 10°C. Lighting conditions also differed, the lighting at PPD being of significantly higher intensity and different spectral quality compared to that at Canterbury (Appendices 6 and 7). A higher level of radiation at temperatures between 15 and 20°C was noted by Headford (1962) and Borah and Milthorpe (1963) to promote earlier tuber initiation in *S. tuberosum* cultivars. In contrast Chapman (1958) noted that a long day, or continuous light, delayed tuber formation for several weeks.

Growth room availability at PPD was at a time when "seed" tuber material had been stored for 9.5 months, substantially longer than that for seed tubers used in Canterbury experiments 1 and 2 (Table 4.19). Increasing age of *S. tuberosum* "seed" tuber material is reported to lead to earlier tuber formation (eg Madec and Perennec, 1959; Iritani, 1968; Wurr, 1978).

PPD experiment 2 was designed to test some of the above possibilities although insufficient growth rooms were available to test all possible combinations of temperature and lighting conditions required and so some results are necessarily confounded. The tubers used in PPD experiment 2 were planted one week following harvest (Table 4.20). The early tuber formation in PPD experiment 1 may have been in part attributable to the age of the tubers although this comparison is confounded by temperature. However, LD did not prevent tuber formation

in PPD experiment 2 and tuber formation indeed was more rapid than occurred in experiment 2 at Canterbury in which the seed tubers were of a similar age and the temperature regime was comparable (Table 4.20). An elevated night temperature did not prevent tuberisation occurring during either experiment 2 at PPD (Table 4.21) or experiment 3 at Canterbury (Table 4.23).

Plants exposed to LD and SD conditions in the growth rooms at Canterbury necessarily were exposed to differing amounts of PAR whereas at PPD the lighting conditions could be programmed so that both LD and SD plants received the same amount of PAR and the day length could then be extended by low intensity lighting. Tuberisation was actually hastened when plants were exposed to 16 hours PAR at PPD (Table 4.22) which was of significantly higher intensity than the PAR at Canterbury. The plants were shorter and the leaves darker green in colour compared to the plants receiving eight hours of PAR and eight hours low intensity lighting.

To determine if the light intensity or spectral characteristics were the critical factor in promoting tuber formation at PPD, a further set of plants were grown at Canterbury under a 16 hour photoperiod with a 20°C night temperature. The plants grown in experiment 3 at Canterbury tuberised just as readily as at PPD (Table 4.23).

The plants grown in the Canterbury growth rooms in 1977, 1978 and 1979 showed an increasing tendency towards earlier tuber initiation (Table 4.20).

Simmonds (1971, 1980) has shown that it is possible to select plants from a SD obligate *S. andigena* line that within a few generations exhibit day neutrality. The variety of potato initially supplied by Dr Bedi (DSIR) had been backcrossed to *S. tuberosum* and it was possible that under the field growing and harvesting programme there was an unconscious selection of plants tending to exhibit a longer critical photoperiod.

The growth rooms at Canterbury were not available for longer than the seven and eight weeks used for experiments 1 and 2 respectively, which in retrospect was an insufficient length of time to determine whether the LD conditions were inhibiting or merely slightly delaying tuber formation.

The conditions under which "seed" potato tubers are grown can also affect the performance of the plants grown subsequently from that "seed" (eg Went, 1959; Wurr, 1978; Perennec and Madec, 1980). Little control could be exerted over this feature.

To repeat the experiment successfully it will be necessary to obtain a clone of potato which has a very short critical photoperiod and it is obviously important to conduct trial experiments under the growth conditions to be employed for the major experiment.

CHAPTER V

TUBER FORMATION IN VITRO

5.1 INTRODUCTION

Much of the work on tuber initiation in sterile cultures has used stem cuttings excised from whole plants which were growing under environmental conditions inductive to tuber formation. Such cuttings were referred to as induced stem cuttings as opposed to non-induced stem cuttings removed from non-tuberising plants (*eg* Gregory, 1956; Chapman, 1958; Forsline and Langille, 1976).

Etiolated sprouts will often develop from the eyes of tubers stored in the dark. Sprouts developed in this manner were used as the stock material in the experiments reported in this chapter and have been used similarly by Palmer and Smith (1969a,b, 1970), Smith and Palmer (1970), Stalknecht (1972), Mauk and Langille (1978) and Stalknecht and Farnsworth (1979).

Segments of sprouts transferred onto suitable media will develop negatively geotropic axillary shoots which are referred to by Palmer and Smith (1969a, 1970) as stolons. The elongating axillary shoots may then be sub-cultured onto fresh media, the sub-culture referred to in this thesis as the second transfer. The potential of sprout segments to form tubers *in vitro* without added plant growth regulators is substantially higher than that of sub-cultured shoots (Mingo-Castel *et al*, 1976b).

Palmer and Smith (1969a,b, 1970) and Smith and Palmer (1970) suggested that there was an absolute requirement for the presence of a cytokinin, in addition to an adequate supply of sucrose, to induce tuber formation in sub-cultured stolons. Tizio and Biaiñ (1973), however, suggested that the observed tuber formation, stimulated *in vitro* by cytokinin, was not a direct result of cytokinin promotion but an indirect result of root inhibition and it was suggested that a factor present in growing roots inhibited tuber formation.

Papers by Stalknecht (1972), Stalknecht and Farnsworth (1979) and

Mingo-Castel *et al* (1974) indicated that the cytokinin requirement for tuberisation of sub-cultured stolons is not absolute. There have also been suggestions that, at certain concentrations, auxins may stimulate tuberisation *in vitro* (Okazawa, 1958, 1967; Harmey *et al*, 1966; Tizio and Biaiñ, 1973; Kumar and Wareing, 1974).

This chapter reports on a very preliminary investigation into tuber formation *in vitro*. Consideration was given to the effect of an auxin alone, or in combination with a cytokinin, on tuber formation of sprout sections and sub-cultured shoots to further investigate the apparent cytokinin requirement for *in vitro* tuber formation. The influence of the presence of roots on tuber formation was noted specifically (*eg* Tizio and Biaiñ, 1973).

5.2 MATERIALS AND METHODS

5.2.1 Tuberisation *in vitro*. Experiment 1

5.2.1.1 First Transfer When tubers from field grown *S. andigena* cv 165 plants (section 3.1.2.2) were stored for eight months at 4°C groups of sprouts developed on each tuber (31/3/78). Sprouts which were 20 to 30 mm long were removed and sterilised. Initially, the sprouts were washed in running tap water for about 10 minutes. Then, working under sterile conditions, the sprouts were dipped in 70% ethanol for ca two seconds and immediately placed in a 10% solution of commercial Janola (3.5% active hyperchlorite). The solution was stirred continuously for 30 minutes after which the sprouts were removed and washed in five changes of sterile distilled water.

Each sterilised sprout was cut into segments containing one node ("nodal segments"). The apical and basal 5 mm of each sprout were discarded. The morphologically lower portion of each sprout section was embedded in 50 ml of a modified Murashige and Skoog (1962) medium in a 100 ml Erhlenmyer flask. The node on each sprout remained above the agar surface.

The basic Murashige and Skoog (Appendix 8) medium was supplemented with 1.862 mg l⁻¹ naphthalene acetic acid, 0.8% Difco-bacto agar and varying amounts of sucrose and kinetin (Table 5.1). A total of six different combinations of sucrose and kinetin were prepared.

Sprout segments were incubated in darkness at 27°C. Frequent detailed observations were made with respect to callus, root, shoot and tuber formation.

TABLE 5.1

Tuberisation *in vitro*. Experiment 1. Combinations of sucrose and kinetin with which the modified Murashige and Skoog nutrient agar was supplemented.

Medium	Sucrose (%)	Kinetin (mg l ⁻¹)
1	0	0
2	0	2.5
3	2	0
4	2	2.5
5	6	0
6	6	2.5

5.2.1.2 Second Transfer After 46 days, shoots which had developed from the nodes of cultured sprout segments on media 1 and 3 were excised under sterile conditions and dissected into apical and basal segments, each containing several nodes. A total of eleven segments were transferred onto fresh media ("second transfer"), four segments being transferred onto Medium 5 and seven onto Medium 6 (Table 5.1). All flasks were incubated at 27^oC in darkness and final observations recorded 34 days after transfer.

5.2.2 Tuberisation *in vitro*. Experiment 2

5.2.2.1 First Transfer The sprouts used in this experiment (15/7/79) had developed on *S. andigena* cv 165 tubers which had been stored in darkness at room temperature from the time of harvest (section 4.2.4.2). Two to three etiolated sprouts had formed on each tuber. Unbranched sprouts up to 200 mm in length were removed and sterilised by the procedure described for Experiment 1 (section 5.2.1.1). Sprouts were sectioned under sterile conditions and divided into two categories. Sprouts with one node, in addition to the intact apex, are subsequently referred to as "apical segments". The "medial segments" each had two nodes and were 40 to 50 mm in length. All segments were embedded in a modified Murashige and Skoog agar medium (Appendix 8). The node on each apical segment remained above the agar surface but the lower of the two nodes on the medial segment was immersed beneath the surface of the medium. Table 5.2 lists the number of apical and medial segments which were initially cultured in agar media supplemented with differing amounts of sucrose and kinetin.

TABLE 5.2

Tuberisation *in vitro*. Experiment 2. Culture details listing the number of apical and medial segments incubated on Murashige and Skoog nutrient agar supplemented with different combinations of sucrose and kinetin.

Medium	Sucrose (%)	Kinetin (mg l ⁻¹)	Number of Segments	
			Apical	Basal
1	0	0	2	5 (1) ^α
2	0	2.5	3	5
3	2	0	6 (1)	18 (1)
4	2	2.5	8 (1)	16 (3)
5	6	0	8	14 (1)
6	6	2.5	8	16 (1)

^α Numbers in parentheses indicate the number of contaminated and subsequently discarded cultures.

Cultures were incubated for 79 days in darkness at 27°C and frequent detailed observations were made.

5.2.2.2 Second Transfer After 11 days in culture on Medium 3 (Table 5.1) shoots which had developed from the node above the agar surface were excised from the original sprout segment. The shoots were dissected into apical and basal segments each containing a single node. A total of three apical and three basal segments were transferred onto Medium 5, while four apical and four basal segments were transferred onto Medium 6 (Table 5.1). Cultures were maintained in darkness at 27°C for 90 days and observations recorded at increasing time intervals from four days after transfer.

5.2.3 Tuberisation *in vitro*. Experiment 3

5.2.3.1 First Transfer Etiolated sprouts had developed on tubers of *S. andigena* and *S. tuberosum* which had been stored in darkness at room temperature for six months from the time of harvest in late March 1979.

Sprouts were removed from the tubers (29/9/79) and sterilised by the procedure described for Experiment 1 (section 5.2.1.1). Each sprout was dissected into single nodal segments, the apical and basal 5 mm of tissue discarded and the segments embedded in Medium 3 (Table 5.1) as described for experiment 1 (section 5.2.1.1). Two segments were

embedded in 50 ml of medium in each 100 ml Erhlenmyer flask. A total of 39 flasks were prepared: 20 flasks contained *S. tuberosum* sprout segments and 19 flasks contained *S. andigena* sprout segments.

5.2.3.2 Second Transfer Shoots developing from the nodes of cultured segments were excised after 23 days. Excised shoots were either trimmed to a length of 25 mm leaving the apex intact (subsequently referred to as "whole shoots"), or cut into "single nodal segments". The apical and basal nodes were discarded. Whole shoots or nodal segments were transferred onto Medium 5 or 6 (Table 5.3). All nodes were positioned above the agar surface and cultures were incubated at 27°C in the darkness for 80 days. Observations were recorded frequently from three days.

TABLE 5.3

Tuberisation *in vitro*. Experiment 3. Culture details listing the number of whole segments and single-nodal segments of *S. tuberosum* and *S. andigena* transferred onto Media 5 and 6.

Subspecies	Medium	Sucrose (%)	Kinetin (mg l ⁻¹)	Number of Cultures	
				Whole Shoots	Nodal Segments
<i>tuberosum</i>	5	6	0	11	12
	6	6	2.5	9	12
<i>andigena</i>	5	6	0	13	10
	6	6	2.5	12	7

5.3 RESULTS

5.3.1 Tuberisation *in vitro*. Experiment 1

5.3.1.1 First transfer When tubers of *S. andigena* were stored at 4°C for eight months, etiolated sprouts developed. If nodal segments of the sterilised sprouts were placed on medium devoid of sucrose, little growth occurred regardless of the kinetin concentration (Table 5.4). Only a trace of callus was present after 26 days while after 80 days both the sprout segment and the small amount of callus were brown. The callus developed on the surface of the agar at the point of contact between the sprout and the agar.

TABLE 5.4

Tuberisation *in vitro*. Experiment 1. *In vitro* culture of sprout segments removed from *S. andigena* tubers. Sprout segments were cultured on modified Murashige and Skoog medium, or medium supplemented with sucrose and/or kinetin. Observations were recorded after 26 days in culture.

Supplemented medium	Number of segments cultured	Callus formation	Callus and root formation	Shoot formation
1. 0 kinetin 0 sucrose	10	trace	0	0
2. 2.5 mg l ⁻¹ kinetin 0 sucrose	14	trace	0	0
3. 0 kinetin 2% sucrose	30	30 (100) ^a	21 (70.0)	1 (3.0)
4. 2.5 mg l ⁻¹ kinetin 2% sucrose	12	12 (100)	2 (16.7)	0
5. 0 kinetin 6% sucrose	14	14 (100)	14 ^b (100)	5 (35.7)
6. 2.5 mg l ⁻¹ kinetin 6% sucrose	14	14 (100)	5 ^c (35.7)	0

^a Number in parenthesis is the percentage of the total cultures

^b Roots long and thin

^c Roots short and fat

When the sprouts segments were maintained on medium with 2% sucrose, large amounts of callus formed within 26 days. A limited number of short roots developed from the callus. A shoot formed from the node of one of the sprout segments. After 80 days in culture root formation was evident but not prolific (Table 5.4). When the low sucrose medium was supplemented with kinetin little callus growth or root formation were evident after 26 days in culture. Callus formation was prolific after 80 days in culture, but little further root formation had occurred (Table 5.4).

Sprouts maintained on a 6% sucrose medium without kinetin formed large callus masses with associated root formation. The extension of an unbranched shoot from the node of the sprout segment occurred in 35.7% of the flasks within 26 days of culture. After 80 days sprouts had formed large amounts of callus. Numerous long thin roots were observed growing from the callus (Table 5.4).

If, in addition to a 6% sucrose content, the medium was supplemented with kinetin, callus growth was again prevalent. Root formation was not prolific and the roots formed were thick. No shoot formation was observed within 80 days (Table 5.4).

5.3.1.2 Second Transfer All shoots formed from the above sprout segments ("stock") within 46 days of culture were cut into apical or basal segments each containing several nodes and transferred onto medium supplemented with sucrose, either with or without the kinetin supplement. Axillary shoots formed at the nodes of all second transfer shoots but the presence of kinetin in the medium had a retarding effect on subsequent shoot elongation as it did on root formation. A sessile tuber formed at the node of one shoot transferred from the medium supplemented with 2% sucrose to medium with 6% sucrose but devoid of kinetin. The tuber sprouted and the shoot formed began to elongate.

5.3.2 Tuberisation *in vitro*. Experiment 2

5.3.2.1 First Transfer The sprout segments used in this experiment were divided into two categories: "apical segments" and "medial segments" as defined in Section 5.2.2.1.

Medium 1 Apical segments maintained on a minimal nutrient medium (without sucrose) produced very little new tissue. Segments became discoloured and were discarded after 22 days. Within four days short shoots and roots were produced by medial segments cultured on the same medium. However, little subsequent growth occurred.

Medium 2 Apical segments maintained on basal medium devoid of sucrose but to which kinetin was added showed no growth within a 30 day period. Shoots and roots produced by medial segments maintained on this medium did not elongate.

Medium 3 All sprout segments maintained on medium supplemented with 2% sucrose were beginning to discolour within 22 days. The apical segments were slow to resume elongation growth but short adventitious roots and small amounts of callus were observed forming at four days. By 30 days the apical segments were elongating. However, the medial segments started to produce new tissues much more rapidly. New shoot formation was apparent within two days. Within nine days the majority of segments had formed shoots arising from the node above the agar surface. The shoots elongated rapidly and shoots up to 15 mm in length had formed within four days. Callus formation was noticeable by 15 days and by 55 days large masses of callus had formed. Roots with numerous

root hairs grew from many of the calluses. Adventitious roots developed from the node above the agar surface. This root formation was rapid and roots frequently grew long enough to penetrate the agar. Root primordia were evident on the surface of the stock material which was immersed in the agar. By 44 days thick roots had sprouted from the base of the stock. On the 2% sucrose medium, two of the shoots which developed had swollen bases and will be referred to as sessile tubers (Table 5.5). The shoots with swollen bases continued to elongate. By 51 days all stock and shoots were senescing and this part of the experiment was terminated.

Medium 4 Sprout segments maintained on medium supplemented with 2% sucrose and 2.5 mg l^{-1} kinetin remained a golden colour for 22 days. By 30 days the stock above the agar surface was shrivelled and brown while that beneath the agar was very swollen.

Apical segments produced little new growth above the agar surface. By 30 days the apical segments had not elongated and never more than one adventitious root formed although small amounts of callus had formed and the stock beneath the agar had increased in diameter considerably.

Short shoots and adventitious roots formed from the medial segments maintained on medium with 2% sucrose and kinetin. No shoots had grown longer than 15 mm within 30 days. Roots were generally short and only traces of callus were formed. The most noticeable growth was the increase in diameter of the stock embedded in the agar.

Medium 5 Adventitious roots and callus formed readily from apical sprout segments maintained on medium supplemented with 6% sucrose. Roots had differentiated from the callus by 15 days. By 22 days the apices had started to elongate, axillary shoots were appearing, and roots were growing from the base of the stock. Tubers forming on the tips of shoots were visible by 71 days. These tubers are referred to subsequently as "apical" tubers to distinguish them from "sessile" tubers. Tubers did not form on the apex of the original sprout segments. Tubers collected after 120 days were slightly elongated and the largest tuber weighed 387 mg.

The majority of medial segments grown on the 6% sucrose medium produced a shoot from the node above the agar surface. Shoot growth was rapid except when a sessile tuber formed. Over 50% of the sprouts formed sessile tubers within 15 days (Table 5.5). Branching of shoots occurred readily. Tuber formation also occurred on branches sessile to the main shoot. All sessile tubers sprouted and the shoots formed elongated rapidly. Apical tuber formation occurred within 44 days (Table 5.5) and sprouting of some of these tubers was occurring by 79 days. Root formation was prolific and adventitious roots elongated rapidly from the nodes above

TABLE 5.5

Tuberisation *in vitro*. Experiment 2. Tuber formation by medial sprout segments cultured on medium supplemented with different concentrations of sucrose and kinetin

Medium (number of cultures)	Total (and percentage) Tuberisation				
	Days in Culture				
A. <u>Sessile tuber formation</u>	4	9	15	22	79
3. 2% sucrose 0 kinetin (17)	0	1 (5.9)	2 (11.76)	2 (20) ^a	-
4. 2% sucrose 2.5 mg l ⁻¹ kinetin (13)	0	0	0	0	-
5. 6% sucrose 0 kinetin (13)	0	4 (30.7)	7 (53.9)	7 (70) ^b	7 (70)
6. 6% sucrose 2.5 mg l ⁻¹ kinetin (15)	0	11 (73.3)	10 (71.4) ^c	10 (71.4)	10 (71.4)
B. <u>Apical tuber formation</u>	22	30	44	58	79
3. 2% sucrose 0 kinetin (10)	0	0	0	1 (10) ^d	1 (10)
4. 2% sucrose 2.5 mg l ⁻¹ kinetin (13)	0	0	0	0	0
5. 6% sucrose 0 kinetin (10)	0	1 (10)	5 (50)	5 (50)	6 (60)
6. 6% sucrose 2.5 mg l ⁻¹ kinetin (14)	0	0	0	3 (21.4)	6 (42.9)

a, b Number of cultures reduced to 10

c Number of cultures reduced to 12

d Appearance of culture quite unlike all other cultures on Medium 3 but in appearance like culture 5.

the agar surface. Callus formed readily and roots developed from the callus after 15 days as they also did from the base of the sprout segments. An increase in diameter of the stock immersed in the agar was observed. By 79 days the callus was a dark brown but masses of fine roots originating from the callus were encircling the base of the flask.

After 120 days in culture it was apparent that several tubers had resprouted and retuberised. Shoots bearing tubers were brown, the callus was dark brown and roots were numerous. The largest tuber formed weighed 700 mg. Most of the tubers formed were elongated rather than spherical in shape.

Medium 6 Apical segments maintained in basal media supplemented with 2.5 mg l^{-1} kinetin in addition to 6% sucrose formed adventitious roots readily but the roots remained short. Small amounts of callus were produced. After 30 days most of the apices had started to elongate and axillary shoots were also developing. By 15 days it was apparent that stock beneath the agar surface was becoming swollen. Roots did not develop from the base of the stock for 44 days. Tuber formation on the ends of the shoots began to occur by 58 days. After 79 days in culture multiple tuber formation on some shoots was apparent. The longest tuber to form after 120 days in culture weighed 97 mg.

Axillary shoots developed rapidly from medial segments. At nine days most of the shoots had formed sessile tubers with the subsequent, but temporary, cessation of shoot elongation (Table 5.5). After 12 days, one culture was removed and the swelling tested with iodine/potassium iodide solution. A positive starch test was observed. The sessile tubers resprouted and long, often branched, shoots developed. The first apical tubers were visible by 58 days (Table 5.5). Little callus formation occurred and few roots developed from the callus that did form. Adventitious root formation did occur but the roots formed were short and not numerous. Stock embedded in the agar became very swollen and roots developed from the base of the stock by 30 days. After 120 days in culture a large number of small, pear-shaped tubers had been produced. The largest tuber weighed 220 mg.

Summary of observations made during Experiment 2. First transfer

Growth of stock or of shoots and roots at nodes above the agar surface could not be maintained on medium without sucrose. The presence of kinetin in medium without sucrose stunted growth even further. Rapid shoot appearance and elongation occurred when the medium had a 2% sucrose content, and root formation was prevalent. The addition of kinetin to the low sucrose medium appeared to retard shoot growth and root growth. An

increase in the diameter of the stock beneath the agar surface was very apparent on the low sucrose medium especially when kinetin was added to the medium.

A higher sucrose level (6%) stimulated shoot growth but when sessile tuber formation occurred shoot growth was temporarily retarded. Following recommencement of shoot growth, apical tuberisation occurred (Table 5.5). Many of the apical tubers subsequently sprouted. Root formation was prolific although it was noticeably less prolific if kinetin was added to the medium already containing 6% sucrose. A more rapid and pronounced formation of sessile tubers was noted on the high sucrose medium supplemented with kinetin. Recommencement of shoot elongation occurred although not as rapidly as on medium devoid of kinetin. Apical tuber formation occurred slightly later on medium supplemented with kinetin (Table 5.5). Sprouting of these apical tubers also occurred. In both high sucrose media the stock embedded in the agar was visibly swollen.

A greater number of smaller whiter tubers were produced when sprout segments were grown on the higher sucrose medium supplemented with kinetin than were grown on medium devoid of kinetin. On this medium a smaller number of larger tubers were produced.

Apical sprout segments generally followed the pattern described above for medial segments. However, the initial growth was not as rapid and consequently all subsequent development occurred after longer incubation periods than were observed for medial segments.

5.3.2.2 Second Transfer

Medium 5 Shoot formation and elongation occurred following transfer of shoot segments onto high sucrose (6%) medium. Extensive branching occurred when shoots developed from the node of basal segments. Swelling of the stock embedded in the agar was apparent only in transferred apical segments but not basal segments. No roots formed from the base of the stock. Callus formation was prolific as was root formation from the callus. Adventitious roots were also formed. Four of the six apical and basal segments transferred to medium without cytokinin produced new shoots. Within 68 days all four shoots had formed tubers.

Medium 6 Shoot elongation was slow from apical segments and did not occur from basal segments transferred to media containing kinetin. Adventitious root formation was apparent but not prolific. Callus began to form after 28 days and roots subsequently developed from the callus. The diameter of the shoot section embedded in the agar increased but no roots formed from the base of the shoot beneath the agar. Tuber formation

had not occurred by 90 days.

5.3.3 Tuberation *in vitro*. Experiment 3

5.3.3.1 Second Transfer All shoots developed from sprout segments cultured on Medium 3 were excised and transferred onto Medium 5 or Medium 6, either with the apex intact ("whole shoots") or as single nodal segments (section 5.2.3.2).

Medium 5 *S. tuberosum* Whole shoots transferred onto high sucrose medium began to elongate rapidly. If the apex was damaged during transfer an axillary shoot rapidly elongated from a sub-apical node. All shoots formed branches readily. However, the growth of axillary shoots from the single nodal segments was less rapid. Branching of new shoots did not occur readily. Both whole shoots and nodal segments formed roots near the agar surface. Callus formed more rapidly on the whole shoots than on the nodal segments and thin roots extended from the callus in abundance. The stock became swollen beneath the agar and root primordia were evident.

Tuber formation occurred on both second transfer whole shoots and nodal segments (Table 5.6). Sessile tubers formed in the axils of transferred nodal segments but shoot elongation still continued. Apical tubers had formed by 49 days on both transferred nodal segments and whole shoots. Apical tubers formed on 54.6% of the axillary shoots developed from transferred whole shoots but on only 25% of the axillary shoots developed from nodal segments.

Medium 6 *S. tuberosum* Following the transfer of whole shoots and nodal segments onto medium supplemented with kinetin, it was noticeable that few new shoots developed from the nodal segments and those that did elongated very slowly and did not branch. The transferred whole shoots did not elongate rapidly and few axillary shoots developed. The few roots which developed from either transferred nodal segments or whole shoots were short and thick. Root primordia were more noticeable on transferred nodal segments than on transferred whole shoots. The stock became swollen beneath the agar surface but callus developed very slowly. Only one of the nine transferred whole shoots formed a tuber and only two of the 12 transferred nodal segments developed shoots which ultimately formed tubers (Table 5.6).

Medium 5 *S. andigena* The transferred whole shoots elongated rapidly and axillary shoots also developed. The transferred nodal segments also supported rapidly elongating shoots. Both whole shoots and nodal segments formed little callus but root formation was prolific. Twenty-three percent of the whole shoots developed axillary shoots which produced apical tubers

TABLE 5.6

Tuberisation *in vitro*. Experiment 3. Tuber formation by second transfer shoots obtained from sprout segments cultured *in vitro*.

Subspecies	Medium	Transferred Material (number of cultures)	Tuber Form	Total (and percentage) Tuberisation				
				Days in Culture				
				3	21	28	49	80
<i>tuberosum</i>	5. 6% sucrose 0 kinetin	Whole shoots (11)	Apical	0	0	1 (9.1)	2 (18.2)	2 (18.2)
			Sessile	0	0	1 (9.1)	6 (54.5)	6 (54.5)
	Nodal segments (12)	Apical	0	0	0	3 (25.0)	4 (33.3)	
		Sessile	1 (8.3)	1 (8.3)	2 (16.7)	4 (33.3)	4 (33.3)	
	6. 6% sucrose 2.5 mg l ⁻¹ kinetin	Whole shoots (9)	Apical	0	0	0	1 (11.1)	1 (11.1)
			Sessile	0	0	0	0	0
Nodal segments (12)	Apical	0	0	0	0	0		
	Sessile	1 (8.3)	2 (16.7)	2 (16.7)	2 (16.7)	2 (16.7)		
<i>andigena</i>	5. 6% sucrose 0 kinetin	Whole shoots (13)	Apical	0	0	0	1 (7.7)	3 (23.1)
			Sessile	0	0	0	0	0
	Nodal segments (10)	Apical	0	0	0	0	1 (10)	
		Sessile	0	3 (30)	3 (30)	3 (30)	3 (30)	
	6. 6% sucrose 2.5 mg l ⁻¹ kinetin	Whole shoots (12)	Apical	0	0	0	2 (16.7)	2 (16.7)
			Sessile	0	0	0	0	0
Nodal segments (7)	Apical	0	0	0	0	0		
	Sessile	0	0	0	0	0		

(Table 5.6). Only one of the shoots formed from the 10 nodal segments developed an apical tuber although sessile tubers were formed on three of the 10 nodal segments.

Medium 6. *S. andigena*. The addition of kinetin to the medium inhibited shoot development on the transferred nodal segments. Elongation of whole shoots and axillary shoot formation was very slow. Root growth was sparse and the few roots which formed were short and fat. Callus formation was noticeable after 49 days. No tubers formed on the transferred nodal segments and tuberisation occurred in only two of the 12 flasks containing transferred whole shoots. One of the two tuberising shoots formed multiple tubers (Table 5.6).

Summary of observations made during Experiment 3. Second Transfer

Shoots produced from sprouts grown on medium supplemented with 2% sucrose were transferred onto fresh medium containing 6% sucrose with, or without, a kinetin supplement. Response of the two potato subspecies was essentially the same. A concentration of 2.5 mg l^{-1} kinetin added to medium containing sucrose had a retarding effect on root development and on shoot development and elongation. It appeared to have an inhibitory effect on tuber formation. *S. tuberosum* formed tubers more readily *in vitro* than did *S. andigena* (Table 5.6).

5.4 DISCUSSION

S. andigena and *S. tuberosum* tubers held at room temperature in the dark developed etiolated sprouts from the eyes of the tuber. Segments of these sprouts were used as the initial stock material for *in vitro* cultures.

The sterilisation procedure finally found to be successful was an adaptation of that used by Stalknecht (1972). After removal from the mother tuber the sprouts were rinsed in running tap water. The brief immersion of the sprouts in 70% ethanol was found to be essential prior to immersion in a 10% solution of commercial Janola. Undiluted Janola, containing 3.5% sodium hypochlorite, damaged the sprout tissues. At least five rinses in sterile distilled water were necessary to remove adhering Janola.

The cultures were maintained in darkness at 27°C with brief exposures to the light when the flasks were examined. Lawrence and Barker (1963) suggested that exposure to intermittent spells of low intensity light had no effect on subsequent tuber formation. Although

Okazawa (1958) had earlier suggested that 27°C was inhibitory to tuber formation, Lawrence and Barker (1963) suggested that 26°C was optimum for vegetative growth and that tuber formation occurred as readily at 25°C as at 20°C. As the experiments had to be run concurrently with callus bioassays in the same incubator, the higher temperature was used. Palmer and Smith routinely maintained cultures at 25°C but also noted that tuber formation occurred as readily at 25°C as at 20°C, although 35°C was found to be inhibitory to tuber formation (Palmer and Smith, 1970).

The sucrose concentration used to promote shoot growth prior to sub-culturing and the concentration of both sucrose and kinetin used during the subsequent second transfer experiments were based on the report by Palmer and Smith (1969a).

As noted in experiments 1 and 2 the presence of sucrose was required in the medium to stimulate growth and a higher sucrose content supported more extensive branching. Gregory (1956) noted a similar requirement for stem cuttings. The number of flasks available to do these experiments was limited and second transfers were passed onto medium with a 6% sucrose content. No other sucrose concentration was tested. Palmer and Smith (1969a) reported that 6% sucrose was optimum for the cytokinin promotion of tuber formation.

In contrast to the photograph shown by Barker (1953) of stolons growing diageotropically from cultured segments of etiolated sprouts, the shoots emerging from the sprout segments used in these experiments were mostly negatively geotropic and grew only occasionally diageotropically or turned to become positively geotropic. Axillary shoot growth was similar to the description given by Mes and Menge (1954) for shoots developing from cultured stem segments rather than the description of stolons emerging from cultured etiolated segments (Barker, 1953). New shoots developing from the axillary buds of second-transfer shoots were generally positively geotropic and appeared essentially as the controls pictured by Palmer and Smith (1969a) and Stalknecht (1972).

There are numerous reports in the literature indicating that a sufficient concentration of sucrose was required in the medium before tuber formation would occur, although sucrose alone was not responsible for tuber induction (*eg* Mes and Menge, 1954; Okazawa, 1955; Gregory, 1956; Lawrence and Barker, 1963; Harmey *et al.*, 1966; Palmer and Smith, 1970).

This feature was also apparent in the experiments reported here as virtually no growth occurred in the total absence of sucrose as

discussed above, and little tuber formation was observed in cultures on media supplemented with 2% sucrose (Tables 5.4, 5.5) while it was more apparent on media supplemented with 6% sucrose (Table 5.5). All second transfers were onto media supplemented with 6% sucrose and tuber formation was again apparent (Table 5.6).

The most rapidly formed tubers were described as sessile (Mes and Menge, 1954) forming directly at the node of the sprout segments (Table 5.5). Apical tubers were formed on shoot tips often not till 30 to 40 days in culture whereas under the same conditions sessile tuber formation was noted within nine days (Table 5.5).

Tuber shapes varied from spherical to elongated and pear-shaped as described by Mes and Menge (1954). Regrowth of both sessile and apical tubers was frequently noted. Mes and Menge (1954) and also Chapman (1958) both noted that sessile tubers which formed on induced stem segments later grew out.

The majority of work investigating the effect of auxin in the medium was performed using induced stem segments and related to the timing of initiation (Harmey *et al*, 1966; Kumar and Wareing, 1974). Since all media used in these experiments contained NAA no comparison can be made relating to the timing of tuber formation. Chapman (1955), however, using cultured sprout nodes, found that the presence of 2,4-D or IAA in combination with 2% sucrose promoted active cell proliferation and a circular mass of tissue formed which was several times the diameter of the original explant. Although primordial roots frequently differentiated there was neither stem nor stolon growth from sprout nodes. Okazawa (1967) suggested that NAA had a promotory effect on tuberisation of sprout tips cultured *in vitro*.

Active cell proliferation below the agar surface was a very noticeable feature of the tissues immersed in the agar, especially in agar supplemented with 2% sucrose and kinetin, and 6% sucrose with or without kinetin. In contrast to Chapman's observations but in agreement with those of Harmey *et al* (1966), the presence of an auxin in the media did not inhibit shoot growth, and shoot growth, root growth and tuber formation were readily observed (Tables 5.4, 5.5, 5.6). However, the presence of kinetin in the media had a retarding effect on both shoot and root growth and this was apparent in all experiments.

The retardation of root growth by kinetin has been reported previously (*eg* Tizio and Biaiñ, 1973; Mingo-Castel *et al*, 1974; Mingo-Castel *et al*, 1976b), Palmer and Smith (1969b) suggested there was little reduction in the elongation of sub-cultured stolons on

kinetin media. However, branching and stem elongation are obviously more prolific in the stolons shown by Palmer and Smith (1969a) sub-cultured on medium free from cytokinin compared to tuberising cultures on kinetin-containing media. Moreover, Forsline and Langille (1976) suggested that kinetin reduced the elongation of cultured non-induced stem segments to those of induced stem segments.

Forsline and Langille (1976) reported that kinetin had a promotory effect on tuber formation of non-induced stem segments cultured *in vitro* and that the difference in tuberisation potential between induced and non-induced stem segments could be eliminated if kinetin was added to the medium. The promotion of tuber formation by kinetin was reflected to some extent in the tuber formation on sprout segments in experiment 2. Sessile tubers formed slightly more rapidly on segments cultured on media supplemented with kinetin (Table 5.5A). However, apical tubers tended to form more readily on the kinetin-free medium although this may be a reflection of the earlier regrowth of the sessile tubers formed (Table 5.5B). Tizio and Biaiñ (1973) showed that there was no antagonism between kinetin or 6-benzylaminopurine and NAA on tuber formation and that at similar concentrations, or in combination, tuber formation was accelerated to the same extent compared with the controls. The slight promotion of sessile tuber formation by kinetin may have been a reflection of the higher kinetin concentration used. Although smaller, tubers were also more numerous if the medium was supplemented with kinetin. While a high sucrose concentration *per se* may promote tuber formation of induced stem segments (*eg* Gregory, 1956; Harmey *et al*, 1966; Forsline and Langille, 1976), and in etiolated sprout segments (*eg* Lawrence and Barker, 1963; Mingo-Castel *et al*, 1976a; Peterson and Barker, 1979), this "tuberisation potential" was diminished if the cultures were initially maintained on very low sucrose media and then transferred onto high sucrose media (Lawrence and Barker, 1963). Palmer and Smith (1969a, 1970) reported that sucrose alone did not promote tuber formation in sub-cultured "stolons" and suggested that the requirement for kinetin was absolute. Mauk and Langille (1978) have shown a similar promotion of tuber formation by sub-cultured stolons using zeatin riboside.

It was observed in experiment 2 that the shoots transferred onto medium supplemented with NAA and 6% sucrose tuberised whereas those on media with the additional supplement of kinetin did not. A larger number of second transfer cultures was set up in experiment 3, and although many of the cultures did not form tubers there was a noticeably greater proportion of the *S. tuberosum* cultures which formed tubers on media without

kinetin than on media supplied with kinetin (Table 5.6). The number of tuberising *S. andigena* cultures was less than observed for *S. tuberosum* but again it was apparent that tubers formed more readily on media with NAA only (Table 5.6).

From the above preliminary results using sub-cultured shoots it would appear that the statement by Palmer and Smith (1969a, 1970) warrants reinvestigation. Indeed, Stalknecht (1972) had earlier indicated that coumarin could induce tuber formation in sub-cultured shoots slightly faster than kinetin. Moreover, Mingo-Castel *et al* (1976a) showed, that while CO₂ was necessary for tuber formation in sprouts cultured *in vitro*, CO₂ actually stimulated tuber formation of sub-cultured stolons growing on medium not supplemented with kinetin (Mingo-Castel *et al*, 1974). Tizio and Biaiñ (1973) further showed that tuber formation of cultured sprouts stimulated by NAA was associated with an increase in endogenous cytokinin-like activity but that tuber formation stimulated by CCC occurred without a concomitant increase in cytokinin-like activity.

