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A STUDY OF THE FACTORS CONTROLLING BASAL INTERNODE LENGTH IN PEAS (*Pisum sativum* L.)

SYLVIE C. SEGUIN

A thesis

presented to the University of Ottawa

in partial fulfillment of the

requirements for the degree of

Master of Science

in

Biology

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UNIVERSITY OF OTTAWA

DEDICATION

I wish to dedicate this thesis to my parents,

Thérèse and Marcel, whose support and

encouragement permitted the successful

completion of this project

Je dédie cette thèse à mes parents,

Thérèse et Marcel, leur appui et

encouragement m'aidèrent à

complèter avec succès ce projet

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ABSTRACT

A study of factors controlling early stem development (e.g. the first two internodes) in etiolated peas was made. Several lines were selected for the study: a tall line (WL1267) with a short first internode and three dwarf lines with long (WL1415, WL1451) or short (WL1570) first internode were used in most experiments.

Measurements of cell size in mature internodes demonstrated that cell division is the major process determining final internode length.

In general, root excision, removal of the testa and excision of one cotyledon had no significant effect upon the growth of seedlings from the dwarf WL1570. Excision of the root reduced first internode growth in the tall line WL1267. Removal of the testa and the excision of one cotyledon caused an increase in internode length of seedlings from WL1267. Inhibitor(s) may be present in the testa and cotyledons of the tall WL1267 but not in the dwarf WL1570. Excision of cotyledons decreased growth in both lines since the energy source necessary for continued growth had been removed. Excision of the apex induced the growth of one lateral branch with increased first internode length.

The effect of exogenous growth regulators upon cut segment growth was determined. All segments showed an increase in length in the presence of IAA. The tall WL1267 responded more than the dwarf lines (WL1570, WL1415, WL1451). The segments from the growing third internode grew more than the segments from the growing second internode. These in turn grew more than the segments from the growing first internode.

Segment growth was not influenced by ethylene, kinetin, and abscisic acid. Only segments cut from the growing third internode of the dwarf WL1570 showed an increase in segment length upon addition of GA3. The endogenous levels of GA and IAA may interact within the segment to influence growth.

Peroxidase and IAA oxidase activities were determined in two dwarf lines, WL1570 and WL1415. IAA oxidase activity decreased along the stem (WL1570) and in the segment below the apex as the tissues aged. Levels were higher in line WL1415. IAA oxidase activity did not correlate well with internode growth since line WL1415 had the longest first internode but the highest enzyme activity. Peroxidase activity followed the same distribution pattern along the stem in both lines. However activity was higher in WL1570. At the base of the stem peroxidase may be involved in lignification. The low levels in the elongating zone of the internode suggest that this enzyme has little to do with elongation. Activity increased in the region near the apex. Peroxidases may be involved in some aspects of cell division. Peroxidase activity in the dividing zone correlates with growth since first internode growth is reduced in internodes with high peroxidase activity.

A procedure for the purification of IAA, ABA, zeatin and zeatin riboside from a small amount of plant tissue was devised. It utilized SepPak C18 cartridges to separate IAA, ABA and zeatin, zeatin riboside into two separate fractions. These fractions were then further separated by HPLC.

Using this procedure it was possible to estimate endogenous amounts of ABA and zeatin in the apices of etiolated pea seedlings of two lines at two stages of development. IAA if present was in amounts too low to be detected. Zeatin riboside levels could not be estimated by HPLC due to problems of detection in the presence of contaminants. However, both zeatin riboside and zeatin were detected by the *Amaranthus* bioassay. In

8 day-old seedlings of WL1570, 18.7 ng/g fr wt of ABA were present. The level went up to 25.2 ng/g fr wt in 11 day-old seedlings. In line WL1415, 15.3 ng/g fr wt were detected at 8 days and 24.0 ng/g fr wt at 11 days.

Zeatin riboside levels as determined by HPLC were 8.3 ng/g fr wt in 8 day-old seedlings, levels could not be estimated in 11 day-old seedlings of WL1570 because of contaminants. In WL1415, 18.8 ng/g fr wt were present at 8 days, and 6.7 ng/g fr wt were found in 11 day-old seedlings. Amounts of ZR and Z estimated by bioassay were 10.7 ng/g fr wt of ZR and undetectable in 8 day-old seedlings of WL1570. In 11 day-old seedlings, 8.7 ng/g fr wt of ZR and 7.1 ng/g fr wt of Z were found. In WL1415, 54.5 ng/g fr wt of ZR and 36.8 ng/g fr wt of Z were detected at 8 days and 8.1 ng/g fr wt of ZR and 4.2 ng/g fr wt of Z were found at 11 days. Thus cytokinins levels differed between the two lines and between the stages of development and may affect growth.

RÉSUMÉ

Une étude des facteurs controllant le développement des deux premiers entrenoeuds chez les pois étiolés fut faite. Plusieurs lignées furent sélectionnées pour cette étude: une lignée normale (WL1267) ayant un premier entrenoeud court et trois lignées naines ayant un premier entrenoeud long (WL1415, WL1451) ou court (WL1570).

Les mesures de la dimension des cellules dans un entrenoeud parvenu à maturité démontrèrent que la division cellulaire est le processus majeur déterminant la longueur finale des entrenoeuds.

En général, l'excision de la racine, l'enlèvement du testa et l'excision d'un cotylédon n'ont eu aucun effet significatif sur la croissance des plantules de la lignée naine WL1570. L'excision de la racine a réduit la croissance du premier entrenoeud chez la lignée normale WL1267. L'enlèvement du testa et l'excision d'un cotylédon ont causé un accroissement de la longueur des entrenoeuds des plantules de la lignée WL1267. Un ou des inhibiteurs peuvent être présents dans le testa et les cotylédons de la lignée normale WL1267 mais pas chez la lignée naine WL1570. L'excision des cotylédons a diminué la croissance chez les deux lignées puisque la source de nutriment nécéssaire pour la continuité de la croissance avait été enlevée. L'excision de la plumule a induit la croissance d'une branche latérale ayant un premier entrenoeud d'une longueur accrue.

L'effet des régulateurs de croissance exogènes sur la croissance de segments coupés fut déterminée. Tous les segments montrèrent un accroissement en longueur en présence de l'AIA, les segments de la lignée normale WL1267 ayant plus allongé que ceux des lignées naines (WL1570, WL1415, WL1451). En cours d'élongation, les segments provenant du

troisième entrenoeud ont grandi plus que les segments provenant du deuxième entrenoeud et ces derniers plus que les segments provenant du premier entrenoeud. La croissance des segments ne fut pas influencée par l'éthylène, la kinétine et l'acide abscisique. Seuls les segments provenant du troisième entrenoeud en cours d'élongation de la lignée naine WL1570 ont montré un accroissement de la longueur finale des segments après l'addition de l'acide gibbérellique. Les niveaux endogènes de l'acide gibbérellique et de l'acide indoleacétique peuvent intéragir à l'intérieur du segment et donc influencer la croissance.

Les activités de la péroxydase et de l'AIA oxydase furent déterminées chez deux lignées naines, WL1570 et WL1415. L'activité de l'AIA oxydase a décru le long de la tige (WL1570) et dans le segment situé sous la plumule durant le vieillissement des tissus. Les niveaux étaient plus élevés chez la lignée WL1415. L'activité de l'AIA oxydase ne correspond pas bien avec la croissance de l'entrenoeud puisque la lignée WL1415 avait un entrenoeud plus long mais une activité enzymatique plus haute.

L'activité de la peroxydase est répartie pareillement le long de la tige chez les deux lignées. Cependant l'activité était plus élevée chez la lignée WL1570. À la base de la tige, la péroxydase peut être impliquée dans la lignification. Les niveaux assez bas dans la zone d'élongation suggèrent que cette enzyme n'influence pas l'élongation. L'activité accrue dans la région près de la plumule semble impliquer les péroxydases dans certains aspects de la division cellulaire. L'activité de la péroxydase dans la région de division cellulaire correspond bien avec la croissance des entrenoeuds puisqu'au celle-ci est réduite chez les entrenoeuds ayant une activité péroxydasique élevée.

Une procédure pour la purification de l'AIA, de l'acide abscisique, de la zéatine et du riboside de zéatine à partir d'un petit montant de tissu végétal fut élaborée. Cette méthode utilise les cartouches SepPak C18 pour la séparation de l'AIA, de l'acide abscisique et de la zéatine (Z), du riboside de zéatine (ZR), dans deux fractions séparées.

Ces fractions furent séparées par chromatographie à phase liquide (HPLC).

En utilisant cette procédure, il fut possible d'estimer les niveaux endogènes de l'acide abscisique et de la zéatine dans le tissu apical des pois étiolés de deux lignées, à deux stades de développement. La quantité d'AIA était trop basse pour être détectée. Les niveaux du riboside de zéatine ne purent être estimés par HPLC à cause de la présence de contaminants. La zéatine ainsi que le riboside de zéatine furent détectés en employant le test biologique de l'*Amaranthus*.

Chez la lignée WL1570, 18.7 ng/g poids frais d'acide abscisique sont présents dans les plantules âgées de 8 jours. Les niveaux s'élevèrent à 25.2 ng/g poids frais dans les plantules âgées de 11 jours. Chez la lignée WL1415, 15.3 ng/g poids frais sont détectés de deux lignées à 8 jours et 24.0 ng/g poids frais de deux lignées à 11 jours.

Les niveaux de riboside de zéatine déterminés par HPLC furent de 8.3 ng/g poids frais dans les plantules âgées de 8 jours, le niveau ne put être estimé dans les plantules âgées de 11 jours à cause de contaminants. Les niveaux de zéatine et de riboside de zéatine estimés par test biologique étaient, 10.7 ng/g poids frais de ZR et aucune cytokinines n'a été détectées dans les plantules âgées de 8 jours de la lignée WL1570. À 11 jours, 8.7 ng/g poids frais de ZR et 7.1 ng/g poids frais de Z furent trouvés. Chez la lignée WL1415, 54.5 ng/g poids frais de ZR et 36.8 ng/g poids frais de Z furent détectés après 8 jours et 8.1 ng/g poids frais de ZR et 4.2 ng/g poids frais de Z furent trouvés après 11 jours. Les niveaux de cytokinines diffèrent entre les lignées et entre les stades de développement et peuvent alors affecter la croissance.