In 1979, Stalknecht and Farnsworth published a paper showing that tuberisation of sub-cultured shoots which had been obtained from cultured etiolated sprouts, could be enhanced or inhibited by manipulating the carbon/nitrogen balance provided a tuberisation stimulus, such as coumarin, was present. The authors further suggested that a high nitrogen medium such as the Murashige and Skoog medium, inhibited tuberisation of sub-cultured shoots induced by IAA, 2,4-D or NAA, but that kinetin was very effective at stimulating tuberisation of sub-cultured shoots grown on the high nitrogen Murashige and Skoog medium, precisely the medium used by Palmer and Smith (1969a,b, 1970).

It is possible then, that the low level of tuberisation detected in the sub-cultured shoots in experiment 3 was the result of a negative interaction between NAA and kinetin (Table 5.6) which is in contrast to the lack of antagonism observed by Tizio and Biaiñ (1973) using sprout segments.

Tizio and Biaiñ's (1973 and references cited therein) suggestion that a substance formed in actively growing roots may inhibit tuber formation was not confirmed in these experiments. Paupardin and Tizio (1970) had earlier presented work which showed that a range of phenolic compounds promoted tuberisation *in vitro* despite the obvious production of roots. Mingo-Castel *et al* (1974) found no correlation between rooting and tuberisation in experiments in which interactions among CO₂, ethylene and kinetin on tuber formation were being observed. Moreover, root formation was inhibited by ethylene (Mingo-Castel *et al*, 1976a) and by

ABA (Claver, 1970), as also was tuber formation.

On the basis of the preliminary investigation reported here and the reports in the literature, it is apparent that much of the published work on *in vitro* tuberisation needs to be reinvestigated and consideration given to the interaction of the plant growth regulators and the nitrogen concentration of the medium.

Tubers cultured *in vitro* have often been used as model systems for tuberisation *in vivo*. Peterson and Barker (1979) cultured induced stolons *in vitro* to study the cytological events occurring at tuber initiation. Tubers cultured *in vitro* do not seem to exceed about 10 mm in diameter (eg Mes and Menge, 1954; Chapman, 1958; Peterson and Barker, 1979). Peterson and Barker (1979) reported that there was a noticeable lack of cell division in tubers 6 mm in diameter cultured on medium with 12% sucrose but devoid of cytokinin. Six mm was the largest diameter attained under the above culture conditions. This investigation is worth repeating in view of the limited tuber growth *in vitro* since cell division occurs very soon after tuber formation in field grown potatoes (eg Cutter, 1978).

Tuber initiation *in vivo* has been correlated with high soluble sugar levels in the stolon tips and this is ascribed a causative role by several workers (eg Borah and Milthorpe, 1963; Burt, 1964; Slater, 1968). In *in vitro* cultures, the reducing sugar levels were reported to be higher in kinetin-treated cultures than in control cultures (Palmer and Barker, 1973). Coincident with the stage of rapid starch accumulation the levels of reducing sugar decreased slowly in kinetin-treated cultures. However, reducing sugar levels in CEPA-treated cultures were similarly high but remained high, as did the controls, with no tuber formation occurring. The level of sucrose increased in kinetin-treated cultures in contrast to the somewhat constant levels of those treated with CEPA, although levels in the controls fluctuated. High sugar levels, therefore, do not appear to correlate closely with the onset of tuber formation *in vitro*.

Stolons sub-cultured on kinetin containing media began to accumulate starch before the first detectable signs of tuber formation (Palmer and Smith, 1969a; Smith and Palmer, 1970; Palmer and Barker, 1973). Accumulation of starch prior to visible tuber formation has been reported in this thesis (Table 4.11) and by Lovell and Booth (1967) and is considered to be a reliable indicator of tuber initiation.

Palmer and Smith (1969b, 1970) and Smith and Palmer (1970) considered that tuber formation of sub-cultured stolons was at least

partially a consequence of the stimulating effect of kinetin on starch synthesizing enzymes. In addition, it has been suggested that the continued accumulation of starch in developing tubers is caused by the cytokinins stimulating enzymes involved in starch biosynthesis (Obata-Sasamoto and Suzuki, 1979a). The closest correlation between the cytokinin changes *in vivo* reported in this thesis and a specific enzyme involved in starch biosynthesis is with ADPG-pyrophosphorylase activity (see pp173) which increases (*in vivo*) following visible tuber formation (Hawker et al, 1979). *In vitro*, the activity of ADPG-pyrophosphorylase was reported to be very weak in the stolon tips at the time of sub-culturing but was also negligible after six days in culture regardless of the presence or absence of kinetin in the medium (Mingo-Castel *et al*, 1976b). After six days on medium containing cytokinin, the starch content was reported to have increased 4.5-fold (Mingo-Castel *et al*, 1976b, citing Palmer, 1969) and kinetin was also reported to be accumulating prior to visible signs of tuber formation whereas an increase in ADPG-pyrophosphorylase activity was detected at a later stage of tuber development *in vitro* (Mingo-Castel *et al*, 1976b). Obata-Sasamoto and Suzuki (1979b) detected little activity *in vitro*.

The accumulation of a cytokinin at the tip of sub-cultured stolons did not appear to stimulate the activity of ADPG-pyrophosphorylase directly. In fact, Mingo-Castel *et al* (1976b) suggested that the stimulation of the enzyme by kinetin could be indirect, through an increase in the availability of sucrose (attracted by kinetin) at the locus of tuberisation *in vitro*. However, it should be considered that tuberisation of sub-cultured stolons can be induced by compounds other than cytokinins, notably coumarin (Stalknecht, 1972; Stalknecht and Farnsworth, 1979) and CO₂ (Mingo-Castel *et al*, 1974). It is, therefore, quite probable that the changes in enzyme activities noted during tuber development (*eg* Mingo-Castel *et al*, 1976b; Hawker *et al*, 1979; Obata-Sasamoto and Suzuki, 1979a,b) are an indirect effect of tuber initiation and a consequence of the stage of development of the tuber and are not affected directly by the cytokinin in the tissues.

CHAPTER VI

WHEAT

6.1 INTRODUCTION

Plants of *Triticum aestivum* L. cultivar Kopara were used for all analyses. Prior to fertilisation the entire pistil was extracted although all figures refer to "grains" instead of pistils and grains. The wheat "grain" is a caryopsis (achenes in which the pericarp and rudimentary testa are fused; Bewley and Black, 1978). The caryopsis, containing both endosperm and embryo, is derived from the fertilised ovule and it is the total caryopsis (grain) which is extracted following fertilisation.

6.2 MATERIALS AND METHODS

6.2.1 Wheat Field Trial 1

6.2.1.1 Wheat (cv Kopara) was planted on 16/6/77 at a density of 500 seeds m^{-2} and grown under prevailing field conditions at Lincoln, mid-Canterbury, New Zealand. Two parallel plots each 50 m x 1.65 m were assigned to the experiment. Each plot was drilled with 10 rows of seeds. The two 50 m long plots were subdivided into 10 m blocks making a total of 10 blocks, each with ten, 10 m long rows. The 100 rows were randomly assigned a number from 1 to 100 inclusive. At any one harvest three independent samples were collected, rows being assigned in increasing numerical order to sample one, two and three sequentially during the harvest period, omitting rows on edges of plots. The dates of harvests and the developmental stages selected are listed in Table 6.1. Harvesting commenced at "ear emergence". At this stage of development the central portion of the ear was visible but the tip was still enclosed by the leaf sheath. The ears selected at Harvest 2 were considered to be at pollination. Field anthesis was beginning at this stage but the anthers were not exposed in those ears selected. In addition, only grains

from florets within which the pollen was visible on the enclosed anthers were selected for extraction. The ears selected at Harvest 3 were considered to be at peak anthesis: pollen-bearing anthers were exposed along two-thirds of the ear. At Harvest 4 spikes were selected which had anthers loosely attached along the length of the spike but these were no longer bearing pollen. By the fifth harvest no anthers remained attached and selection of ears during this and the remaining harvests was based on an objective estimate of size.

TABLE 6.1

Wheat Field Trial 1. Harvest schedule listing the dates, developmental stage relative to ear emergence and anthesis, the number of ears dissected in the field per sample, and the number dissected in the laboratory as a "bulk sample" at each respective harvest

Harvest number and date	Developmental Stage ^a		No of spikes dissected in the field per sample	"Bulk sample"	
	Days after ear emergence	Days after anthesis			
1 17/11/77	ear emergence	-	-	200	-
2 27/11/77	pollination	10	-	200	-
3 28/11/77	anthesis	11	-	200	1,000
4 1/12/77		14	3	200	1,000
5 5/12/77		18	7	200	1,000
6 9/12/77		22	11	200	1,000
7 14/12/77		27	16	200	-
8 21/12/77		34	23	200	-
9 28/12/77		41	30	200	-
10 4/1/78		48	37	200	-
11 11/1/78		55	44	30	-
12 18/1/78		62	51	200	-

^a Refer to text for selection criteria

Harvesting involved selection of primary and secondary tillers from the assigned rows and excision of the stem beneath the flag leaf node. Up to ten ears would be collected before the middle six spikelets were excised and immediately plunged into liquid N₂ or liquid air.

No more than five minutes elapsed between excision of the stem and flash freezing of the selected portion of the spike. At each harvest 100 spikes would be processed for sample one, followed by those for sample two and then three. This sequence was repeated and a total of 200 ears selected per replicate. An additional 1,000 ears were collected at the end of selected harvests (Table 6.1). In these cases the selected stems were cut near ground level and the plant material placed in a "Chilly Bin". Liquid N₂ (or air) was then poured onto the material to freeze it.

Harvesting in the field began by 9.30 am and was normally complete by mid-day - the precise time varied depending on the number of ears collected and the number of field assistants available to help dissect the material in the field.

A selection of ears was also collected just prior to the return to the laboratory. In the laboratory these ears were immediately dissected and primary and secondary grains from the central six spikelets weighed to provide fresh weight data.

The spikelets immersed in liquid N₂ (or air) were transferred to plastic bags and immediately stored at -20°C prior to freeze drying. The central six spikelets of the additional 1,000 whole ears were dissected in the laboratory and immediately placed in liquid N₂ (or air). Following dissection all spikelets were transferred into plastic bags and stored at -20°C prior to freeze drying. The spikelets remained frozen throughout the time required for dissection. Following freeze drying storage of all material was in a cold room at 4°C. Just prior to extraction the primary and secondary grains from each of the six central spikelets were removed. Any obviously larger or smaller grains were discarded as were the grains from the remaining florets. A total of 500 grains from each of the three samples collected at each harvest were weighed to provide dry weight data, before being ground to a fine powder using a mortar and pestle (Table 6.2).

6.2.1.2 Analysis of tissue for cytokinin content The ground tissue was suspended in 80% methanol (-20°C) and extracted by stirring for 24 hours in darkness at 4°C (Table 6.2). The extract was then filtered under reduced pressure through one layer of Whatman 3 MM filter paper and the residue washed with measured amounts of 80% methanol and water before being resuspended in 80% methanol and extracted as described above for a further 24 hours. The extract was again filtered through Whatman 3 MM filter paper, the residue washed (Table 6.2) and the filtrates and washes subsequently pooled.

TABLE 6.2

Wheat Field Trial 1. Extraction sequence detailing the number of grains extracted, the dry weight of sample extracted, the volume of 80% methanol used for extraction of individual samples over each 24 hour period, and the amount of solvent used to wash the retained residue following each filtration (refer also to text)

Harvest Number (Developmental Stage)	Sample	Number of Grains Extracted	DW (g) Extracted	Extracting Solvent (ml) (1st 24h)	First Residue Wash (ml) ^a	Extracting Solvent (ml) (2nd 24h)	Second Residue Wash (ml) ^a
2 (pollination)	A	500	0.272				
	B	500	0.415	40	10	40	15
	C	500	0.300				
3 (anthesis)	A	500	0.791				
	B	500	0.882	50	10	50	15
	C	500	0.871				
4 (3d post-anthesis)	A	500	1.524				
	B	500	1.540	50	10	50	15
	C	500	1.529				
5 (7d post-anthesis)	A	500	3.910				
	B	500	-	75	15	75	30
	C	500	3.759				

^a Each residue was washed with the stated volume of 80% methanol and this was followed immediately by an equal volume of water.

The solution was evaporated to dryness at 35°C under reduced pressure using a rotary film evaporator, the residue redissolved in methanol/NH₄OH (2N) (70:30 v/v) and evaporated to dryness on a hot plate at 37°C under a stream of air. The extract was then redissolved in 1 ml 35% ethanol and filter centrifuged through a Sinta glass vial of porosity No. 3. The residue was washed with a further 0.5 ml 35% ethanol and the combined filtrates applied to a column of Sephadex LH-20 (section 3.2.3.1).

A further two samples, each of 500 grains, from Harvest 3 (anthesis) (with dry weights of 0.928 g and 0.800 g respectively) were extracted as described previously. The extract was reduced to the aqueous phase by evaporation at 35°C under reduced pressure and the total volume increased to 50 ml by the addition of water. The pH of the extract was adjusted to 7 using 0.05 M NaOH and the extract partitioned against four equal volumes of water-saturated butan-1-ol

over 24 hours. Both aqueous and butan-1-ol phases were evaporated to dryness at 35°C under reduced pressure and the residues redissolved in methanol/NH₄OH (2N) (30:70 v/v). The solvent was evaporated at 35°C under reduced pressure using a test tube evaporator until the volumes of extract were reduced to convenient amounts for strip-loading onto paper chromatograms. Aqueous- and butanol-soluble components were strip-loaded onto 3 MM chromatography paper in 70 mm wide bands. Loaded chromatograms, and three control chromatograms, were developed for 350 mm by descending chromatography in solvent 1 (section 3.2.2).

6.2.2 Wheat Field Trial 2

6.2.2.1 Wheat (cv Kopara) was sown in mid-June, 1978 as described for Wheat Field Trial 1. Six 50 m x 1.65 m plots were allocated to the experiment. Each plot was subdivided into three 10 m lengths, leaving 10 m buffer zones at each end of the plot. The 180 rows were randomly assigned a number from one to 180 inclusive and at each harvest three independent samples were collected as described for Wheat Field Trial 1. Rows on the edges of plots were again excluded. The dates of harvests, the developmental stages selected and the number of spikes dissected per sample are listed in Table 6.3.

The same criteria were applied to selecting wheat spikes at emergence, pollination and anthesis as were applied during the first field harvest. Spikes selected two days post-anthesis showed pollen-bearing anthers protruding from the most apical and basal florets while those protruding from the central spiklets had shed their pollen. The spikes collected four days following anthesis were selected on the basis of the attached anthers having shed their pollen and remaining only loosely attached to the florets.

By Harvest 6 (seven days after anthesis), no anthers remained attached to the spike and selection of ears during this and the remaining harvests was based on an objective estimate of size.

Treatment of excised ears was as described for Field Trial 1 except storage following freeze drying was at room temperature.

6.2.2.2 Analysis of tissue for cytokinin content

6.2.2.2A Solvent extraction using 80% methanol (v/v) Freeze dried tissue was ground and extracted as described for Field Trial 1; the amounts of solvent used during extraction and washing of residues are listed in Table 6.4. Each methanolic extract was evaporated to dryness at 35°C under reduced pressure, redissolved in a small volume of methanol/NH₄OH

(2N) (70:30 v/v). The extract was evaporated to dryness at 37°C under a stream of air. Extracts from all harvests except 8 and 11 were resuspended in 2 ml 35% ethanol and centrifuged for 30 minutes at 3000 g. The pellet was resuspended in 1.0 ml of 35% ethanol and again centrifuged. This process was repeated. The extracts of material from Harvests 8 and 11 were suspended in 3 ml 35% ethanol prior to centrifuging and the pellets resuspended in 2 mls of 35% ethanol. The combined supernatants were evaporated to dryness on a hot plate at 37°C under a stream of air, resuspended in 1.5 ml (or 2.0 ml, Harvests 8 and 11) of 35% ethanol and applied to a column of Sephadex LH-20 (section 3.2.3.1).

TABLE 6.3

Wheat Field Trial 2. Harvest schedule listing the date, developmental stage relative to ear emergence and anthesis, the number of ears dissected in the field per sample, and the number dissected in the laboratory as a "bulk sample" at each respective harvest

Harvest number and date	Developmental Stage ^α		No of spikes dissected in the field per sample	"Bulk sample"
	Days after ear emergence	Days after anthesis		
1 14/11/78	Ear emergence	-	200	-
2 23/11/78	Pollination	9	200	-
3 24/11/78	Anthesis	10	80	-
4 26/11/78		12	200	300
5 28/11/78		14	200	-
6 1/12/78		17	200	-
7 5/12/78		21	200	-
8 12/12/78		28	200	-
9 19/12/78		35	200	-
10 27/12/78		43	200	-
11 5/1/79		52	200	-
12 19/1/79		66	200	-

^α Refer to text for selection criteria

TABLE 6.4

Wheat Field Trial 2. Extraction sequence detailing the number of grains extracted, the dry weight of sample extracted, the volume of 80% methanol used for extraction of individual samples over each 24 hour period, and the amount of solvent used to wash the retained residue following each filtration (refer also to text)

Harvest Number (Develop- mental Stage)	Sample	Number of Grains Extracted	DW (g) Extract- ed	Extract- ing Solvent (ml) (1st 24h)	First Residue Wash (ml) ^a	Extract- ing Solvent (ml) (2nd 24h)	Second Residue Wash (ml) ^a
1 (ear emergence)	A	500	0.095				
	B	500	0.091	20	5	15	10
	C	500	0.091				
2 (pollin- ation)	A	500	0.371				
	B	500	0.342	25	5	20	10
	C	500	0.356				
3 (anthesis)	A	500	0.529				
	B	500	0.573	25	5	20	10
	C	500	0.566				
4 (2d post- anthesis)	A	500	0.960				
	B	500	0.976	50	10	40	15
	C	500	1.000				
	A	100	0.191				
	B	100	0.180	25	5	20	10
	C	100	0.196				
4 ("Bulk Sample")		1500	2.863	150	30	120	50
5 (4d post- anthesis)	A	500	1.840				
	B	500	1.784	100	15	80	25
	C	500	1.942				
	A	100	0.400				
	B	100	0.402	25	5	20	10
	C	100	0.377				
6 (7d post- anthesis)	A	500	3.128				
	B	500	3.112	150	20	120	30
	C	500	3.239				

Continued ...

TABLE 6.4 (continued)

Harvest Number (Developmental Stage)	Sample	Number of Grains Extracted	DW (g) Extracted	Extracting Solvent (ml) (1st 24h)	First Residue Wash (ml) ^α	Extracting Solvent (ml) (2nd 24h)	Second Residue Wash (ml) ^α
8 (18d post-anthesis)	A	500	8.243				
	B	500	8.262	500	100	400	150
	C	500	8.135				
11 (42d post-anthesis)	A	250	10.760	450	60	360	100

^α Each residue was washed with the stated volume of 80% methanol and this was followed immediately by an equal volume of water.

6.2.2.2B Extraction of 1500 grains from the "bulk sample" collected two days after anthesis was as described in section 6.2.2.2A (Table 6.4). Following extraction, however, the pooled filtrates and washes were divided into three equal volumes which were subsequently evaporated and treated as described previously.

6.2.2.2C Solvent extraction using methanol-chloroform-formic acid (7N) (12:5:3) (Bielecki, 1964). A total of 300 freeze-dried grains (550 g DW; 2.136 g eq FW) from the bulk sample collected two days post-anthesis (Table 6.3) were placed at -20°C for 60 minutes. Bielecki's extraction procedure as described by Horgan (1978) was then followed. The cold grains were placed in 20 ml methanol-chloroform-formic acid (7N) (12:5:3) and left for 24 hours at -20°C. The tissue was subsequently homogenised in the solvent and centrifuged at 15,000 g for 15 minutes at -10°C. The pellet was suspended in 10 ml methanol-formic acid (7N)-H₂O (60:40:1) for one hour at -20°C and then centrifuged. The combined supernatants were evaporated to dryness at 35°C under reduced pressure. The residue was redissolved in methanol/NH₄OH (2N) (70:30 v/v) and divided into three equal volumes. Each was evaporated to dryness on a hot plate at 37°C under a stream of air, and subsequently treated as described in section 6.2.2.2 prior to column chromatography.

A further 300 grains from the same bulk sample (0.520 g DW) were

ground and suspended in 30 ml 80% methanol. The extraction procedure described in section 6.2.2.2A was followed: 10 ml 80% methanol and 10 ml water were used to wash the residue after the first 24 hours and the residue was then resuspended in 20 ml 80% methanol. Following filtration the residue was washed in 15 ml 80% methanol and 15 ml water. After evaporation to dryness the residue was dissolved in methanol/ NH_4OH (2N) (70:30 v/v), divided into three equal volumes and each portion evaporated to dryness on a hot plate at 37°C under a stream of air. The extracts were redissolved and subsequently treated as described in section 6.2.2.2A prior to column chromatography.

6.2.2.2D β -glucosidase treatment Duplicate sets of 100 grains from each of the three samples collected two days post-anthesis were extracted as described in section 6.2.2.2A and Table 6.4. Each of the six samples was dissolved in 1.5 ml 20% ethanol and applied sequentially to a column of Sephadex LH-20 which was eluted with 20% ethanol (Smith and van Staden, 1978). Three of the extracts (samples 1a, 2a, 3a) were then bioassayed. Fractions 3 to 9 from the remaining samples (1b, 2b, 3b) were pooled, evaporated to dryness on a hot plate at 37°C under a stream of air and subjected to β -glucosidase treatment by a modification of the technique introduced by Yoshida and Oritani (1972).

Almond β -glucosidase (Sigma Chemical Co) was solubilised in 0.05 M sodium acetate buffer (pH 5.2) at a concentration of $0.6 \text{ mg enzyme ml}^{-1}$. The selected portions of extract were also dissolved in the buffer and the enzyme solution added to give a final concentration of enzyme of 0.3 mg ml^{-1} of extract (van Staden, 1976d). The solutions were incubated at 25°C in the dark for 22 hours and stirred occasionally. The reaction was stopped by the addition of 10 ml 80% ethanol (Smith and van Staden, 1978). The solution was then evaporated to dryness on a hot plate at 37°C under a stream of air and subsequently redissolved in 1.5 ml 20% ethanol prior to column chromatography.

Duplicate sets of 100 grains from each of the three samples collected four days after anthesis were extracted as described in section 6.2.2.2A and Table 6.4. Each of the six samples was dissolved in 1.5 ml 20% ethanol and resolved by upward flow elution on a column of Sephadex LH-20. As described above, three of the extracts were then bioassayed and selected fractions from the remaining extracts pooled and subjected to β -glucosidase treatment using twice the enzyme concentration (0.6 mg ml^{-1}) for these particular extracts.

Samples of 500 grains from Harvests 2 and 6 were similarly extracted, chromatographed using 20% ethanol as eluant and treated with

β -glucosidase at a concentration of 0.3 mg ml^{-1} .

6.2.2.2E Permanganate Oxidation A total of 100 freeze-dried grains from each of the three samples collected four days after anthesis (0.370 g, 0.386 g and 0.387 g DW respectively) was extracted as described in section 6.2.2.2A and Table 6.4. Following centrifugation each extract was evaporated to dryness on a hot plate at 37°C under a stream of air. The residue was treated with a 0.1% cold aqueous solution of potassium permanganate (KMnO_4) (Smith and van Staden, 1978) which was added until a pink colour persisted for at least 30 seconds (Sondheimer and Tzou, 1971). A total of 10 ml was added after which ethanol (95%) was added to the sample to complete the decomposition of the KMnO_4 (Tegley *et al.*, 1971).

The treated samples were subsequently filtered through Whatman Number 111 filter paper, the paper and residues washed with 80% and 20% ethanol and the combined filtrates evaporated to dryness on a hot plate at 37°C under a stream of air. The treated samples were redissolved in 1.5 ml of 20% ethanol and applied to a column of Sephadex LH-20 (section 3.2.3.1).

6.2.2.3 Soluble sugar and starch analyses The analyses were performed as outlined in section 4.2.4.1C and in Table 6.5.

6.3 RESULTS

6.3.1 Wheat Field Trial 1

A field plot of wheat was sampled during the 1977/78 growing season to provide pistils and grains at different stages of development. Ten days after ear emergence the dry weight per grain began to increase very rapidly (Fig 6.1). However, the fresh weight increased more slowly, showing a defined lag period followed by a rapid increase after anthesis (11 days after ear emergence). The maximum fresh weight was approximately 48 days after ear emergence.

Grains harvested 10, 11, 14 and 18 days after ear emergence (pollination, peak anthesis, three days and seven days after anthesis respectively) were extracted and analysed for cytokinin-like activity (Table 6.2). A series of kinetin standards was run with each bioassay. The cytokinin-like activity was estimated by reference to the standards and to the regression line fitted to the straight line portion of the standard curve (section 2.2.3.3).

Wheat grains harvested 10 days after ear emergence contained a sufficient amount of cytokinin-like activity to stimulate callus growth

TABLE 6.5

Wheat Field Trial 2. Starch and Soluble Sugar Analyses. Details of the analyses showing the dry weight, fresh weight and number of grains ground per sample, and the dry weight extracted per replicate. The volumes to which starch hydrolysates were diluted and the aliquots removed for incubation with glucose oxidase are also indicated

Harvest Number (Developmental Stage)	Amount of material ground per sample		DW extracted (mg)	Volume hydrolysate diluted to (ml)	Aliquot tested for starch (μ l)
	Number of grains	DW (g)			
1 (ear emergence)	520	0.134	41.2	10	250
			40.2	"	"
2 (pollination)	304	0.260	49.4	"	"
			51.3	"	"
			55.0	"	"
3 (anthesis)	293	0.350	54.4	100	"
			53.2	"	"
			53.2	"	"
4 (2d post- anthesis)	252	0.500	53.5	"	"
			55.5	"	"
			55.9	"	"
5 (4d post- anthesis)	136	0.500	56.0	"	"
			52.5	"	"
			53.6	"	"
6 (7d post- anthesis)	98	0.500	50.9	"	"
			52.5	"	"
			52.5	"	"
7 (11d post- anthesis)	71	0.500	55.0	"	"
			50.9	"	"
			52.0	"	"
8 (18d post- anthesis)	35	0.500	53.5	"	"
			52.5	"	"
			55.9	"	"
9 (25d post- anthesis)	19	0.500	50.9	"	"
			56.1	"	"
			50.3	"	"
10 (33d post- anthesis)	14	0.500	51.2	"	"
			51.6	"	"
			52.8	"	"
11 (42d post- anthesis)	20	1.000	55.7	"	"
			53.9	"	"
			53.0	"	"
12 (56d post- anthesis)	21	1.000	55.0	"	"
			52.3	"	"
			51.6	"	"

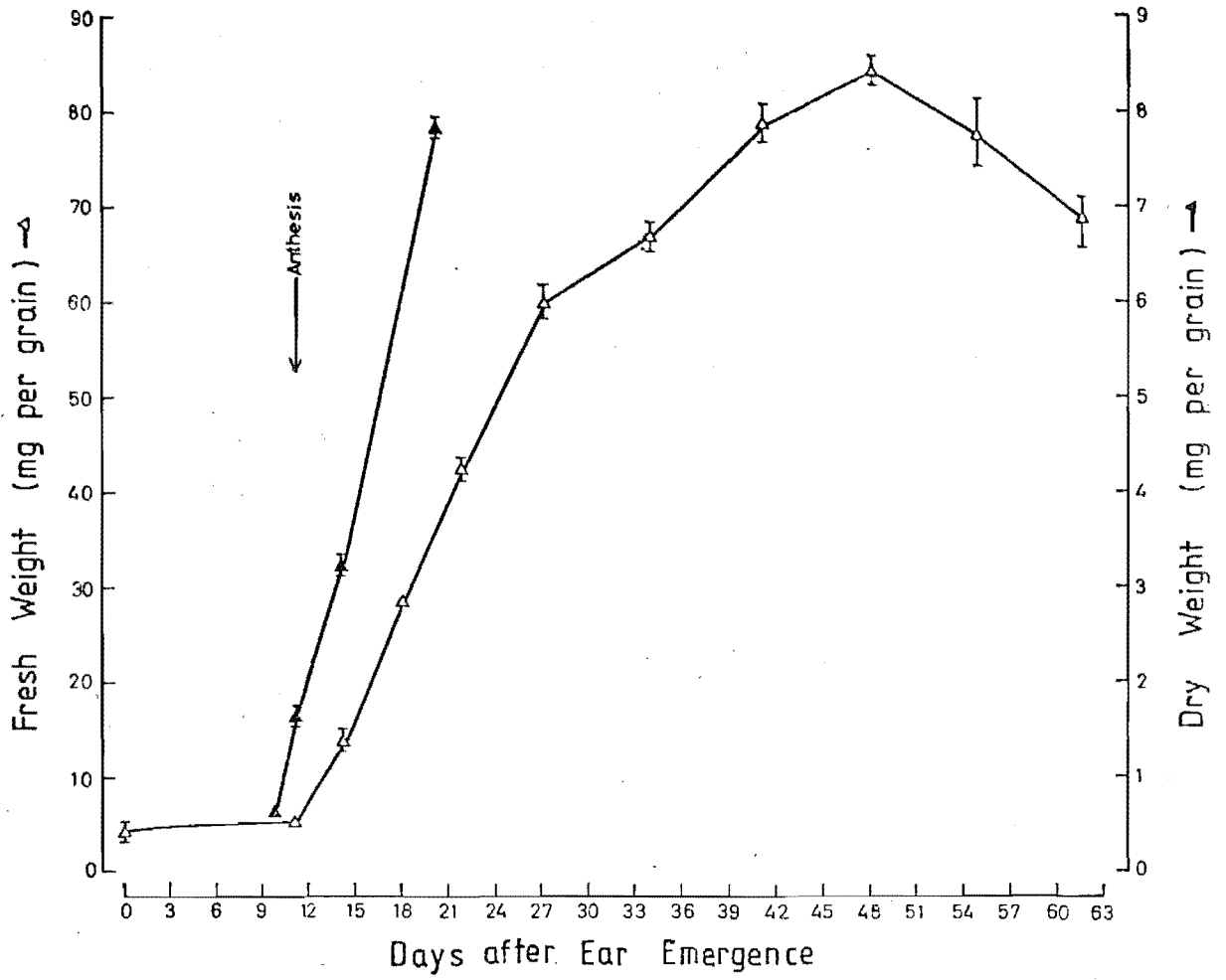


FIGURE 6.1

Wheat Field Trial 1. Fresh weight and dry weight of grains as a function of days from ear emergence

in the bioassay (Fig 6.2). Cytokinin-like activity was detected in fraction 7 in all three samples and in fraction 11 in two of the three samples. Most of the activity from the zeatin riboside standard was collected in fraction 9 and the majority of the zeatin standard was detected in fraction 11. The results are presented in Table 6.6.

TABLE 6.6

Wheat Field Trial 1. Quantification of cytokinin-like activity in 500 wheat pistils collected at pollination. Values in kinetin equivalents were obtained from the regression line $Y = 1.53X - 2.08$

Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)					Total
	5 - 8	9	<u>Fractions</u> 10	11 - 13	23	
A	27.87	ns ^a	25.69	52.01	ns	105.57
B	129.74	25.58	25.08	25.55	25.39	231.34
C	113.30	25.43	ns	72.71	ns	211.44

^a ns = not significant

The three samples harvested at anthesis all showed cytokinin-like activity which eluted from the column faster than expected for zeatin riboside (Fig 6.3). Sample C, however, showed substantially more cytokinin-like activity in fraction 7 than did samples A and B. The value lay above the straight line portion of the standard curve and was estimated from the non-linear region of the curve. Cytokinin-like activity was detected at the elution volumes corresponding to zeatin riboside and zeatin, respectively (Fig 6.3, Table 6.7).

Two extracts of wheat grains at anthesis were partitioned against butan-1-ol (pH 7) and resolved using paper chromatography (solvent 1). The results of the subsequent bioassay are presented in Figure 6.4. In both samples cytokinin-like activity was detected in the organic fraction at R_f 0 to 0.2 and R_f 0.4 to 0.5. In sample A, activity was also detected at R_f 0.5 to 0.6. Cytokinin-like activity was detected in the aqueous fraction of sample A at R_f 0 to 0.1. A straight line could not be drawn to link more than two consecutive points obtained from the kinetin standards and therefore estimates of the total cytokinin-like activity are not presented.

Fourteen days after ear emergence (3 days after peak anthesis) most of the apparent cytokinin-like activity was collected in fractions

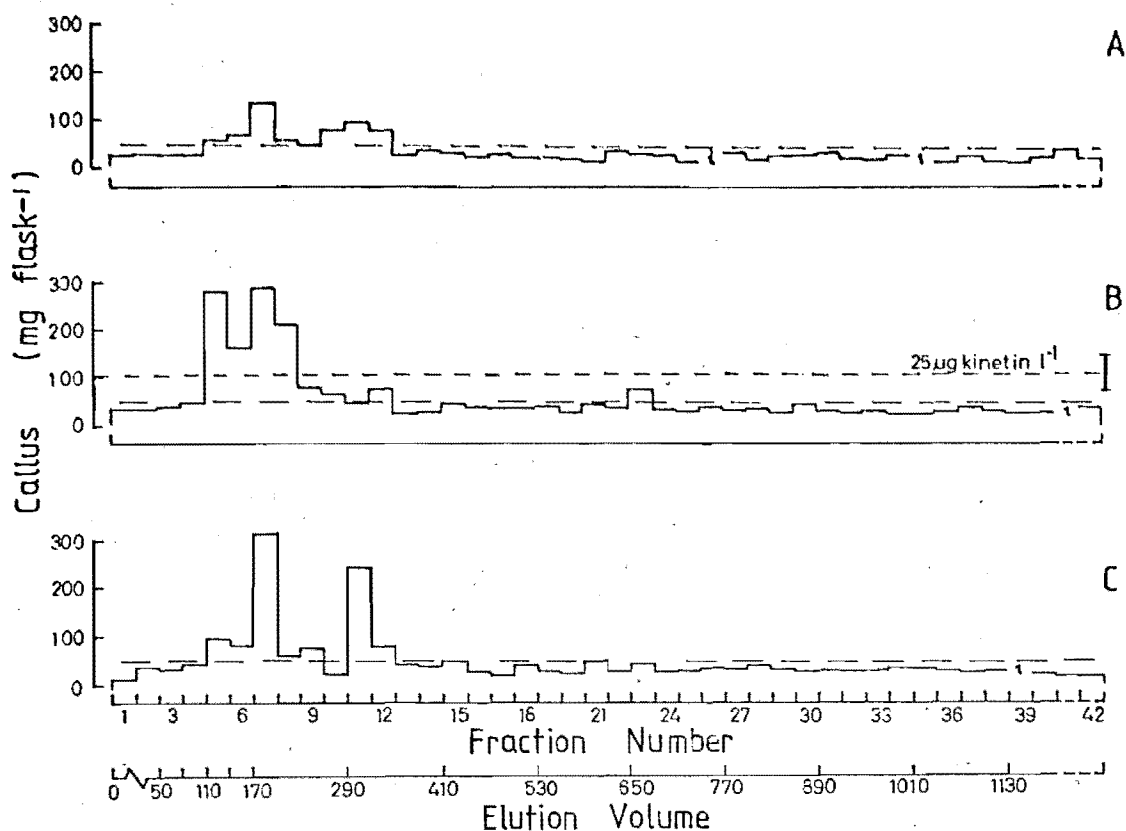


FIGURE 6.2

Wheat Field Trial 1. Soybean callus bioassay of extracts of 500 wheat pistils collected 10 days after ear emergence (pollination). The extracts were resolved by partition chromatography on Sephadex LH-20

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 41 (60 ml) and fraction 42 (50 ml)

Callus: Kit A (37)

Confidence limits: $n = 9$

99% limit — — —

Regression line: $Y = 1.53X - 2.08$

Lowest detectable level of kinetin: $10 \mu\text{g l}^{-1}$ ($42.3 \pm 5.9 \text{ mg callus flask}^{-1}$)

Kinetin standard: as indicated on Figure

FIGURE 6.3

Wheat Field Trial 1. Soybean callus bioassay of extracts of 500 grains collected 11 days after ear emergence (anthesis). The extracts were resolved by partition chromatography on Sephadex LH-20.

i: contaminated and discarded flask

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml)

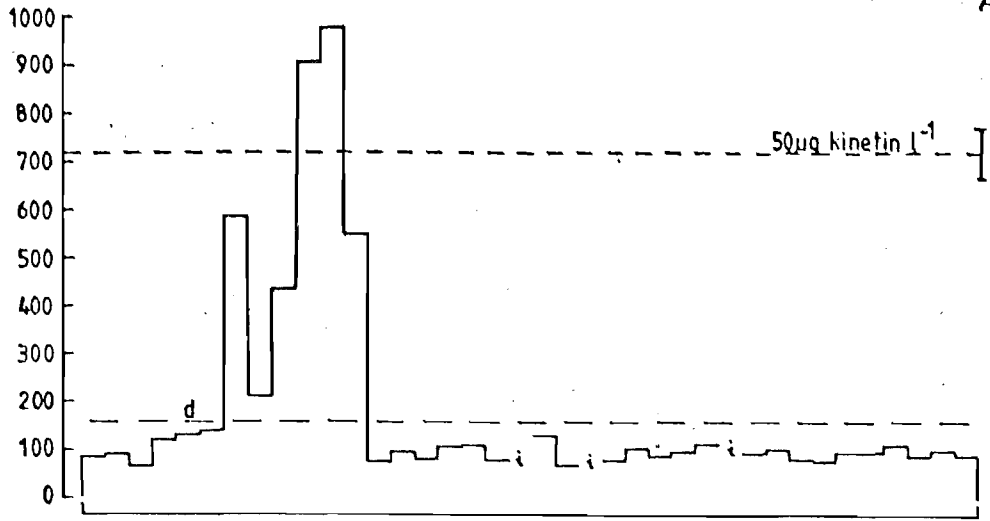
Callus: Kit A (54)

Confidence limits: $n = 8$

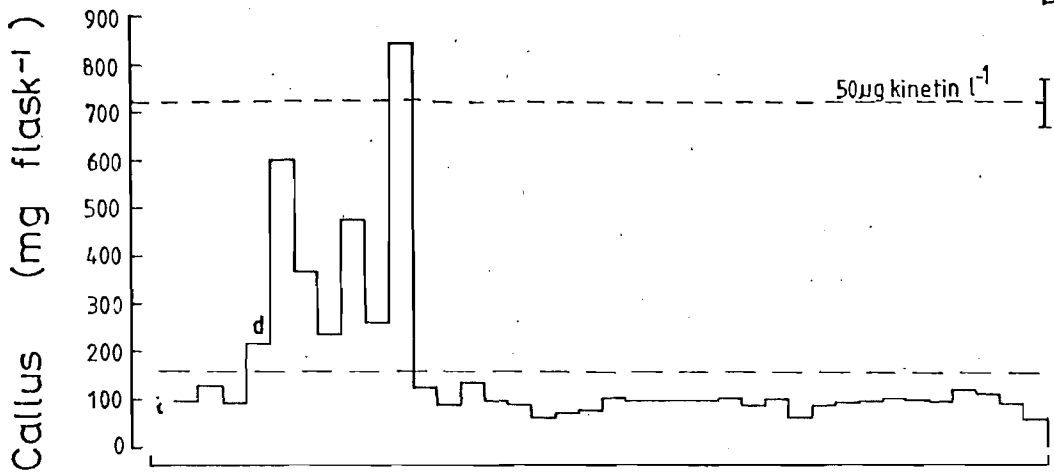
Regression line: $Y = 2.09X - 2.78$

Lowest detectable amount of kinetin: $25 \mu\text{g l}^{-1}$ ($157 \pm 16 \text{ mg callus flask}^{-1}$).