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CHAPTER I

INTRODUCTION

1.1 IMPORTANCE AND DISTRIBUTION OF PEAS

The pea, *Pisum sativum* L., is an annual herbaceous plant, member of the family Fabaceae, tribe Viceae (Duke, 1981). Peas have been grown since neolithic times (Marx, 1977), the primary centers of origin are thought to be in northwest Asia from where the pea species spread to the mountainous regions of southwest Asia (Makasheva, 1983). The secondary center of variability of the cultivated forms is found in the Mediterranean region, where intense cultivation ultimately led to the development of pea varieties for vegetable purposes (Makasheva, 1983).

The use of peas as a crop has many advantages. The pea seed has a rich protein content (20%) with a relatively balanced amino acid composition, even if it is limiting in the sulphur-containing amino acids, cysteine and methionine (Wright, 1985) (Table 1). It has a high digestibility; trypsin inhibitors and lectins are present in pea but these compounds are heat-labile so the digestibility value increases with cooking (Wright, 1985). As a forage crop, yields are similar to those obtained with grass but with a much lower fertilizer cost (Gane, 1985). In animal rations pea plants help to reduce fodder consumption and increase output of animal productivity (weight gain and milk production). Since the pea plant is a nitrogen-fixing legume it has been used to improve soil fertility in crop rotation (Makasheva, 1983, Pipe, 1985, Pate, 1977).

TABLE 1

Amino acid content in seeds of some common legumes.

Legume	Essential amino-acid content (as % of FAO/WHO (1973) amino-acid score)									
	Isoleucine	*Leucine*	*Lysine*	*Cysteine/methionine*	*Phenylalanine/tyrosine*	*Threonine*	*Tryptophan*	*Valine*	*Total essential amino acids (per g N)*	*NPU*[a] (%)
Common bean (*Phaseolus* spp.)	105	108	132	54	127	99	105	93	2.39	42
Broad bean (*Vicia faba major*)	100	101	119	44	124	84	90	89	2.20	48
Chick pea (*Cicer arietinum*)	111	106	126	63	142	94	90	92	2.43	58
Pea (*Pisum sativum*, *P. arvense*)	107	97	138	58	121	102	93	95	2.35	56
Lentil (*Lens esculenta*)	108	108	132	49	140	99	100	101	2.46	38
Peanut (*Arachis hypogaea*)	84	90	65	68	146	65	108	84	2.03	47
Soyabean (*Glycine max*)	114	110	118	74	133	96	133	97	2.46	66

[a] Net Protein Utilization = biological value × digestibility
Adapted from Burr (1975)

WRIGHT, 1985

Today, peas are grown mostly in temperate regions of the world, and at higher altitudes or in cooler seasons of warmer regions (Gane, 1985). About nine million hectares are devoted to the production of dry peas worldwide, three quarters of them in China and the USSR (Table 2). Peas rank third or fourth in worldwide production amongst the grain legumes (Pate, 1977). They are utilized as dried seeds, vegetables, and fodder, the production of dried peas being the most important aspect. Green peas are principally grown for processing by freezing and secondarily for canning (Hartmann et al., 1981) (Table 3). Some cultivars (snow peas) form an important part of the diet in China, Japan and Europe (Hartmann et al., 1981). Green pea production is increasing, especially in the more developed countries where the demand for frozen and canned peas exceeds production (Makasheva, 1983). In the USSR, peas occupy more than 66% of the total area under grain legumes. They are used as a food crop, although their significance as a fodder crop has increased in recent years (Makasheva, 1983).

1.2 PROBLEMS ASSOCIATED WITH PEA CULTIVATION

Lodging, which results from weak stems and a rambling growth habit (Heath & Hebblethwaite, 1985), is one of the most serious problems encountered by pea growers, especially in dried pea cultivation (Hedley et al., 1983). The crop yield is unpredictable from year to year because of the difficulty in harvesting seeds and because of the disease problems which occur when air movement through the crop is restricted (Hedley et al., 1983). Yields are reduced because less light reaches the leaves and photosynthesis is not as efficient (Kaatz & Gritton, 1975). Shaded pods are a lighter green color which is undesirable, because it reduces color uniformity which makes the product less appealing to the consumer (Kaatz & Gritton, 1975). The growth of the pea seedlings, and also

TABLE 2

Production of dry peas worldwide during two 3-year periods.

Region/Country	*Area* (1000 ha)		*Yield* (metric t ha^{-1})		*Production* (1000 metric t)	
	1969–71	*1979–81*	*1969–71*	*1979–81*	*1969–71*	*1979–81*
Africa	416	433	0.66	0.77	275	335
Burundi	38	31	0.80	1.19	30	37
Ethiopia	105	131	0.65	0.98	68	128
Morocco	61	49	0.64	0.48	39	25
Rwanda	76	60	0.85	0.78	64	47
Zaire	61	73	0.63	0.63	39	47
N. and C. America	160	131	1.64	1.95	262	255
Canada	32	47	1.35	1.76	44	84
USA	118	70	1.80	2.29	212	162
South America	138	146	0.73	0.71	101	103
Colombia	44	56	0.68	0.59	30	33
Asia	3138	2160	0.97	1.25	3055	2692
China	2100	1533	1.03	1.50	2167	2300
India	982	571	0.85	0.57	835	330
Iran	21	25	1.23	1.26	26	31
Europe	404	273	1.57	2.15	636	590
Czechoslovakia	14	31	1.62	2.11	23	64
France	12	26	3.31	4.13	38	107
Hungary	80	53	1.40	2.03	111	104
Poland	50	42	1.24	1.55	62	65
UK	25	36	2.96	2.96	75	107
Oceania	51	71	1.57	1.73	81	122
Australia	29	47	0.97	1.17	28	55
New Zealand	22	24	2.35	2.82	53	67
USSR	3316	4181	1.51	1.05	4989	4339
World	7624	7395	1.23	1.15	9399	8434

GANE, 1985

TABLE 3

Production of green peas worldwide during two 3-year periods.

Region/Country	*Area* (1000 ha)		*Yield* (metric t ha^{-1})		*Production* (1000 metric t)	
	1969–71	*1979–81*	*1969–71*	*1979–81*	*1969–71*	*1979–81*
Africa	19	21	4.65	5.96	89	126
N. and C. America	192	171	6.52	7.57	1254	1300
Canada	20	19	2.89	3.67	57	69
Mexico	13	16	2.23	3.19	29	54
USA	160	136	7.32	8.64	1168	1177
South America	45	42	2.48	2.93	111	122
Asia	132	151	3.96	4.00	523	603
China	33	42	5.47	5.38	181	225
India	75	88	2.87	2.88	215	253
Europe	280	295	6.88	6.96	1926	2054
France	48	60	8.36	7.50	400	447
Hungary	30	35	5.03	6.09	152	215
Romania	16	28	2.66	2.56	41	71
UK	52	56	10.56	12.03	546	679
Oceania	30	23	4.96	6.83	147	154
Australia	21	14	5.58	7.83	117	111
USSR	57	67	3.04	3.73	174	251
World	755	770	5.59	5.99	4224	4610

GANE, 1985

other legumes (e.g. beans), differs from other plants such as cereals, and flax. During hypogean germination the epicotyl elongates rapidly, to bring the apex to the soil surface. The base of the pea stem is most vulnerable to lodging. As the plant grows taller, the base provides a weak link between root and shoot and a natural hinge for the stem when the crop collapses (Snoad, 1985). Menoux et al. (1982) have determined that resistance to lodging in flax is essentially due to short basal internode length, associated with a slow elongation rate during early growth. Pea plants with short and thick stems and short internodes, having abundent supporting tissue, should be less susceptible to lodging.

1.3 GENETIC AND ENVIRONMENTAL CONTROL OF STEM LENGTH IN PEAS

In peas, total height is made up of two independent components: internode number and internode length (Marx, 1974). Internode number is influenced by the time required to reach maturity, late flowering varieties tend to have more nodes than early flowering varieties. Internode length varies between successive internodes (Marx, 1974), and varies with the plant genotype.

Eight or more loci control internode length in *Pisum*. Possible genotypes and phenotypic descriptions are found in Table 4. The Le locus, on chromosome 4, possesses two alleles which, depending on the alleles found at other loci, give, when expressed, tall (Le) and dwarf (le) plants. Tall plants have longer internodes (3rd internode and up) than dwarf plants.

The Na locus, on chromosome 6, possesses two alleles. It is epistatic to the Le locus since the tall phenotype is not expressed in nana plants (Murfet, 1978). These plants have very short internodes, resulting in plants only a few centimeters high.

TABLE 4

Description of the main internode length phenotypes in peas.

Phenotype	*Phenotypic description*	*Genotype*
Tall	Normal long internodes above node 4, internodes 5–6 onwards approx. 10 cm, probably wildtype	*Le La Cry Na Lm* *Le La cry^c Na Lm* *Le La cry^s Na Lm* *Le la Cry Na Lm*
Dwarf	Internodes 40–60% of tall types, zig-zag appearance of stem. Pleiotropic effects on flowering	As for tall except *le*
Nana (= Compactum)	Extreme shortening of internodes (usually less than 1 cm), darkly coloured leaves, reduced yield	As for tall and dwarf except *na*
Cryptodwarf	Long, thin lower internodes, higher internodes still larger than dwarf	*le la cry^c Na Lm* *le la cry^c na Lm*
Cryptotall	Long, thin lower internodes but above node 7 slightly shorter than for tall	*Le la cry^c Na Lm*
Slender	Long, thin stems from node 0, pale foliage, malformed flowers, parthenocarpic pods and reduced yield	*Le la cry^s Na Lm* *le la cry^s Na Lm* *Le la cry^s na Lm* *le la cry^s na Lm*
Microdwarf Microcryptodwarf Microslender Microtall	Smaller than corresponding dwarf, tall, cryptodwarf or slender in all parts including internode length	As for corresponding dwarf, tall, cryptodwarf or slender, except *lm*

MURFET AND REID, 1985

The La locus, on chromosome 3, possesses two alleles and the Cry locus, on chromosome 2, has three alleles, two of them recessive, and giving the phenotypes, slender (cry^s) and crypto (cry^c)(Marx, 1978). Recessive alleles must be present at both loci for the phenotypic expression. Crypto-dwarf (genotype le la cry^c) possesses longer first and second internodes than tall, however, succeeding internodes are shorter which results in a shorter plant when considering total height. Crypto-tall (Le la cry^c) also has long early internodes; however, a doubling of internode length occurs between nodes 4-9 (Reid et al., 1983). Slender plants (le la cry^s) possess elongated internodes, longer than those of tall plants. Final height is greatest for slender plants (Marx, 1977).

Plants with the phenotype micro (lm) show a size reduction of the whole plant, not just the internodes. Phenotypes such as microtall, microdwarf, microslender, etc. are obtained, depending on the alleles found at other loci (Reid et al., 1983). Lm has two alleles on chromosome 6.

Other genes, recently studied by Reid (1986), reduce internode length. Lh and Ls plants are found within the dwarf/nana range, whereas Lk gives the erectoide phenotype. Erectoide plants have short internodes, dark green foliage, reduced yields, brittle stems of increased diameter, very short peduncles and petioles, and an increased apical dominance (Reid, 1986).

Stem growth of etiolated pea plants is influenced by the genotype (Le, Na, La, Cry), in a similar fashion as light-grown pea plants (Reid, 1986). The effect of the Le/le difference is less pronounced because the Le locus has less influence on early basal internode development (Reid, 1983). The maximum effect of this gene occurs during the development of internodes 6-7. Etiolated plants are able to grow to the sixth internode in darkness, afterwards growth stops because the cotyledons are depleted (Low, 1971).

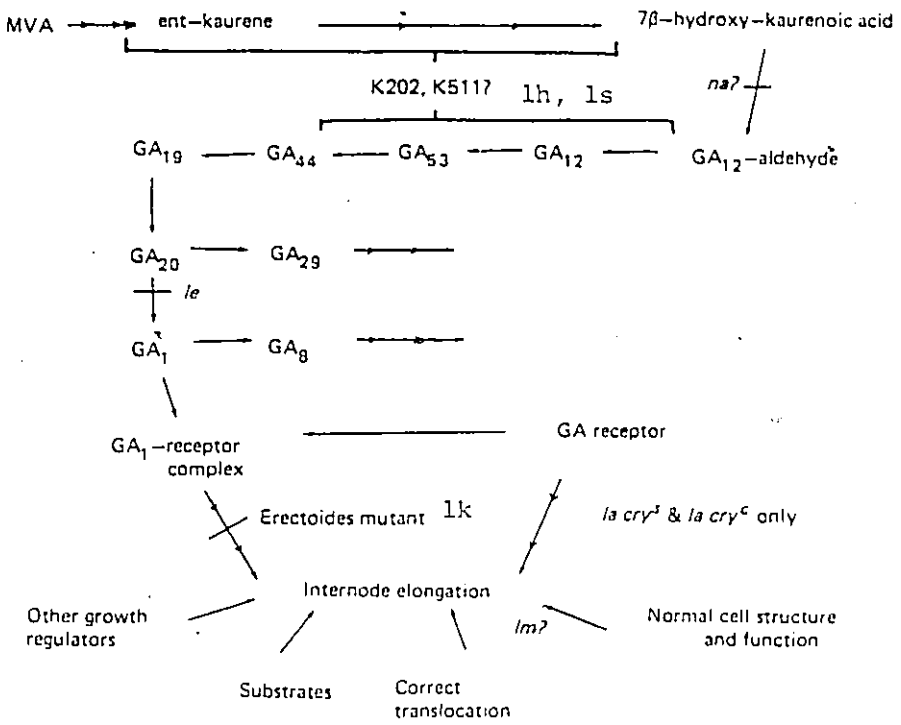
The environment plays a major role in plant development and internode length. The presence or absence of light brings about important changes in stem growth (Priestley, 1926). In darkness, internodes are much longer, whereas in light they are shortened. Two photoreactions induced by red and blue light inhibit internode elongation (Sale & Vince, 1960). Short periods of red light are effective and the effect is reversed by far red light. Blue light is also effective in reducing stem length and its effect is not reversed by far red light.

Changes in growth responses to the environment are mediated by growth factors (e.g. gibberellins, auxins, cytokinins, etc.) which act by influencing the pattern of cell elongation and cell division. Gibberellins are synthesized from mevalonic acid in both seeds and shoots of pea plants (Table 5). Differences are found in levels and types of GAs within different plant organs (Sponsel, 1985). Gibberellins promote stem elongation in both tall and dwarf plants. Dwarf plants respond more than tall plants, mimicking the appearance of slender plants (Reid et al., 1983). The effect is observed in both light and dark (Reid, 1983) although it is more prominent in light-grown plants. Gibberellin levels have not been found to differ between tall and dwarf pea plants (Jones & Lang, 1968). High levels of an endogenous polar gibberellin-like substance were found by Potts and coworkers (Potts et al., 1982) in the apical portion of the shoot of tall pea. This substance is absent or present in very small quantities in dwarf pea plants (Potts & Reid, 1983). It thus appears that a difference in the GA types rather than in endogenous levels induces the dwarf or tall phenotypes.

The nana phenotype can be induced by the inhibitor AMO-1618, (Potts & Reid, 1983), and restored to normality by the application of GA3 (Murfet, 1978). Also, no gibberellin-like substances were detected by bioassay in extracts from nana plants (Potts & Reid, 1983). These results and also the fact that the Na gene is epistatic to Le,

TABLE 5

Possible sites of actions in the gibberellin biosynthetic pathway of some genes responsible for altering internode length in *Pisum*.



MURFET AND REID, 1985

indicate that Na affects a step very early in the GA synthetic pathway, whereas Le acts later. The gene Le has been suggested as promoting the conversion of GA20 to the highly active GA1 (Potts & Reid, 1983).

Other phenotypes, such as slender (la cry^s) and microcryptodwarf (la cry^clm) are not inhibited by AMO-1618 at concentrations which affect tall and nana (McComb & McComb, 1970), do not respond to exogenous GA and thus may not implicate a step in the gibberellin biosynthetic pathway. The allele lk is incompletely epistatic to the Le, Na, la cry^s, and la cry^c combination (Reid, 1986). Reid (1986) suggested that lh and and ls may partially block steps in the gibberellin biosynthetic pathway, while lk acts after gibberellin reception (Reid, 1986). It is thus recognized that gibberellins play an important role in pea internode growth (Table 5). However, other factors are also of importance.

Auxins are synthesized from tryptophan and have been detected in pea extracts by various workers (McComb, 1977). Recently, auxins such as phenylacetic acid, 3-indoleacetic acid and 4-chloro-3-indoleacetic acid were quantified from roots, cotyledons, and epicotyls of etiolated pea seedlings (Schneider et al., 1985). Also, 3-indolepropionic acid was detected in roots and 3-indolebutyric acid was detected in both roots and epicotyls (Schneider et al., 1985). Auxins promote cell elongation and cause an increase in the extension of isolated segments of pea internodes floating in a sucrose-containing buffer with increasing concentrations up to an optimum (McComb, 1977). In intact plants, applied auxins do not affect growth presumably because the endogenous level is near optimum (McComb, 1977).

Ethylene, synthesized from methionine, is normally produced by the hook region and plumule of etiolated pea epicotyl (Goeschl et al., 1967) and at very low levels by pea roots (Chadwick & Burg, 1967). Ethylene inhibits stem growth of etiolated pea seedlings

by altering growth in the subhook region of the pea epicotyl and by inhibiting growth in the apical hook region (Stewart et al. 1974, Eisinger, 1983). Intact plants respond more to ethylene than do segments (Eisinger, 1983).

Abscisic acid is a growth-inhibiting substance which has been found to occur in immature pea seed, in shoots, in roots, and in mature leaves (McComb, 1977). It is formed from either mevalonic acid or more directly from a C14 carotenoid-type precursor (Walton, 1980). Abscisic acid usually inhibits the growth of whole plants, excised organs or seeds (Milborrow, 1974). It has not been directly linked to the control of stem growth and no differences in ABA content have been detected between tall and dwarf plants (Simpson & Saunders, 1972). However ABA may still affect growth since it can counteract the stimulatory effects of natural growth promoting compounds (Milborrow, 1974).

Cytokinins, such as zeatin, zeatin riboside, 2iP[6-(3-methyl-2-butenylamino)purine] and its riboside, have been extracted from pea root t-RNA (Babcock & Morris, 1970). These have also been reported in t-RNA from pea shoots (Vreman et al., 1972). Adenine and adenosine are likely precursors of cytokinins, with the side chain originating from mevalonate. Cytokinins are thought to be involved in cell division processes in plant cells. When applied through the root system to intact light-grown plants, cytokinins inhibit shoot elongation (McComb, 1977). When applied exogenously to epicotyls of dark-grown bean seedlings, stem elongation is promoted (Miller, 1956).

Interaction between various endogenous growth factors occurs during growth. Gibberellin and auxins act together to promote stem elongation in both dark- and light-grown seedlings (Ockerse & Galston, 1967, Purves & Hillman, 1959). Auxin-induced stem elongation is inhibited by exogenous cytokinins (Miller, 1956, Vanderhoef & Stahl, 1975) and ethylene (Brian & Hemming, 1957). Ethylene reduces polar auxin movement

and stops lateral auxin transport in pea stem sections (Burg & Burg, 1967). In intact seedlings, gibberellins counteract the action of ethylene in the subhook region but have no effect on the inhibition of elongation and division in the hook region and in the apical meristem (Stewart et al., 1974). Supraoptimal levels of auxins cause an increase in ethylene production (Burg & Burg, 1968). Cytokinins induce ethylene production in 3-day-old etiolated tall pea seedlings (Gertman & Fuchs, 1972), but not in dwarf pea seedlings (Fuchs & Lieberman, 1968). Gibberellins have no effect on ethylene production (Fuchs & Lieberman, 1968). ABA inhibits ethylene production (Gertman & Fuchs, 1972) as well as the auxin -induced ethylene production (Kondo et al., 1975).