Further details are tabulated with Figure 6.2.



B



C

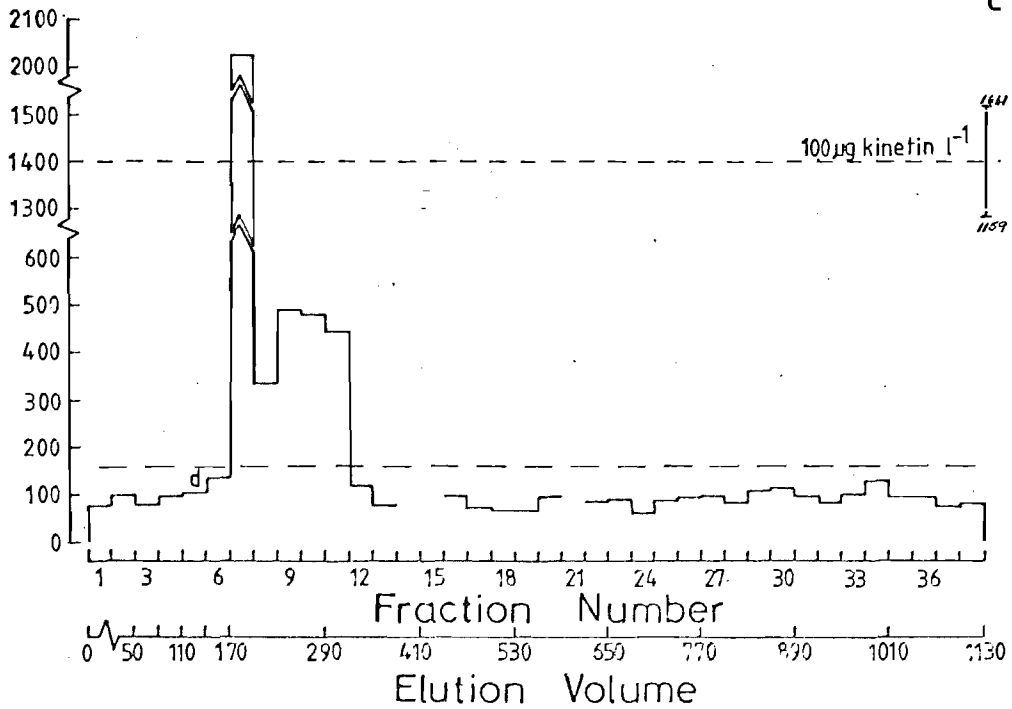


FIGURE 6.3

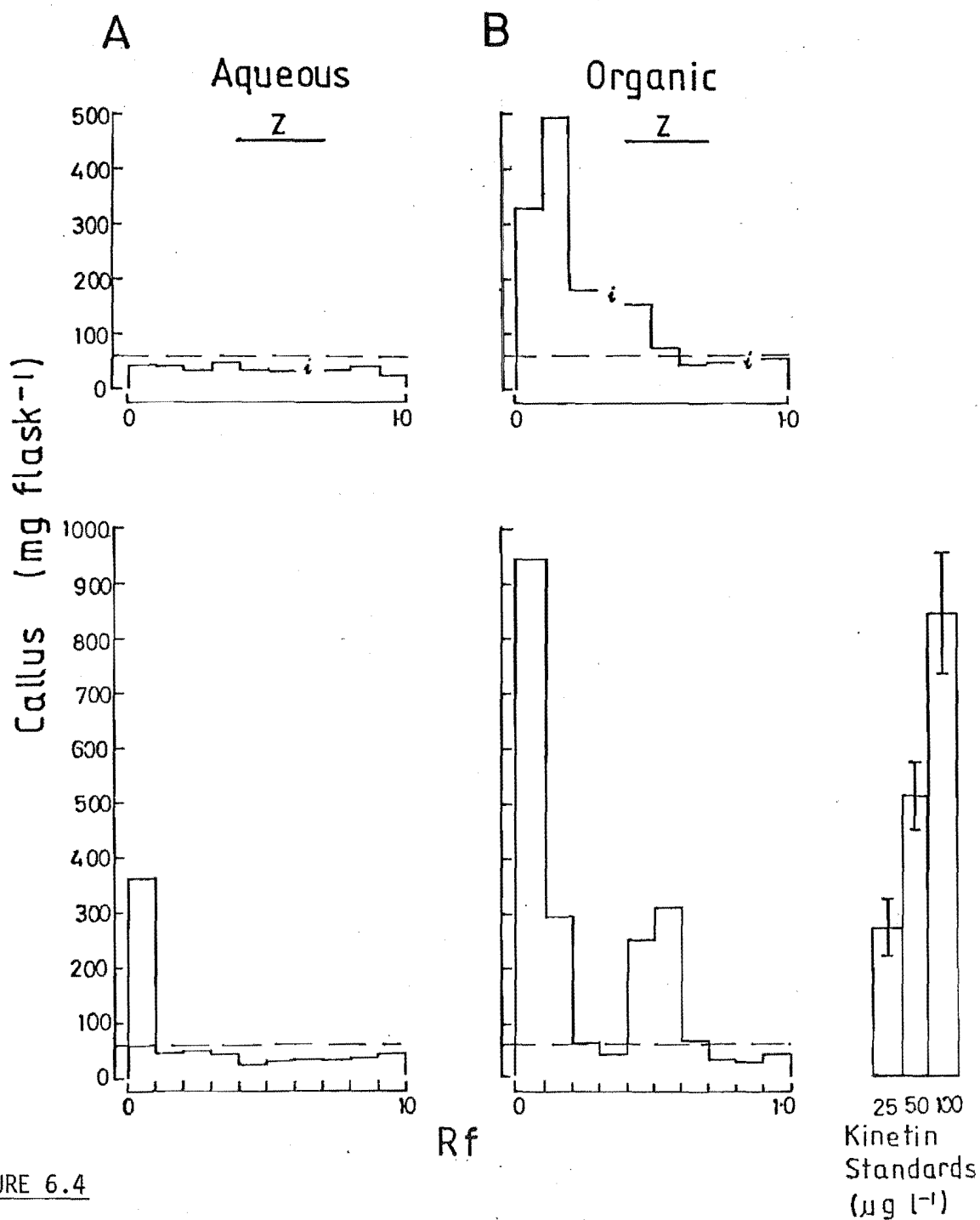


FIGURE 6.4

Wheat Field Trial 1. Soybean callus bioassay of paper chromatograms loaded with extract of 500 grains collected 11 days after ear emergence (anthesis).

Solvent: propan-2-ol-ammonia-water (10:1:1 v/v)

Callus: Kit A (38)

Confidence limits: $n = 30$

Lowest detectable amount of kinetin: $10 \mu\text{g l}^{-1}$ ($63 \pm 10 \text{ mg callus flask}^{-1}$).

Further details are tabulated with Figure 6.2.

10 and 11 (Fig 6.5). A portion of the total activity eluted from the column earlier than authentic zeatin riboside. In sample B little activity was detected eluting in the same volume as the expected peak elution volume for zeatin riboside (fraction 9). The bioassay of fractions 9 from samples A and B respectively were affected by fungal contamination. In all samples the callus growth stimulated by compounds in fraction 10 lay above the upper limit of the regression line. The level of activity was estimated for these values from a fitted curve (Table 6.8).

TABLE 6.7

Wheat Field Trial 1. Quantification of cytokinin-like activity in 500 wheat grains collected at anthesis. Values in kinetin equivalents were obtained from the regression line $Y = 2.09X - 2.78$.

Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)				Total
	5 - 8	9	Fractions 10	11 - 12	
A	96.67	34.48	58.16	101.95	287.26
B	128.79	36.19	28.62	54.43	248.03
C	ca 325.86 ^a	36.80	36.35	34.89	ca 433.90

^a Value estimated from non-linear portion of the standard curve

TABLE 6.8

Wheat Field Trial 1. Quantification of cytokinin-like activity in 500 wheat grains collected three days post-anthesis. Values in kinetin equivalents were obtained from the regression line $Y = 1.01X - 1.29$

Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)				Total
	5 - 8	9	Fractions 10	11 - 12	
A	37.23	inf ^a	ca 297.2 ^b	28.41	ca 362.8
B	50.62	ns	ca 132.8 ^b	ca 134.9 ^b	ca 318.3
C	37.23	inf	ca 912.0 ^b	89.43	ca 1028.7

^a inf: contaminated culture discarded

^b value estimated from non-linear portion of the standard curve

FIGURE 6.5

Wheat Field Trial 1. Soybean callus bioassay of extracts of 500 wheat grains collected 14 days after ear emergence (3 days post-anthesis). Extracts were resolved by partition chromatography on Sephadex LH-20.

Solvent: 35% ethanol.

Fraction volume: 30 ml with the exception of fraction 1 (20 ml) and fraction 41 (60 ml).

Callus: Kit A (27)

Confidence limits: $n = 10$

Regression line: $Y = 1.01X - 1.29$

Lowest detectable amount of kinetin: $5 \mu\text{g l}^{-1}$ ($16.8 \pm 1.9 \text{ mg callus flask}^{-1}$).

Further details are tabulated with Figure 6.2.

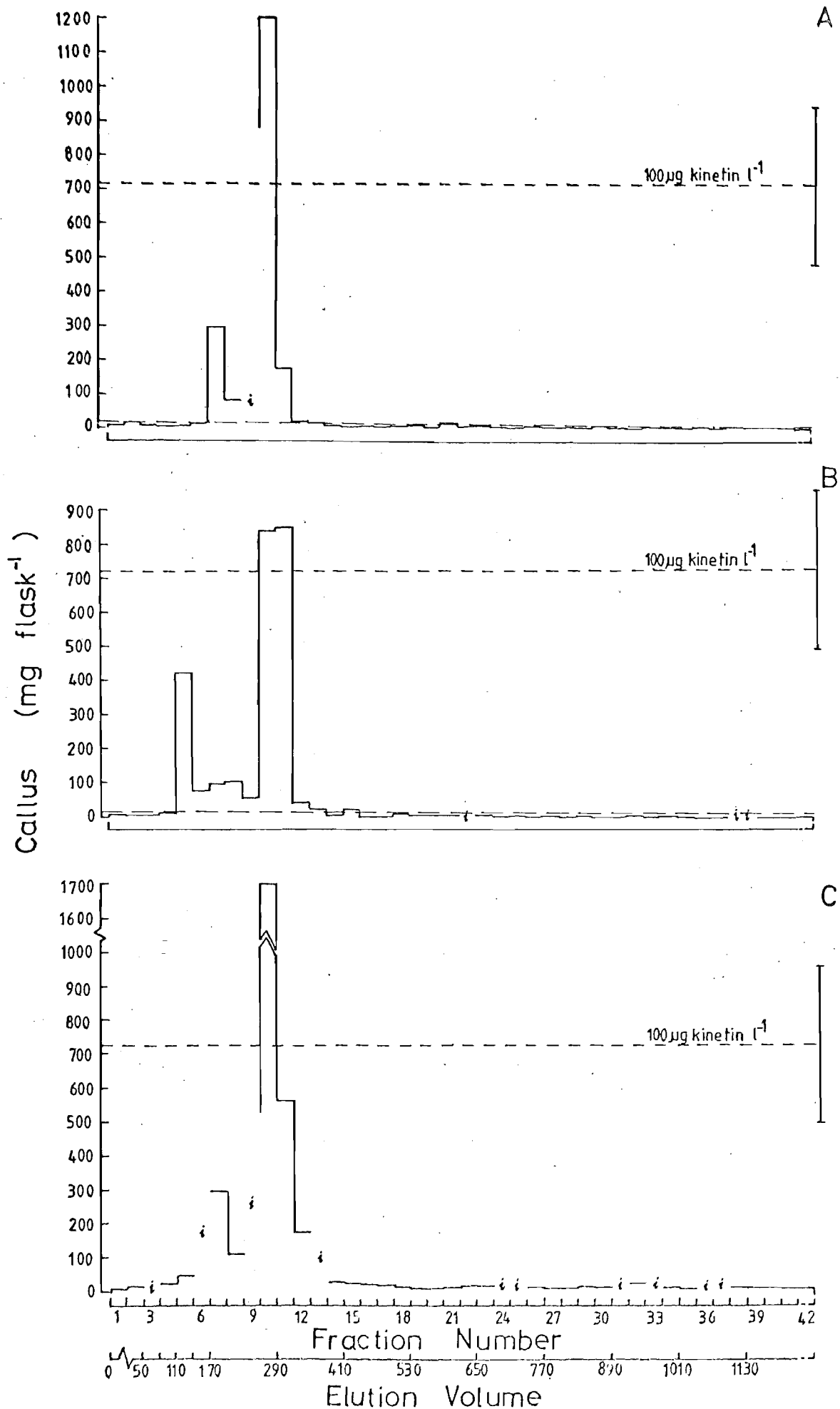


FIGURE 6.5

The three samples of grain extracted seven days after anthesis contained detectable cytokinin-like activity (Fig 6.6). Cytokinin-like activity was estimated in kinetin equivalents from the regression line for samples B and C and calculated with reference to the standards for sample A. The majority of activity detected was in fractions 8 and 10 (Table 6.9).

TABLE 6.9

Wheat Field Trial 1. Quantification of cytokinin-like activity in 500 wheat grains collected seven days post-anthesis. Values in kinetin equivalents were obtained from the regression line $Y = 1.30X - 1.64$.

Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)				Total
	5 - 8	9	Fractions 10	11 - 12	
A	ca 10^{α}	ca 10^{α}	ns	ns	ca 20
B	30.80	ns	26.65	25.31	82.76
C	57.64	ns	24.74	ns	82.38

α Value estimated by direct reference to the cytokinin standards.

The change in the total cytokinin-like activity in the grain from pollination to seven days post-anthesis is presented in Figure 6.7. A rapid increase in the cytokinin-like activity per grain occurred immediately after pollination. This increase appeared due mainly to an increase in the level of cytokinin-like activity collected in fractions 10, 11 and 12 (Tables 6.6, 6.7, 6.8). Within seven days of peak anthesis the cytokinin-like activity had decreased to a low level. On a per unit dry weight basis the activity decreased slightly between pollination and anthesis, changed little over the three days following anthesis, but decreased to a low level by seven days after anthesis (Fig 6.7).

6.3.2 Wheat Field Trial 2

A field plot of wheat was sampled during the 1978/79 growing season at more frequent intervals than during the first field trial.

The fresh weight of grains began to increase rapidly nine days after ear emergence which was assessed to be pollination and had reached a maximum 51 days from ear emergence (Fig 6.8). The relative rate of the increase was high until 17 days after ear emergence after which it decreased. The growth, as determined by dry weight increase, was slow

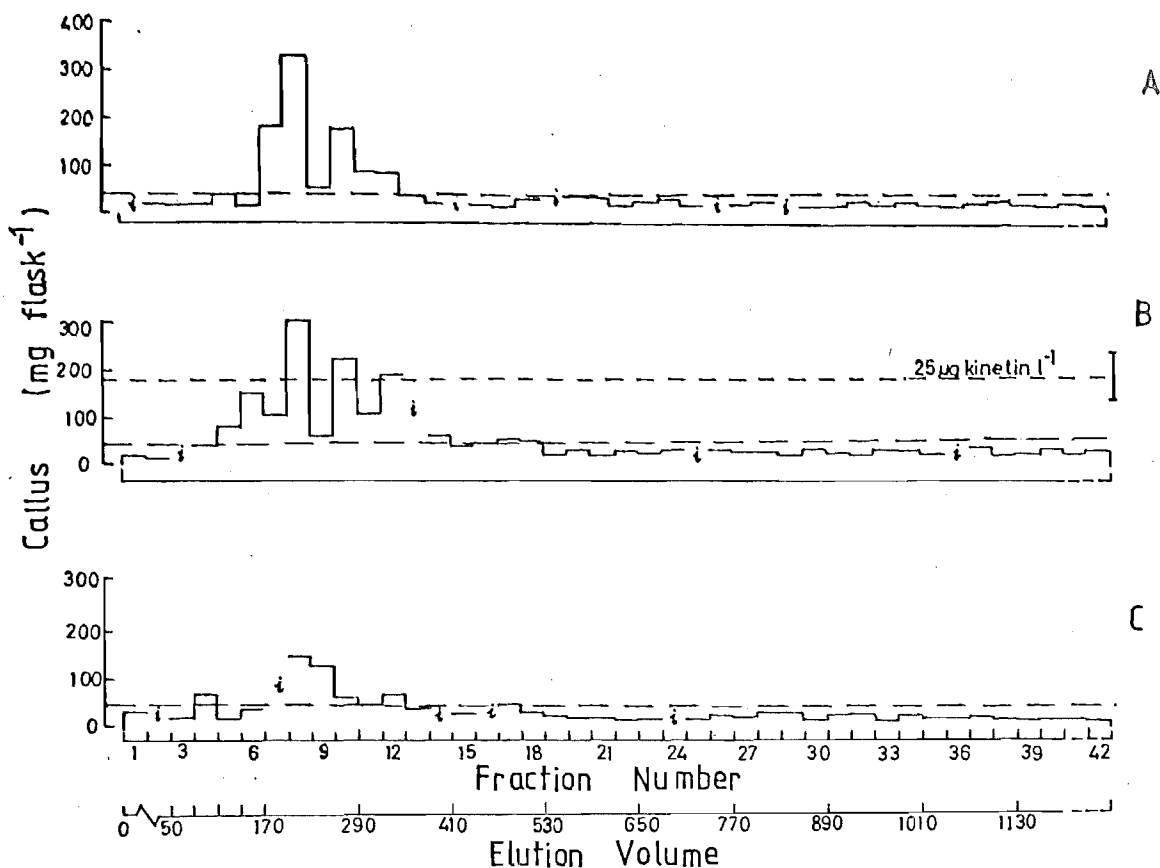


FIGURE 6.6

Wheat Field Trial 1. Soybean callus bioassay of extracts of 500 wheat grains collected 18 days after ear emergence (7 days post-anthesis). Extracts were resolved by partition chromatography on sephadex LH-20.

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml), fraction 41 (60 ml) and fraction 42 (50 ml).

Callus: Kit A (35)

Confidence limits: $n = 8$.

Regression line: $Y = 1.30X - 1.64$

Lowest detectable amount of kinetin: $10 \mu\text{g l}^{-1}$ (35 mg callus flasks⁻¹).

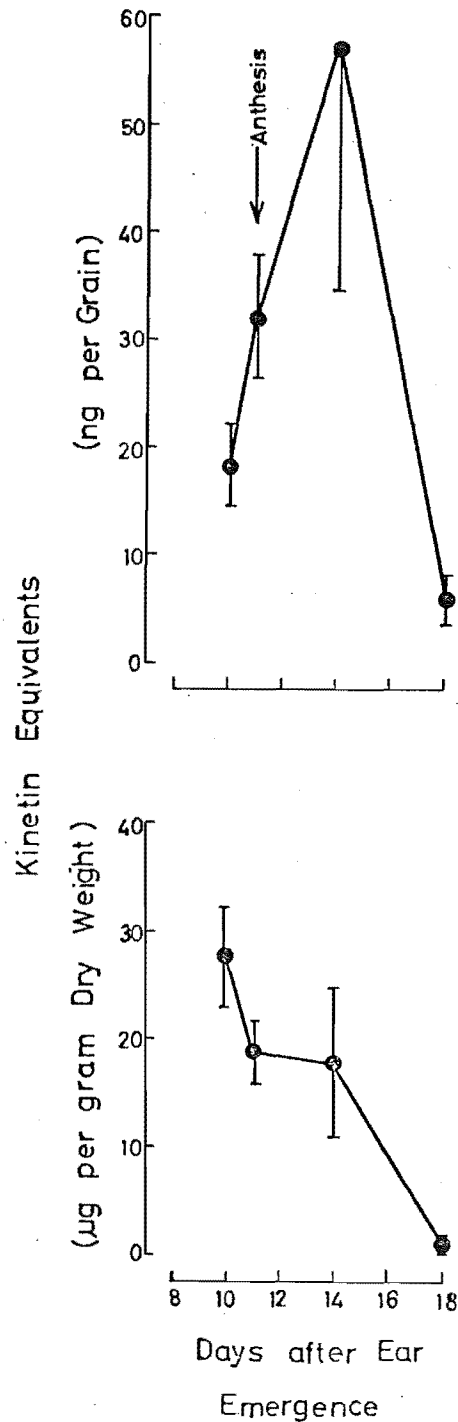


FIGURE 6.7

Wheat Field Trial 1. Total cytokinin-like activity in wheat grains between pollination and seven days post-anthesis (10 and 18 days after ear emergence). The data are expressed as kinetin equivalents per grain and per unit dry weight. Vertical bars show one standard error above and/or below the line. The time of anthesis is indicated

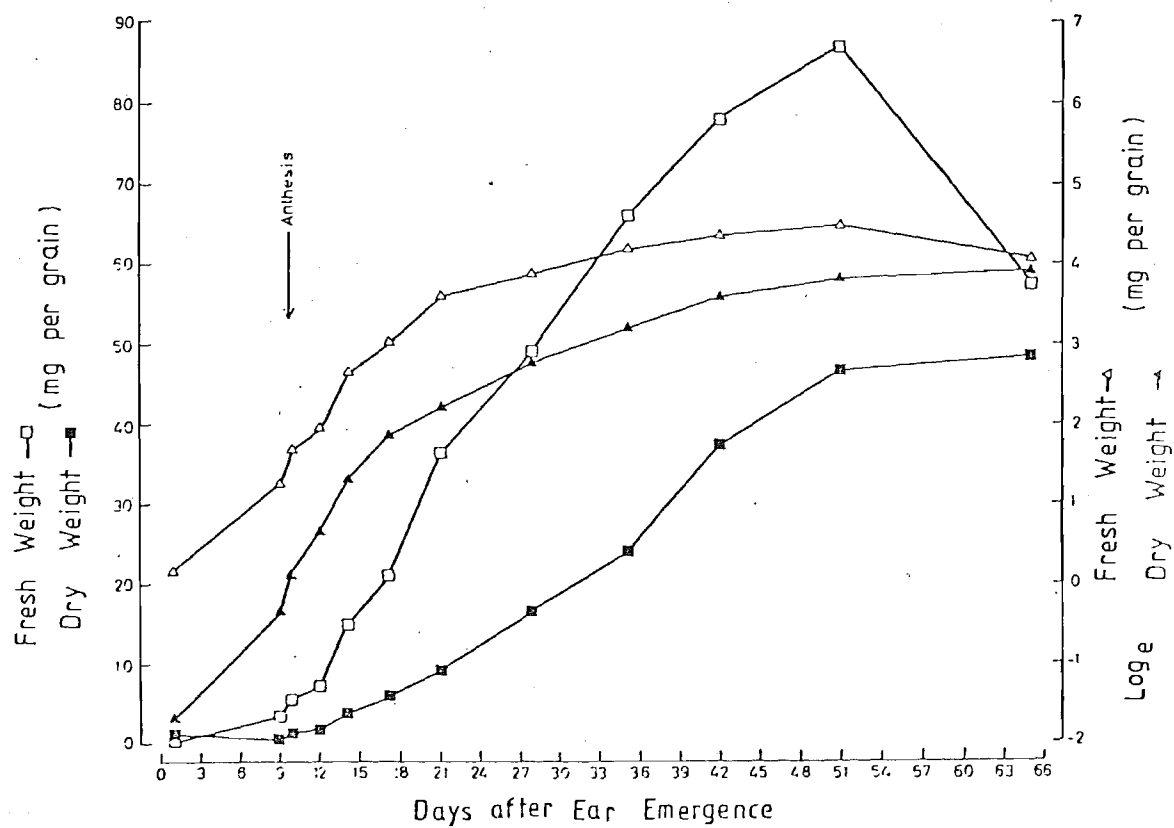


FIGURE 6.8

Wheat Field Trial 2. Fresh weight and dry weight of grains as a function of the time from ear emergence

until 14 days after ear emergence although the relative growth rate was at its highest until this time.

Following pollination (nine days after ear emergence) the amount of starch, calculated as a percentage of the total dry weight, increased rapidly although a temporary decrease occurred 21 days after ear emergence (Fig 6.9). The starch content had reached 50% of the dry weight 42 days after ear emergence.

When starch was calculated as a percentage of the fresh weight an increase was apparent nine days after ear emergence (pollination) (Fig 6.9). Twenty-eight days after ear emergence starch accounted for an increasing proportion of the fresh weight and this relative increase continued thereafter.

The rate at which starch accumulated in the grains was slow until 28 days after ear emergence. Between 28 and 51 days after ear emergence accumulation was rapid but it ceased within 51 days of ear emergence. However, the relative rate of starch increase was highest soon after pollination. It remained high for 35 days after ear emergence and subsequently decreased (Fig 6.9).

The contribution of soluble sugar to the total fresh weight of the grains was small (Fig 6.10). The proportion of the dry weight attributable to soluble sugar increased following pollination (nine days after ear emergence) and achieved a maximum of 35% of the dry weight 21 days after ear emergence. When the soluble sugar content is calculated as the amount per grain, an increase is apparent prior to pollination. Following pollination the amount of soluble sugars continued to increase rapidly until 28 days after ear emergence, after which a decrease occurred. The relative rate of increase in soluble sugar per grain was extremely high shortly before and after anthesis. The relative rate began to decrease within 21 days of ear emergence (11 days after anthesis) (Fig 6.10).

Grains (or pistils) were selected from eight different wheat harvests for cytokinin analysis. Cytokinin-like activity was quantified in kinetin equivalents from the regression line compiled from kinetin standards run with each bioassay (section 2.2.3).

Extracts of pistils removed from spikelets at ear emergence contained a trace of cytokinin-like activity (Fig 6.11). The activity was detected at an elution volume similar to that of the zeatin standard (fractions 10 and/or 11). By reference to the kinetin standards, the bioassay response indicated that the level of cytokinin-like activity was more than $1 \mu\text{g KE l}^{-1}$ (ca $0.1 \text{ ng KE grain}^{-1}$; $0.152 \mu\text{g KE g DW}^{-1}$).

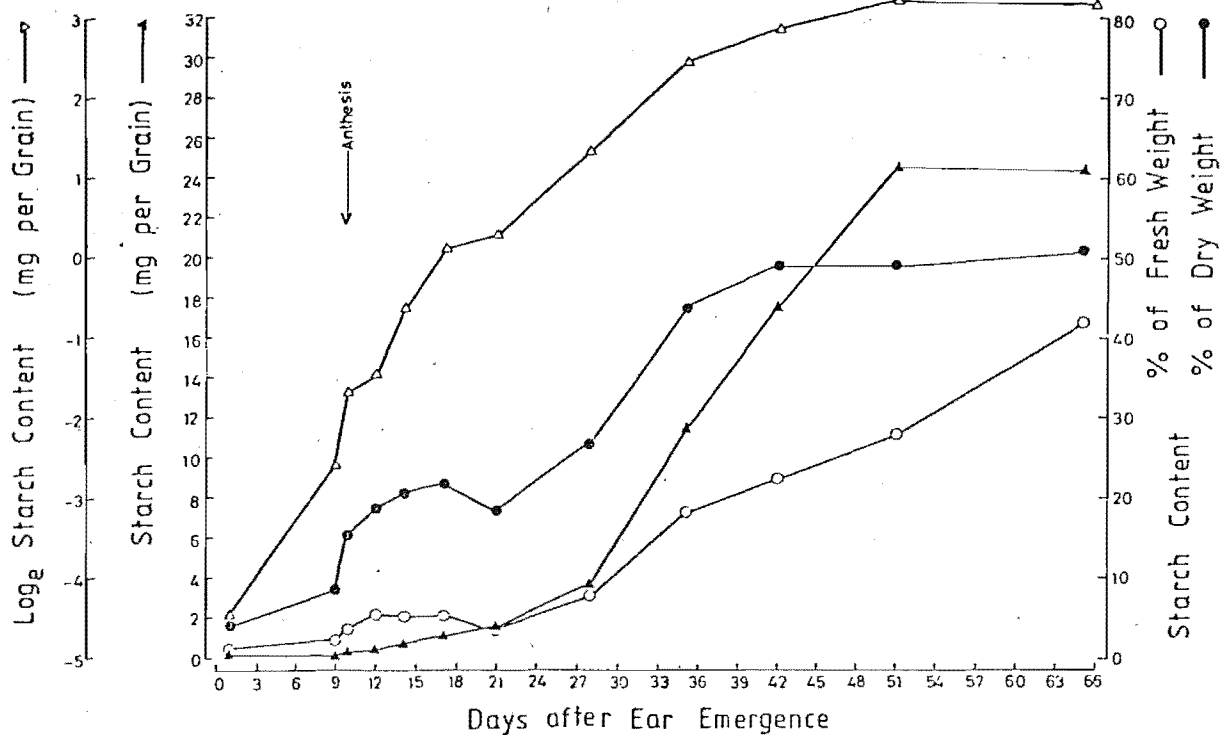


FIGURE 6.9

Wheat Field Trial 2. Starch content of developing wheat grains estimated either as the amount per grain or as a proportion of the total fresh or dry weight of the grain. The coefficients of variation were less than 10% for all analyses.

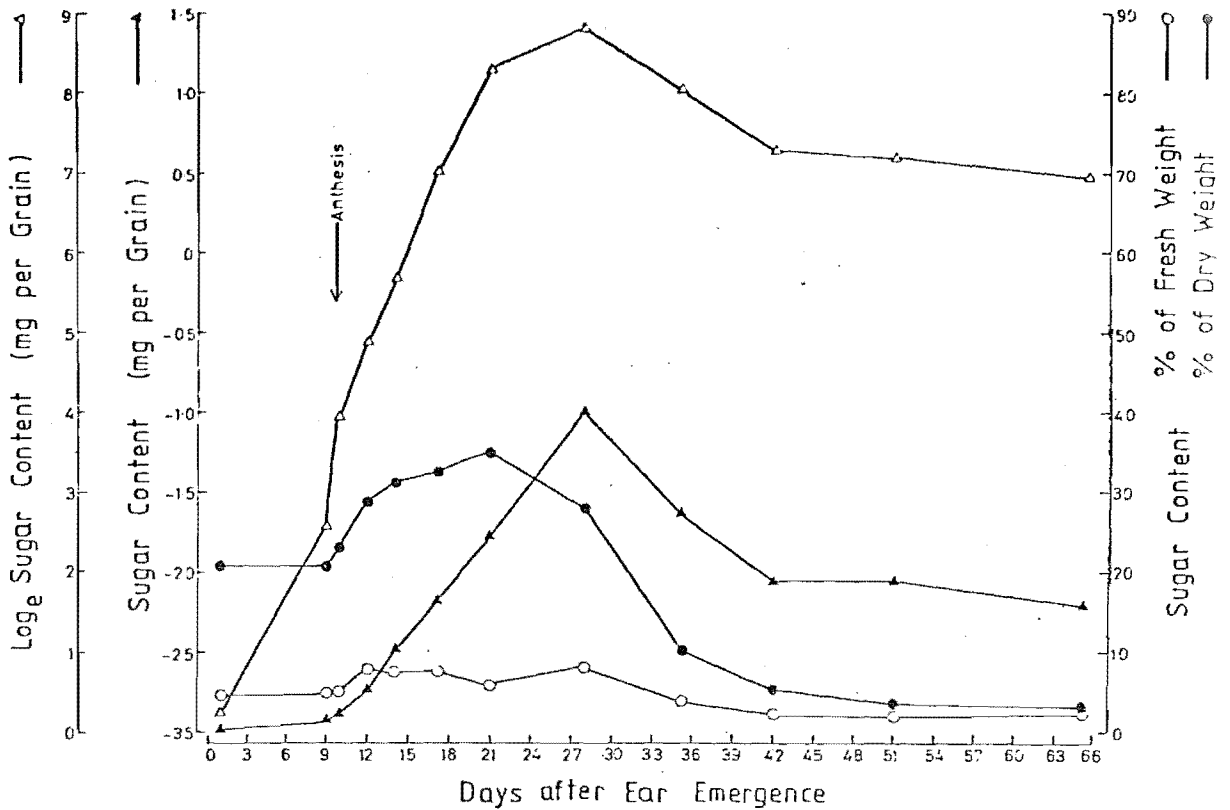


FIGURE 6.10

Wheat Field Trial 2. Soluble sugar content of developing wheat grains estimated either as the amount per grain or as a proportion of the total fresh or dry weight of the grain. The coefficients of variation were less than 6.6% for all but the final analysis when it was 11.8%

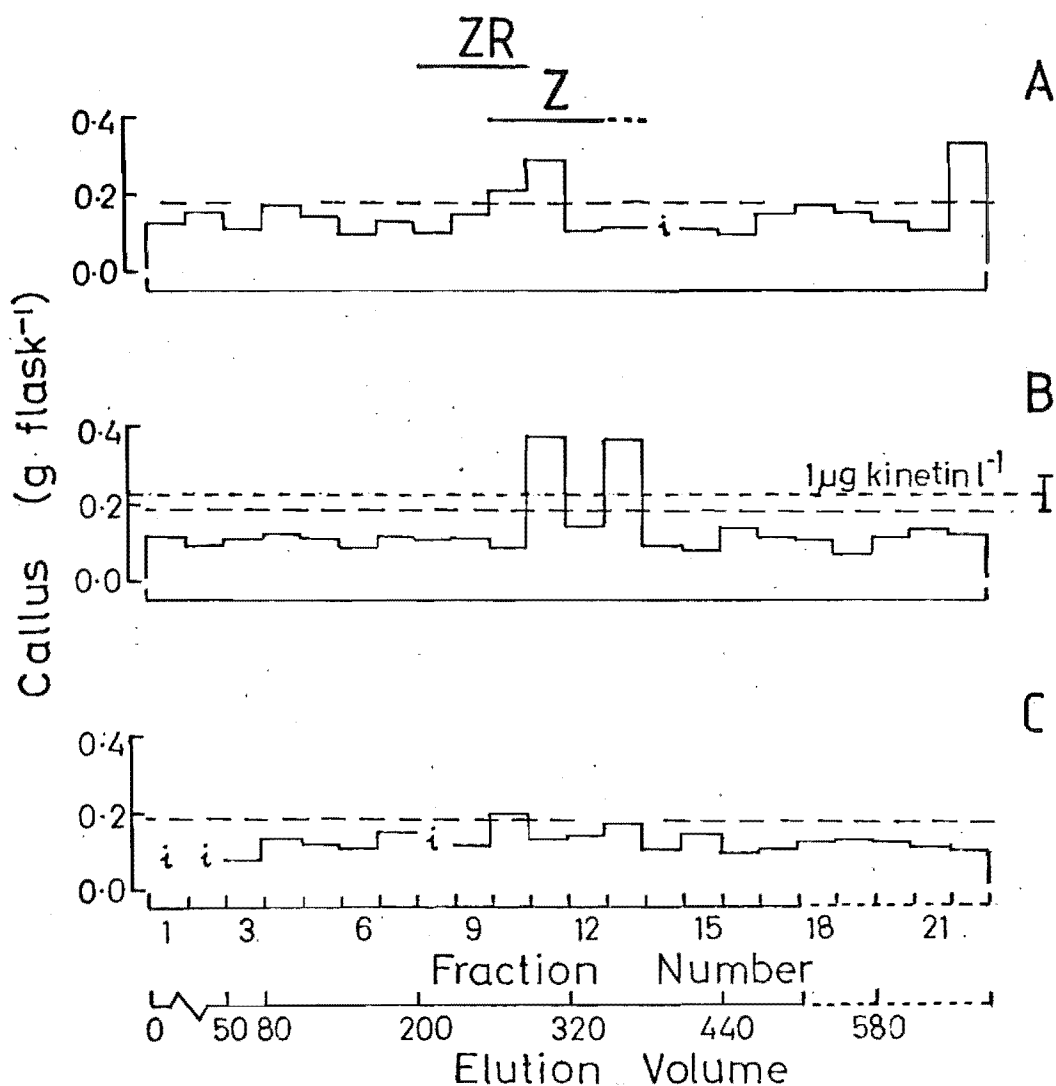


FIGURE 6.11

Wheat Field Trial 2. Soybean callus bioassay of 500 wheat pistils collected at ear emergence. Extracts were resolved by partition chromatography on Sephadex LH-20.