Less auxin is present in dark-grown than in light-grown pea seedlings (Scott & Brigg, 1960). Auxin transport is faster in darkness, thus elongation in tissue farther from the auxin source is enhanced, whereas in light-grown plants, tissues nearer the apex are elongating more (Briggs, 1963). Jones and Lang (1968) found no differences in GA levels of light-grown and dark-grown pea stem tissue. In bean seedlings, lower levels of ABA are found in the dark (Tillberg, 1974). Cytokinin content is higher in roots which have not been exposed to light (Woolley & Wareing, 1972). Ethylene production is higher in dark-grown seedlings (Burg & Burg, 1966).

1.4 OBJECTIVE OF THE THESIS

Very little information is available on the development of the first and second internode and on lodging resistance in peas. The development of such resistance appears desirable since it could improve yielding ability, quality, and efficiency of production (Kaatz & Gritton, 1975). The present study is concerned with the early internode development in darkness. Most studies (dark- and light-grown plants) have dealt with

third or higher internodes and few have looked at the two basal internodes. It is relevant to study etiolated peas since in field conditions, seeds can be sown to quite a depth under the soil surface, thus initial growth would occur in darkness.

The establishment of the pea seedling is determined by the development of the basal internodes. It was noticed (Nozzolillo & Seguin, 1984) that basal internode development varied amongst pea lines. In some, the first internode would elongate and bring the apex to the soil surface. In others, the first internode would stop elongating at some point below the surface. The second internode would then bring the apex to the soil surface. An attempt at understanding, how the development of the basal internodes is controlled, is of some interest because it is vital to the seedling that at least the third leaf-bearing node reaches sunlight so that photosynthesis can begin.

The purpose of the present study was to define factors affecting extension growth of etiolated first and second internodes of pea seedlings. Pea lines, showing differences in the etiolation response of these internodes, were selected. The experiments were divided into three phases. 1) A study with intact seedlings comprising a determination of internode length, an analysis of the activity of IAA metabolizing enzymes in the stem and a determination of cell size in mature internodes. 2) A study of the response of excised segments to exogenous growth regulators and a determination of seedling growth following organ excision. Finally, 3) an analysis of growth factors in the plumule of etiolated pea seedlings performed at two stages of development.

CHAPTER II

MATERIAL AND METHODS

2.1 PLANT MATERIAL

Pea seeds (*Pisum sativum* L.) were obtained from S. Blixt, Weibullsholm, Sweden. Some lines were multiplied at the Ottawa Research Station, Agriculture Canada, during the summers of 1982/83 to obtain fresh seed for subsequent experiments. Lines WL1415, WL1570, WL1451 and WL1267 were used for most of the experiments. Lines WL1415 and WL1570 are wild-type dwarfs originating from Afghanistan and Ethiopia, respectively. WL1451 is a cross-derivative dwarf and WL1267 is a cross-derivative tall. When grown in the field, the mean internode length was 3.8 cm for WL1415, 3.4 cm for WL1570, 3.1 cm for WL1451 and 6.3 cm for WL1267 (Nordic Gene Bank Data). The percent germination was 92% for WL1415, 93% for WL1570, 74% for WL1267 and 46% for WL1451.

2.2 CHEMICALS

Indole-3-acetic acid (IAA) was purchased from Eastman Kodak Co., (±)-cis,trans-abscisic acid (ABA), zeatin (Z), zeatin riboside (ZR), adenine, and adenosine from Sigma Chemical Co.. Gibberellic acid (GA3) was obtained from Baker, kinetin from Aldrich and

ethylene from Liquid Carbonic Canada Ltd. [^{14}C]-IAA (48 mCi/mmol) and DL-cis,trans- [G-^{3}H]abscisic acid (22 Ci/mmol) were supplied by Amersham. Solvents were HPLC grade or redistilled prior to utilization.

2.3 INTERNODE LENGTH SURVEY OF VARIOUS PEA LINES

Fifteen seeds were used for each test. The seeds were surface-sterilized with a 1% solution of sodium hypochlorite for 5 minutes, then rinsed extensively with distilled water. They were subsequently soaked in distilled water for 24 hours in darkness at 15 ± 1 ^{0}C. They were then transferred under a sterile hood, to aluminum soil cans (diameter 9 cm) containing a shallow layer of heat sterilized, moistened sand (31 ml of sand, 20 ml water). Each can was covered with a 48 ounce juice can, (diameter 10.5 cm, height 17.5 cm) which effectively prevented light penetration, and placed in a temperature controlled (15 ± 1 ^{0}C) growth chamber. After two weeks, seedling development was checked using a dim green safelight. A small amount of green light has no significant effect on the growth of dark-grown seedlings (Low, 1971). The seedlings at the appropriate stage of growth were removed and the lengths of the fully developed first and second internodes were measured.

2.4 CELL SIZE DETERMINATION

Seedlings with fully expanded first and second internodes were selected. The internodes were cut with a razor blade in many 5 mm segments, with no attempt to keep top and bottom segments separated. They fixed in FAA (ethanol / glacial acetic

acid / formalin / water 50/5/10/35 v/v/v/v) at room temperature for 18-24 hours. This method of fixation is recommended for tissues to be embedded in paraffin wax (McCully & O'Brien, 1981). They were then dehydrated by passing through a series of alcohols (TBA) as described by Jensen (1962), embedded in paraffin and sectioned with a rotary microtome to give sections 10 um thick. The sections were dewaxed, stained with safranin-fast green (O'Brien & McCully, 1981), and mounted in permount. Cortical cell length and diameter were measured using a microscope equipped with a scaled ocular. Five seedlings from each of lines WL1570 and WL1267 were used and at least 100 cells were measured in each internode.

Cell length and diameter were also measured using epidermal strips as described by Arney and Mancinelli (1966). Three seedlings were used and at least 30 intact cortical cells selected at random cells were measured in each internode. The stem diameter was measured at different positions throughout the internode and the mean was calculated for each internode. This was done for line WL1570 and WL1415.

2.5 GROWTH RATE DETERMINATION

Growth rates had to be determined so that seedlings could be sampled for subsequent experiments with minimum disturbance. Seeds (800 or more) were surface-sterilized with concentrated sulphuric acid for 7 minutes. The acid was poured off, the seeds were dropped in 1 L of cold distilled water and subsequently rinsed 10x with distilled water. The rest of the procedure was as described above in section 2.3. Groups of 15 seeds were grown in each can and 3 cans were measured every time. Seedlings were removed on each of days 5,7,9,12,14,16,19 and 20. Internode length, total stem length (from cotyledon attachment to apical hook), root length (from cotyledon attachment to primary root tip)

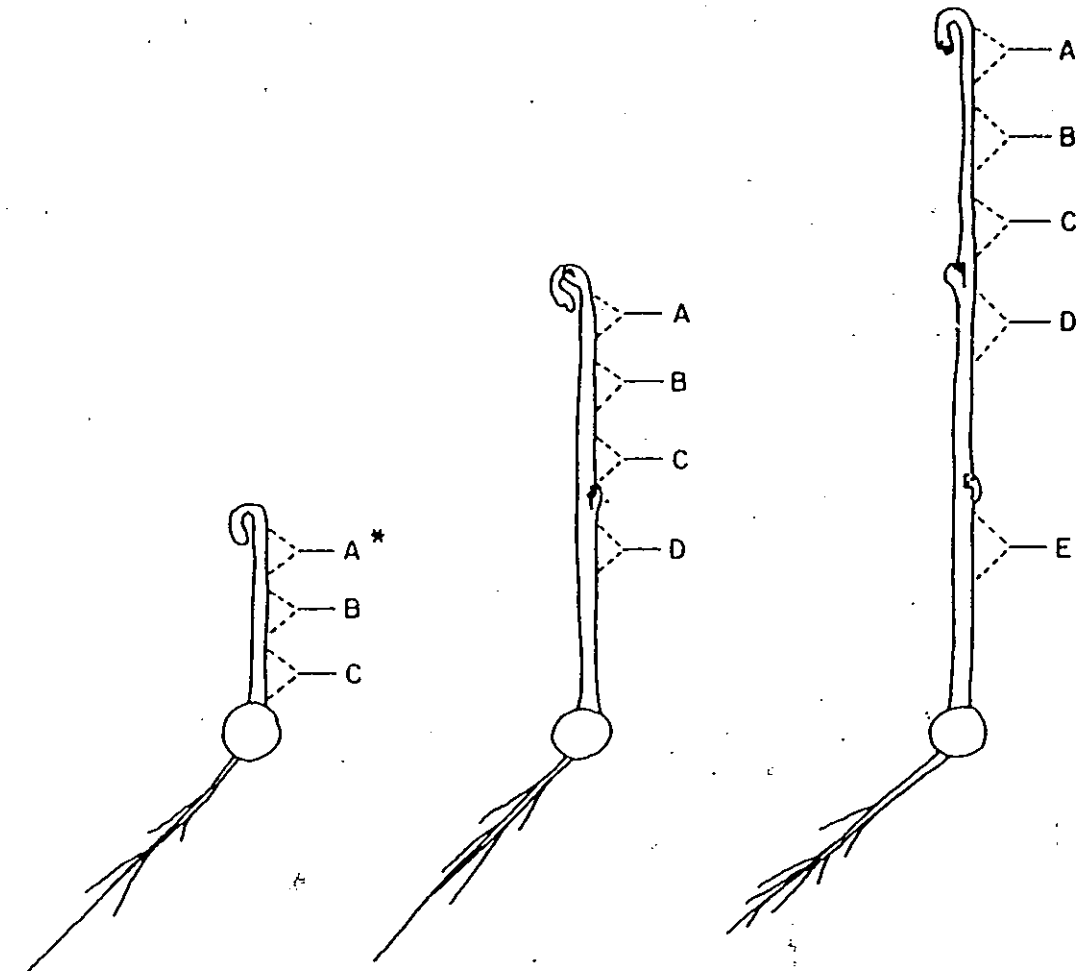
and lateral root number were all measured for each of three lines (WL1267, WL1415, WL1570). Graphs of growth versus time were plotted (Appendix 1 and 2). Seedlings used for subsequent experiments were harvested at times of maximum internode elongation.