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 22 (40 ml) and fraction 23 (40 ml)

Callus: Kin (4)

Confidence limits: $n = 13$

Regression line: $Y = 1.87X - 0.74$

Lowest detectable amount of kinetin: $1 \mu\text{g l}^{-1}$ ($232.5 \pm 52.5 \text{ mg callus flask}^{-1}$)

Further details are tabulated with Figure 6.2

Pistils removed from spikelets nine days after ear emergence contained a different pattern of cytokinin-like activity (Fig 6.12). A major portion of the cytokinin-like activity eluted from the column earlier than the zeatin riboside standard. A lesser amount of activity was detected in fraction 11. The cytokinin-like activity collected in specified fractions and calculated in kinetin equivalents is presented in Table 6.10.

TABLE 6.10

Wheat Field Trial 2. Quantification of cytokinin-like activity in extracts of 500 pistils collected at pollination (nine days after ear emergence)^a. Values in kinetin equivalents were obtained from the regression line $Y = 1.87X - 0.74$

Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)		
	7	11	Total
A	30.33	9.75	40.08
B	61.84	8.37	70.21
C	47.08	6.73	53.81

^a Chromatography and bioassay details tabulated with Figure 6.12

Grains collected at peak anthesis (10 days after ear emergence) contained increased amounts of cytokinin-like activity (Fig 6.13). Complete resolution of cytokinin-like components was not achieved although it was again apparent that a proportion of the activity was eluting earlier than the zeatin riboside fraction. Activity was collected in the same volumes of eluant as zeatin and zeatin riboside standards. Compounds present in fraction 5 caused the callus to turn dark brown during the bioassay. The cytokinin-like activity in specified fractions is summarised in Table 6.11.

Extracts of 500 grains collected two days post-anthesis (12 days after ear emergence) contained very large quantities of cytokinin-like activity (Fig 6.14). The response of the callus in some instances exceeded the range of the standards and could not be estimated quantitatively. Compounds collected in fraction 6 (sample A) and fractions 4 and 5 (sample B) caused the callus to become brown during the bioassay, while compounds in fraction 6 (sample C) caused two pieces of callus to turn black.

FIGURE 6.12

Wheat Field Trial 2. Soybean callus bioassay of 500 wheat pistils collected 9 days after ear emergence (pollination). Extracts were resolved by partition chromatography on Sephadex LH-20.

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml)

Further details are tabulated with Figure 6.11.

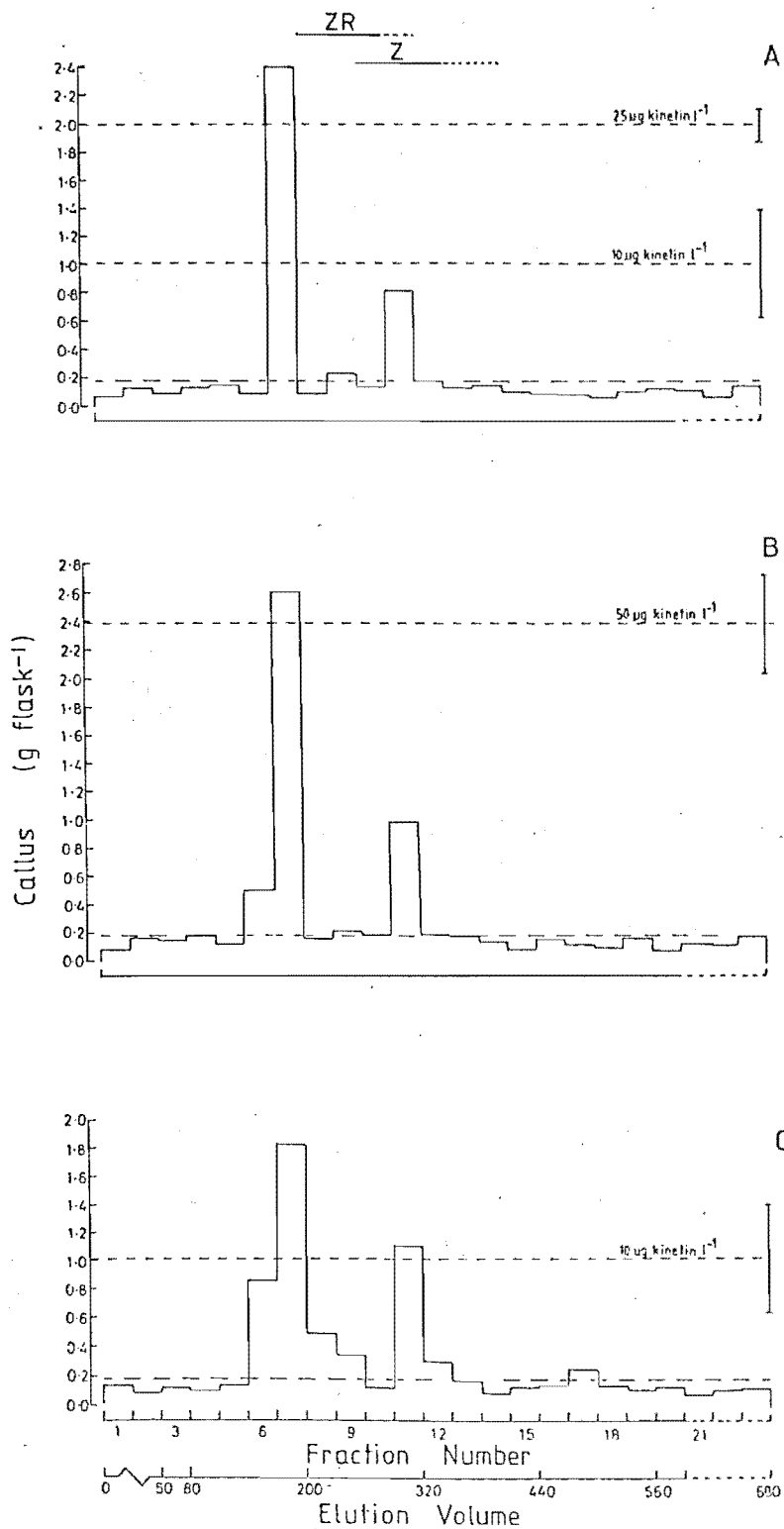


FIGURE 6.12

FIGURE 6.13

Wheat Field Trial 2. Soybean callus bioassay of extracts of 500 wheat grains collected 10 days after ear emergence (anthesis). Extracts were resolved by partition chromatography on Sephadex LH-20.

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 21 (50 ml) and fraction 22 (60 ml)

Callus: Kin (3)

Confidence Limits: $n = 10$

Regression line: $Y = 1.87X - 0.74$

Lowest detectable amount of kinetin: $5 \mu\text{g l}^{-1}$ ($626 \pm 138 \text{ mg callus}$
 flask^{-1})

Further details are tabulated with Figure 6.2

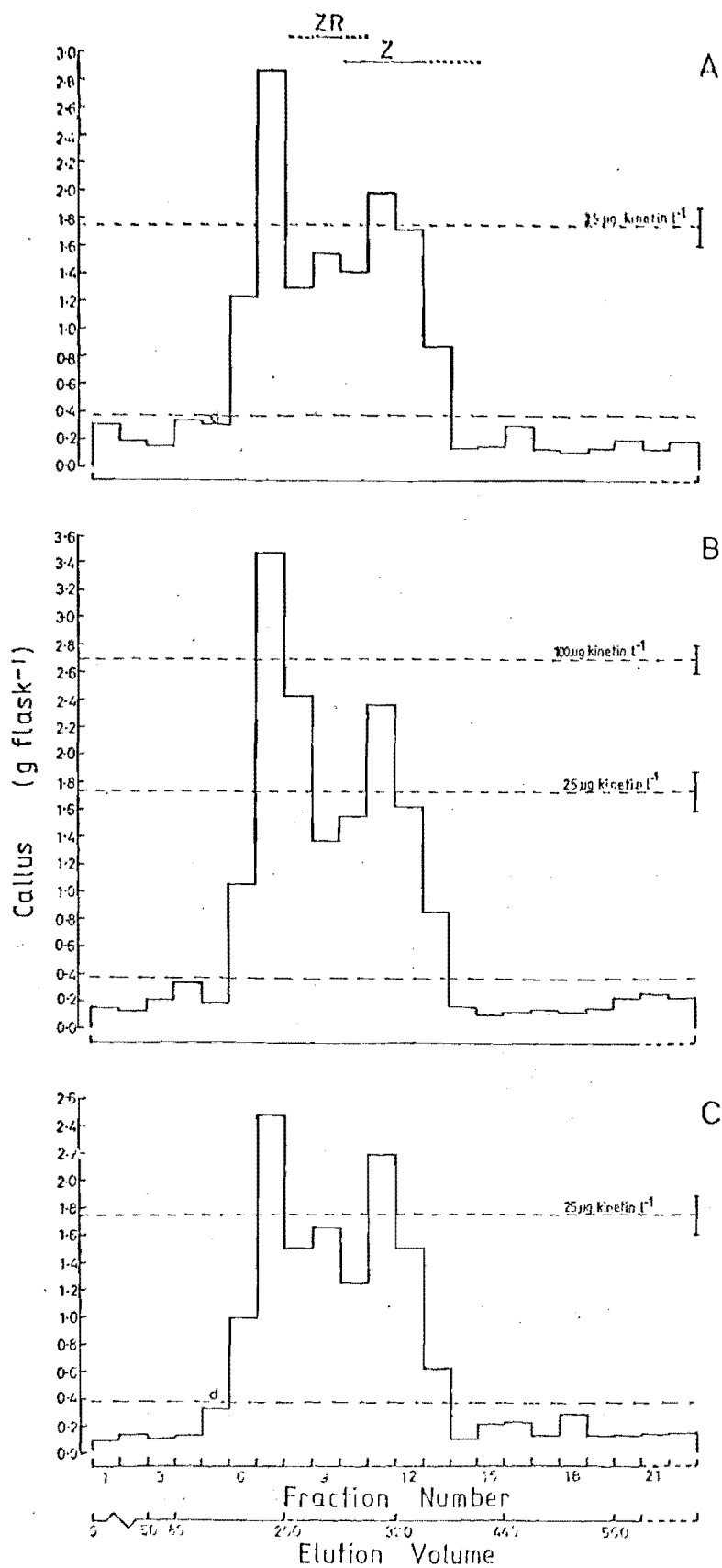


FIGURE 6.13

FIGURE 6.14

heat Field Trial 2. Soybean callus bioassay of 500 wheat grains collected 12 days after ear emergence (2 days post-anthesis). Extracts are resolved by partition chromatography on Sephadex LH-20.

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml), fraction 21 (50 ml) and fraction 22 (50 ml).

Callus: Kin (2)

Confidence limits: $n = 9$

Regression line: $Y = 1.87X - 0.74$

Lowest detectable limit amount of kinetin: $5 \mu\text{g l}^{-1}$ ($555 \pm 145 \text{ mg callus flask}^{-1}$).

Further details are tabulated with Figure 6.2.

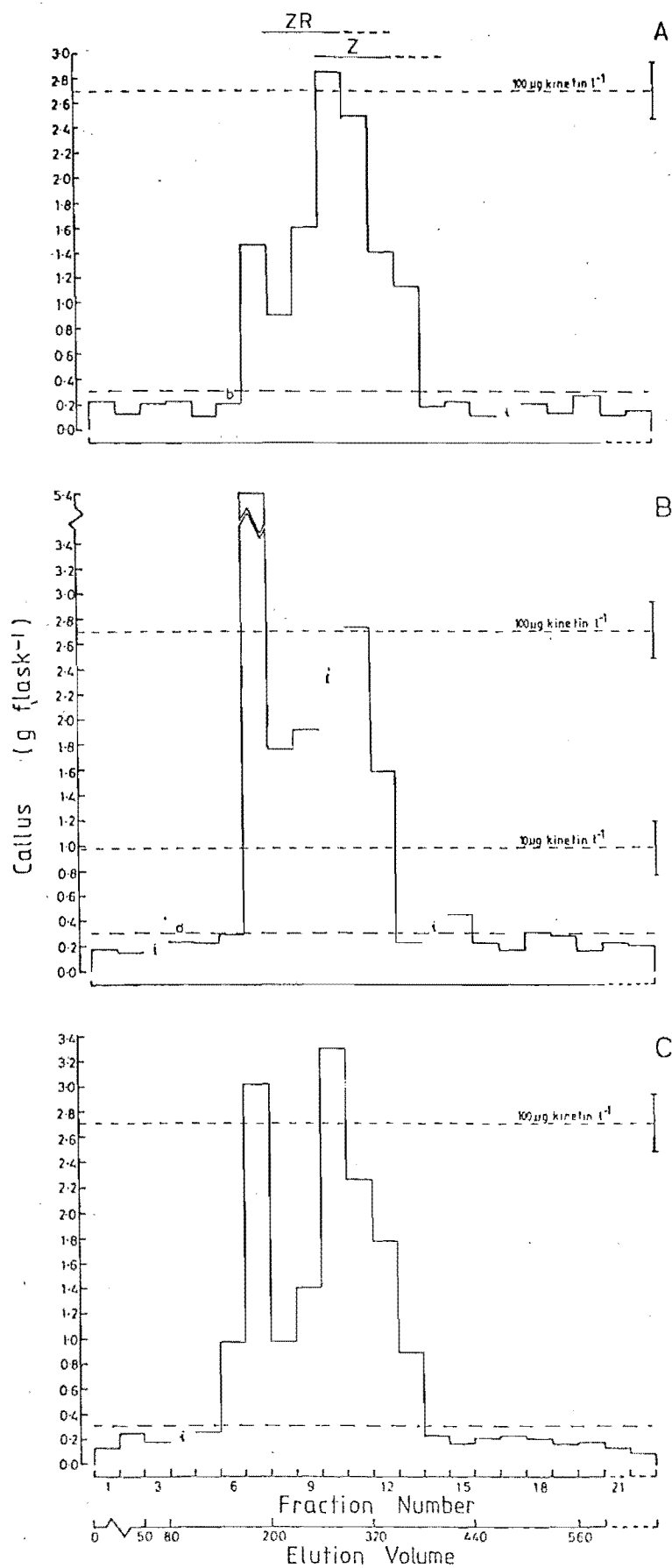


FIGURE 6.14

TABLE 6.11

Wheat Field Trial 2. Quantification of cytokinin-like activity in extracts of 500 wheat grains collected at anthesis (10 days after ear emergence)^α. Values in kinetin equivalents were obtained from the regression line $Y = 1.87X - 0.74$

Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)				Total
	6 - 8	Fraction 9	10	11 - 13	
A	139.55	15.96	13.16	56.89	225.56
B	327.59	12.44	16.77	77.35	434.15
C	83.35	18.74	10.33	55.36	167.78

^α Chromatography and bioassay details are tabulated with Figure 6.13

Three extracts, each of 500 grains, of the "bulk sample" (Table 6.3) were resolved by column chromatography, 10 ml fractions being collected and bioassayed (Fig 6.15). Baseline resolution of cytokinin-like components was achieved in replicates A and B. In all three samples activity was detected in the same fractions as were expected to contain zeatin and zeatin riboside. However, in only one sample was significant cytokinin-like activity detected eluting earlier from the column than zeatin riboside (replicate B, fraction 15). In all three replicates a trace of cytokinin-like activity is present in fraction 12. During the bioassay of fraction 15 (replicate A), fraction 14 (replicate B) and fraction 14 and 15 (replicate C) the callus rapidly turned dark brown. The results are summarised in Table 6.12.

Extracts of 100 grains collected two days post-anthesis contained variable but substantial amounts of cytokinin-like activity eluting from the column earlier than zeatin riboside (Fig 6.16) but little activity was detected in the fractions expected to contain zeatin riboside. Activity was detected in the fractions expected to contain zeatin. The results are summarised in Table 6.13.

Prior to β -glucosidase treatment six extracts of 100 grains collected two days after anthesis were resolved by column chromatography using 20% ethanol as the eluting solvent. Three samples were subsequently bioassayed. Fractions 3 to 9 of the remaining three samples were pooled and treated with β -glucosidase. In the untreated samples the cytokinin-like activity was resolved into three discrete components. The two later eluting peaks occurred in similar elution volumes as zeatin riboside and

FIGURE 6.15

Heat Field Trial 2. Soybean callus bioassay of 500 wheat grains collected in the "bulk sample" 12 days after ear emergence (two days post-anthesis). Extracts were resolved by partition chromatography on Sephadex LH-20

Solvent: 35% ethanol

Fraction volume: 10 ml with the exception of fraction 1 (20 ml), fraction 2 (30 ml), fraction 3 (20 ml), fraction 4 (150 ml), fraction 45 (250 ml) and fraction 46 (190 ml)

Callus: Kin (13)

Confidence limits: $n = 10$

Regression line: $Y = 1.46X - 1.05$

Lowest detectable amount of kinetin: $7.5 \mu\text{g KE l}^{-1}$ ($189 \pm 80 \text{ mg callus flask}^{-1}$)

Further details are tabulated with Figure 6.2

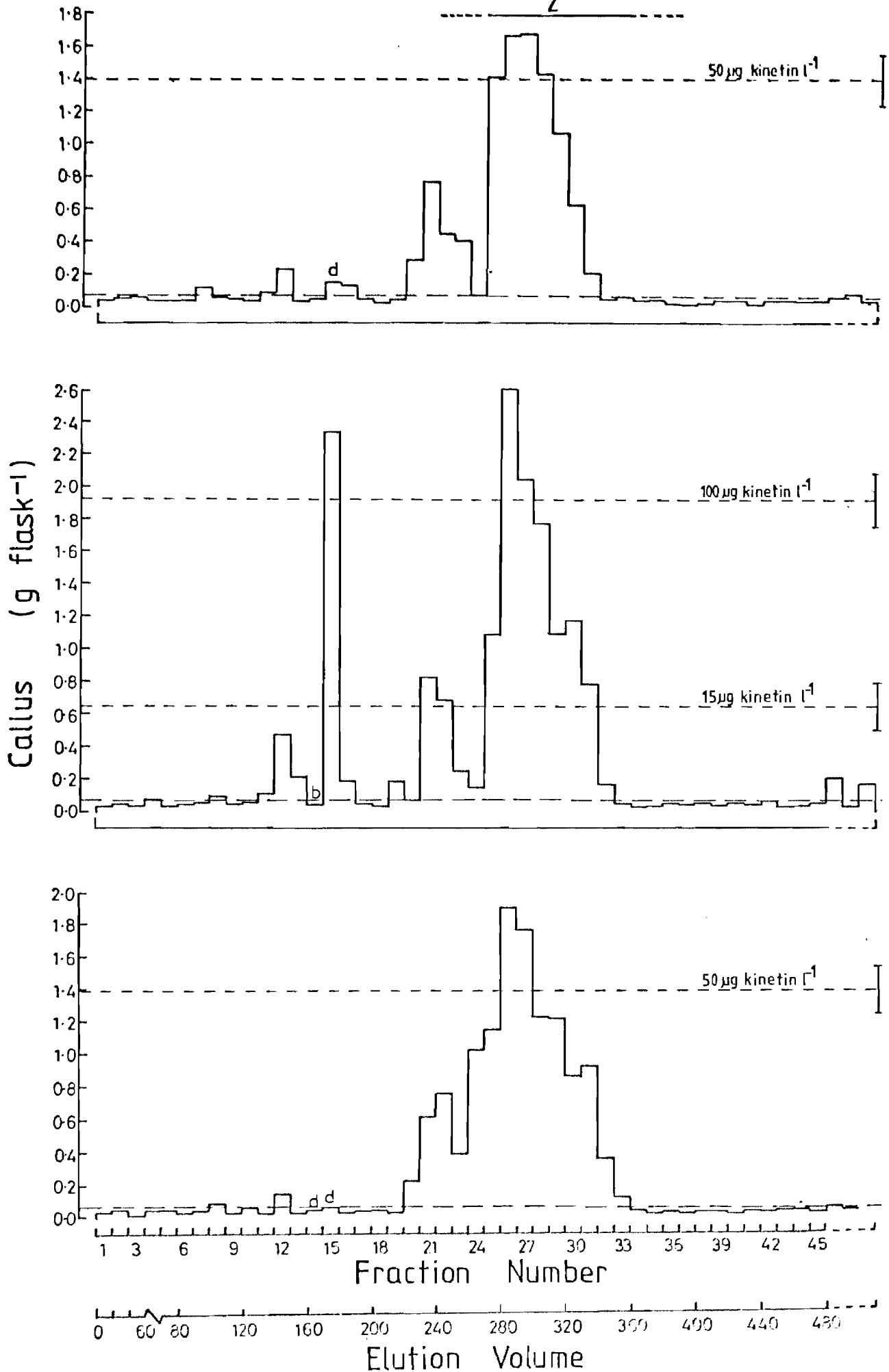


FIGURE 6.15

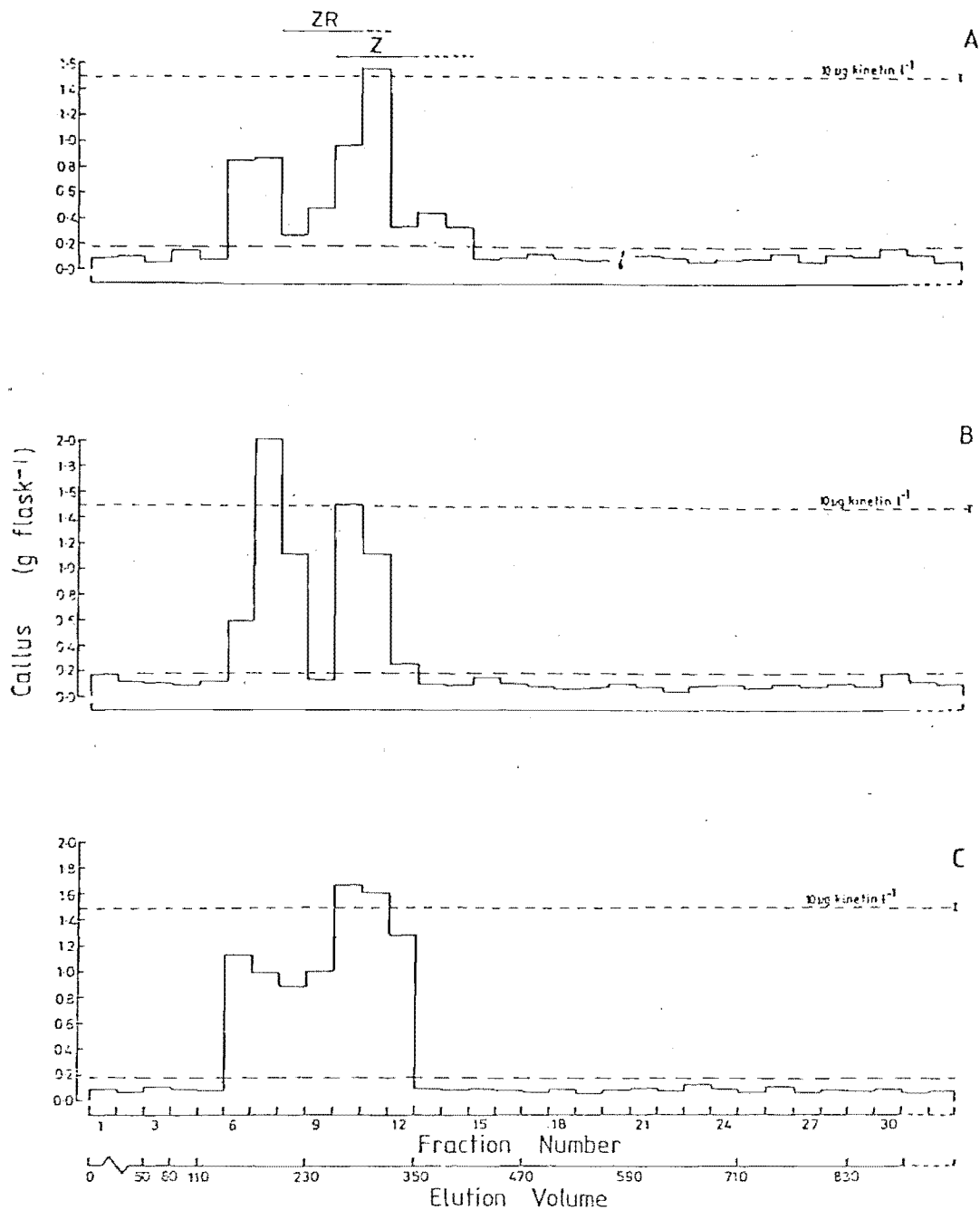


FIGURE 6.16

Wheat Field Trial 2. Soybean callus bioassay of extracts of 100 wheat grains collected 12 days after ear emergence. Extracts were resolved by partition chromatography on Sephadex LH-20.

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 31 (50 ml) and fraction 32 (50 ml)

Callus: Bap (9)

Confidence limits: $n = 10$

Regression line: $Y = 2.04X - 0.78$

Lowest detectable amount of kinetin: $2 \mu\text{g l}^{-1}$ ($206 \pm 95 \text{ mg callus flask}^{-1}$)

Further details are tabulated with Figure 6.2

zeatin, respectively (Fig 6.17). More rapidly eluting cytokinin-like activity was detected in fractions 7, 8 and 9 in samples B and C. In addition to this activity, cytokinin-like activity was detected in fractions 4, 5 and 6 in sample A. The results are summarised in Table 6.14.

TABLE 6.12

Wheat Field Trial 2. Quantification of the cytokinin-like activity in extract of 500 wheat grains collected in the "bulk sample" two days post-anthesis (12 days after ear emergence).^a Values in kinetin equivalents were obtained from the regression line $Y = 1.46X - 1.05$.

Replicate	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)				
	12 - 19	Fractions		25 - 31	Total
		20 - 23	24		
A	7.48	46.33	ns ^b	387.52	441.33
B	218.77	42.38	ns	626.66	887.81
C	ns	41.31	26.56	405.66	473.52

^a Chromatography and bioassay details are tabulated with Figure 6.15

^b ns = not significant

TABLE 6.13

Wheat Field Trial 2. Quantification of the cytokinin-like activity in extracts of 100 wheat grains collected two days post-anthesis (12 days after ear emergence).^a Values in kinetin equivalents were obtained from the regression line $Y = 2.04X - 0.78$

Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)				
	6 - 8	Fractions		11 - 13	Total
		9	10		
A	12.73	4.13	7.14	13.89	37.89
B	36.35	ns ^b	12.38	8.40	57.13
C	22.60	7.47	15.91	25.01	70.99

^a Chromatography and bioassay details are tabulated with Figure 6.16

^b ns = not significant

FIGURE 6.17

Wheat Field Trial 2. Soybean callus bioassay of extracts of 100 wheat grains collected 12 days after ear emergence

1. Extracts were resolved by partition chromatography on Sephadex LH-20
2. Soybean callus bioassay of the first zone of activity in "1" (fractions 3 to 9) after hydrolysis with β -glucosidase and further separation on Sephadex LH-20

Solvent: 20% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml), fraction 24 (60 ml) and fraction 25 (350 ml)

Callus: (1) Bap 26
(2) Bap 29

Confidence limits: $n = 10$

Regression line: (1) $Y = 0.87X - 0.09$
(2) $Y = 0.84X + 0.13$

Lowest detectable amount of kinetin:

(1) $3 \mu\text{g l}^{-1}$ (255 ± 63 mg callus flask $^{-1}$)
(2) $3 \mu\text{g l}^{-1}$ (563 ± 145 mg callus flask $^{-1}$)

Further details are tabulated with Figure 6.2

FIGURE 6.17

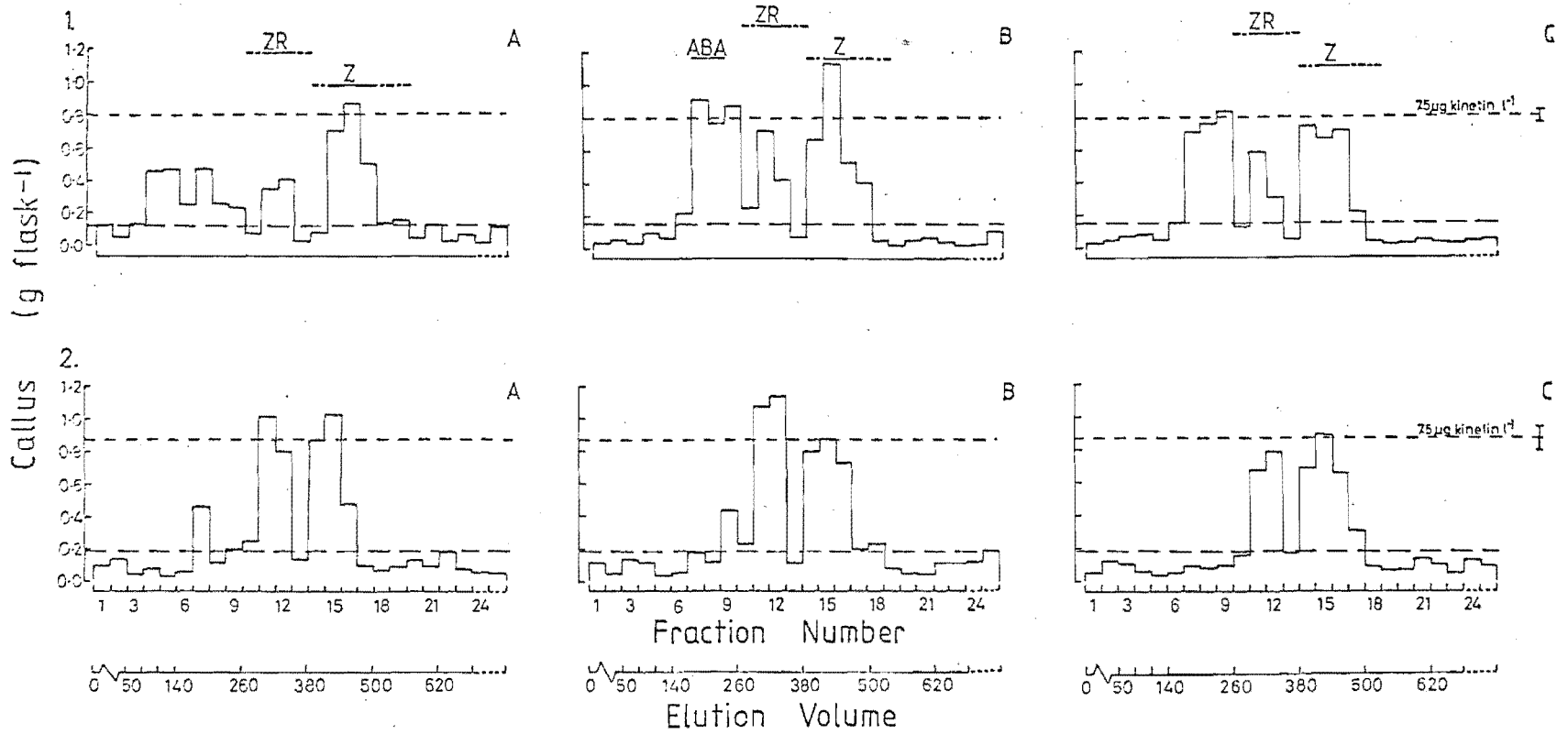


TABLE 6.14

Wheat Field Trial 2. Quantification of the cytokinin-like activity in extracts of 100 wheat grains collected two days post-anthesis (12 days after ear emergence). The Sephadex LH-20 chromatography eluant was 20% ethanol.^a Values in kinetin equivalents were obtained from the regression line $Y = 0.87X - 0.09$

Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)					Total
	4 - 9	10	Fractions 11 - 12	13	14 - 17	
A	13.61	ns	6.85	ns	30.31	50.77
B	35.92	ns	12.34	ns	40.74	89.00
C	28.90	ns	5.94	ns	25.30	60.14

^a Further chromatography and bioassay details are tabulated with Figure 6.17

Following β -glucosidase treatment the extracts were again resolved using Sephadex LH-20 and bioassayed (Fig 6.17). Two peaks of cytokinin-like activity were detected at elution volumes similar to those for zeatin riboside and zeatin. Only traces of cytokinin-like activity remained in samples A and B eluting earlier than the zeatin riboside standard. The cytokinin-like activity in specified fractions is summarised in Table 6.15.

TABLE 6.15

Wheat Field Trial 2. Quantification of the cytokinin-like activity collected in fractions 7 to 16 following the treatment of fractions 3 to 9 with β -glucosidase and rechromatography. Extracts of 100 grains from three samples collected two days post-anthesis were treated and bioassayed.^a Values in kinetin equivalents were obtained from the equation of the regression line $Y = 0.84X + 0.13$

Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$) following β -glucosidase treatment					Total
	7 - 9	10	Fractions 11 - 12	13	14 - 16	
A	ns	trace	16.88	ns	18.48	35.36
B	trace	ns	27.79	ns	18.70	46.49
C ^b	ns	ns	8.93	ns	16.67	25.60

^a Further chromatography and bioassay details are tabulated with Figure 6.17

^b Some extract lost

A comparison between the total cytokinin-like activity present in fractions 3 to 9 before and after β -glucosidase treatment is presented in Table 6.16. After β -glucosidase treatment the pooled fractions were re-chromatographed.

TABLE 6.16

Wheat Field Trial 2. Comparison of the total cytokinin-like activity detected in fractions 3 to 9 before and after β -glucosidase treatment of extracts of 100 wheat grains collected two days post-anthesis^a (Fig 6.17)

Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)	
	Fractions 3 - 9	Fractions 3 - 9 treated with β -glucosidase
A	13.61	35.36
B	35.92	46.49
C	28.90	25.60 ^b

^a Further chromatography and bioassay details are tabulated with Figure 6.17

^b Some extract lost

Abscisic acid was applied to the column in 20% ethanol and detected by the UV monitor. The bulk of the abscisic acid was collected in fractions 7 and 8 (Fig 6.17).

Grains from the "bulk" wheat sample collected two days post-anthesis were extracted using the standard 80% methanol procedure and by the procedure described by Bielecki (1964) (section 6.2.2.4). The bioassays of the extracts were run concurrently (Fig 6.18) and the results are summarised in Table 6.17.

The total amount of cytokinin-like activity extracted by both procedures was similar (Table 6.17). The amount of activity detected in the fractions expected to contain zeatin riboside and zeatin appeared greater after extraction using Bielecki's solvent cocktail (155.38 ± 27.28) than after using 80% methanol (105.48 ± 11.23).

A comparison of Table 6.17 with Tables 6.12, 6.13 and 6.14 indicates that the "bulk" wheat sample contained a higher level of cytokinin-like activity than those grains collected earlier in the morning, dissected and placed in liquid N_2 in the field.

Grains collected four days after anthesis (14 days after ear emergence) contained high levels of cytokinin-like activity (Fig 6.19). When extract of 500 grains was bioassayed, a large proportion of the cytokinin-like activity detected was present in fractions 7 and 8 although

FIGURE 6.18

Wheat Field Trial 2. Soybean callus bioassay of extracts of 100 wheat grains from the "bulk sample" collected 12 days after ear emergence.

1. Samples were extracted with 80% ethanol.
2. Samples were extracted using Bielecki's procedure (see text).

Extracts were resolved by partition chromatography on Sephadex LH-20.

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 21 (60 ml), fraction 22 (150 ml) and
fraction 23 (180 ml)

Callus: Kin (18)

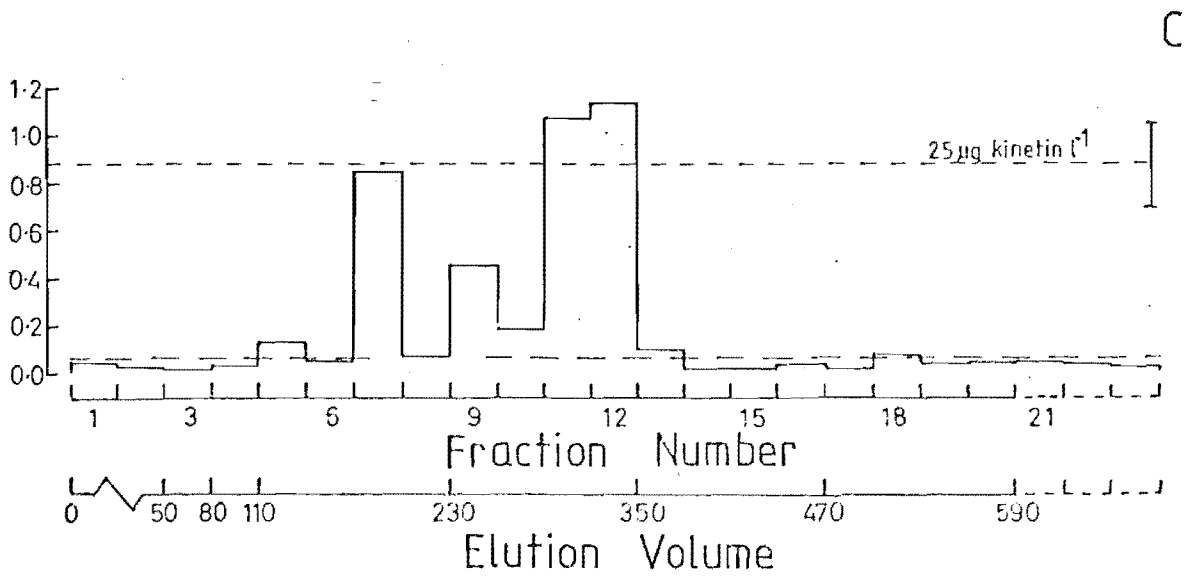
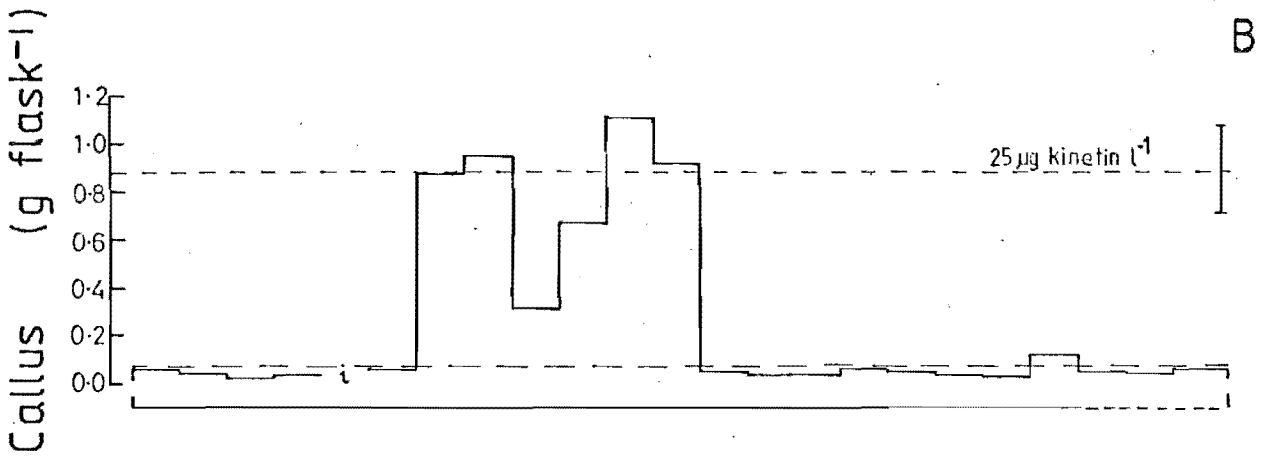
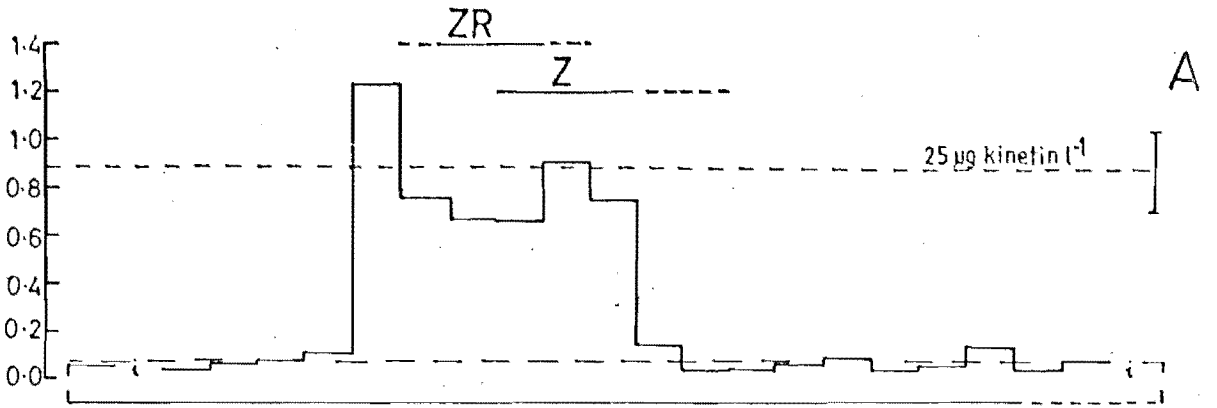
Confidence limits: $n = 13$

Regression lin: $Y = 1.09X - 0.66$

Lowest detectable amount of kinetin: $10 \mu\text{g l}^{-1}$ ($791 \pm 51 \text{ mg callus flask}^{-1}$)

Further details are tabulated with Figure 6.2

1

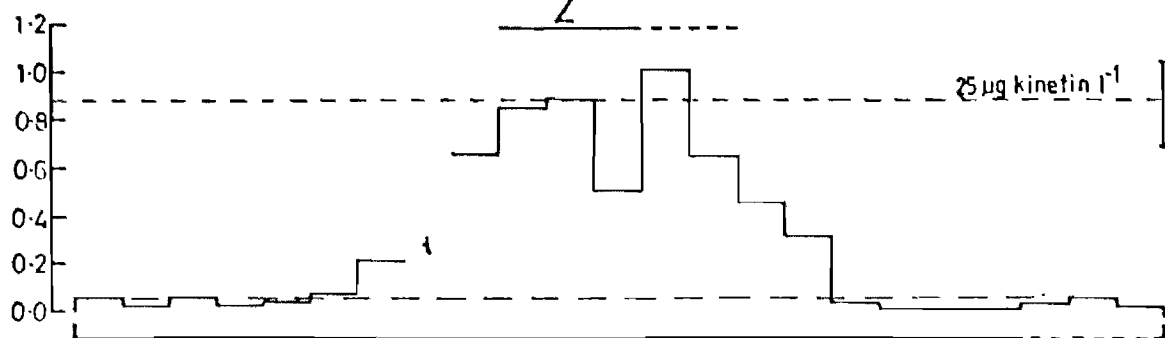


2

ZR

Z

A

Callus (g flask⁻¹)

B

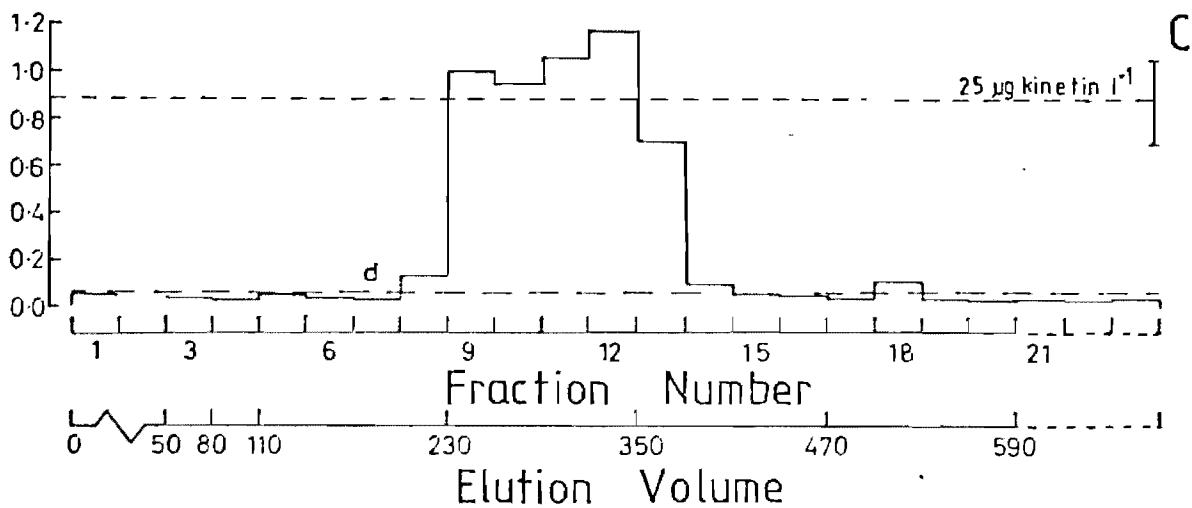
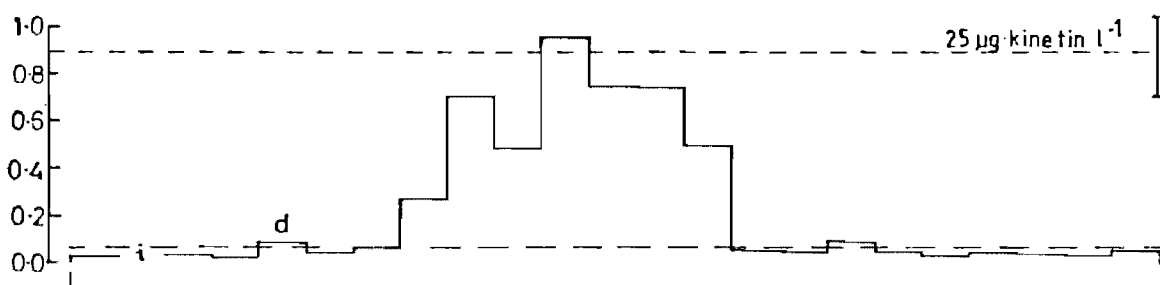
25 μg kinetin l⁻¹

FIGURE 6.19

Wheat Field Trial 2. Soybean callus bioassay of extracts of 500 wheat grains collected 14 days after ear emergence (four days after anthesis). Extracts were resolved by partition chromatography on Sephadex LH-20

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml)
fraction 31 (50 ml) and fraction 32 (50 ml)

Callus: Kin (5)

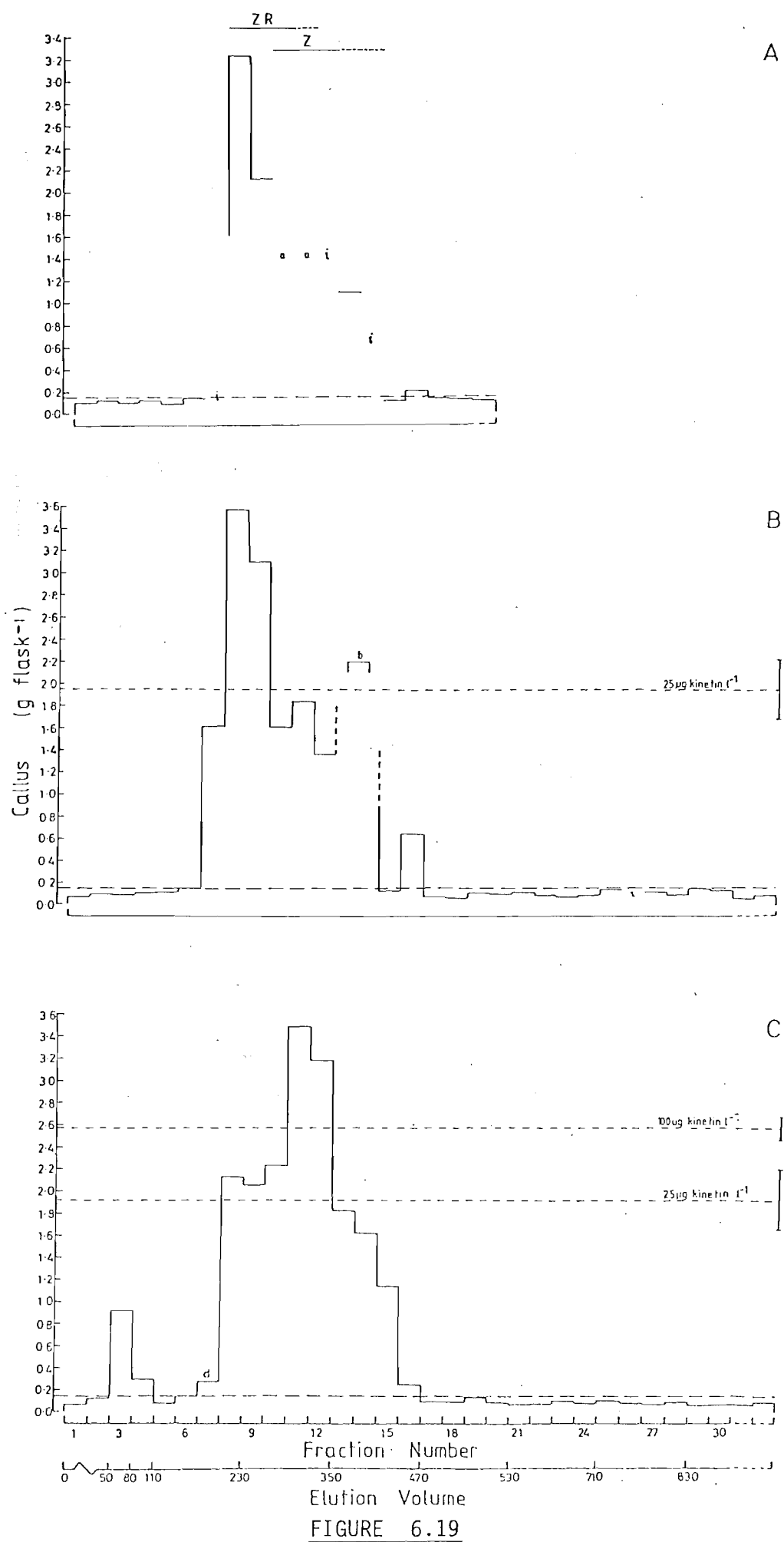
Confidence limits: $n = 8$

Regression line: $Y = 2.07X - 1.34$

Lowest detectable amount of kinetin: $5 \mu\text{g l}^{-1}$ ($342 \pm 215 \text{ mg callus flask}^{-1}$)

b: Fractions inadvertently run with the bioassay depicted in Figure 4.22

Further details are tabulated with Figure 6.2



activity was detected up to at least fraction 13. The results are summarised in Table 6.18.

TABLE 6.17

Quantification of the cytokinin-like activity in extracts of 100 wheat grains collected in the "bulk sample" two days post-anthesis following extraction with either 80% methanol or Bielecki's solvent cocktail.^a Values in kinetin equivalents were obtained from the regression line $Y = 1.09X - 0.66$

Solvent	Replicate	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)				Total	Average (\pm SE)
		7 - 8	Fractions		11 - 15		
			9	10			
80% methanol	A	72.80	16.39	65.10	46.44	200.73	
	B	55.17	7.66	16.53	69.48	148.86	
	C	24.53	10.68	ns ^b	84.17	119.38	156.32 \pm 23.78
Methanol-Formic Acid (7N)-H ₂ O (12:5:3)	A	ns	16.45	25.00	109.63	151.08	
	B	ns	17.95	11.27	81.21	110.43	
	C	ns	33.54	29.98	141.11	204.63	155.35 \pm 27.28

^a Further chromatography and bioassay details are tabulated with Figure 6.18

^b ns = not significant

TABLE 6.18

Wheat Field Trial 2. Quantification of the cytokinin-like activity in extracts of 500 wheat grains collected four days post-anthesis (14 days after ear emergence).^a Values in kinetin equivalents were obtained from the regression line $Y = 2.07X - 1.34$

Sample	6 - 8	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)			Total
		Fractions		11 - 15	
		9	10		
A	162.10	46.80	409.20 ^b	398.30 ^b	1016.40
B	264.77	141.40	26.49	106.80	539.46
C	47.65	43.68	53.44	446.85	591.62

^a Further chromatography and bioassay details are tabulated with Fig 6.19

^b Values obtained from regression line $Y = 2.51X - 0.70$ (Fig 4.22)

When extract of 100 grains collected four days post-anthesis was bioassayed cytokinin-like activity was detected in all three samples (Fig 6.20). However, activity eluting earlier than the zeatin riboside fraction was high in sample C, low in sample B and not apparent in sample A. Cytokinin-like activity was detected in fractions 9 to 12 in all three samples. The amount of cytokinin-like activity in specified fractions is summarised in Table 6.19.

TABLE 6.19

Wheat Field Trial 2. Quantification of the cytokinin-like activity in extracts of 100 wheat grains collected four days post-anthesis (14 days after ear emergence).^a Values in kinetin equivalents were obtained from the regression line $Y = 1.01X - 0.07$

Sample	7 - 8	Cytokinin -like activity ($\mu\text{g KE l}^{-1}$)			Total
		9	10	11 - 12	
A	ns ^b	8.47	ns	180.13	188.60
B	ca 5 ^e	23.75	51.63	260.77	341
C	ca 500 ^e	ns	ns	186.44	686

^a Further chromatography and bioassay details are tabulated with Figure 6.20

^b ns = not significant

^c Values estimated by direct reference to kinetin standards

Cytokinin-like activity eluting from the column earlier than the zeatin riboside standard was detected in all three samples when extracts of 100 grains (from wheat collected 4d post-anthesis) were resolved using 20% ethanol as the eluting solvent (Fig 6.21). Baseline resolution was achieved in sample B, indicating three discrete peaks of activity. The results are summarised in Table 6.20.

No cytokinin-like activity was detected following permanganate oxidation of extract of wheat grains collected four days post-anthesis (section 6.2.2.5). However, the sensitivity of the callus used in the bioassay was low and it did not respond to less than $15 \mu\text{g l}^{-1}$ kinetin. Consequently up to $7.5 \text{ ng KE grain}^{-1}$ could have been present in each fraction bioassayed.

Wheat grains extracted seven days after anthesis (17 days after ear emergence) contained cytokinin-like activity which corresponded in elution volume with the zeatin riboside standard (Fig 6.22). No cytokinin-

FIGURE 6.20

Wheat Field Trial 2. Soybean callus bioassay of extracts of 100 wheat grains collected 14 days after ear emergence. Extracts were resolved by partition chromatography on Sephadex LH-20

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 31 (50 ml) and fraction 32 (50 ml)

Callus: Bap (10)

Confidence limits: $n = 10$

Regression line: $Y = 0.90X + 0.06$

Lowest detectable amount of kinetin: $5 \mu\text{g l}^{-1}$ (459 ± 166 mg callus
flask $^{-1}$)

Further details are tabulated with Figure 6.2

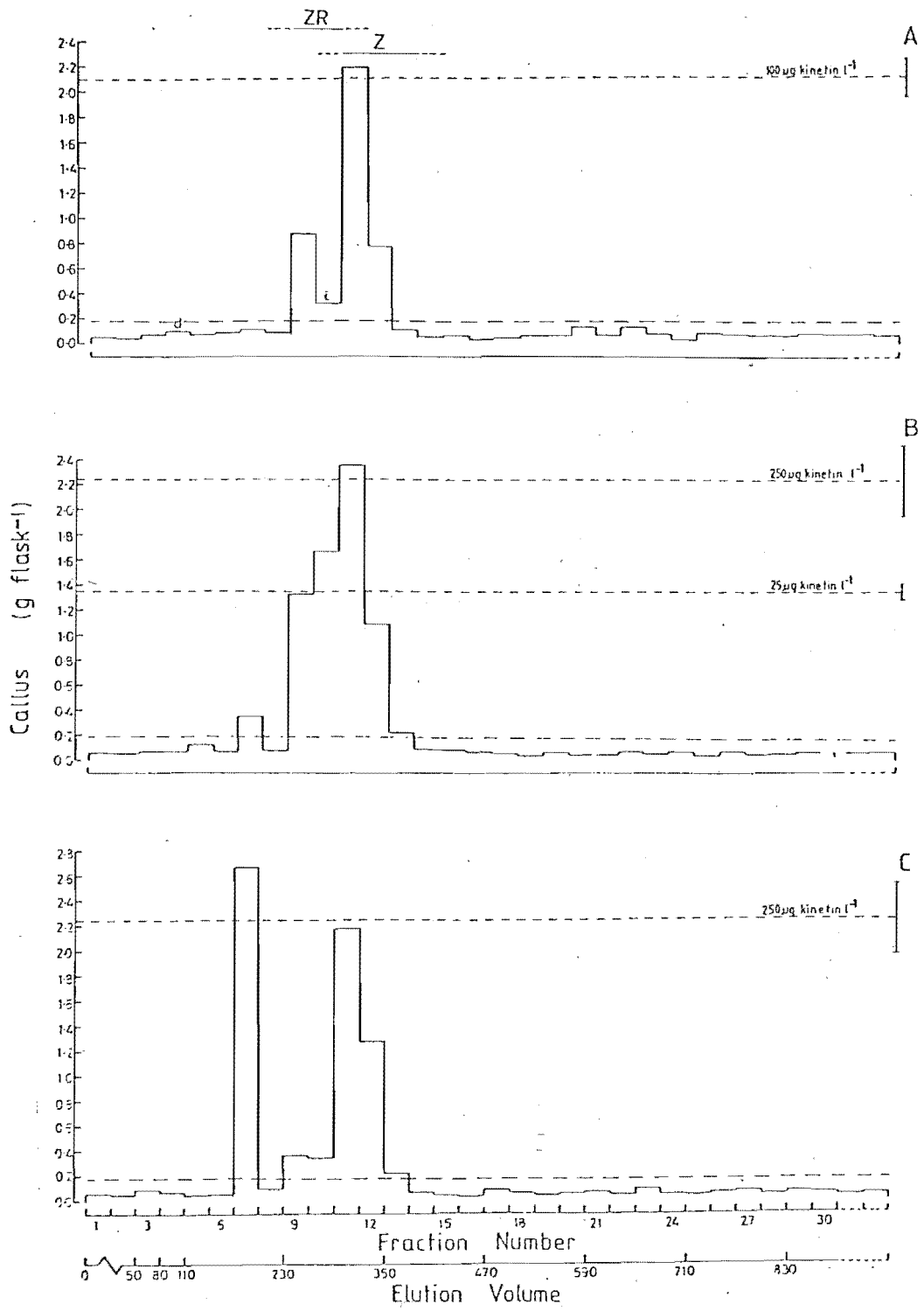


FIGURE 6.20

FIGURE 6.21

Heat Field Trial 2. Soybean callus bioassay of extracts of 100 wheat rains collected 14 days after ear emergence. Extracts were resolved by partition chromatography on Sephadex LH-20

Solvent: 20% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml)
and fraction 26 (260 ml)

Callus: Bap (34)

Confidence limits: $n = 10$

Regression line: $Y = 1.33X - 0.64$

Lowest detectable amount of kinetin: $1 \mu\text{g l}^{-1}$ ($560 \pm 79 \text{ mg callus flask}^{-1}$)

Further details are tabulated with Figure 6.2

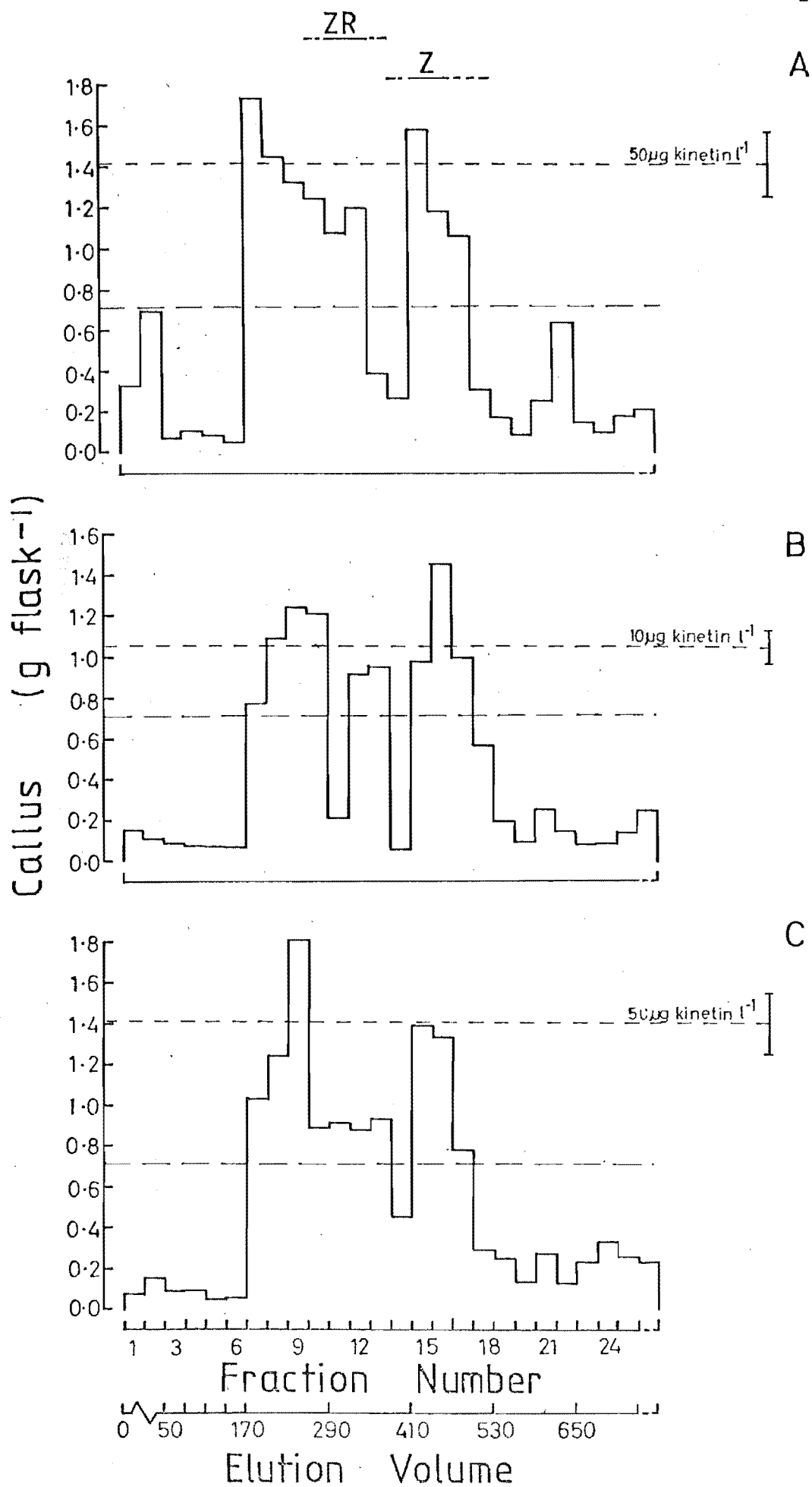


FIGURE 6.21

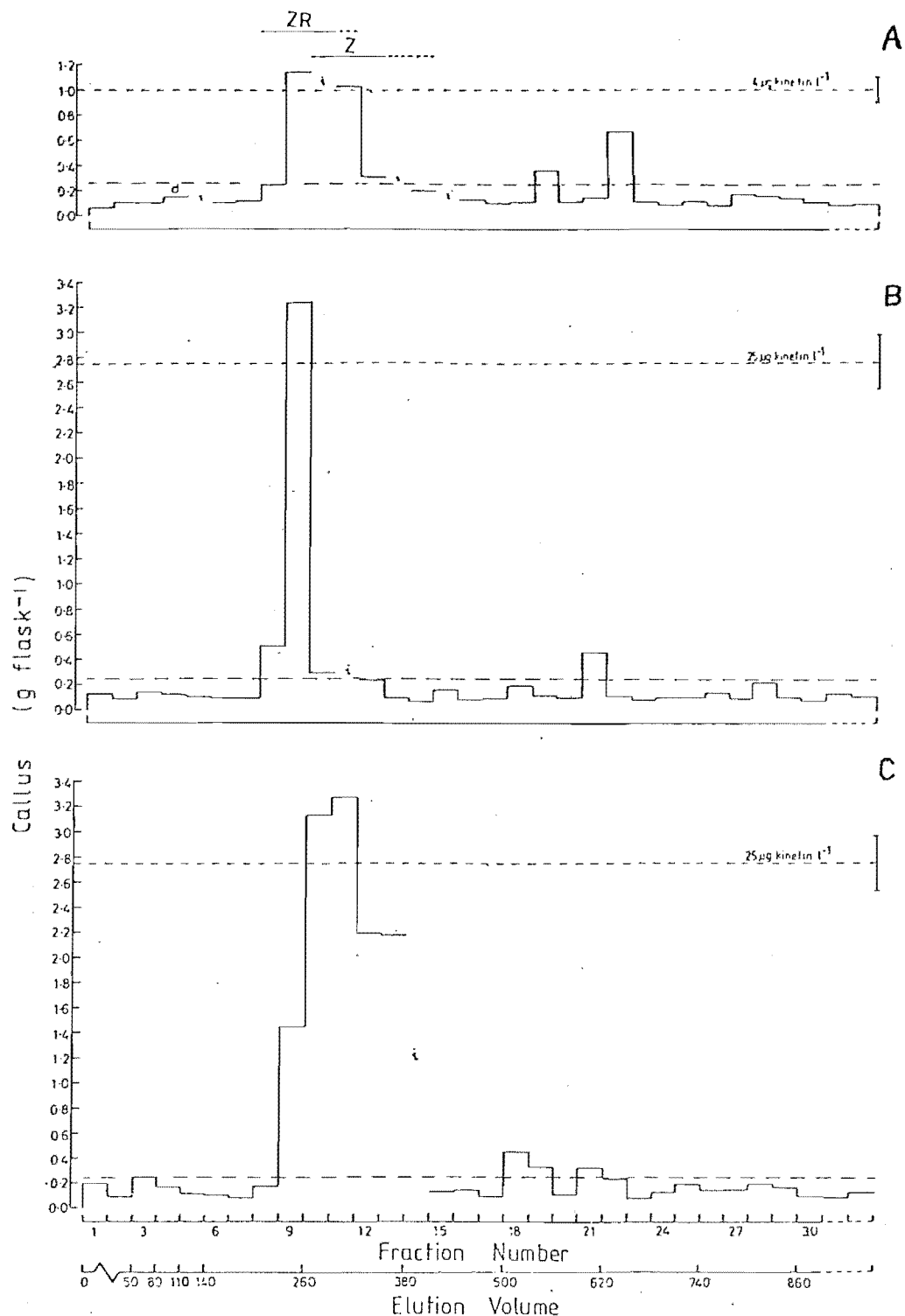


FIGURE 6.22

Wheat Field Trial 2. Soybean callus bioassay of extracts of 500 wheat grains collected 17 days after ear emergence (seven days post-anthesis)

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml)
fraction 31 (50 ml) and fraction 32 (50 ml)

Callus: Bap (7)

Confidence limits: $n = 10$

Regression line: $Y = 1.78X + 0.05$

Lowest detectable amount of kinetin: $1.5 \mu\text{g l}^{-1}$ ($409 \pm 164 \text{ mg callus flask}^{-1}$)

Further details are tabulated with Figure 6.2

like activity was detected eluting earlier than the zeatin riboside standard. Fractions which may have contained cytokinin-like activity eluting later than the zeatin riboside standard were contaminated and consequently discarded. Activity was, however, detected in fraction 11 in samples A and C and at much higher elution volumes in all three samples. The results are summarised in Table 6.21.

TABLE 6.20

Wheat Field Trial 2. Quantification of the cytokinin-like activity in extracts of wheat grains collected four days post-anthesis (14 days after ear emergence). The Sephadex LH-20 chromatography eluant was 20% ethanol.^a Values in kinetin equivalents were obtained from the equation of the regression line $Y = 1.33X - 0.64$

Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)					Total
	7 - 10	11	Fractions 12 - 13	14	15 - 18	
A	109.80	ca 5 ^b	16.67	ns ^c	48.54	180
B	62.63	ns	15.43	ns	70.43	148.49
C	112.21	ca 5	15.17	ns	63.34	196

^a Further chromatography and bioassay details are tabulated with Fig 6.21

^b Calculated by direct reference to the kinetin standards

^c ns = not significant

A second bioassay of wheat grains collected seven days after anthesis was set up with callus which proved to be unstable (Fig 6.23). However, all three extracts showed some activity in elution volumes similar to zeatin riboside and to zeatin both before and after β -glucosidase treatment of fractions 3 to 9. Critical fractions were lost through contamination.

Extract of 500 grains collected 11 or 18 days after anthesis did not contain consistently detectable levels of cytokinin-like activity (Figs 6.24 and 6.25, respectively).

No cytokinin-like activity was detected in extract of 250 grains harvested 51 days after ear emergence.

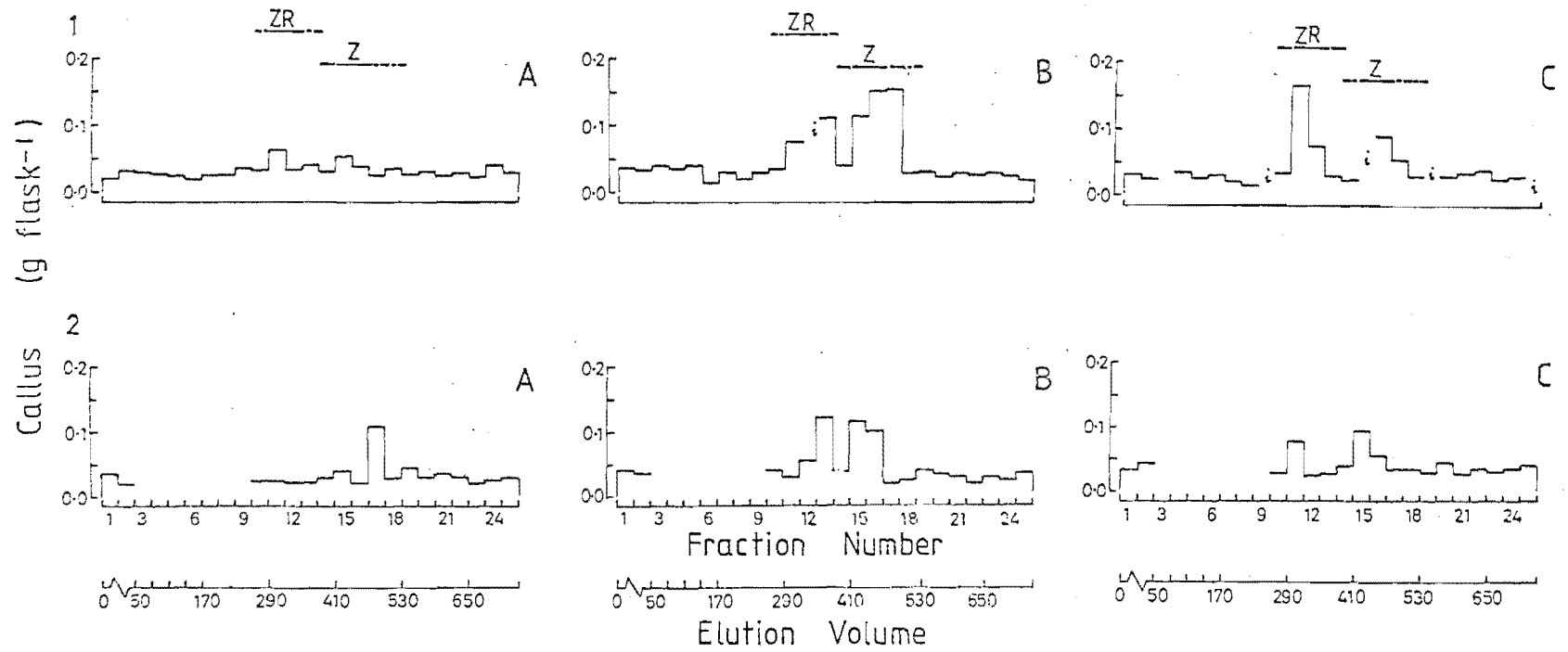
FIGURE 6.23

Wheat Field Trial 2. Soybean callus bioassay of extracts of 500 wheat grains collected 17 days after ear emergence. Extracts were resolved by partition chromatography on Sephadex LH-20.

1. Soybean callus bioassay fractions 3 to 9 after hydrolysis with β -glucosidase and further separation on Sephadex LH-20
2. Soybean callus bioassay of the remaining non-hydrolysed fractions

Refer to test for further information

FIGURE 6.23



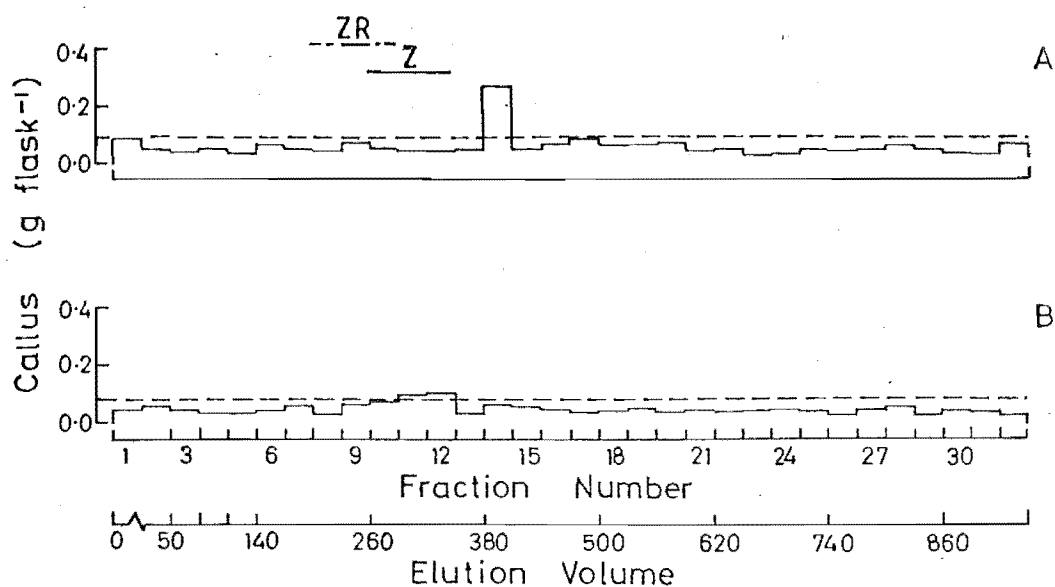


FIGURE 6.24

Wheat Field Trial 2. Soybean callus bioassay of extract of 500 wheat grains collected 21 days after ear emergence (11 days post-anthesis). Extracts were resolved by partition chromatography on Sephadex LH-20

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 31 (50 ml) and fraction 32 (50 ml)

Callus: Bap (13)

Confidence limits: $n = 10$

Regression line $Y = 0.84X + 0.02$

Lowest detectable amount of kinetin: $3 \mu\text{g l}^{-1}$ (226 ± 90 mg callus
flask⁻¹)

Further details are tabulated with Figure 6.2

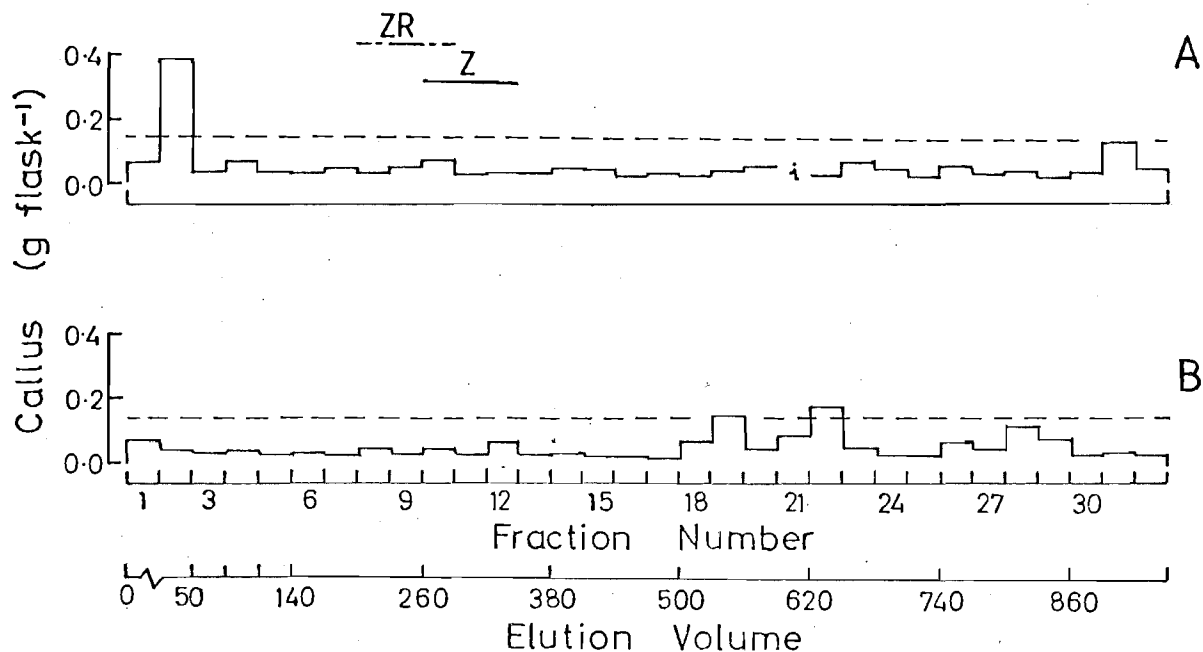


FIGURE 6.25

Wheat Field Trial 2. Soybean callus bioassay of extract of 500 wheat grains collected 28 days after ear emergence (18 days post-anthesis). Extracts were resolved after partition chromatography on Sephadex LH-20.