2.6 EXCISION EXPERIMENTS

Seedlings were grown as described in section 2.5. Plant parts (apex, root, testa, cotyledons) were excised at three different stages of development for lines WL1267 and WL1570. Excision was performed under a green safelight. Seedlings were returned to the growth chamber to resume their development. This experiment was done once with line WL1267 and twice with line WL1570.

2.7 EFFECT OF EXOGENOUS GROWTH REGULATORS

Thirty segments, 1 cm long, were cut (as in Fig. 1, stage 1, 2, 3, position A) 3 mm below the apex with a two-bladed guillotine and transferred to petri dishes (10 segments/dish) containing 10 ml of incubation solution (.5 M sodium potassium phosphate buffer pH 6.8, 2% sucrose, growth regulator 10^{-4}M) (J. Phipps, personal communication). The segments were incubated in darkness at 15 ^{0}C for 24 hours. All manipulations were done in darkness with a green safelight. After incubation, the segments were weighed and measured for length and diameter. Dry weights were also determined, after segments were air dried for one week on top of an oven (T ^{0}C = 30). Each experiment was repeated at least twice.



STAGE 1
(day 8)

STAGE 2
(day 11)

STAGE 3
(day 14)

Figure 1. Diagram of 3 developmental stages of etiolated pea seedlings. The positions where segments were cut for the enzyme assay and segment growth (1A, 2A, 3A) are shown.

A - cut 3 mm below the apical hook

B - cut in the middle of the internode

C - cut 3 mm above cotyledon (stage 1) or 3 mm above node (stage 2 & 3)

D & E - cut 3 mm below node

2.7.1 EFFECTS OF GROWTH REGULATOR CONCENTRATION

A concentration range of 10^{-7} to 10^{-2} M was used; the growth regulators used singly were gibberellic acid (GA3), abscisic acid (ABA), kinetin and indole-3-acetic acid (IAA). Gibberellic acid, abscisic acid and indole-3-acetic acid were dissolved in a small amount of 95% ethanol whereas kinetin was dissolved in a small amount of dilute NaOH solution before being added to the buffer. The controls were prepared in the same way but without the growth regulator. Seedlings were cut on days 8 and 11 as described above. Line WL1415, WL1570, WL1267 and WL1451 were utilized for experiments with IAA. WL1415 and WL1570 were used for experiments with other growth regulators.

2.7.2 ETHYLENE

Segments were prepared and cut in the same manner as above. Instead of being transferred to petri dishes they were transferred to 100 ml Erlenmeyer flasks containing the incubation solution. These were subsequently closed with vaccine caps. Enough ethylene was injected with an airtight syringe through the vaccine cap to make a final concentration of 50 ppm. The segments were incubated and measured as described above. Before opening the Erlenmeyer the concentration of ethylene was assessed by injecting a 5 ul sample into a 5830A Hewlett-Packard GC connected to a 18850A GC Terminal Hewlett-Packard recorder and equipped with a flame ionization detector. Analyses were performed isothermally at 30 ^{0}C on a 1.33m x 2 mm i.d. Poropak N glass column

(80-100 mesh). The carrier gas was nitrogen at a flow rate of 30 ml/min. Ethylene was detected after 1.5 minutes. The ethylene peak areas were calculated and the absolute quantities determined from a known stock concentration of 2% ethylene in argon.

2.8 STATISTICAL ANALYSIS

Data from cell size measurements, excision experiments and segment growth experiments were subjected to an analysis of variance and to Duncan's multiple range test for means.

2.9 ENZYME ACTIVITIES

Segments 1 cm long were cut as shown in Fig.1 , separated in three groups and weighed. The soluble enzymes were extracted following the method of Galston and Dalberg (1954). IAA oxidase activity was determined with the Salkowski colorimetric method as used by Stenlid (1963) except for the addition of $MnCl_2$ (.1mM) and 2,4-diclorophenol (.05 mM) in the buffer (Galston & Dalberg, 1954). The guaiacol method used to determine peroxidase activity is as described by Maehly (1954). A Pye Unicam SP 1800 ultraviolet spectrophotometer equipped with a Unicam AR 25 linear recorder was used to record the increase in absorbance. The experiment was repeated at least three times with 3 replicates each.

2.10 ENDOGENOUS GROWTH REGULATOR ANALYSIS

2.10.1 DEVELOPMENT OF EXTRACTION METHOD

Development of the separation procedure used in the present study required considerable experimental testing before a satisfactory system was worked out. The goal was to purify IAA, ABA, adenine, adenosine, zeatin and its riboside from the same plant tissue. SepPak C18 cartridges were tested for their ability to retain these growth regulators. The elution of the SepPak C18 cartridges with various organic solvents was also studied. Steps which included filtration with Celite, fractionation with diethyl ether, slurry with polyvinylpyrrolidone (PVP), fractionation with n-butanol (for cytokinins) were tried and assessed for purification of the plant extract and for recovery of the growth regulators.

2.10.2 SAMPLE PREPARATION

The growth regulators were extracted from the apex at two stages of development, in two dwarf lines, WL1570 and WL1415, 1) at 8 days when the first internode was actively elongating and 2) at 11 days when the second internode was elongating. In the method finally selected the tissues were excised under a green safelight, weighed and homogenized with a mortar and pestle in acetone (final concentration 80% acetone/water) over ice (step 1,Fig. 2). Growth regulator standards if desired, were added at this step. The homogenate was filtered, the residue was rinsed with acetone and filtered. The filtrate was reduced to water (<10 ml) on a R110 Buchi rotovapor, at 35 ^{0}C (step 2),

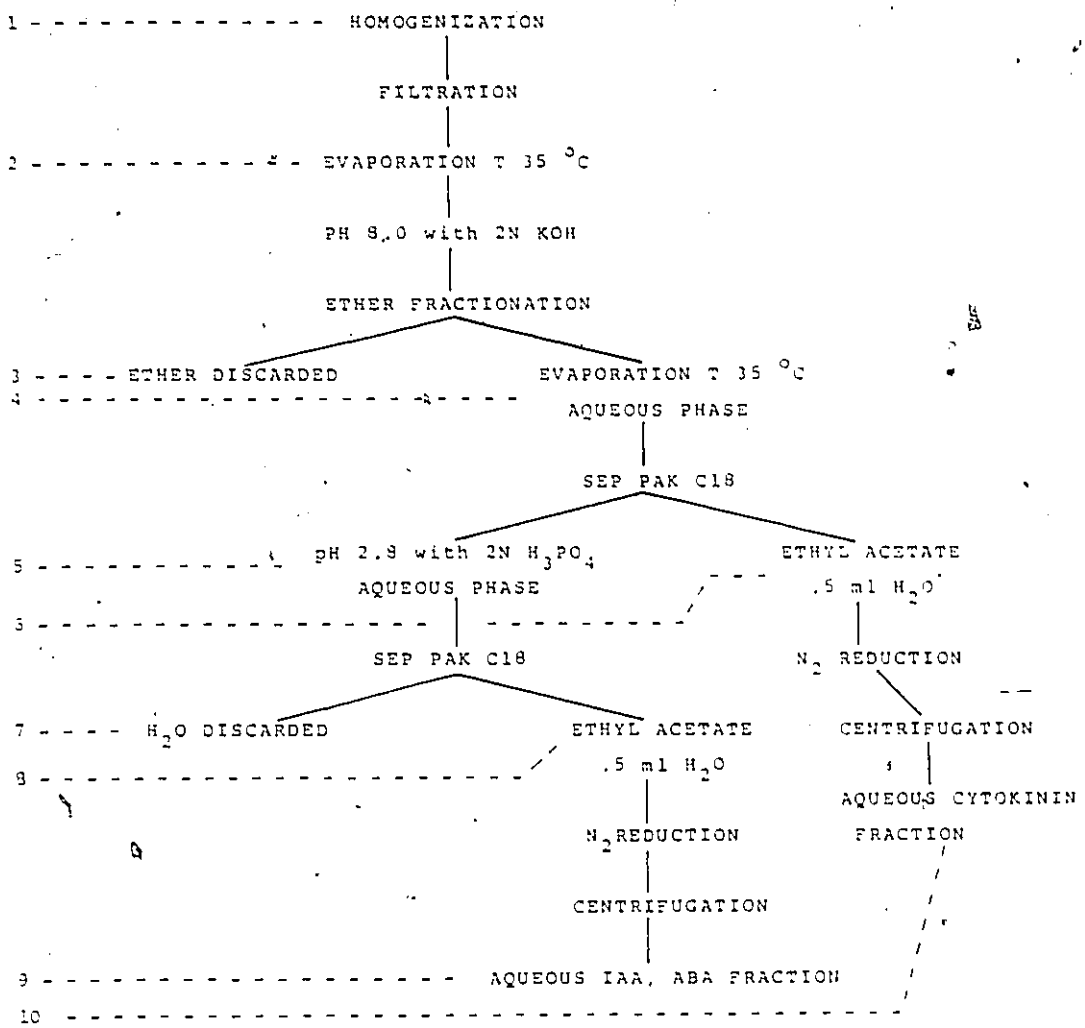

1
HOMOGENIZATION
FILTRATION
2
EVAPORATION T 35 °C
PH 8.0 with 2N KOH
ETHER FRACTIONATION
3
ETHER DISCARDED
EVAPORATION T 35 °C
4
AQUEOUS PHASE
SEP PAK C18
5
pH 2.8 with 2N H_3PO_4
ETHYL ACETATE
AQUEOUS PHASE
.5 ml H_2O
6
SEP PAK C18
N_2 REDUCTION
7
H_2O DISCARDED
ETHYL ACETATE
CENTRIFUGATION
8
.5 ml H_2O
AQUEOUS CYTOKININ
FRACTION
N_2 REDUCTION
CENTRIFUGATION
9
AQUEOUS IAA, ABA FRACTION
10

Figure 2. Flow chart for the extraction and purification of IAA, ABA, zeatin and zeatin riboside from etiolated pea apices. Standards were added at step 1.

and transferred to a beaker. The rotovapor flask was rinsed with 5 ml of hot water (70-80 ^{0}C) and added to the aqueous solution in the beaker. The pH of this aqueous solution was adjusted to 8.0 with 2*N* KOH and fractionated with diethyl ether (3X, equal volume) which was subsequently discarded (step 3). The aqueous phase was reduced with a rotavapor, T= 35 ^{0}C (step 4), to remove any traces of diethyl ether. This step was essential since preliminary experiments showed that Z and ZR were not retained on the first SepPak C18 cartridge if ether was present. The aqueous solution was subsequently applied to a SepPak C18 cartridge previously washed with methanol (2 ml) and water pH 8.0 (5 ml).

The aqueous sample, plus the washings, were pooled after their passage through the cartridge, and adjusted to a pH of 2.8 with 2*N* phosphoric acid (step 5). This solution was applied to a second SepPak C18 cartridge (prewashed with 2 ml methanol, 5 ml water pH 2.8) and the aqueous eluate was discarded (step 7). The cartridge was washed with water and then eluted with 5 ml ethyl acetate (step 8). Water (.5 ml) was added to the ethyl acetate solution which was subsequently reduced under a nitrogen stream, centrifuged in an Eppendorf centrifuge (10 min.) and kept for HPLC analysis (step 9).

The first SepPak C18 cartridge was eluted with 10 ml ethyl acetate (step 6) to which .5 ml water was added. This eluate was reduced to .5 ml water under a stream of nitrogen, centrifuged in an Eppendorf centrifuge (10 minutes) and kept for high performance liquid chromatography (HPLC) analysis (step 10).

2.10.3 HPLC CONDITIONS

HPLC chromatography was done on a Varian 5000 LC equipped with a Varian

4270 integrator. The detector was set at 280 nm when analysing the ABA and IAA containing fraction because it is the wavelength where IAA absorbs the most (Appendix 3). Maximum absorbance of IAA is obtained at this wavelength, however, ABA is still detected much more than IAA. The wavelength of detection for Z and ZR was 270 nm which is where both compounds absorb maximally. Zeatin absorbs less than zeatin riboside (Appendix 4).

The separation was done through a 25 X 4.6 mm I.D. Supelcosil LC-18 column (5 um). The mobile phase was 0.1% glacial acetic acid in water (A) and methanol (B) with an elution gradient of 70% to 55%A in 30 minutes for the cytokinin analysis. For IAA and ABA analysis, solvent B was acetonitrile. The gradient was 75% to 60%A in 15 minutes. The flow rate for both was 1 ml/minute. The samples were injected through a high pressure loop injector fitted with a 100 ul sample loop. Amounts were determined from peak area, and the peaks were identified with spiked injection of standards. To calculate the amount of IAA and ABA present in HPLC traces, known amounts of both growth regulators were injected and chromatographed by HPLC. The peak area was obtained for each amount and plotted against the amount injected. A best-fitted line was obtained for IAA (Appendix 5) and for ABA (Appendix 6). Thus by using the peak area, the amount of growth regulator present could be calculated. The same was done with zeatin (Appendix 7) and zeatin riboside (Appendix 8). The peaks with a larger peak area in the spiked trace were taken as being either IAA or ABA depending upon their retention time.

2.10.4 BIOASSAY FOR CYTOKININS

Fractions from the cytokinin analysis were collected from the HPLC and subjected to the *Amaranthus* bioassay (Bigot, 1968) as modified by Biddington and Thomas (1973). In *Amaranthus caudatus* seedlings, grown in darkness, cytokinins stimulate the production of betacyanin in the presence of tyrosine (Bigot, 1968). This bioassay permits the detection of cytokinins (Z and ZR) at concentrations of 0.5 ng/ml or more (Fig. 3). Collecting the HPLC fractions for the bioassay is better than subjecting the unseparated sample to the bioassay. Prior HPLC separation gives an idea of how many cytokinins are present and each can be identified by comparison with appropriate standards. Furthermore, the separation reduces or removes completely the amounts of contaminating, potentially inhibitory compounds within each fraction.

2.10.5 RECOVERY DETERMINATION

^{14}C-IAA and ^{3}H-ABA were utilized to determine losses during the extraction procedure. Aliquots were taken at different steps (Fig. 2) throughout the procedure and placed into scintillation vials to which 20 ml of ScintiVerse I (Fisher Sci. Ltd.) was added. The vials were stoppered and shaken thoroughly. The radioactivity present was measured with a Beckman LS-3133P scintillation counter, the external standard ratio method was used to determine the number of desintegrations per minute (dpm). The dpm value was also corrected for quenching.

For Z and ZR, total loss was determined by HPLC at different steps during the procedure. Plant tissue was not present in the samples used to measure recovery since no labeled zeatin or labeled zeatin riboside were available.

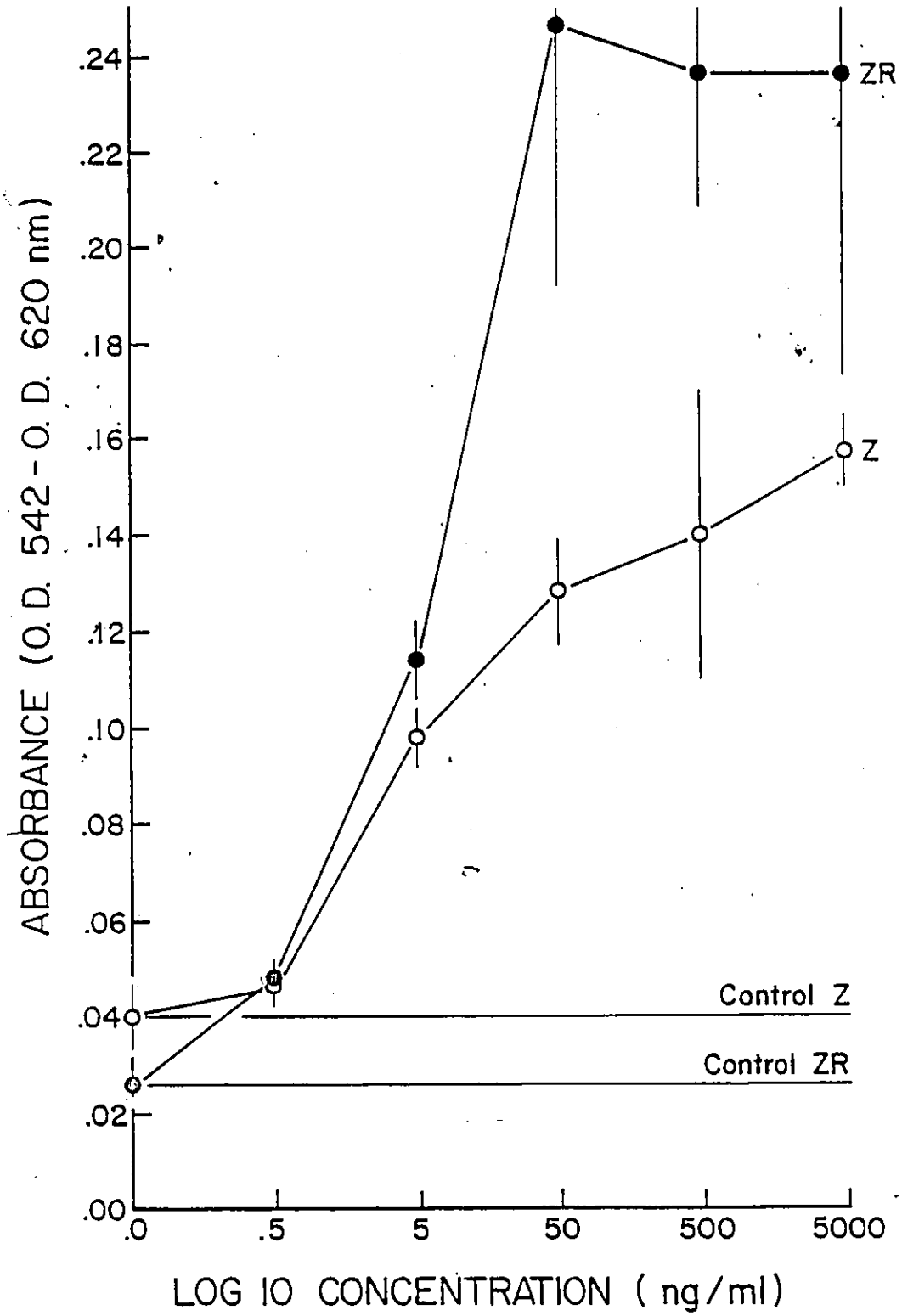

ABSORBANCE (O.D. 542 - O.D. 620 nm)
.24
.22
.20
.18
.16
.14
.12
.10
.08
.06
.04
.02
.00
ZR
Z
Control Z
Control ZR
.0
.5
5
50
500
5000
LOG 10 CONCENTRATION (ng/ml)

Figure 3. Production of betacyanin by *Amaranthus* seedlings in response to the presence of increasing concentrations of zeatin and zeatin riboside as measured by net absorbance of betacyanin extract at 542 nm. Seedlings grown at 25 ^{0}C in darkness as described by Biddington and Thomas (1973). Standard deviation is shown.

CHAPTER III

RESULTS

3.1 INTERNODE AND CELL MEASUREMENTS

As shown in the survey, internode lengths vary among lines and between the first and second internodes (Table 6). Dwarf lines with very short first internodes (WL1088, WL1570) and others with much longer ones (WL1402, WL1415) have been observed. The same pattern is also found with the tall lines. Tall lines, such as WL582 and WL1267, possess short first internodes whereas others (WL1433, WL1754) have much longer first internodes.

A relationship between the lengths of the first and second internodes is not apparent. Some lines with a short first internode have long second internode (WL1614, WL1570) while others have a short second internode (WL1088, WL582). Usually, the second internode is longer or equal to the first, however there are exceptions (WL1088). Length variability occurs in both the first and second internodes.