Solvent: 35% ethanol

Fraction volume: 30 ml with the exception of fraction 1 (20 ml),
fraction 31 (50 ml) and fraction 32 (50 ml)

Callus: Bap (14)

Confidence limits: $n = 10$

Regression line: $1.27X - 0.61$

Lowest detectable amount of kinetin: $2 \mu\text{g l}^{-1}$ (193 mg callus per
flask)

Further details are tabulated with Figure 6.2

TABLE 6.21

Wheat Field Trial 2. Quantification of the cytokinin-like activity in extracts of 500 wheat grains collected seven days post-anthesis (17 days after ear emergence).^a Values in kinetin equivalents were obtained from the equation of the regression line $Y = 1.78X + 0.05$

Sample	Cytokinin-like activity ($\mu\text{g KE l}^{-1}$)				
	7 - 8	9	10	11 - 13	Total
A	ns ^b	4.12	i ^c	3.53	7.65
B	ns	62.55	ns	i	62.55
C	ns	6.13	54.30	97.31	157.74

^a Further chromatography and bioassay details are tabulated with Fig 6.22

^b ns = not significant

^c i = contaminated flask

Changes in the total cytokinin-like activity in wheat grains with time are presented in Figure 6.26. The amount of cytokinin-like activity within the wheat grain increased rapidly after pollination which occurred nine days after ear emergence. The activity reached a peak 14 days after ear emergence (four days after anthesis) and dropped rapidly over the next three days. Cytokinin-like activity was barely detectable 11 days after peak anthesis.

The cytokinin-like activity increased rapidly on both a fresh weight and dry weight basis between pollination and anthesis (Fig 6.27). The amount of activity then fluctuated until it decreased rapidly seven days after anthesis and remained at a low level from 11 days after anthesis.

Changes in the relative levels of the three cytokinin-like compounds are presented in Figures 6.28 and 6.29. The activity eluting earliest was first detected at pollination. The amount per grain increased between pollination and anthesis and finally decreased to a low level seven days after anthesis. Activity with an elution volume similar to zeatin riboside was not detected until anthesis. A steady increase in this activity occurred until a maximum level was achieved four days after peak anthesis. A decline then occurred to undetectable levels by 11 days after anthesis. Activity with an elution volume similar to zeatin was detected at ear emergence but showed no marked increase until after pollination. The activity then increased very rapidly to a peak level four days after anthesis and this was followed by an equally rapid decrease

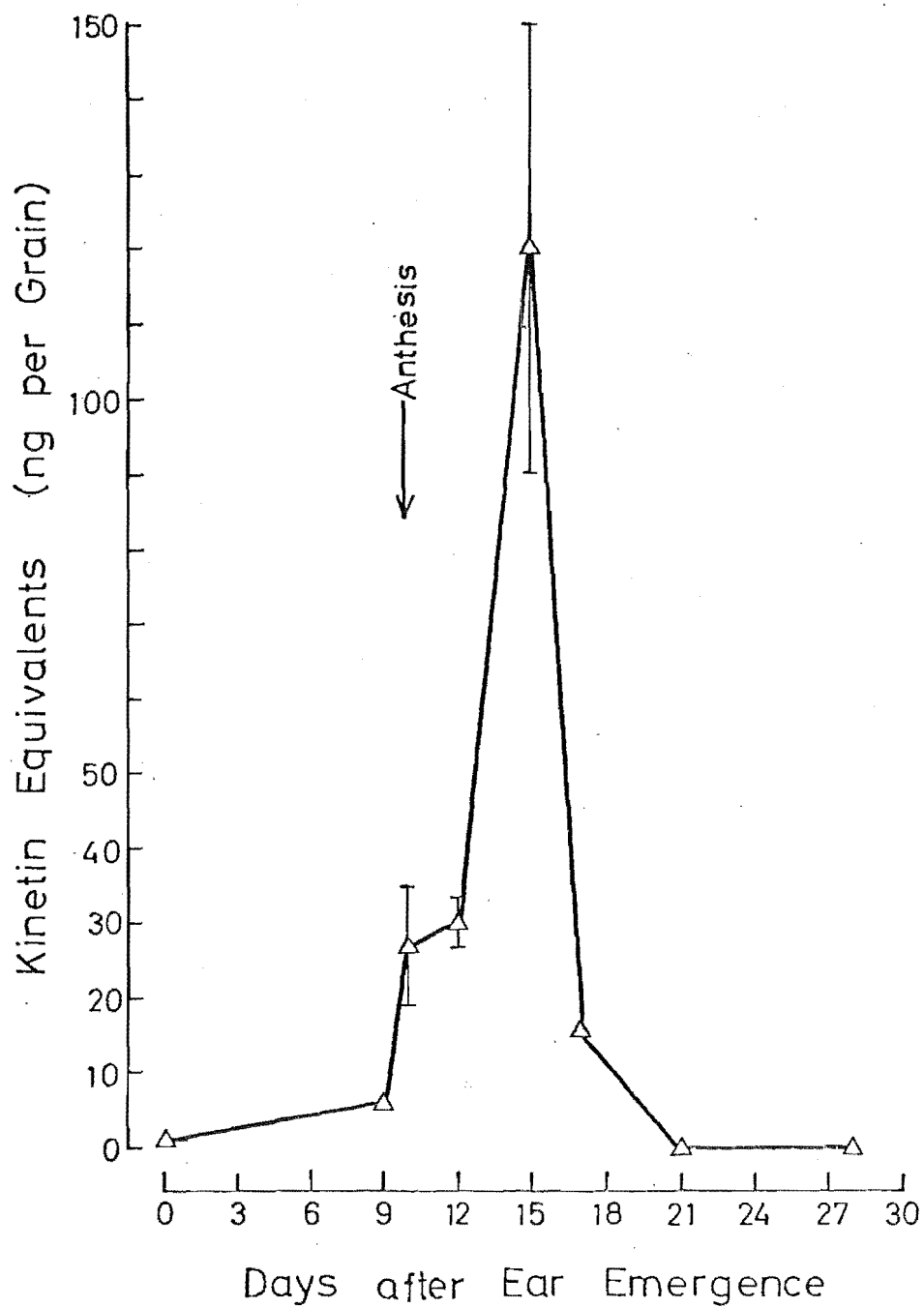


FIGURE 6.26

Wheat Field Trial 2. Total cytokinin-like activity in wheat grains from ear emergence to maturity. The data are expressed as kinetin equivalents per grain. Vertical bars show one standard error above and/or below the lines. The time of anthesis is indicated.

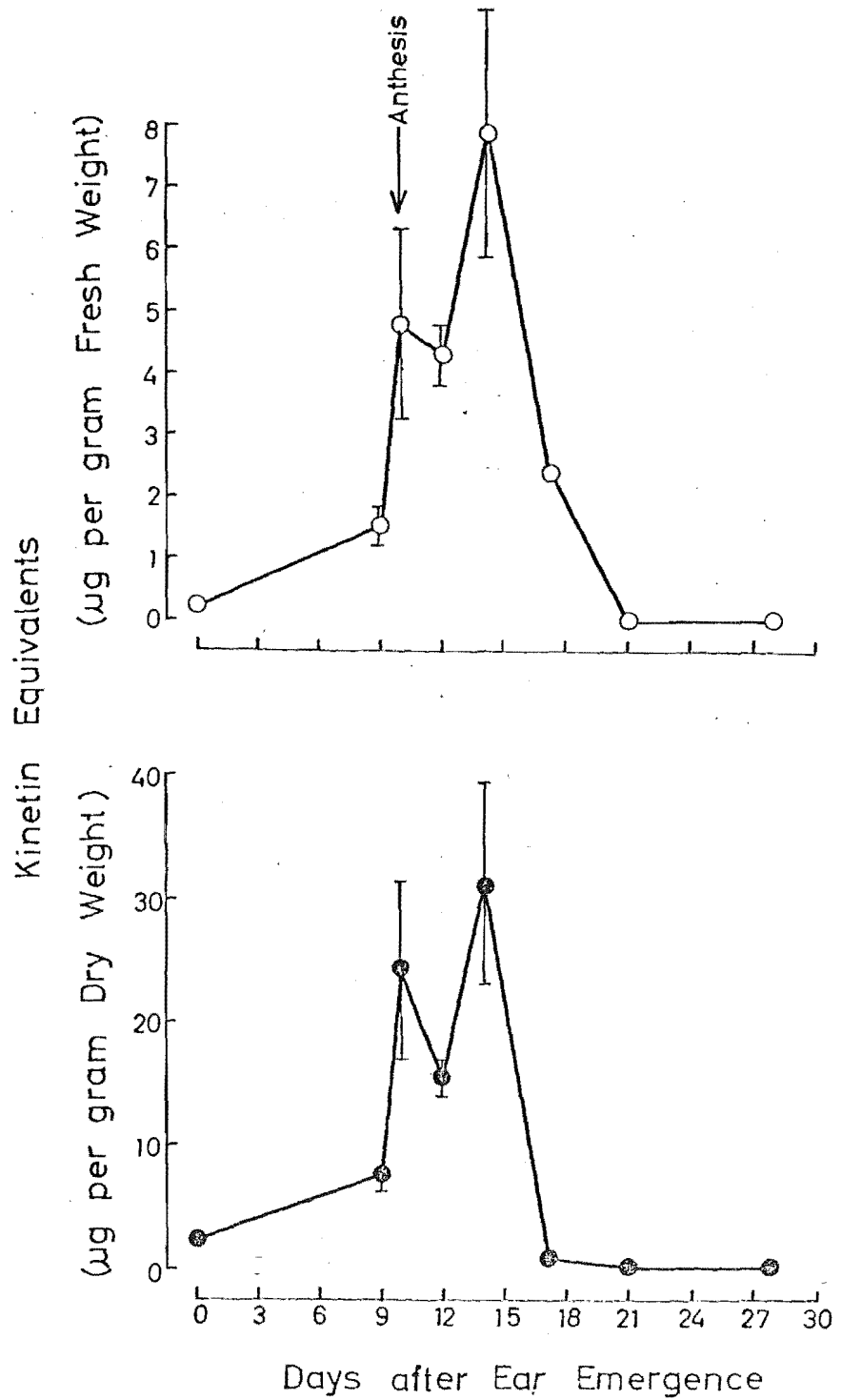


FIGURE 6.27

Wheat Field Trial 2. Total cytokinin-like activity in wheat grains from ear emergence to maturity. The data are expressed as kinetin equivalents either per gram dry weight or per grain fresh weight. Vertical bars show one standard error above and/or below the line. The time of anthesis is indicated.

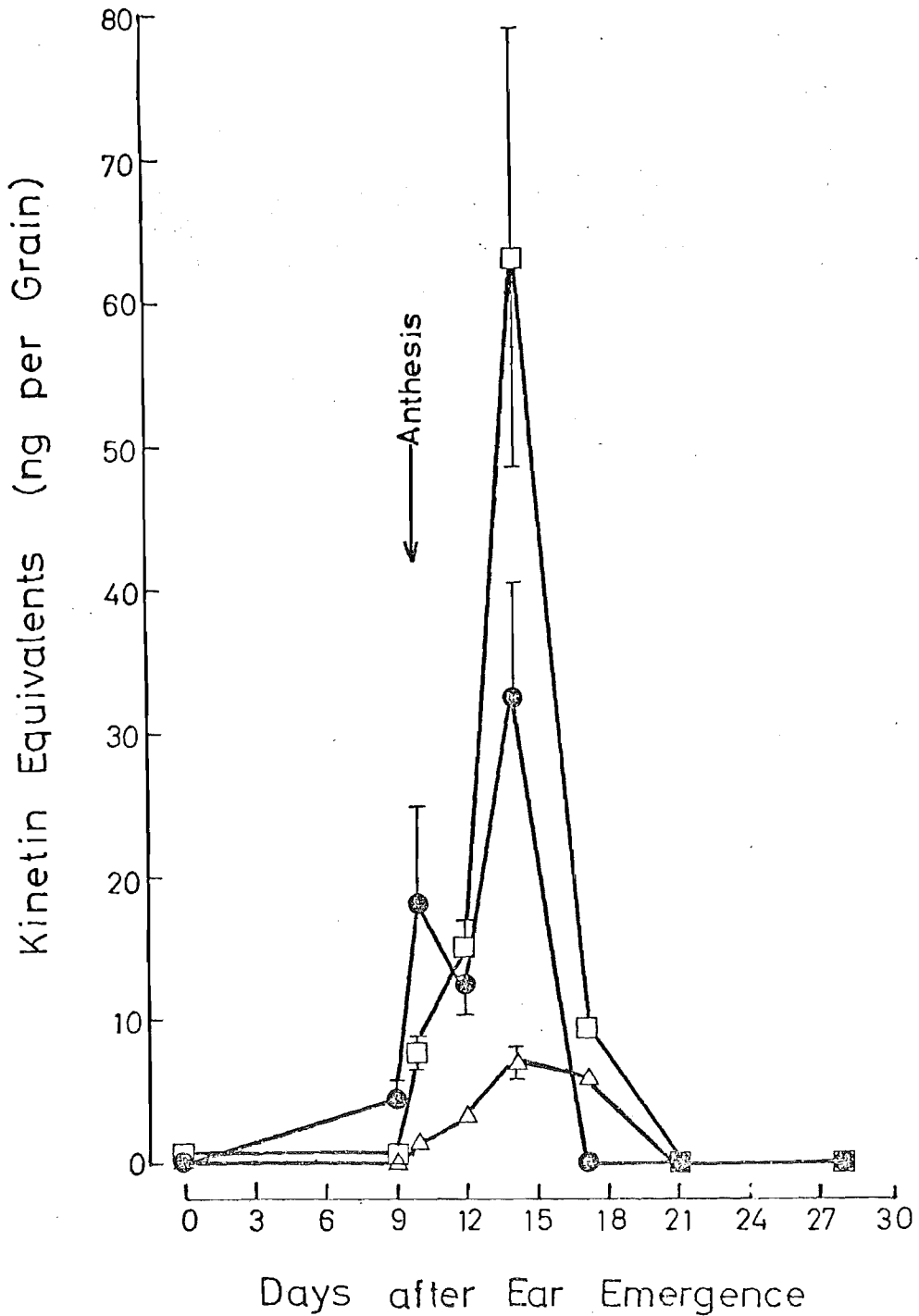


FIGURE 6.28

Wheat Field Trial 2. Changes in the activity of the cytokinin-like compounds in developing grains of wheat between ear emergence and maturity. The data are expressed as kinetin equivalents per grain. The circles represent cytokinin-*O*-glucoside activity, the squares zeatin-like activity and the triangles zeatin riboside-like activity. Vertical bars show one standard error above and/or below the line.

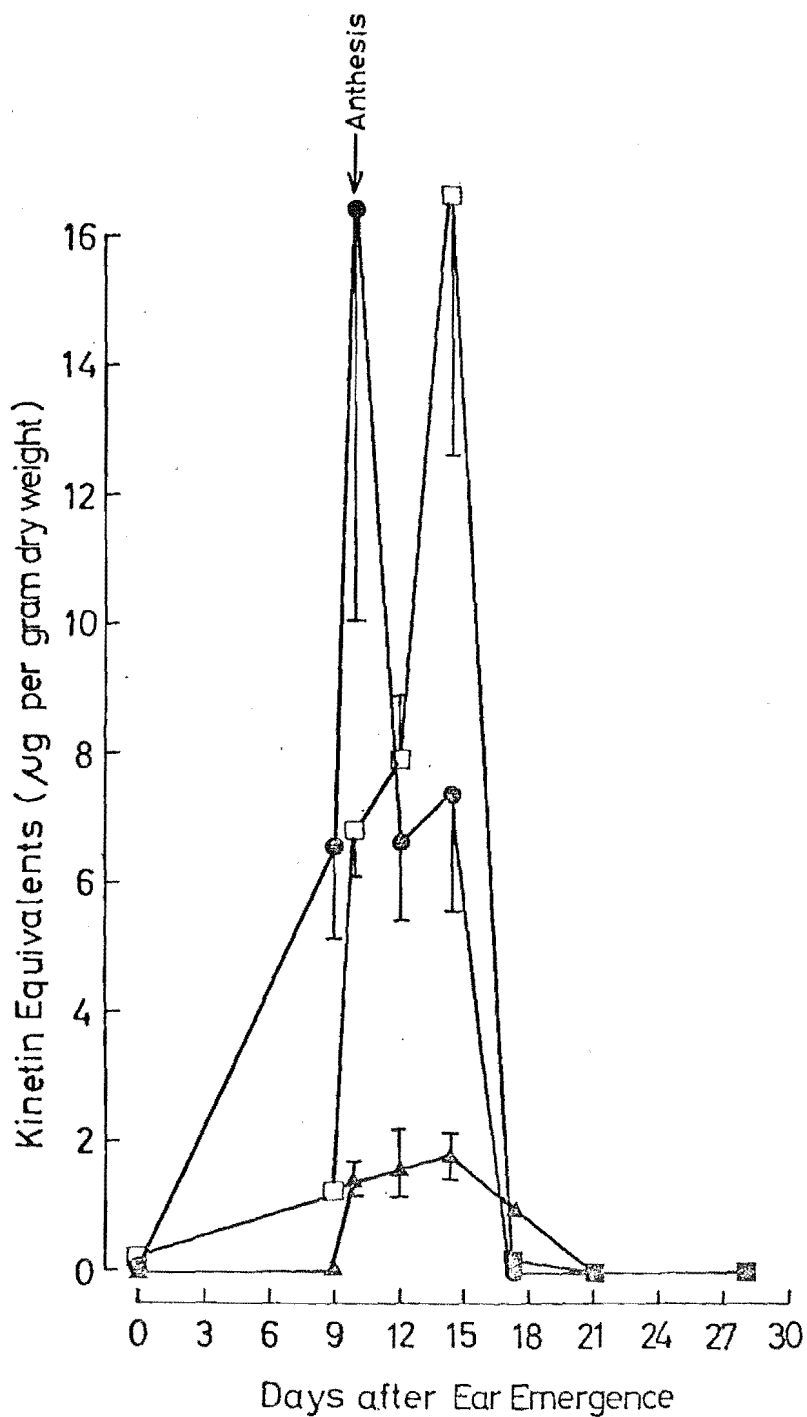


FIGURE 6.29

Wheat Field Trial 2. Changes in the activity of the cytokinin-like compounds in developing grains of wheat between ear emergence and maturity. The data are expressed in kinetin equivalents per unit dry weight. The circles represent cytokinin-*O*-glucoside activity, the squares zeatin-like activity and the triangles zeatin riboside-like activity. Vertical bars show one standard error above and/or below the line.

to an undetectable level by 11 days after anthesis.

On a per unit dry weight basis the earliest eluting cytokinin-like activity increased extremely rapidly until anthesis (Fig 6.29). Following anthesis the amount of this activity decreased to a level which remained constant until four days after anthesis after which it decreased rapidly to a low level. On the same basis the amount of zeatin riboside-like activity increased from pollination to anthesis, remained relatively constant until four days after anthesis and then decreased gradually to a non-detectable level 11 days after anthesis. A rapid increase in zeatin-like activity per unit dry weight occurred from pollination until four days after anthesis. This was followed by a sharp decrease to a low concentration seven days after anthesis.

6.4 DISCUSSION

The development within the ear of a wheat plant is not uniform. The central spikelets reach anthesis earlier than the upper or lower spikelets (*eg* Percival, 1921, cited by Asana and Bagga, 1966; Rawson and Evans, 1970; Pinthus and Millet, 1978). Development within a spikelet is also progressive. Rawson and Evans (1970) noted that there was a four day interval between anthesis in the first and fourth florets, with anthesis occurring progressively from basal to upper florets within a spikelet (*eg* Percival, cited by Asana and Bagga, 1966; Rawson and Evans, 1970; Pinthus and Millet, 1978; Simmons and Crookston, 1979). Anthesis within a whole ear normally takes longer than five days (Evans *et al.*, 1972). Grains in the middle spikelets of the ear grow faster than those in apical or basal spikelets and grains in more distal florets tend to grow faster than in basal florets (Sofield *et al.*, 1977, and references cited therein; Simmons and Crookston, 1979). At maturity, grains in the central spikelets are larger than equivalent grains in basal spikelets, these in turn usually being larger than grains in upper spikelets. Within central spikelets, at least, grain weight increases from the basal floret to the second, and decreases thereafter to the apical floret (*eg* Bremner and Rawson, 1978, and references cited therein; Pinthus and Millet, 1978; Simmons and Crookston, 1979). Simmons and Crookston (1979) noted that kernels formed in the proximal florets had established a substantial weight advantage over the distal kernels even before the linear dry matter accumulation period (Fig 6.8).

All the above features were considered when grains were selected for analysis although the actual age of the grains selected for cytokinin

analysis was based on the reports by Wheeler (1972, 1976) who suggested that the cytokinin content of wheat grains was maximal at the end of anthesis. Although the rows from which the three samples were collected at each harvest were randomly assigned, sampling of the ears themselves within the rows was subjective. Only primary and secondary tillers were selected and at the time of dissection of the spike any obviously more or less advanced ears were discarded. Only the central six spikelets were retained and flash frozen in liquid air. Following freeze drying, when the two most basal grains in each floret were being removed prior to extraction, any obviously larger or smaller grains in a sample were discarded. These precautions were taken to ensure that each sample of grains was at a similar developmental stage. The importance of this was emphasised by Wheeler (1976) who showed that changes in cytokinin-like activity within the upper, middle and lower thirds of an ear were not in phase.

Although removal of stigmas was not possible at ear emergence and pollination, and hence the entire pistil was extracted, care was taken to remove anthers prior to extraction. The presence of cytokinin-like compounds in *Cymbidium* anthers was reported by van Staden (1979).

For the reasons discussed previously, with relation to potato tubers (pp 151-155) it was again decided to follow a basic extraction and purification technique and to employ column chromatography to resolve the cytokinin-like activity in extracts of wheat grains. Incomplete partitioning of highly polar cytokinin-like activity prior to paper chromatography was apparent in extracts of grains at anthesis (Fig 6.4).

The same general pattern of cytokinin activity changing with time and peaking shortly after anthesis shown by Wheeler (1972) and Herzog and Geisler (1977) for wheat grains was verified following extraction and bioassay of grains obtained from the first field trial (Fig 6.7). Wheeler (1976) suggested that the peak of cytokinin-like activity in the middle third of the ear occurred within seven days of anthesis, since the sample at seven days showed low amounts of cytokinin-like activity. The cytokinin-like activity in grains from the middle third of the ear was highest in grains sampled three days after anthesis (Fig 6.7) and is in agreement with Wheeler's supposition. Wheeler (1972, 1976) suggested that the most abundant cytokinin after anthesis exhibited highly polar characteristics and in this study it was noted that some of the cytokinin-like activity in the grains eluted from the column faster than zeatin riboside (Figs 6.2 to 6.5). The actual amount of this

activity, however, was frequently inconsistent among the samples (*eg* Fig 6.3).

It was apparent that wheat grains developed very rapidly following anthesis and that similarly rapid changes were occurring in the cytokinin content of the grain. More samples were selected during the second harvest. The sampling was timed to coincide with the specific developmental events within the floret and ovule: pollination, fertilisation, free nuclear divisions, cellularisation and cell division (*eg* Bennett *et al*, 1973; Morrison and O'Brien, 1976).

The initial cytokinin analysis was performed on 500 grain samples and the chromatography eluant was 35% ethanol. The same general pattern was observed as at Harvest 1 with the total cytokinin content of the grains reaching a peak shortly after anthesis followed by a rapid decline (Fig 6.26). However, the relative levels of some of the individual components was less well defined and a more detailed analysis of the cytokinin-like activity was performed on grains from selected harvest dates.

Wheeler (1972) suggested that two cytokinin-like compounds were present in extracts of grains from whole ears at ear emergence, the first exhibiting properties similar to zeatin. However, the second component, which was reported to have more cytokinin activity, was a highly polar compound. A similar cytokinin complement was not detected in grains collected at ear emergence during Field Trial 2 (Fig 6.11). Cytokinin-like activity was present in fraction 11 which would be expected to contain zeatin but no highly polar components were detected. There was, however, a noticeable increase in the total cytokinin-like activity at pollination, mainly due to the appearance of a second component eluting from the column more rapidly than the zeatin riboside standard. In later work, albeit using extracts of ears rather than grains, Wheeler (1976) reported that the most abundant cytokinin at ear emergence (seven days pre-anthesis) exhibited chromatographic properties similar to zeatin. It is possible that the discrepancy in the results reported by Wheeler (1972, 1976) is due to ears being selected at slightly different developmental stages in 1972 and 1976.

Wheeler (1972) suggested that the majority of cytokinin-like activity in grains sampled from whole ears after anthesis was due to a highly polar cytokinin and this was also noted in extracts from whole ears (Wheeler, 1976). However, in this thesis a compound of this type was not observed consistently. Extracts of 500 grains collected two days after anthesis contained large amounts of cytokinin-like activity

(Fig 6.14) but the amount of activity detected in fraction 7 in the three samples was quite different. To determine whether the variation was between the independent samples or the result of technique, three replicate extracts were resolved by column chromatography and 10 ml fractions bioassayed (Fig 6.15). It was apparent that the major active component exhibited chromatographic properties similar to zeatin while only a relatively small proportion of the activity had a similar elution volume to the zeatin riboside standard. Again, the amount of activity detected eluting faster than zeatin riboside was highly variable. It appeared that either the amount of activity present was at such a level as to be inhibitory to the callus growth (Palni and Horgan, in Press) or material capable of masking the callus response was co-eluting with the cytokinin-like activity. However, the observation that some pieces of callus were blackened or dark-brown suggested more strongly the latter possibility.

When samples of only 100 grains were extracted, cytokinin-like activity was detected in both fractions 6 and 7 in all three samples (Fig 6.16). To achieve more complete resolution of the components the extracts were resolved on Sephadex LH-20 eluted with 20% ethanol (Dyson and Hall, 1972; Smith and van Staden, 1978). Again, there was more cytokinin-like activity with chromatographic properties similar to zeatin than to zeatin riboside but equally noticeable was the consistent detection of cytokinin-like activity eluting rapidly from the column (Fig 6.17). ABA was shown to co-elute with this activity (Fig 6.17). However, low levels of ABA may actually have a positive synergistic rather than an antagonistic interaction with cytokinins in the soybean callus bioassay if the synergistic effect noted between kinetin and ABA on the growth of soybean callus (Blumenfeld and Gazit, 1970a) is applicable to cytokinins generally. Abscisic acid is reported to be at a very low level in grains immediately after anthesis (King, 1976) and, consequently, is unlikely to be causing the observed inhibition of callus growth (eg Fig 6.15).

Since the polar cytokinin-like activity was not eluting immediately after the void volume it was unlikely to represent nucleotide activity (see p. 152). However, it has been suggested that the *O*- β -D-glucosides of both zeatin and zeatin riboside and their dihydro derivatives elute from a Sephadex LH-20 column faster than zeatin riboside (eg Stuchbury *et al*, 1979; Palni and Horgan, in Press). In addition, Scott *et al* (1980b) have reported that zeatin-*O*-glucoside, zeatin-9-glucoside and zeatin-7-

glucoside all have similar mobilities on Sephadex LH-20 eluted with 35% ethanol.

It is generally considered that cytokinin-like compounds which are readily hydrolysed by β -glucosidase are the side chain glucosides (eg van Staden and Davey, 1979; Palni and Horgan, in Press). Since the 7- and 9-glucosides of zeatin have been reported to be relatively resistant to β -glucosidase (Deleuze *et al*, 1972; Letham *et al*, 1975; Parker *et al*, 1975). It is probable that the *o*- β -D-glucosides of both zeatin and zeatin riboside (and possibly also of their dihydro derivatives) are present in developing wheat grains because treatment with β -glucosidase and subsequent chromatography revealed that the single polar peak had been resolved into two components with elution volumes corresponding with zeatin riboside and zeatin standards, respectively (Fig 6.17).

The bioassay response to the cytokinin-like compounds released by the β -glucosidase treatment was greater than to the untreated compounds (Table 6.15). Authentic samples of the side-chain glucosides were not available to test the relative activities of these cytokinins against zeatin and zeatin riboside in the soybean bioassay, and it is possible that the observed increase may simply be due to the greater activity of zeatin and zeatin riboside in the soybean callus bioassay as indeed has been suggested by van Staden and Papaphilippou, 1977. In addition, a shifting of the cytokinin-like activity away from the effect of inhibitory compounds may partly account for the increased activity detected.

Cytokinin nucleotides would not be extracted in a quantitative manner by the procedures used routinely (see p 151). Consequently an attempt was made to determine if there was nucleotide activity present by using the experimental technique developed by Bielecki (1964) which reportedly inhibits the activity of non-specific phosphatase enzymes. However, the technique was not used on freshly frozen material, but on material flash frozen, freeze dried and subsequently stored at room temperature (section 6.2.2.4) although the grains were chilled to -20°C prior to immersion in the solvent. No cytokinin-like activity was detected in fractions eluting immediately after the void volume. Jenner (1968a) suggested that there was a high level of phosphatase in wheat grains, but also commented that Bielecki's solvent cocktail may not penetrate the relatively bulking grains, collected 10 and 20 days post-anthesis, rapidly enough to prevent degradation of ATP and UTP. If the cytokinin nucleotides had been metabolised prior to the inactivation of phosphatase enzymes, the relatively small proportion of activity detected

in the fractions expected to contain zeatin riboside would indicate that there could have been, initially, only a similarly small amount of nucleotide present.

The total amount of cytokinin-like activity extracted by 80% methanol and by Bieleski's procedure appeared to be similar (Table 6.16) although the poor standard curve must lead to some reservation concerning quantification especially since Parker and Letham (1973) suggested that less activity (measured as radioactivity) was extracted by Bieleski's procedure than by 80% methanol.

The chromatographic properties of the cytokinin-like activity in samples extracted by Bieleski's procedure appeared quite different to that extracted by 80% methanol, with only small amounts of activity being detected in fractions 7 or 8 (Fig 6.18). A substantial amount of the activity was present in fractions normally expected to contain zeatin riboside and zeatin. Moreover all the cytokinin-like activity was collected by fraction 12 in the control samples, but activity was detected in fractions 13, 14 and 15 in samples extracted using Bieleski's procedure. There would appear to be a "loss" of detectable activity in fractions normally containing the side chain glucosides and an "increase" in activity at greater elution volumes if Bieleski's solvent is used for the initial extraction. Vonk and Davelaar (1981) suggested that the apparent loss of cytokinin glucoside activity during the extraction of *Yucca* leaves (Vonk, 1979) was the result of hydrolysis of *O*- β -D-zeatin glucoside caused by low pH during extraction whereas Palni (pers comm) suggested that the side chain glucosides were quite stable during extraction using Bieleski's procedure. In addition van Staden (1976d) reported that the cytokinin-*O*-glucosides were not hydrolysed during ion-exchange chromatography using Dowex 50 (H^+ form), a procedure which has been reported to promote hydrolysis of zeatin riboside (see p 154), and Yokota *et al* (1981) suggested that while OGZR may degrade to OGZ during purification little degradation of the glucosides themselves occurred.

Alternatively, samples extracted using Bieleski's procedure may have had a different pH when applied to the column and consequently may have altered the chromatographic properties of the column. This might also explain the reduced resolution and the delayed elution. However, a substantial increase in the amount of zeatin and zeatin riboside (as the result of hydrolysis of the respective glucosides) could also have led to poor resolution and a similar tailing of the zeatin

peaks. This problem needs further evaluation as Bielecki's procedure is recommended for the extraction of cytokinins from plant tissues (Horgan, 1978b; Letham, 1978).

The highest cytokinin levels were detected four days after anthesis. The cytokinin-like activity in extracts of 500 grains was poorly resolved but consistently good callus growth was stimulated by compounds in fraction 7 (Fig 6.19), whereas if only 100 grains were extracted the amount of the activity detected in fraction 7 was quite variable (Fig 6.20). This tends to confirm the suggestion that variable detection was due to the presence of interfering compounds rather than to a concentration of cytokinin inhibitory to callus growth. Moreover, van Staden and Papaphilippou (1977) suggested that comparatively high levels of *O*- β -D-zeatin glucoside were less toxic than zeatin or zeatin riboside to soybean callus.

Baseline resolution of the three cytokinin-like components was not achieved in all three samples following chromatography using 20% ethanol (Fig 6.21), although the results confirmed those obtained with 35% ethanol (Fig 6.20). Generally the amount of the cytokinin-like activity present in the fractions expected to contain zeatin riboside was small relative to the other two components. It was apparent also that substantial amounts of the putative side chain glucosides were present four days after anthesis. However, the callus used to test for the presence of activity following β -glucosidase treatment became autotrophic for cytokinin and no information was obtained. However, Wheeler's statement that the polar compound is maximal after anthesis is not supported (Figs 6.27, 6.28).

Although zeatin, zeatin riboside and the side-chain glucosides can be separated on Sephadex LH-20, these compounds do not readily separate from their respective dihydro derivatives in either 35 or 20% ethanol (*eg* Davey and van Staden, 1978 ; Smith and van Staden, 1978; van Staden and Dimalla, 1980; Palni and Horgan, in Press). However, their presence can be distinguished following permanganate oxidation (*eg* Sondheimer and Tzou, 1971). This procedure cleaves the allylic double bond in the side chain of the cytokinin molecule and consequently eliminates the activity of the compound in the bioassay (*eg* Miller, 1965; Davey and van Staden, 1978a). Meanwhile, the activity of the dihydro derivatives remains unaffected (*eg* Sondheimer and Tzou, 1971; Davey and van Staden, 1978a,b). Gordon *et al* (1974) suggested that there were compounds present in relatively unpurified extracts which may interfere with the oxidation of cytokinins. It was found with the wheat samples

that 10 ml of 0.1% potassium permanganate were required before a pink colour persisted for more than 30 seconds. Obviously care must be taken to ensure complete oxidation before chromatography and bioassay. Samples of wheat collected four days after anthesis were subjected to permanganate oxidation. The callus used in this bioassay was quite insensitive and did not respond to kinetin below $15 \mu\text{g l}^{-1}$. No cytokinin-like activity was detected during the bioassay of permanganate-treated samples. However, it cannot be concluded that dihydro derivatives were not present but simply that, of the total activity detected in 100 g wheat grains (Table 6.19), less than one quarter could be present as the dihydro derivatives, assuming similar activities in the soybean callus bioassay (Henson, 1978).

Wheat grains collected seven days following anthesis still contained readily detectable amounts of cytokinin-like activity although in the initial bioassay, several key fractions were lost through contamination (Fig 6.22). No activity eluting faster than the zeatin riboside standard was detected, although the presence of compounds inhibiting callus growth must be considered. A subsequent bioassay using callus of limited sensitivity indicated that cytokinin-like activity was present in fractions expected to contain zeatin and zeatin riboside (Fig 6.23). The presence of cytokinin-*O*-glucosides was shown indirectly following the treatment of fractions 3 to 9 with β -glucosidase and the detection of cytokinin-like activity with similar elution volumes to zeatin riboside and zeatin (Fig 6.23). The callus in the flasks containing the standards was autotrophic for cytokinin so little comment can be made concerning the amounts of cytokinin-like activity present. A substantial decrease in the total amount of cytokinin-like activity seven days after anthesis was observed in Field Trials 1 and 2 (Figs 6.7 and 6.26, respectively) although the total amount of activity is uncertain.

In some extracts, small amounts of cytokinin-like activity were detected which exhibited chromatographic properties similar to isopen-tenyladenine (eg Fig 6.13, samples A and C; Fig 6.18, replicates A and B; Fig 6.22, samples A, B and C). However, this and other small peaks of activity detected (eg Fig 6.22) comprise little of the total cytokinin-like activity in any sample.

Figure 6.26, representing the total amount of cytokinin-like activity during early grain development, has been compiled from the average of several determinations at each harvest date. The value at ear emergence represents the minimum cytokinin content (see p 222) while the values at nine and 10 days after ear emergence were obtained from the average total values

in Tables 6.9 and 6.10, respectively. Six values were used to calculate the total average cytokinin content 12 days after ear emergence. These were obtained from Table 6.12 and 6.13 and are in close agreement.