The differences in internode length among the various lines can result from differences in cell length and cell number. Measurements of stem and cortical cell dimensions of three lines of peas possessing internodes of different lengths are given in

TABLE 6

Mean lengths of the first and second internodes of etiolated pea seedlings from 21 lines selected for study. Growth at 15 ^{0}C in air.

Pea Line	Phenotype	Length of the 1st internode ± S.D. mm	Length of the second internode ± S.D. mm	Sample size
WL58	T*	44 ± 11c**	56 ± 14c	49
WL59	T	50 ± 7B	60 ± 11c	35
WL582	T	30 ± 7D	32 ± 5D	11
WL1088	D	33 ± 11c	24 ± 6D	10
WL1241	T	52 ± 10B	54 ± 11c	11
WL1267a	T	33 ± 18c	56 ± 24c	71
WL1369	D	31 ± 8D	50 ± 14c	14
WL1402	D	60 ± 12B	63 ± 14B	11
WL1415a	D	64 ± 9B	75 ± 13A	67
WL1416	T	63 ± 12B	86 ± 11A	15
WL1422	T	63 ± 10B	81 ± 14A	15
WL1433	T	64 ± 12B	84 ± 9A	15
WL1451a	D	69 ± 13A	76 ± 22A	27
WL1458	T	45 ± 9c	44 ± 10c	12
WL1464	T	56 ± 10B	70 ± 10A	38
WL1467	T	47 ± 19c	73 ± 25A	21
WL1503	T	32 ± 12c	39 ± 10D	11
WL1508	D	34 ± 13c	36 ± 9D	14
WL1570a	D	41 ± 11c	70 ± 12A	51
WL1614	T	32 ± 6c	64 ± 7B	14
WL1754	T	80 ± 7A	74 ± 10A	15

* T= TALL, D= DWARF

** MEANS WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT. DATA ANALYSED WITH DUNCANS MULTIPLE RANGE TEST, $\alpha = .05$

a LINES SELECTED FOR FURTHER STUDY

S.D. = STANDARD DEVIATION

TABLE 7

Mean cortical cell diameter and length measurements in first and second internodes of etiolated seedlings of 3 pea lines grown at 15 ^{0}C.

	Pea line	Internode	
		1st	2nd
Internode length ± S.D. (cm)	WL1267	3.1 ± 1.3	7.2 ± 1.1
	WL1570	4.7 ± 2.9	5.5 ± 1.0
	WL1415	6.0 ± 1.1	7.1 ± 1.6
Internode diameter ± S.D. (mm)	WL1267	1.84 ± .07	1.43 ± .11
	WL1570	1.93 ± .06	1.81 ± .08
	WL1415	1.89 ± .15	1.48 ± .07
Cortical cell length ± S.D. (mm)	WL1267	.40 ± .02	.43 ± .06
	WL1570	.42 ± .04	.45 ± .02
	WL1415	.41 ± .02	.47 ± .03
Cortical cell diameter ± S.D. (mm)	WL1267	.069 ± .010	.061 ± .009
	WL1570	.062 ± .008	.069 ± .010
	WL1415	.060 ± .007	.068 ± .004
Total cell number estimation (x 10^{5})	WL1267	5.5	9.1
	WL1570	10.6	8.4
	WL1415	14.5	7.2

Cell lengths and diameters were measured using two methods (see Materials and Methods). Five seedlings were used in the first method (75 cells measured/seedling) and three in the second method (25 cells measured/seedling).

S.D. = STANDARD DEVIATION

Table 7. First internode lengths ranged from short (WL1267) to quite long (WL1415), WL1570 having an intermediate length as also shown in Table 6. The second internodes were long in all lines, with WL1570 having an internode slightly shorter than the other two lines. Internode diameter is similar among the lines, although WL1570 tends to be thicker, especially the second internode. The diameter is less in the second internode.

Cortical cell lengths and cell diameters did not differ significantly among lines nor among internodes. However, in the second internode there was a trend toward longer cortical cells. Lines with longer internodes would have more cells. Therefore tall line WL1267 (with the shortest first internode) has the least number of cells and the dwarf line WL1415 has the most cells in the first internode. A larger number of cells are found in WL1267. However in WL1415, fewer cells are found despite a similar length because cells are somewhat longer.

3.2 SEEDLING GROWTH IN RESPONSE TO EXCISION

The effects of organ excision upon the growth of the first two internodes of the tall line WL1267 are given in Table 8. The excision of the root at the first stage of development reduced first internode growth as compared to the control. When the root was excised at imbibition, the growth of the first internode was not affected whereas the second internode growth was increased at the imbibition stage but not at the second internode stage. The removal of the testa at the first internode stage reduced first internode growth. The removal of the testa at the imbibition stage caused an increase in the length of the second internode but did not affect significantly the growth of the first internode. Removal of the testa at the second stage had no effect on internode growth. The excision of one cotyledon at the first and second internode stages increased the

TABLE 8

Effect of organ excision at 3 stages of development on the lengths of the first and second internodes of etiolated seedlings of tall pea WL1267.

STAGE*	TREATMENT	SAMPLE SIZE	MEAN FIRST INTERNODE LENGTH ± S.D. (CM)	MEAN SECOND INTERNODE LENGTH ± S.D. (CM)	MEAN LENGTH OF LATERALS (CM)
IMB	ROOT	6	3.6 ± 1.5A**	7.9 ± 1.4A	-
1ST		8	1.4 ± 0.4B	2.4 ± 0.6B	-
2ND		4	2.3 ± 1.0A	2.4 ± 0.9B	-
IMB	TESTA	6	5.3 ± 3.1B	9.8 ± 2.7B	-
1ST		10	2.8 ± 1.2A	3.7 ± 2.1B	-
2ND		5	4.9 ± 1.4A	4.9 ± 0.9A	-
1ST	COTYL	9	5.4 ± 1.2B	10.0 ± 2.3B	-
2ND		4	5.0 ± 0.6B	5.3 ± 0.3A	-
2ND	2COTYL	4	1.6 ± 0.3B	2.1 ± 0.9B	-
	CONTROL	34	3.8 ± 1.6A	6.5 ± 3.0A	-
IMB	APEX	7	-	-	14.5 ± 4.9
1ST		10	-	-	15.4 ± 1.4
2ND		5	2.8 ± 0.6A	-	18.5 ± 2.3

* STAGE DURING WHICH THE TREATMENT WAS PERFORMED; AFTER IMBIBITION OR DURING THE GROWTH OF THE FIRST OR THE SECOND INTERNODE

** DUNCAN'S ANALYSIS, $\alpha = .01$, LENGTHS WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT FROM THE CONTROL

S.D. = STANDARD DEVIATION

length of the first internode and of the second internode for the first internode stage. The excision of both cotyledons reduced significantly the growth of the internodes.

Excision of the apex had a dramatic effect on stem growth. Main stem growth was stopped. The two lateral buds of the first node extended when the apex was removed at the second internode stage, whereas the two cotyledonary nodes elongated at the other two stages. In both cases one bud soon took precedence over the other. The first internode produced by the dominant node was much longer than the first internode of the main stem.

The effects of organ excision on the growth of the first and second internodes of the dwarf line WL1570 are summarized in Table 9. The excision of the root, the removal of the testa, and of one cotyledon, had no significant effect on the growth of either internode at any stage of development. The removal of both cotyledons at the first internode stage reduced significantly the growth of both internodes, this reduction was not significant when the treatment was performed at the second stage.

Upon removal of the apex, this line reacted in a fashion similar to WL1267. The cotyledonary or node buds were stimulated and produced lateral stems of increased internode lengths.

3.3 RESPONSE OF STEM SEGMENTS TO GROWTH FACTORS

3.3.1 EXOGENOUS GROWTH REGULATORS AND STEM SEGMENT GROWTH

Treatment with 10^{-4}M of GA3, ABA and kinetin did not affect significantly the

TABLE 9

Effect of organ excision at 3 stages of development on the lengths of the first and second internodes of etiolated seedlings of dwarf pea WL1570.

STAGE*	TREATMENT	SAMPLE SIZE	MEAN FIRST INTERNODE LENGTH ± S.D. (CM)	MEAN SECOND INTERNODE LENGTH ± S.D. (CM)	MEAN LENGTH OF LATERALS (CM)
IMB	ROOT	31	3.2 ± 1.0A	4.4 ± 1.6A	-
1ST		42	2.2 ± 0.6A	3.6 ± 1.1A	-
2ND		42	2.6 ± 0.6A	3.6 ± 0.7A	-
IMB	TESTA	48	2.3 ± 0.9A	4.2 ± 1.8A	-
1ST		36	2.4 ± 0.6A	3.6 ± 0.8A	-
2ND		24	2.5 ± 0.8A	4.2 ± 0.9A	-
IMB	COTYL	47	2.3 ± 0.9A	5.6 ± 1.8A	-
1ST		30	2.6 ± 0.9A	4.0 ± 1.1A	-
2ND		19	3.3 ± 1.2A	4.3 ± 1.1A	-
1ST	2COTYL	15	1.1 ± 0.3B	1.5 ± 0.3B	-
2ND		10	2.2 ± 0.4A	2.8 ± 0.7A	-
	CONTROL	74	3.0 ± 1.0A	5.2 ± 1.9A	-
IMB	APEX	14	2.6 ± 0.3A	-	16.4 ± 3.4
1ST		11	-	-	13.3 ± 1.9
2ND		16	-	-	17.2 ± 1.1

* STAGE DURING WHICH THE TREATMENT WAS PERFORMED; AFTER IMBIBITION OR DURING THE GROWTH OF THE FIRST OR THE SECOND INTERNODE

** DUNCAN'S ANALYSIS, $\alpha = .01$, LENGTHS WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT FROM THE CONTROL

S.D. = STANDARD DEVIATION

extension of segments excised at different stages of growth of either WL1267 (tall) or WL1570 (dwarf), although segments from the third internode of both lines tended to grow more with GA3 (Table 10). Treatment with indole-3-acetic acid (10^{-4}M) on the other hand caused an elongation of the excised segments in every case (Table 10). Segments cut from the first internode extended less in the presence of IAA than segments from the second internode and in turn these segments extended less than segments from the third internode. The segments of tall line 1267 were more responsive to the growth regulator than the segments from dwarf line 1570.