The total value obtained four days post-anthesis was from nine samples (Tables 6.17, 6.18, 6.19). Two fractions from sample A (Fig 6.19) were run in a different bioassay, but indicate substantial amounts of cytokinin-like activity (Table 6.1). However, the total value must represent a minimum because of the variability in fraction 7 (Table 6.20). Regardless of the variability between samples analysed four days post-anthesis (Tables 6.17 to 6.19), each indicates independently that these grains contained the highest cytokinin content of all the samples dissected in the field and subsequently analysed.

The value used 17 days after ear emergence is that of sample C (Table 6.20) as key fractions are missing from the other samples (Fig 6.22).

Incomplete resolution of many samples following column chromatography has made it more difficult to assign accurate values to the three major components contributing to the total cytokinin-like activity. However, where good resolution has been obtained it is apparent that activity with similar chromatographic mobilities to zeatin riboside was substantially less at all harvest dates relative to the slower moving band of activity expected to contain zeatin and its dihydro derivative (*eg* Fig 6.12, sample A; Fig 6.15; Fig 6.17; Fig 6.20, samples A and C; Fig 6.21, sample B). The possible exception is that of samples selected seven days after anthesis (Fig 6.22).

Activity present in fraction 9 (35% ethanol) was ascribed to zeatin riboside and its dihydro derivative but in some instances this may have included some side chain glucoside activity. Attributing activity in sample 10 to zeatin and its dihydro derivative may have resulted in some underestimation of zeatin riboside and dihydrozeatin riboside activity.

A very noticeable decrease in the cytokinin content of wheat grains, both on a per grain (Fig 6.26) and per unit weight basis (Fig 6.27), occurred between four and seven days after anthesis. A similarly sharp decrease was not noted by Wheeler (1972) but this could be attributed to the extraction of grains from whole ears as sections of ears were later shown to contain different cytokinin levels depending on their position (Wheeler, 1976).

Although Herzog and Geisler (1977) mentioned taking grains of the same mean physiological status from parts of ears for the determination

of gross yield there was no definitive statement that a similar selection of grains was used for cytokinin analysis. The increase in cytokinin-like activity close to anthesis on both a fresh weight and per kernel basis was pronounced. On a fresh weight basis the activity decreased rapidly to very low but detectable levels within seven days of the peak cytokinin activity, while on a per grain basis the decrease in activity from seven days after the peak was less pronounced.

Dua and Bhardwaj (1979) detected only a trace of cytokinin-like activity in ears within two days of anthesis, whereas ears sampled at eight days contained more activity than those sampled at four days. This difference may be related simply to the range of stages present within an individual ear.

The total cytokinin content per grain did not increase in the two days following anthesis (Fig 6.26) and in fact decreased when calculated on a per unit weight basis (Fig 6.27). This decrease is attributable to a decrease in detectable glucoside activity (Figs 6.28, 6.29). If the values obtained from Tables 6.12 and 6.13 are replaced by those obtained following β -glucosidase treatment (Table 6.14, samples A and B), a slight increase is then observed in the glucoside content on a per grain basis within two days of anthesis. However, a decrease is still observed on a per unit weight basis indicating that the concentration of glucoside activity does decrease shortly after anthesis and the observed decrease is not solely the result of inhibitor interference in the bioassay.

However, the presence of inhibitory material may prevent detection of cytokinin-like activity from 11 to 18 days after anthesis if, in fact, the majority of cytokinin-like activity detected by Wheeler (1972) after anthesis is attributable to side-chain glucoside activity.

Dry matter accumulation in wheat grains is reported to begin three to five days after anthesis and requires several more days to achieve its maximum rate (*eg* Jenner, 1979 and references cited therein). Following the initial lag after anthesis, the dry weight of grains selected from the first or second florets of the central spikelets is reported to increase at a constant absolute rate for a considerable time (*eg* Sofield *et al*, 1977; Pinthus and Sar-Shalom, 1978; Simmons and Crookston, 1979). A similar lag followed by a very rapid increase was noted in the dry weight of the two basal grains of the six central spikelets collected during Field Trial 2 (Fig 6.8). A more pronounced linear increase was noted for the fresh weight beginning very soon after anthesis and is in agreement with Rijven and Cohen (1961) and Donovan *et al* (1977a).

The maximum dry weight of the grains was attained about the time the fresh weight began to decrease as found by others (*eg* Turner, 1969; Simmons and Crookston, 1979). However, Rijven and Cohen (1961) suggested that the maximum dry weight was attained shortly after the peak fresh weight, although this may be a feature of variety (*eg* Donovan *et al*, 1977a).

Few chemical analyses of either the soluble sugar or starch content of wheat grains have been performed on grains immediately pre- and post-anthesis and the apparent increase in starch as a proportion of the dry weight immediately following anthesis has not been reported (Fig 6.9).

Sandstedt (1946) reported that minute starch granules were present in the unfertilised wheat ovary and this was confirmed by chemical analysis (although the value obtained was for extraction of the entire pistil). Fischer and Stockman (1980) reported an increase in the starch content of carpels between nine and one day pre-anthesis.

The initial growth following fertilisation is reported to occur in the maternal outer pericarp and the rest of the grain coat (*eg* Rijven and Cohen, 1961; Jennings and Morton, 1963a). Sandstedt (1946) indicated that starch developed rapidly in the pericarp but that enzymatic digestion of this starch was apparent within four to five days after fertilisation. Jenkins *et al* (1974) showed that a mass of small amyloplasts was present in the pericarp 18 days after ear emergence (ca nine days post-anthesis), while Jenner (1968b) suggested that the amount of starch in the pericarp increased slightly between five and eight days after anthesis and then remained constant.

Jennings and Morton (1963a) suggested that the amount of starch in the testa-pericarp remained relatively constant from ca five days after anthesis (the first sampling date) although as a proportion of the dry weight it declined with time. However, Sandstedt (1946) suggested that the pericarp was practically devoid of starch within 16 days of pollination and Campbell *et al* (1981) suggested that by 27 days post-anthesis the pericarp was devoid of starch.

Jenner and Rathjen (1978) considered that dry matter accumulation in the endosperm did not begin at an appreciable rate until the pericarp was almost fully grown and after analysis of the endosperm with the pericarp removed it was suggested that there was little starch present in the endosperm until 17 days after flowering (Jenner and Rathjen, 1977), although Jennings and Morton (1963a) suggested that the endosperm starch, as the amount per endosperm, increased rapidly and almost linearly from about 12 to 35 days after flowering, and as a percentage of the

endosperm dry weight, reached a maximum about Day 35. However, it has been shown that starch granules are present in endosperm cells by five to six days after anthesis (*eg* Sandstedt, 1946; Mares *et al*, 1977; Briarty *et al*, 1979; Campbell *et al*, 1981).

Starch accumulation in whole grains from Field Harvest 2, was rapid between 11 and 25 days after anthesis and became a major component of the dry weight about Day 32, a pattern similar to that noted by Kumar and Singh (1980). On a per grain basis, Turner (1969) noted that the content was low at the first analysis, five days post-anthesis, and that there was little change till between 14 to 19 days after which starch accumulated rapidly till 33 days post-anthesis. No further accumulation occurred after 38 days. This pattern was also reported by Kumar and Singh (1981). A similar trend is outlined in Figure 6.9. Jenkins *et al* (1974) showed a very rapid increase in starch accumulation between Days 23 and 41 after ear emergence for wheat grown in a similar area in New Zealand. Unfortunately the first analysis was subsequent to the apparent increase (% DW) shown in Figure 6.9.

It is proposed that the early increase in starch as a proportion of the dry weight (Fig 6.9) is related to starch formation in the pericarp tissues while the subsequent more linear increase in starch accumulation is related to starch formation in the endosperm.

The soluble sugar content of wheat grains consists predominantly of sucrose, fructose and fructosans with glucose in very small quantities (*eg* Jennings and Morton, 1963a; Johnson *et al*, 1964; Jenner, 1970; Abou-Guendia and D'Appolonia, 1972, 1973; Cerning and Guilbot, 1973). However, 65% methanol probably extracted a high proportion of the ethanol-soluble carbohydrates such as the glucofructosans (Cerning and Guilbot, 1973; Escalada and Moss, 1976).

The soluble sugar content as a percentage of the dry weight increased immediately following pollination and reached a maximum level 11 days after anthesis (Fig 6.10). However, the rapidly increasing starch content masked the fact that the amount of soluble sugar per grain was still increasing to a maximum at Day 18 (Fig 6.9, cf Fig 6.10).

The earliest analyses performed by Jennings and Morton (1963a) were at five days after anthesis, and it was shown that while the sugar levels in the endosperm (% DW) subsequently decreased rapidly, a similar decrease did not occur within the testa-pericarp tissues until at least 10 days after anthesis. This led to an overall increase in the amount of sugars per grain between eight and 12 days after anthesis whereas over the same period total sugars declined markedly as a percentage of the dry

weight. From day 20 to maturity the soluble sugars formed a constant proportion of the dry weight.

The results reported in this thesis are supported by the observations of Turner (1969) who noted that the total sugars per grain showed a maximum about 16 days after anthesis then decreased to a low level at 45 days, and also by Cerning and Guilbot (1973) and Kumar and Singh (1981) who showed that total alcohol-soluble sugars increased to a maximum per kernel up to 12 to 19 days after anthesis, depending on variety, then decreased to a reasonably constant value.

The changes in cytokinin content per grain or per unit weight do not correlate closely with any of the changes observed in soluble sugars or starch in whole grains. Starch was accumulating at a decreasing relative rate while the cytokinin content was changing rapidly. Starch continued to increase in the grain for a substantial time after the cytokinin-like activity was no longer detectable. The transient increase in starch close to anthesis could be related to pericarp development and correlated more closely with the rapid changes in cytokinin-like activity. Soluble sugars continued to increase for some days after the cytokinin peak had decreased to low levels.

As noted for potato tubers there is a general agreement that the scheme involving the sugar-nucleotide pathway for the biosynthesis of starch is ubiquitous (Jenner, 1980) and that starch synthesis is catalysed by the enzymes ADPG-pyrophosphorylase and starch synthase (Mares and Marschner, 1980), while the first reaction in the sequence involves the production of UDP-glucose by the action of sucrose synthase and its subsequent metabolism by UDPG-pyrophosphorylase (*eg* Jenner, 1980; Mares and Marschner, 1980).

Turner (1969) showed that the level of ADPG-pyrophosphorylase was initially very low and changed little until 10 days after anthesis. A very sharp rise occurred 14 to 19 days after anthesis and the activity remained at a high level until 33 days from anthesis, after which it decreased rapidly. Turner (1969) noted that the maximum rate of starch synthesis coincided with the maximum activity of ADPG-pyrophosphorylase. The sharp increase in the enzyme occurred when the cytokinin level in the grain as found in this thesis is very low (Fig 6.26). The activity of UDPG-pyrophosphorylase was low at five and seven days post-anthesis and increased steadily after 10 days to a maximum at 33 days. Again, the level of enzyme increased when grain cytokinin had decreased to very low levels.

Kumar and Singh (1980) used a slightly more refined selection of

grains from central spikelets compared with Turner (1969) who randomly selected grains from whole ears. Kumar and Singh (1980) monitored changes in the enzyme levels of four varieties of wheat and suggested that the changes in both UDPG- and ADPG-pyrophosphorylase tended to parallel grain size and starch content, generally observing an increase in activity from seven days to 28 days from anthesis followed by a decline to the final sampling at Day 42. The enzyme activities did not parallel the changes in cytokinin-like activity reported in wheat grains (Figs 6.26, 6.27). The activity of invertase was shown to be declining from seven days in three of the varieties. Unfortunately no samples were taken at an earlier date.

The endosperm of grasses is described as being of the nuclear type in which the early mitoses occurring in the developing endosperm are not accompanied by cytokinesis (*eg* Mares *et al*, 1975; Brocklehurst *et al*, 1978). Fertilisation can occur within a few hours of pollination (*eg* Hoshikawa, 1959) and divisions of the endosperm nuclei can begin as early as six hours after pollination and continue in a synchronous manner until about 72 hours after pollination at which time the endosperm becomes cellular tissue in wheat grown at 20°C (Bennet *et al*, 1973). Morrison and O'Brien (1976) and Morrison *et al* (1978) considered that cellularisation of the free nuclear endosperm lining the inner periphery of the embryo sac wall was initiated within one to two days after anthesis and that the initial cellularisation was completed within two to three days after anthesis. Buttrose (1963) and Briarty *et al* (1979) also considered that cell walls formed within two days of fertilisation and subsequently the endosperm rapidly became cellular.

In contrast to all the above reports, Mares *et al* (1975) suggested that no cell wall development was apparent three to four days after anthesis and that cellularisation did not occur until four to five days post-anthesis. In a more recent paper, Mares *et al* (1977) suggested that endosperm cell wall formation commenced within two to three days after anthesis and by five days cellularisation was complete.

Following cellularisation, further increase in cell number is by normal cell division (*eg* Buttrose, 1963). Morrison and O'Brien (1976) and Campbell *et al* (1981) suggested that within four days of anthesis multiple tangential and radial divisions were occurring in the peripheral layer of cells resulting in cellularisation of the entire embryo sac. Briarty *et al* (1979) also suggested that following the initial cellularisation, the mass of cellular material was rapidly broken down by cell divisions and that division was progressing faster than expansion between

days four to five post-anthesis. Evers (1970) observed that the increase in cell number up to four days was a result of radial divisions in the meristematic layer but by Day 6 both tangential and radial divisions were occurring. Bennett *et al* (1973) suggested that the rate of division was actually faster before cellularisation occurred.

The maximum cytokinin concentration per unit weight or per grain occurred four days post-anthesis at the stage when the majority of reports suggest normal cell divisions are occurring rapidly. At the time of the initial cellularisation the *o*-glucoside concentration had decreased, but the zeatin content of the grains was steadily increasing as free nuclear divisions gave way to normal cell divisions.

However, all cytokinin-like activity had decreased in the grains by seven days after anthesis and by 11 days little activity could be detected, but the time at which cell division within the wheat grain ceases is being debated. The early cytological work of Sandstedt (1946) indicated that cell divisions were decreasing within 14 days of pollination although some cell divisions still occurred in the cells of the aleurone layer.

Jennings and Morton (1963b) monitored the amount of DNA per endosperm and noted that, although DNA increased from Day 8 to Day 19 there was little change after Day 14 indicating that few cell divisions occurred after Day 14. They suggested, on the same basis, that the number of cells in the pericarp was essentially constant from Day 5 (the first experimental reading). Asana and Bagga (1966) monitored the DNA content of the whole grain in two varieties and noted that the DNA content increased to 14 days but that there was no further increase up to 20 days post-anthesis, whereas Donovan *et al* (1977b) suggested that the increase in DNA content per grain did not begin to slow until between 18 and 20 days after anthesis and reached a maximum value at 25 days. Donovan (1979) again indicated that DNA reached maximum levels between 21 and 28 days post-anthesis. However, he cautions that chemical measurement of DNA may reflect changes other than cell division, as for example, a change in the DNA content per nucleus (Donovan, 1979; pers comm). However, Radley (1978) suggested there was some evidence for the occurrence of cell division in the third grain of spikelets more than 30 days after anthesis, although anthesis in the third floret would have been a few days later than in the lower florets (Radley, 1978).

Morrison *et al* (1975) noted that some cell divisions were still occurring at 14 days and Evers (1970) suggested that the increase in size of the endosperm involved only cell enlargement and not division

from 16 days post-anthesis, while Hoshikawa (1961) suggested cell division of the endosperm was complete by 18 to 20 days.

Briarty *et al* (1979) used two different techniques to calculate the change in the number of cells per unit volume of endosperm tissue. Cell numbers appeared to have stopped increasing by 12 days after anthesis if the stereologically-derived data is considered while data obtained from macerated preparations indicated that cell division had ceased between 16 and 20 days after anthesis.

Brocklehurst (1977) suggested that cell division had ceased by 14 days. Selecting the two lower grains of the four central spikelets for cell number counts, Brocklehurst *et al* (1978) suggested that cell division in the endosperm had ceased between 11 and 13 days after anthesis for two field grown cultivars while cell division in plants grown in pot experiments ceased by 14 days.

It would appear that the cytokinin content of the grain has decreased to a low level probably prior to a decrease or cessation of cell division. However, the time at which cell division ceases may well be influenced by environmental parameters (*eg* Wardlaw, 1970; Brocklehurst *et al*, 1978) or position in the ear (Radley, 1978). Consequently, the cytokinins and cell division need to be monitored in the same sample of grain before a more definitive statement can be made concerning the decrease in cytokinin-like activity and the decrease in cell division.

Kretching *et al* (1978) considered that the cytokinin accumulation in developing seeds is the result rather than the cause of sink activity in the seeds. The relatively early and very rapid decrease in the cytokinin content of the developing caryopses is difficult to explain by such an hypothesis. Moreover, Wheeler (1976) noted that the ears of wheat in which fertilisation of the ovules was prevented were lighter but contained more cytokinin activity than normal ears. He suggested that this may have occurred because there were fewer developing grains which used less cytokinin during growth, for example in cell division. Witham and Miller (1963) had also noted that unfertilised ears of *Zea mays* contained high concentrations of cytokinin-like activity.

A correlation between the increase in the *O*-glucoside activity prior to pollination and any particular developmental event is difficult to establish (Figs 6.28, 6.29). Wheeler (1972) detected the presence of cytokinins in ovules prior to fertilisation and at ear emergence the upper third of the ear contained most cytokinin-like activity but the amount in the ovules alone was not determined. Wheeler, however, considered this accumulation in the apex of the ear may have been a

result of surplus cytokinin moving in the transpiration stream. Michael *et al* (1970) suggested that cytokinins moved in the transpiration stream of barley stems and Wheeler (1972) in fact detected two cytokinin-like components in root exudate of wheat plants. He reported that maximal cytokinin was collected from the root exudate of field grown wheat before anthesis, but at anthesis for glass-house grown wheat. Most of the activity was attributable to a highly polar cytokinin. It is tempting to speculate that the cytokinin is moving in the transpiration stream as the *O*-glucoside.

It has been shown in this thesis that a large proportion of the polar cytokinin-like activity detected in pistils and grains can be attributed to the *O*- β -D-glucosides of zeatin and zeatin riboside. It may be that the polar cytokinins detected by Wheeler (1972) in the ovules, grains and root exudate are the same (but see p. 151).

Henson and Wareing (1976) considered that the formation of glucosides represented inactivation products of excess amounts of cytokinin arriving in the transpiration stream. Until recently (see references cited in Duke *et al*, 1979, and in van Staden and Davey, 1979), cytokinin glucosides have not been detected in the xylem and the general consensus has been that the cytokinin glucosides detected were formed from the cytokinins commonly found in the xylem exudate, namely zeatin and zeatin riboside (see references cited in van Staden, 1980). Most of the cited work was performed using mature leaves (see references cited by van Staden and Davey, 1979).

Cytokinin glucosides have been detected in roots (*eg* Yoshida and Oritani, 1972; Henson and Wheeler, 1977; van Staden and Dimalla, 1977b; van Staden and Smith, 1978) but only recently have cytokinin-*O*-glucosides been detected in root exudate (Davey and van Staden, 1978a; van Staden and Dimalla, 1980) although polar cytokinin-like activity has been detected in numerous root exudates (*eg* Banko and Boe, 1975 and references cited therein). Since the cytokinin-*O*-glucosides are present in roots and have been detected in phloem sap (*eg* van Staden, 1976b; Davey and van Staden, 1978a; van Staden and Brown, 1978), van Staden and Dimalla (1980) considered that the possibility that their presence in root sap was due to phloem transport, could not be disregarded. However, Davey and van Staden (1978b) reported that the sap passing into *Lupinus* fruits contained compounds that "co-chromatographed" with zeatin, zeatin riboside and the glucoside cytokinins, and Salama and Wareing (1979) have detected a polar cytokinin in root exudate of sunflower plants. It will be of interest to determine the nature of

the cytokinin in wheat stem exudate especially since the activity was highest just prior to the grains attaining maximal concentrations of cytokinins (Wheeler, 1972).

Michael and co-workers have shown a close association between transpiration, the cytokinin content of barley grains and grain size. De-awned barley plants had a shorter ripening period and lower cytokinin content compared with control plants. Moreover, the differences in cytokinin content were noticeable in the early stages of development when grains were still almost the same size (Michael and Seiler-Kelbitsch, 1972). In addition, Michael *et al* (1969) have shown that detached stalks with immature ears and intact awns incorporated more [8-¹⁴C]-kinetin into the grain and have a higher transpiration rate than do de-awned ears. At a high air humidity, incorporation of [8-¹⁴C]-kinetin into the grain and the effects of awns were small. Michael *et al* (1970) showed that the removal of awns led to a decrease in grain weights and that spraying of kinetin onto ears increased the grain weight especially when the awns were removed.

These results with barley lead to the suggestion that the cytokinin in the developing barley grain is arriving in the transpiration stream and is not arriving indirectly via the leaves and the phloem as suggested for the inflorescences of *Yucca* (Vonk, 1979; Vonk and Davelaar, 1981). Seiler-Kelbitsch *et al* (1974) considered that a relationship existed between the cytokinin activity of barley grains during maturation and grain size and that the root system participated in the regulation of grain size by the production of hormones in the root tips.

It is of importance to consider whether all the cytokinin detected in the grain is derived, via the transpiration stream from the roots, or if some is synthesized within the grain. It is generally accepted that roots, and in particular their apices, are sites of cytokinin biosynthesis (refer to Letham, 1978; van Staden and Davey, 1979). Even newly formed root primordia are considered by Forsyth and van Staden (1981) to produce cytokinin soon after being initiated. Maaß and Klämbt (1981) consider also, that cytokinins are produced by roots, but claim that cytokinin production by roots in intact bean plants is entirely the result of RNA-hydrolysis and that no *de novo* synthesis occurs.

In contrast to the production of cytokinins by excised root tips *in vitro* (eg van Staden and Smith, 1978; Koda and Okazawa, 1978), no evidence was found by van Staden and Button (1978), Kretching *et al* (1978) or Srivastava *et al* (1980) for cytokinin production *in vitro* by developing pea seeds, which is opposite to the conclusion drawn by Hahn *et al* (1974).

More recently, however, Summons *et al* (1981) reported that immature seeds of *Lupinus luteus* biosynthesized at least two cytokinins from [³H]-adenosine *in vitro*.

Van Staden and Button (1978) concluded that seeds were dependent for cytokinins on the rest of the plant but had the capacity to metabolise or convert imported cytokinins. Rodgers (1981) considered that the appearance of cytokinins in the cotton ovules at a time prior to fertilisation raised the possibility that these substances were being produced elsewhere in the plant and transported to the developing ovules.

The cytokinin-glucosides are generally considered to be storage forms (*eg* Parker and Letham, 1973; van Staden and Davey, 1979) and the cytokinin-*O*-glucosides to be readily hydrolysed for utilisation when required (van Staden and Papaphilippou, 1977). The accumulation of the cytokinin-*O*-glucosides at pollination (Figs 6.18, 6.29) and the subsequent rapid accumulation of zeatin are indicative of the conversion of the less active *O*-glucosides to zeatin and zeatin riboside (van Staden and Papaphillipou, 1977), and the turnover of zeatin riboside to zeatin at the early stages of endosperm development. Maaß and Klambt (1981) recently suggested that the cytokinins would be present in the free base instead of the glucosyl- or ribosyl-derivatives if the plant was in a "state of high physiological activity". This is in accordance with the suggestions by Engelbrecht (1971) and more recently by Lorenzi *et al* (1978), that the less polar activity detected was the more active form. Consequently, the high level of cytokinin-*O*-glucosides present until four days after anthesis may indicate an excess of cytokinin until the time when normal cell divisions begin to occur rapidly. As Wheeler (1972) suggested, if the cytokinin consumed in grain growth cannot be replaced, then that present after anthesis may regulate the early stages of grain growth and possibly grain size providing photosynthate is adequate.

It would be interesting to determine the cytokinin content of grains at or close to anthesis while manipulating environmental parameters to cause a reduction in cell number (*eg* Wardlaw, 1970; Brocklehurst, 1977; Brocklehurst *et al*, 1978).

CHAPTER VII

IDENTIFICATION OF CYTOKININS
IN WHEAT GRAINS

7.1 INTRODUCTION

The unequivocal identification of a cytokinin has usually involved extensive chemical and mass spectral analyses prior to comparison with an unambiguously synthesized compound (*eg* Letham *et al*, 1967; Horgan *et al*, 1975; Duke *et al*, 1979). However, the comparison of spectra obtained from cytokinin-containing extract with spectra obtained from known cytokinins has been used to confirm the presence of a particular cytokinin in an extract (*eg* Shindy and Smith, 1975; Watanabe *et al*, 1978a; Arteca *et al*, 1980; Scott *et al*, 1980a). Furthermore, Summons *et al* (1979b) obtained confirmation of the presence of zeatin-9-glucoside in extract from *Zea mays* by spiking a selected HPLC fraction with zeatin-9- β -D-glucoside and noting an increase in the intensity of the relevant ions in the mass spectrum obtained. An enhancement of the diagnostic ions in the mass spectrum of TMS- $^{2}\text{H}_2$ -raphanatin (TMS-zeatin-7-glucoside) was obtained from unlabelled TMS-raphanatin and used as confirmation of the presence of raphanatin in radish seed (Summons *et al*, 1977).

In situations where the amount of compound available is too small, or the contamination too great, to give definitive spectra, the selection of specific ions can increase sensitivity although selectivity will be reduced (*eg* Taylor *et al*, 1974; Young, 1977; Salama and Wareing, 1979). However, the reported detection of only a single diagnostic ion was considered by Scott *et al* (1980a) as indicative of the presence of zeatin in extract of tobacco crown gall callus, and by Salama and Wareing (1979) as indicative of zeatin riboside in sunflower extracts. Young (1977) suggested that the presence of two selected ions in the correct abundance ratio, together with retention time and R_f values in the various chromatographic clean-up steps could be considered sufficient

evidence for the positive identification of a compound. Young (1977) used this procedure to "identify" zeatin and zeatin riboside in extracts from Chinese gooseberry fruit and to confirm the presence of zeatin in extracts from sour cherries (*Prunus cerasus* L) (Hopping *et al*, 1979). Yokota and Takahashi (1980) considered that three ions of the correct relative intensity at the correct retention time established the presence of iPA in extract of shoots of the chestnut tree. Multiple ion monitoring was used by Watanabe *et al* (1981) to identify zeatin riboside and *cis*-zeatin riboside, zeatin and *cis*-zeatin in cones of hop plants and by Hashizume *et al* (1979) to detect the presence of msIPA and zeatin riboside in extract of cabbage hearts.

The technique of multiple ion monitoring (MIM) was used in this thesis in an attempt to confirm the presence of zeatin and zeatin riboside in extracts from wheat grains collected four days after anthesis.

As was discussed previously in Chapter 2, quantification from bioassay data can only indicate activity "like" a particular cytokinin while MIM is selective for one particular compound. The least refined method of quantitation using MIM is a comparison between the peak heights of selected ions from the cytokinin-containing sample and from the standard cytokinin (*eg* Lorenzi *et al*, 1975; Young, 1977; Scott *et al*, 1980a). However, this procedure can only give an indication of the amount of cytokinin present in the sample at the end of sometimes extensive purification procedures and will give no indication of losses during purification. Thompson *et al* (1975) used an internal standard to quantify the losses during purification. However, a standard with a slightly different structure to that of the compound under investigation would not necessarily be given the same recoveries.

The most satisfactory quantification to date has been achieved with isotopically labelled internal standards, a procedure introduced by Letham and co-workers (Summons *et al*, 1977) and subsequently also used by Hashizume *et al* (1979). The technique uses as internal standards deuterated analogues which might be expected to have virtually identical chemical and physical properties, and therefore identical behaviour during extraction, to the compound under investigation.

However, in this work deuterated derivatives were not available nor was labelled zeatin or any other labelled endogenous cytokinin, so no estimation of loss during clean-up procedures could be made. Quantification of the derivatised sample was attempted by comparison with peak heights of selected ions from known quantities of the relevant authentic cytokinins.

7.2 MATERIALS AND METHODS

Wheat grains collected from the field four days after anthesis were extracted and partially purified by column chromatography (eluted with 20% ethanol v/v) as described in Section 6.2.2.2A. Fractions with elution volumes corresponding to those of zeatin and zeatin riboside standards respectively, were collected. Each solution was evaporated to dryness on a hot plate at 37°C under a stream of air prior to dissolution in small volumes of 80% ethanol and transfer to 100 µl capacity Pierce Reactivials.

Standard solutions (100 mg l⁻¹) of zeatin and zeatin riboside were prepared in 20% ethanol. Aliquots of the zeatin and zeatin riboside solutions were transferred to Pierce Reactivials to give final quantities of 30 and 3 µg per vial of each cytokinin.

Extracts and cytokinin standards were dried by one of two methods. Samples to be silylated were held under vacuum over silica gel followed by three azeotropic evaporations with a mixture of 1:1 benzene and ethanol. The alternative procedure (utilised when samples were to be permethylated) was to hold the samples under vacuum over phosphorous pentoxide (P₂O₅) for a minimum of three hours. The vials were sealed with Teflon-backed silicone rubber septa.

7.2.1 Trimethylsilylation

The extracts were dissolved in 10 µl anhydrous pyridine (Lab Supply Pierce NZ Ltd). 20 µl N,O-Bis-(Trimethylsilyl)-trifluoroacetamide (BSTFA: Lab Supply NZ Ltd) was added to the solution and the reaction mixture held at 60°C for 5 to 45 minutes (*eg* Babcock and Morris, 1970; MacLeod *et al*, 1976; Upper *et al*, 1970; Dyson and Hall, 1972; Pierce Chemical Co Catalogue, 1978). Aliquots (1 to 5 µl) of the trimethylsilylated derivatives (TMS-derivatives) were removed for identification by gas chromatography (GC) or combined gas chromatography-mass spectrometry (GCMS).

For direct probe MS analysis the BSTFA-pyridine solvent mixture was removed by evaporation *in vacuo* and replaced by 30 µl anhydrous ethyl acetate.

7.2.2 Permethylation

The methylsulphonyl carbanion was prepared under an atmosphere of dry nitrogen (N₂) using dry solvents. A 50% oil dispersion of sodium hydride (200 mg) was washed with dry diethyl ether (2 x 1 ml) under dry N₂ and 4 ml dry dimethylsulphoxide (DMSO) added (Young, 1977). The

mixture was heated under N_2 (Corey and Chaykovsky, 1967) at $65^\circ C$ until evolution of hydrogen ceased (about 2 h). To ensure completion of the reaction the carbanion solution was decanted under N_2 into a test tube containing a further 0.5 ml dry DMSO. The reaction was considered complete if no further evolution of H_2 occurred. The methylsulphinyl carbanion solution was stored under N_2 at $-20^\circ C$, and used within one month of its preparation.

Dried samples and standards in 2 ml vials were purged with dry N_2 . The methylsulphinyl carbanion solution was added to the Reactivials: 100 μl to vials containing zeatin and zeatin riboside and 300 μl to vials containing wheat samples. The mixture was agitated frequently and kept at room temperature under N_2 for 30 minutes (Young, 1977).

A trace of triphenylmethane dispersed in dry DMSO was added to the reaction mixture at this point. The appearance of a red colour indicated an excess of the methylsulphinyl carbanion (Corey and Chaykovsky, 1962; Young, 1977). Excess methyl iodide was added (10 μl to the standards, 30 μl to the wheat sample) under N_2 . The mixture was agitated frequently and allowed to react for 60 minutes at room temperature. Following the addition of 1 ml H_2O the aqueous solution was partitioned three times against 1 ml chloroform (Young, 1977), allowing 30 minutes per partition to ensure complete phase separation. The combined chloroform layers were then washed three times with 0.5 ml H_2O again allowing 30 minutes per phase separation. The chloroform extract was then evaporated under a stream of N_2 and transferred to a 100 μl Reactivial. The residue was dried *in vacuo* over P_2O_5 and stored dry at $-20^\circ C$. Just prior to use 30 μl dry $CHCl_3$ were added to dissolve the permethylated derivatives; aliquots of 1 to 5 μl were used for GCMS analysis and 1 μl aliquots for direct probe MS

7.2.3 Gas Chromatography and Combined Gas Chromatography-Mass Spectrometry

Gas chromatography was performed on a Hewlett Packard 5710A gas chromatograph fitted with a flame ionisation detector. The columns used were either 2.5% OV-17 or 2.5% OV-101 on chromosorb W/AWDMCS, silanised glass (2.5 mm ID x 1.5 m). The injector and detector temperatures were standardised at $250^\circ C$ and the helium carrier gas flow at 30 ml min^{-1} .

The 5710 gas chromatograph is part of the 5982A GCMS system used in this work. For GCMS work the GC and quadrupole mass spectrometer are connected by a single stage glass jet separator and the MS is used as the GC detector either in total ion current (TIC) or multiple ion monitoring (MIM) mode. The GCMS has a 4-channel multiple ion monitor.

Direct probe samples were run on the same mass spectrometer at an ionising voltage of 70 eV. The instrumental parameters for each analysis are given in the results section. Temperature programming was utilised for GC analysis of all TMS-derivatives (eg Most *et al*, 1968; MacLeod *et al*, 1976; Morris, 1977).

7.3 RESULTS

7.3.1 Zeatin Riboside

The mass spectrum of trimethylsilylated zeatin riboside (TMS-ZR) was obtained from direct probe insertion of the derivatised standard. The relative intensities of the major high mass ions are tabulated in Table 7.1.

TABLE 7.1

Relative intensities of high mass ions in the mass spectrum obtained from direct probe insertion of 1,000 ng trimethylsilylated zeatin riboside. Intensities relative to m/z 73 = 100

m/z	Relative Intensity	m/z	Relative Intensity
641	1.3	538	4.0
640	2.0	537	8.5
639	4.0	536	16.0
626	3.0	509	1.7
625	4.3	508	2.0
624	8.3		
551	3.7	424	1.7
550	8.0		
549	11.0		

GC analysis of TMS-ZR (OV-17, FID) gave a sharp peak (Fig 7.1), but when the MS in total ion mode was used as the detector, a very broad peak was observed with a peak retention time of 6.75 minutes (Fig 7.2). A mass spectrum recorded at the centre of this peak showed prominent high mass ions at m/z 639, 624, 550, 549 and 536 characteristic of TMS₄-ZR. A similarly broad peak also occurred when an OV-101 column was used in the GC (Fig 7.2). A mass spectrum recorded at the peak retention time of 6.5 minutes contained prominent ions at m/z 624, 550, 549, 537 and 536.

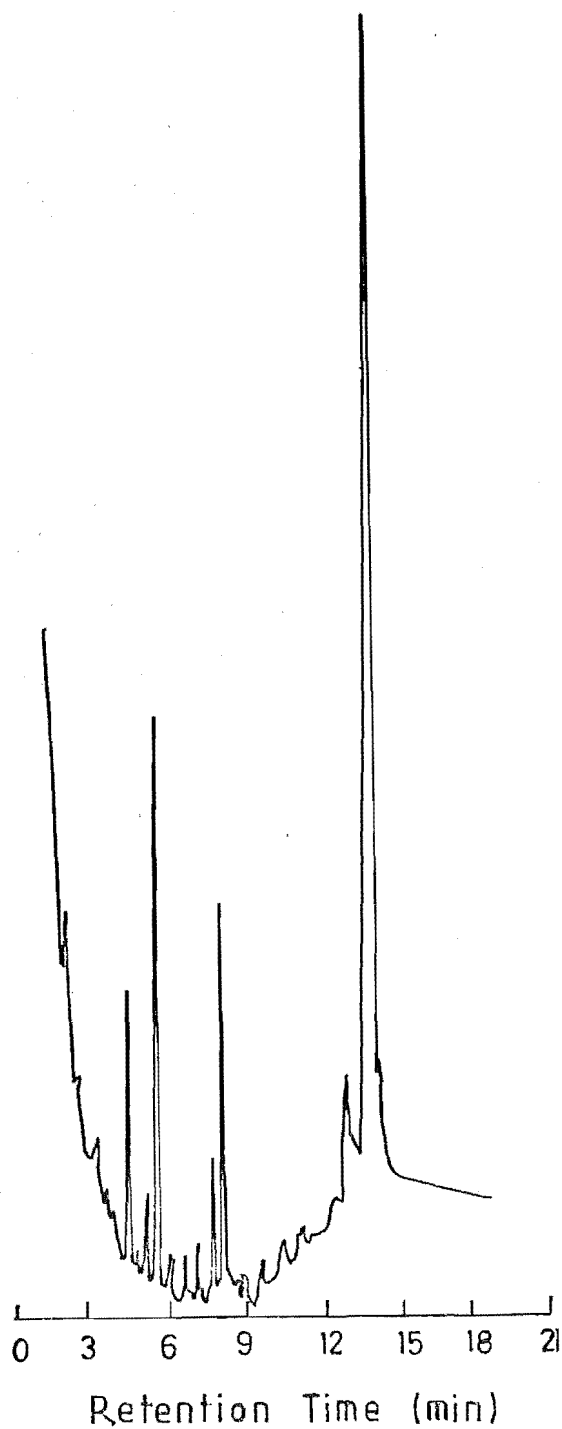


FIGURE 7.1

FID trace of from gas chromatography of 1,000 ng trimethylsilylated zeatin riboside standard

Gas chromatograph operating parameters

Column: 0V-17

Temperature programme: 150-280/16^oC min⁻¹

T_{injector}: 250^oC

T_{detector}: 250^oC

The GC was equipped with an OV-17 column for all subsequent chromatography.

Ions at m/z 639, 624 and 536 were selected for multiple ion monitoring of the TMS-ZR standard. A simultaneous response at a retention time of 6.25 minutes was recorded from the three ions following injection of 1,000 ng of TMS-ZR (Fig 7.3). Relative peak heights are presented in Table 7.2, the most intense peak being that of the ion at m/z 536.

TABLE 7.2

Relative peak heights of ions diagnostic of TMS₄-zeatin riboside obtained following the injection of 1,000 ng of trimethylsilylated zeatin riboside standard and multiple ion monitoring of ions at m/z 639, 624 and 536^α

Ion monitored (m/z)	Retention time (min)	Relative peak height
639	6.25	1.00
624	6.25	6.02
536	6.25	11.48

^α Operating parameters tabulated with Figure 7.3

A sample of wheat extract from the column eluate corresponding to the zeatin riboside marker was monitored for m/z 639, 624, 536, 550 and 549 in two runs - initially m/z 639, 624 and 536 and then m/z 550 and 549 together with 624 were monitored. All masses were found at a retention time of 6.25 minutes (Fig 7.4). Relative peak heights are presented in Table 7.3.

7.3.2 Zeatin

Trimethylsilylation of zeatin appeared to give several derivatives. Ions characteristic of TMS₃-zeatin (m/z 435, 434, 422, 421, 420, 348, 347, 346, 332, 306, 305, 304, 272) and of TMS₂-zeatin (m/z 348, 274, 273, 272, 261, 260, 258 and 232) were detected in the mass spectra recorded during GCMS of the TMS-zeatin standard.

Following permethylation of zeatin and gas chromatography, a single peak at a retention time of seven minutes was detected (Fig 7.5). The mass spectrum was obtained from direct probe insertion of 1,000 ng of the standard. The major high mass ions and their relative intensities are tabulated in Table 7.4.

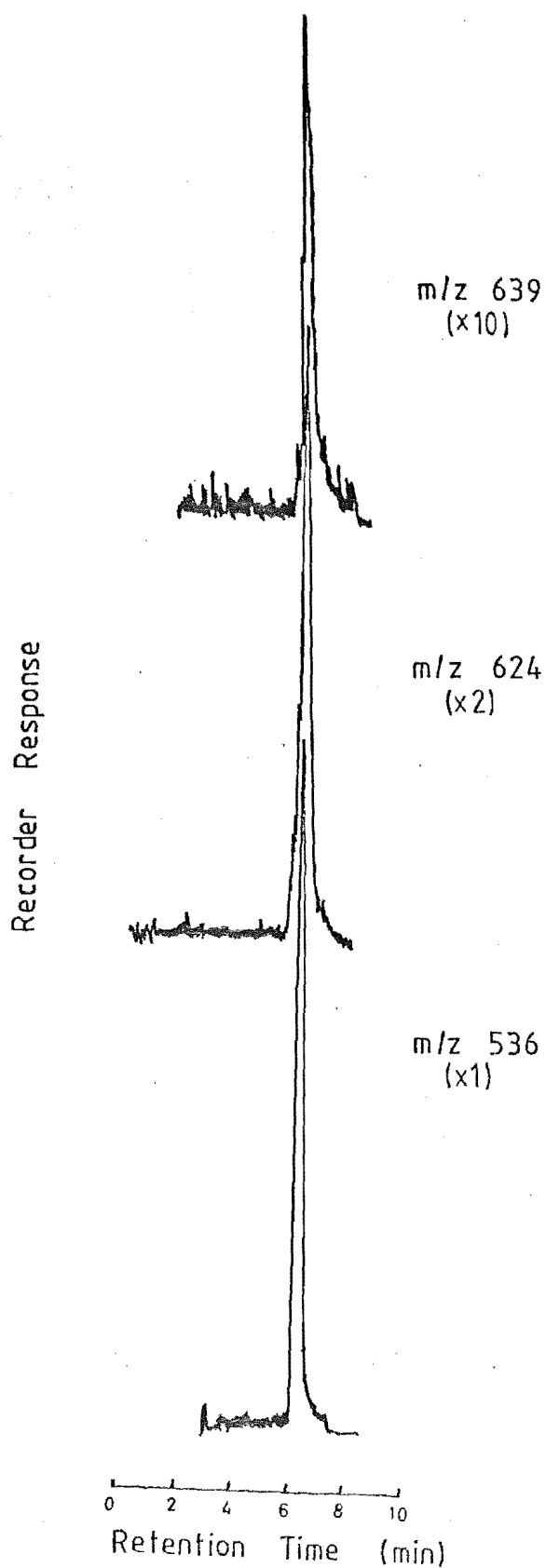


FIGURE 7.3

Multiple ion monitoring of ions characteristic of TMS₄-zeatin riboside. 1,000 ng TMS₄-zeatin riboside standard injected.

GCMS operating parameters.

Column: OV-17

Temperature programme: 150-280/16°C min⁻¹

T_{injector}: 250°C

T_{auxiliary}: 350°C

T_{manifold}: 280°C

T_{source}: 260°C

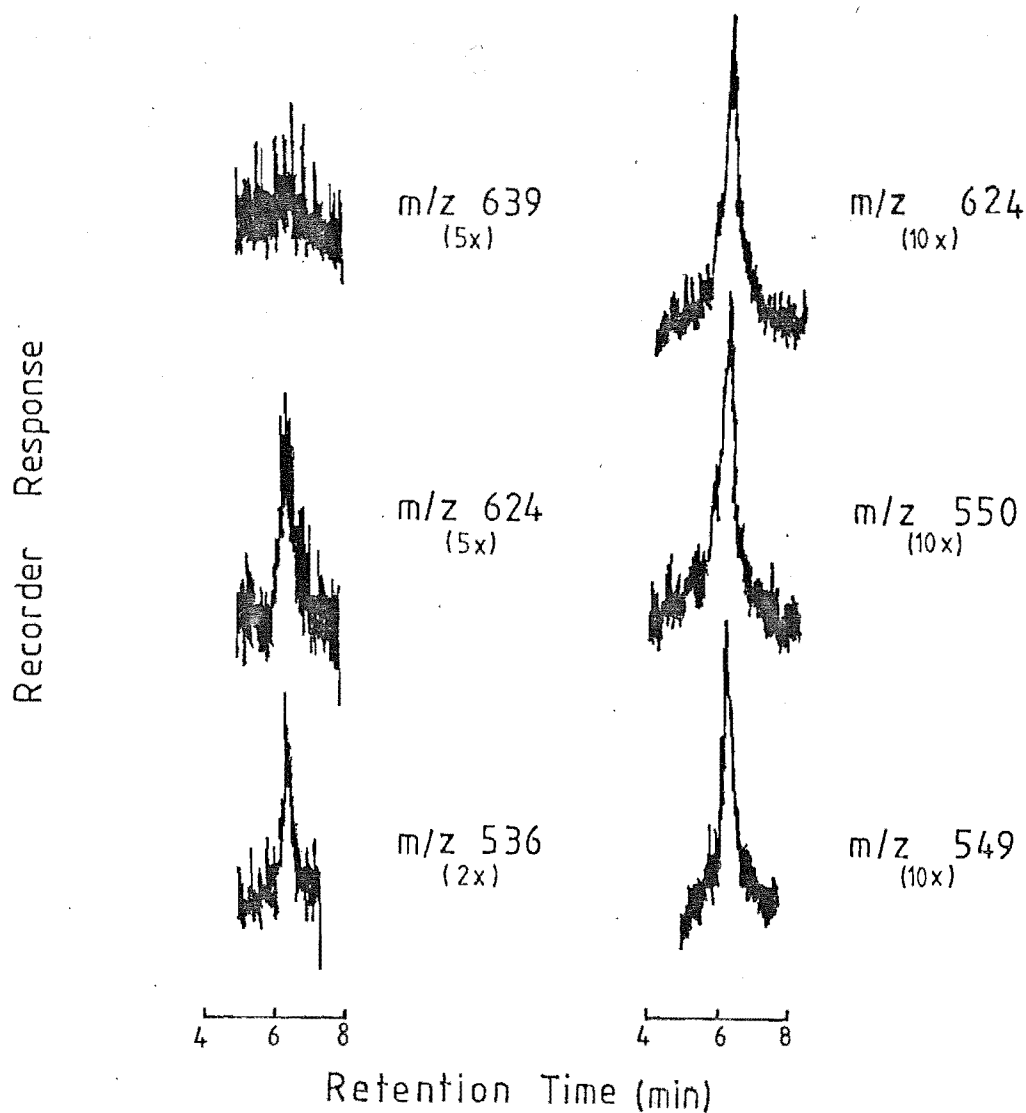


FIGURE 7.4

Multiple ion monitoring of ions characteristic of TMS₄-zeatin riboside. 2 μl aliquot of trimethylsilylated wheat sample injected

GCMS operating parameters

Column: OV-17

Temperature programme: 150-280/16⁰C min⁻¹

T_{injector}: 250

T_{manifold}: 280

T_{axillary}: 350

T_{source}: 260



FIGURE 7.5

FID trace from gas chromatography of 1,000 ng permethylated zeatin standard

Gas chromatograph operating parameters

Column: OV-17

Temperature programme: isothermal at 230°C

T_{injector}: 250°C

T_{detector}: 250°C

TABLE 7.3

Relative peak heights of ions diagnostic of TMS₄-zeatin riboside obtained following the injection of a 2 μ l aliquot of wheat sample and multiple ion monitoring of ions at A: m/z 639, 624 and 536; and B: m/z 624, 550 and 549 ^{α}

	Ion monitored (m/z)	Retention time (min)	Relative peak height
A:	639	6.25	trace
	624	6.25	1.00
	536	6.25	2.36
B:	624	6.25	1.00
	550	6.25	1.00
	549	6.25	0.95

^{α} Operating parameters tabulated with Figure 7.4

TABLE 7.4

Relative intensities of high mass ions in the mass spectrum obtained from direct probe insertion of 1,000 ng of permethylated zeatin standard. Intensities relative to m/z 230 = 100

m/z	Relative intensity
261	5
231	18
230	100
216	6
214	5
200	5
199	12
189	10
188	25

Ions at m/z 261, 230, 199 and 188 were selected and monitored simultaneously. Injection of 100 ng of the permethylated zeatin standard gave all four ions at a retention time of 4.25 minutes (Fig 7.6), the ion at m/z 261 showing additional peaks.

A second permethylation of zeatin and wheat extract gave the results shown in Figure 7.7. Following injection of 100 ng permethylated

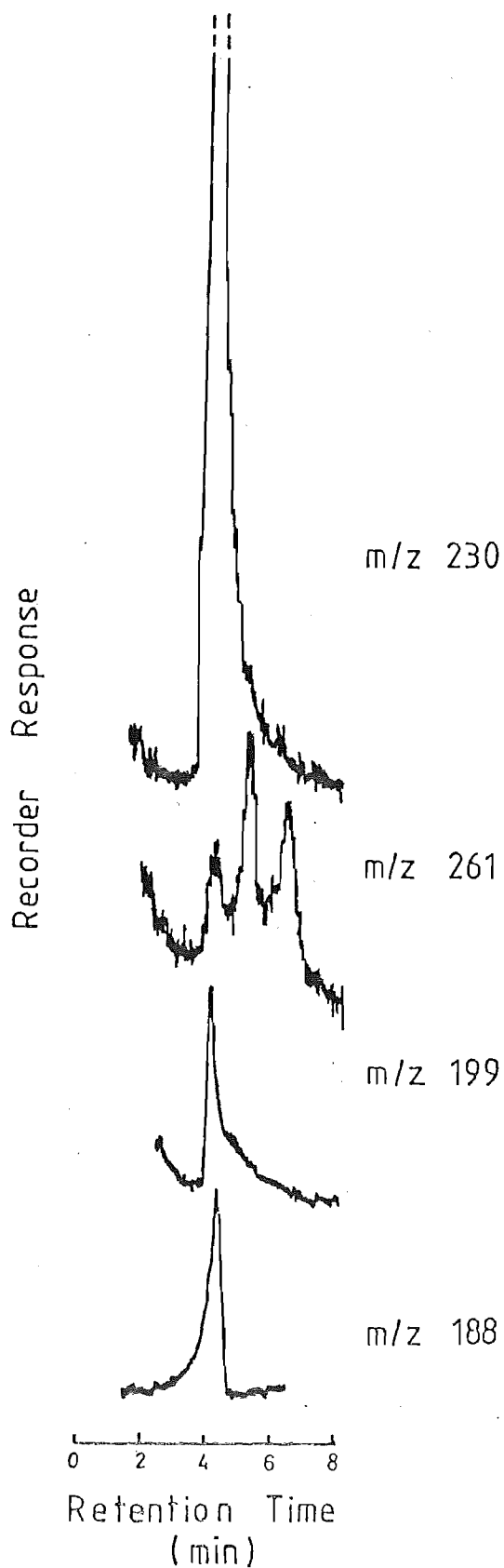


FIGURE 7.6

Multiple ion monitoring of ions characteristic of Me_3 -zeatin. 100 ng permethylated zeatin standard was injected.

Column: OV-17

Temperature programme: isothermal at 230°C

T_{injector} : 250°C

$T_{\text{auxillary}}$: 300°C

T_{manifold} : 280°C

T_{source} : 230°C

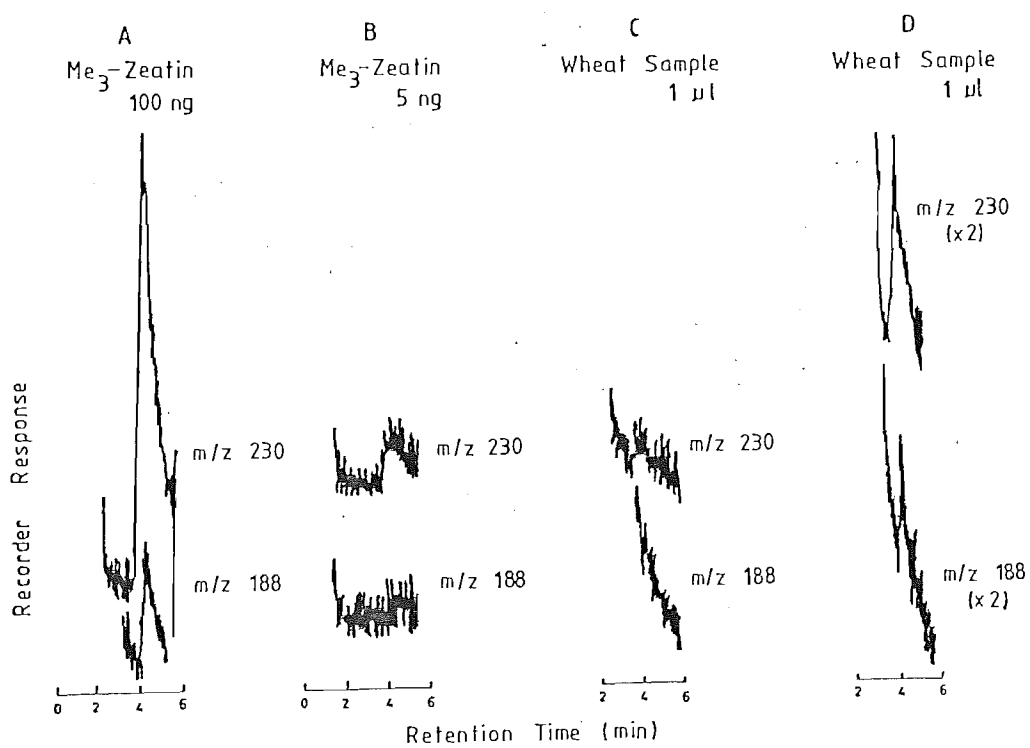


FIGURE 7.7

Multiple ion monitoring of ions characteristic of Me_3 -zeatin. Samples of permethylated zeatin (A, 100 ng and B, 5 ng) and of permethylated wheat samples (1 μl , C and D) were injected.

GC-MS operating parameters as in Figure 7.6.

zeatin a response was detected from ions at m/z 230 and 188 at a retention time of 4.25 minutes. When only 5 ng of standard was injected m/z 230 was detected at 4.25 minutes but no m/z 188 could be seen (Fig 7.7).

Ions at m/z 230 and 188 were monitored following injection of permethylated wheat sample. A small response to the ion at m/z 230 was obtained at a retention time of 4.25 minutes. A more definitive response from m/z 230 was obtained on a higher sensitivity setting (Fig 7.7) and a response from m/z 188 was also then detected.

7.3.3 Quantification

Quantification of zeatin and zeatin riboside was not possible from FID traces. The small amounts of TMS_4 -zeatin riboside and Me_3 -zeatin in wheat extracts were obscured by larger amounts of contaminating compounds. Quantification using peak heights obtained after multiple ion monitoring would suggest that there was less than 5 ng zeatin in 1 μl of extract of 200 wheat grains collected four days post-anthesis (Fig 7.7).

7.4 DISCUSSION

For optimal mass spectral analyses, the quantity of material passing into the mass spectrometer must be minimal. It became apparent as the work progressed that the large quantity of material being injected was, at least in part, contributing to the deterioration in the sensitivity of the mass spectrometer. A further purification step such as HPLC would have reduced this problem substantially but at the time of this study HPLC equipment was not available.

The trimethylsilylation of zeatin riboside resulted in the formation of one major derivative (Fig 7.1). The mass spectra obtained from direct probe insertion of TMS -zeatin riboside (Table 7.1) are consistent with the previously reported spectra for TMS_4 -zeatin riboside for which ions of m/z 639, 624, 550, 549 and 536 are diagnostic (Table 7.1) (eg Upper *et al.*, 1970; Purse *et al.*, 1976; Vreman *et al.*, 1978; Scott *et al.*, 1980a). The formation of TMS_4 -ZR was further verified from mass spectra obtained from GCMS analysis (data not presented).

The peak shape on the total ion traces of TMS_4 -ZR was poor and not improved by the use of a non-polar OV-101 column (Fig 7.2). Since the FID peaks were sharp, the broadening must arise either in the GCMS

interface or within the transfer lines from the interface to the mass spectrometer. The temperature of the interface could be raised to over 300°C but the maximum temperature of the internal transfer lines was 280°C. The broad peak shape was attributed to limitations imposed by the internal transfer lines, both in length and in temperature. An increased source temperature helped refine the MIM peak shapes.

Discrepancies between retention times for compounds determined from the FID trace and on the MS total ion current are attributed to the difference in distance between the column exit and FID and the column exit and the source respectively (eg Figs 7.1 and 7.2). Smaller daily variations in retention times, also noted by Upper *et al* (1970), probably arise from changes in carrier gas flow rates.

The retention time of authentic TMS₄-zeatin riboside (6.25 min under the conditions used) was established by monitoring three characteristic ions (m/z 639, 624 and 536) (Fig 7.3, Table 7.2). These ions, and ions at m/z 550 and 549, were then monitored during analysis of a silylated wheat sample, all but the lowest intensity ion (m/z 639) being observed at 6.25 minutes (Fig 7.4). If these ions arise from TMS₄-zeatin riboside in the wheat sample their relative intensities should be the same as those found in the mass spectrum of authentic TMS₄-zeatin riboside. The agreement was good within experimental error (m/z 624:536:550 = 1.0:2.36:1.0; Table 7.3) for the instrument operating at the extreme limit of sensitivity as it was for this study (Wright, pers comm), and this agreement, coupled with the identical retention times found for the wheat sample and standard, was considered conclusive evidence for the presence of zeatin riboside in the wheat samples.

Multiple derivatisation of zeatin during silylation has been reported previously (eg Purse *et al*, 1976; Dauphin *et al*, 1979; Yokota *et al*, 1981). Two derivatives of authentic zeatin were formed during silylation in this study. The mass spectra obtained were consistent with previously reported spectra for TMS₃-zeatin for which ions at m/z 435, 434, 420, 348, 347, 346, 332, 304 are diagnostic (Purse *et al*, 1976; Dauphin *et al*, 1979), while ions at m/z 348, 274, 273, 272, 261, 260, 258 and 232 are diagnostic of TMS₂-zeatin (eg Purse *et al*, 1976; Scott *et al*, 1980a; Yokota *et al*, 1981). The low sensitivity to TMS-zeatin as noted previously by Young (1977) was also found in this study where the minimum detectable level of TMS-zeatin was greater than 200 ng.

Permethylation of zeatin was the more successful technique. In

comparison to trimethylsilylation, permethylation was the more difficult and lengthy derivatisation procedure, but only one derivative was formed. The mass spectrum data obtained from the direct probe insertion of permethylated zeatin are consistent with previously reported spectra for the trimethylated derivative of zeatin (Me_3 -zeatin) for which ions at m/z 261, 230, 199 and 188 are diagnostic (Table 7.4) (Young, 1977; Morris, 1977; Hopping *et al.*, 1979). The permethylated derivatives were also easier to handle as they are stable in the presence of atmospheric water compared to the TMS-derivatives which are readily hydrolysed (*eg* Sasaki and Hashizume, 1966; Morris, 1977; Young, 1977). They also appear to be thermally more stable under the GCMS conditions used since ions characteristic of Me_3 -zeatin were readily detected after injection of only 100 ng of Me_3 -zeatin (MIM mode; Figs 7.6 and 7.7). The minimum detectable level of Me_3 -zeatin was 5 ng (Fig 7.7) while that of TMS-zeatin was 200 ng (data not presented).

The retention time of authentic Me_3 -zeatin (4.25 minutes under the conditions used) was established by monitoring four characteristic ions (Fig 7.6). In order to obtain maximum sensitivity, only two of these (m/z 230, 188) were monitored during the analysis of a permethylated wheat sample, both appearing at a retention time of 4.25 minutes. If these ions arise from Me_3 -zeatin in the wheat sample, their relative intensities should be the same as those found in the mass spectrum of authentic Me_3 -zeatin. The agreement for ions at m/z 230 and 188 was good (Fig 7.7, Table 7.4) and this agreement, coupled with the identical retention times found for the wheat sample and standard, was considered evidence for the presence of zeatin in the wheat samples.

The deteriorating sensitivity of both source and photomultiplier meant that the actual amounts of zeatin and zeatin riboside present in the wheat samples could not be determined accurately by sequential injections of known amounts of standard and sample (*eg* Young, 1977; Lorenzi *et al.*, 1975). All that could be done was to establish an upper limit for zeatin.

The actual amount of zeatin present in 1 μl of permethylated wheat sample was less than 5 ng (Fig 7.7). This was equivalent to 0.75 ng per grain, whereas the bioassay response to cytokinin-like activity with a similar elution volume to zeatin was 120 ± 30 ng KE per grain.

There is some discrepancy in the literature concerning the effectiveness of kinetin, zeatin and zeatin riboside on callus growth. Engelbrecht (1971) considered that zeatin was about ten times as

effective in promoting soybean callus growth as either zeatin riboside or kinetin, whereas van Staden and Papaphilippou (1977) considered zeatin and zeatin riboside to be fairly similar in effectiveness. The Bap callus was more responsive to zeatin than to zeatin riboside or kinetin (Fig 2.8). Zeatin values calculated in kinetin equivalents will then be overestimates. The (undetected) presence of the dihydro derivative of zeatin could lessen only slightly the discrepancy between the bioassay and GCMS result (see p 241). With the above consideration, in addition to the losses incurred during derivatisation and GCMS, it is not surprising that the bioassay value is substantially higher than that determined by a physico-chemical technique.

Scott *et al* (1980a) also noted that the quantities of TMS₄-ZR and TMS₂-Z detected by SIM and determined by comparison of peak heights with those obtained from known quantities of the authentic compounds, were only 0.6 — 2.7% of those estimated by bioassay following Sephadex LH-20 chromatography.

The results reported in this thesis, and those of Scott *et al* (1980a) illustrate the desirability of using an internal standard in the extraction which can then be monitored concurrently in the MIM analysis to provide an accurate determination of the cytokinin content of the tissue.

CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSIONS

One of the major drawbacks of the soybean callus bioassay is the differential response of the callus to different cytokinins. This and the lack of internal standards means that any reported quantification of the cytokinin-like activity in wheat grains and potato tubers cannot be considered absolute. A second major drawback is that relatively small changes in cytokinin levels are difficult to verify because of the log/linear dose-response relationship between the cytokinins and callus growth in the bioassay. Brenner (1981), however, considers that bioassays, with all their limitations, are probably better than unsubstantiated physicochemical methods. At least the bioassay will indicate biological activity "like-the-substance" being studied, while an incomplete physicochemical analysis might only detect unknown impurities.

Bioassay data provides a good first approximation when determining endogenous cytokinin levels and a first approximation is a prerequisite before methods such as those using isotopically labelled internal standards can be used. At present these techniques are relatively unavailable.

Cell divisions in the potato tuber do not occur at a clearly defined stage of development but together with cell enlargement occur to a varying extent throughout the growth of the tuber (Plaisted, 1957; Reeve *et al.*, 1973a,b). A period of intensive cell division occurs in small expanding tubers according to Reeve *et al.* (1973a,b) but cell divisions decrease markedly in tubers greater than 20 mm diameter.

The data presented in this thesis have shown that while cytokinin-like activity can be detected in tubers greater than 20 mm diameter, the concentration is highest in tubers of the size reported by Reeve *et al.* (1973a,b) to be undergoing rapid cell divisions (*eg* Fig 4.34).

Reeve *et al.* (1973a,b) suggested that tubers growing either more rapidly or more slowly than the average for that cultivar contained more

or less cells per unit volume, respectively. To further strengthen the correlation between the cytokinin concentration and cell division, an investigation should be made of the cytokinin concentration in rapidly and slowly bulking tubers.

A decrease in the soluble sugar levels appears to be a good indication that tuber initiation has occurred (Table 4.11) whereas increasing starch levels indicate imminent tuber formation (Table 4.10). The starch levels in stolon tips excised from plants showing no tuber formation were substantially less than those from tuber-forming plants (Table 4.10). On the basis of low starch content it is assumed that the stolon tips selected from *S. andigena* cv 165 plants for cytokinin analysis were not induced. There was little apparent difference in the cytokinin complement of stolon tips and tubers 5 to 7.5 mm diameter (Figs 4.22, 4.25). There was certainly no evidence for major changes in the cytokinin complement of the stolon tips at the time of tuber formation (Sattelmacher and Marschner, 1978c) nor of any noticeable increase in the concentration of cytokinin-like activity at the time of tuber formation (Figs 4.22 to 4.25) (Smith and Palmer, 1970). The suggestion by Mauk and Langille (1978) that a plant with increased zeatin riboside levels was in a "state to tuberise" was not reflected by detectable increases in zeatin riboside-like activity in stolons or tubers until after tuber formation was established (*eg* Fig 4.33).

According to Jenner (1980) and Mares and Marschner (1980) the enzymes involved in starch accumulation in storage organs are ubiquitous. Although there was a correlation between cytokinin-like activity and ADPG-pyrophosphorylase activity in potato tubers there was no such correlation in wheat grains. It is probable that the enzyme changes noted following the kinetin-induced tuber formation of sub-cultured stolons *in vitro* (*eg* Mingo-Castel *et al*, 1976b) were a consequence, rather than a cause, of tuber formation.

Although the amount of cytokinin-like activity per tuber continued to increase with development (Fig 4.31) as did the starch and sugar contents of the tuber (Figs 4.20, 4.21), this was not the case with the cytokinin content of the wheat grain (Fig 6.26). The very rapid and transient increase in the cytokinin content of the grain correlated most closely with the intensive phase of cell division within the endosperm (*eg* Briarty *et al*, 1979).

This is the first report of the presence in wheat grains of cytokinin-like activity which is susceptible to hydrolysis by the enzyme

β -glucosidase (Fig 6.17). This reaction is indicative of the presence of cytokinin-*O*-glucosides (van Staden and Davey, 1979). The increase in cytokinin-*O*-glucoside activity prior to pollination and its rapid turnover (Fig 6.29) at the commencement of normal cell divisions within the endosperm is suggestive of an active role for the cytokinins at this stage in the development of the wheat grain.

Although Burrows *et al* (1970) identified four cytokinins as components of wheat germ tRNA, the first published report of the identification of free cytokinins in wheat comes directly from the results presented in this thesis (Jameson *et al*, in Press). The identification of zeatin riboside and of zeatin was attempted using GCMS (MIM). The response of the five ions monitored (m/z 639, 624, 550, 549, 536), their retention times and relative peak heights compared to those of TMS₄-zeatin riboside suggested that zeatin riboside was present in the silylated wheat sample. Zeatin riboside must therefore be responsible for at least some of the cytokinin activity detected by bioassay in that fraction of the wheat sample with an elution volume similar to that of authentic zeatin riboside. This is considered to be a positive identification of zeatin riboside.

The identification of zeatin was less definitive being based on the response of only two ions (m/z 230 and 188) characteristic of trimethylated zeatin. However, the ions appeared at the correct retention time and with relative peak heights similar to those established by authentic Me₃-zeatin. These responses would indicate that zeatin was present in wheat grains collected four days post-anthesis.

The data presented in this thesis support a strong positive correlation between the cytokinin concentration and the intensity of cell division in both developing potato tubers and developing wheat grains. The initial accumulation of the cytokinins may be a consequence of increased sink activity (Kretching *et al*, 1978) but the cytokinins themselves will further influence sink activity indirectly through their stimulation of cell division. They are not a mere accompaniment of growth as suggested by Prakash and Maheshware (1970) and may, in fact, be an important yield determinant exerting a major influence on the potential size of the wheat grain and potato tuber.

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APPENDIX 1

A. Basal Medium A

(i) <u>Macronutrients</u>	<u>mg l⁻¹ medium</u>	<u>g l⁻¹</u>	(x 10)
KH ₂ PO ₄	300.0	3.000	
KNO ₃	1,000.0	10.000	
NH ₄ NO ₃	1,000.0	10.000	
Ca(NO ₃) ₂ ·4H ₂ O	500.0	5.000	
MgSO ₄ ·7H ₂ O	71.5	0.715	
KCl	65.0	0.650	
(ii) <u>Micronutrients</u>			(x 100)
H ₃ BO ₃	1.60	0.160	
MnSO ₄ ·4H ₂ O	14.00	1.400	
Cu(NO ₃) ₂ ·3H ₂ O	0.35	0.035	
ZnSO ₄ ·7H ₂ O	3.80	0.380	
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.10	0.010	
KI	0.80	0.080	
NaFeEDTA	13.20	1.320	
(iii) <u>Organic Salts</u>			(x 100)
Thiamine.HCl	0.80	0.080	
Nicotinic acid	2.00	0.200	
Pyridoxine.HCl	0.80	0.080	
Myo-inositol	100.00	10.000	
Sucrose	3.0%		
Difco-Bacto agar	0.8%		
Add	100 ml l ⁻¹ (i)		
	10 ml l ⁻¹ (ii) and (iii)		

Add sucrose and make up volume with water and adjust pH to 5.8. Weigh agar into flasks, add medium and autoclave (104 kPa for 20 minutes).

B. Transfer Medium A

To Basal Medium A add

- (i) 1.862 mg l⁻¹ α-naphthalene acetic acid to give a final concentration of 10⁻⁵ M NAA, and
- (ii) 0.215 mg l⁻¹ kinetin to give a final concentration of 10⁻⁶ M kinetin.

C. Medium B

To Basal Medium A add 1.862 mg l^{-1} α -NAA to give a final concentration of 10^{-5} M NAA.

D. Transfer Medium B

To Basal Medium A add

- (i) 1.862 mg l^{-1} α -NAA, and
- (ii) 0.250 mg l^{-1} 6-benzylaminopurine or 0.250 mg l^{-1} kinetin.

APPENDIX 2

Hoaglund A. Nutrient Solution

A.	<u>Macronutrients</u>	<u>Amount</u> (g)	<u>H₂O</u> (to ml)	<u>Use in preparation of</u> <u>nutrient solution (ml)</u>
	KH ₂ PO ₄	136.09	1,000	1
	KNO ₃	101.10	1,000	5
	Ca(NO ₃) ₂	164.10	1,000	5
	MgSO ₄	120.39	1,000	2

B. Micronutrients

H ₃ BO ₃	2.86
MnCl ₂ .4H ₂ O	1.81
ZnSO ₄ .7H ₂ O	0.22
CuSO ₄ .5H ₂ O	0.08
NaMoO ₄ .2H ₂ O	0.02

Distilled H₂O to 1,000 ml

Use 1 ml in preparation of nutrient solution.

C. Iron solution

Iron tartrate	5
Distilled H ₂ O to	1,000 ml

Use 1 ml in preparation of nutrient solution.

To make one litre of full strength solution, use the required amounts of macronutrient solutions, micronutrient and iron solutions.

Germination of soybean hypocotyls Use 20% Hoaglund A solution.

PPD Experiment 1 Hoaglund A solution, modified by the use of chelated iron (Sequestrene NaFe chelate: stock solution 10.4 g l⁻¹ added at rate of 2 ml l⁻¹) and used at 25% stock solution.

APPENDIX 3

Hypocotyl Bioassay Medium

Prepare at 1.11x concentration.

A.	<u>Macronutrients</u>	<u>mg l⁻¹</u>	<u>g l⁻¹</u>	(x 10)
	KH ₂ PO ₄	333.33	3.33	
	KNO ₃	1111.11	11.11	
	NH ₄ NO ₃	1111.11	11.11	
	Ca(NO ₃) ₂ · 4H ₂ O	555.56	5.55	
	MgSO ₄ · 7H ₂ O	38.88	0.38	
	KCl	72.22	0.72	
B.	<u>Micronutrients</u>			(x 100)
	KI	0.88	0.089	
	MnSO ₄	4.88	0.489	
	H ₃ BO ₃	1.77	0.178	
C.	<u>Organic Salts</u>			(x 100)
	Glycine	2.22	0.20	
	Thiamine.HCl	0.11	0.01	
	Nicotinic acid	0.55	0.05	
	Pyridoxine.HCl	0.11	0.01	
	Myō-inositol	111.11	11.11	

Add 100 ml l⁻¹ A
 10 ml l⁻¹ B,C
 sucrose (33.3 g l⁻¹)

Make up volume with H₂O; adjust pH to 5.8.

Weight agar into dishes (0.8%); add growth regulator solutions at a rate to dilute the basic nutrient solution to 1x; autoclave at 104 kPa for 20 minutes.

APPENDIX 4

Preparation of Dowex 50 ion exchange resin in H⁺ form

1. Wash resin in distilled water until supernatant is clear.
2. Pour slurry into 25 x 300 mm column.
3. Elute column with:

	<u>ml</u>
2N NH ₄ OH	250
H ₂ O	400
2N HCl	250
H ₂ O	300
5N NH ₄ OH	150
H ₂ O	850
2N HCl	250
H ₂ O	700
2N NH ₄ OH	250
H ₂ O	500
2N HCl	400
H ₂ O	300

Column washed with methanol (70%; 250 ml) before application of extract.

APPENDIX 5

Soil Mix

(Adapted from K.F. Baker, The UC System for Producing Healthy Container Grown Plants, pg 73, Table 4. Adaptations as suggested by D.J. Woolley, pers comm.)

<u>Fertiliser</u>	<u>g PB8 bag⁻¹</u>
Uramite	1.44
Dolomite	2.31
KNO ₃	0.28
K ₂ SO ₄	0.28
Superphosphate (single)	2.98
	<u>cm³ PB8 bag⁻¹</u>
<u>Peat</u> (NZ)	1700
<u>Sand</u> (fine)	1700

Grind to a fine powder the uramite, dolomite, KNO₂ and K₂SO₄ and add to the sand.

Grind the superphosphate and add to the sand mixture. Gradually add sand mixture to the presoaked peat.

APPENDIX 6

Canterbury Controlled Growth Rooms

Lighting was provided by a rig of 'Philips' fluorescent tubes suspended above the plants. The light intensity and quality varied depending on the age of the tubes and the number of supplementary incandescent bulbs. Light intensity ranged from 100 to 200 $\mu\text{E m}^{-2}\text{s}^{-1}$ as indicated with the individual experiments. The humidity was uncontrolled.

APPENDIX 7

PPD Controlled Environment Rooms

Lighting was provided by 'Sylvania Metalarc' high pressure discharge lamps, together with 4 x 100 watt 'Philips' tungsten iodine lamps. Light was directed from the lamp rig down into the room through a glass and flowing water thermal barrier ($158 \pm 3 \text{ Wm}^{-2}$). Photoperiod extension lighting was supplied by 6 x 150 incandescent bulbs (7 Wm^{-2}). Air flow through the canopy was 0.3 - 0.9 ms^{-1} , and the relative humidity was controlled at 75%.

APPENDIX 8

Murashige and Skoog (1962) Nutrient Medium

A.	<u>Macronutrients</u>	mg l ⁻¹	g l ⁻¹	(x 10)
	NH ₄ NO ₃	1650	16.50	
	KNO ₃	1900	19.00	
	CaCl ₂ .2H ₂ O	440	4.40	
	MgSO ₄ .7H ₂ O	370	3.70	
	KH ₂ PO ₄	170	1.70	
B.	Na ₂ EDTA	37.3	7.45	(x 200)
	FeSO ₄	27.8	5.57	
C.	<u>Minor Elements</u>			(x 100)
	H ₃ BO ₃	6.2	0.6200	
	MnSO ₄ .4H ₂ O	22.3	2.2300	
	ZnSO ₄ .4H ₂ O	8.6	0.8600	
	KI	0.83	0.0830	
	Na ₂ MoO ₄ .2H ₂ O	0.25	0.0250	
	CuSO ₄ .5H ₂ O	0.025	0.0025	
	CoCl ₂ .6H ₂ O	0.025	0.0025	
D.	<u>Organic Salts</u>			(x 100)
	Glycine	2.0	0.20	
	Myo-inositol	100.0	10.00	
	Nicotinic acid	0.5	0.05	
	Pyridoxine.HCl	0.5	0.05	
	Thiamine.HCl	0.1	0.01	

Add 100 ml l⁻¹ A
 5 ml l⁻¹ B
 10 ml l⁻¹ C
 10 ml l⁻¹ D
 1.862 mg l⁻¹ NAA
 20 mg l⁻¹ sucrose
 10 g l⁻¹ Difco-Bacto agar

Adjust pH to 5.8 and autoclave at 104 kPa for 20 minutes.