The application of 50 ppm of ethylene did not significantly alter the final length of excised segments when compared with control (Table 11), although segments of the dwarf WL1570 were slightly lengthened.

3.3.2 EXOGENOUS GROWTH REGULATORS AT A RANGE OF CONCENTRATION

Concentrations ranging from 10^{-2} to 10^{-6}M of ABA (Table 12) and kinetin (Table 13) had no effect on the extension of the segments. The same range of concentrations of GA3 had no effect upon the growth of segments except for segments cut from the growing third internode of the dwarf WL1570 (Table 14). These segments extended slightly more than the control; this is not so for segments of the tall line WL1267.

The effect of varying concentrations of exogenous IAA upon the growth of excised segments of three dwarf (WL1451, WL1415, WL1570) and one tall (WL1267) lines which were cut at different stages of development are shown in Figures 4,5,6 and 7. Segment growth was enhanced by the addition of increasing concentrations of exogenous IAA up to an optimal concentration. More auxin in the medium reduced and finally

TABLE 10

Extension response of etiolated pea stem segments at 3 different stages of development to 10^{-4}M concentration of various growth regulators.

PEA LINE	STAGE*	TREATMENT				
		CONTROL	GA	ABA	KINETIN	IAA
WL1267	1ST	1.08	1.11	1.12	1.15	1.35
	2ND	1.10	1.15	1.11	1.13	1.42
	3RD	1.08	1.17	1.08	1.17	1.65
WL1570	1ST	1.07	1.13	1.05	1.09	1.17
	2ND	1.16	1.13	1.10	1.14	1.29
	3RD	1.15	1.22	1.18	1.20	1.45

* SEGMENTS WERE CUT FROM THE GROWING FIRST, SECOND AND THIRD INTERNODE

TABLE 11

Extension response of etiolated pea stem segments, at 3 different stages of development, to 50 ppm of ethylene.

PEA LINE	STAGE*	ETHYLENE CONCENTRATION (PPM)	
		0	50
WL1267	1ST	1.13	1.15
	2ND	1.13	1.11
	3RD	1.14	1.09
WL1570	1ST	1.12	1.11
	2ND	1.10	1.18
	3RD	1.14	1.20

* SEGMENTS WERE CUT FROM THE GROWING FIRST, SECOND AND THIRD INTERNODE

TABLE 12

Extension response of etiolated pea stem segments at 3 different stages of development to various concentrations of abscisic acid.

Pea line	Stage*	ABA concentrations (M)			
		10^{-2}	10^{-3}	10^{-5}	10^{-6}
WL1267	1st	1.07	1.09	1.11	1.10
	2nd	1.05	1.08	1.14	1.11
	3rd	1.03	1.10	1.09	1.14
WL1570	1st	1.02	1.04	1.08	1.07
	2nd	1.01	1.02	1.05	1.08
	3rd	1.01	1.02	1.04	1.09

* Segments were cut from the growing first, second and third internode

TABLE 13

Extension response of etiolated pea stem segments at 3 different stages of development to various concentrations of kinetin.

PEA LINE	STAGE*	KINETIN CONCENTRATIONS (M)					
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	0
WL1267	1ST	1.06	1.11	1.08	1.05	1.07	1.05
	2ND	1.12	1.13	1.08	1.05	1.07	1.03
	3RD	1.08	1.05	-	1.14	1.10	1.07
WL1570	1ST	1.05	1.03	1.01	1.03	1.04	1.05
	2ND	1.11	1.10	1.10	1.10	1.16	1.10
	3RD	1.08	1.12	1.14	1.06	1.16	1.12

* SEGMENTS WERE CUT FROM THE GROWING FIRST, SECOND AND THIRD INTERNODE

TABLE 14

Extension response of etiolated pea stem segments at 3 different stages of development to various concentrations of gibberellin (GA3).

PEA LINE	STAGE*	GA_3 CONCENTRATIONS (M)			
		10^{-2}	10^{-3}	10^{-5}	10^{-6}
WL1267	1ST	1.11	1.09	1.07	1.12
	2ND	1.14	1.13	1.12	1.13
	3RD	1.11	1.11	1.14	1.07
WL1570	1ST	1.01	1.07	1.09	1.10
	2ND	1.13	1.15	1.09	1.08
	3RD	1.24	1.22	1.26	1.28

* SEGMENTS WERE CUT FROM THE GROWING FIRST, SECOND AND THIRD INTERNODE

inhibited growth.

The segments cut from the growing second internodes of the tall line WL1267 which has a short first internode (33mm) and a long second internode (56mm), responded more to the presence of exogenous IAA than segments from the first and third internodes (Fig. 4). Segments from the third internode grew the least in response to IAA. At optimal concentrations, segments from the third internode elongated by 30%, segments from the second internode by 69% and segments from the first by 48%. The optimum concentration for the elongation of the segments is found between 5 X 10^{-3} and 5 X 10^{-5}M, the concentration of 5 X 10^{-2}M was inhibitory to segment growth.

The segments cut from the growing second and third internodes of the dwarf line WL1570, which has a short first internode (41mm) and a long second internode (70mm), elongated more than segments from the first internode (Fig.5). The response of the segments from the third and second internodes are similar. The maximum growth occurred around 10^{-6} to 10^{-5}M of IAA. The segments of the third internode grew by 38%, the segments of the second by 30-32% and the segments from the first internode by 28%. Growth was not significantly different from control in the presence of 5 X 10^{-2}M IAA.

The growth of segments from the dwarf line WL1415, which has long first (64mm) and second (75mm) internodes, in response to IAA is found in Fig.6. Segments obtained from the growing internode of older seedlings responded more to the applied IAA than segments from growing internodes of younger seedlings (third>second>first). The maximum response occurred at 10^{-5}M, the segments from the third, second and first internodes grew by 45%, 38% and 25% respectively. Growth was inhibited in the presence of 10^{-2}M of IAA.

WL 1267

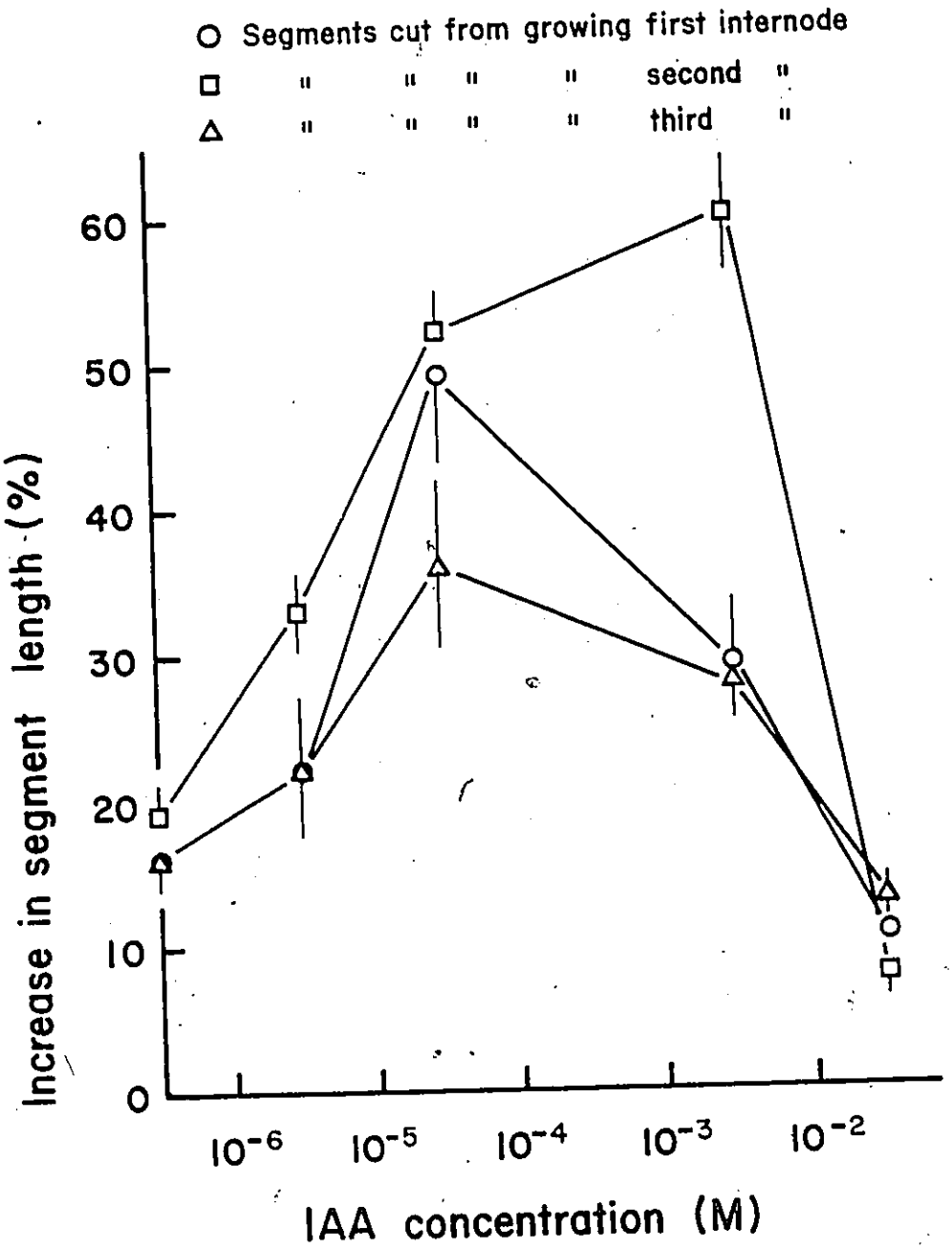

○ Segments cut from growing first internode
□ " " " " second "
△ " " " " third "
60
50
40
30
20
10
0
Increase in segment length (%)
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
IAA concentration (M)

Figure 4. Extension of etiolated pea stem segments of tall WL1267 in response to various concentrations of indole-3-acetic acid. The segments were excised at three stages of development and incubated in darkness at 15 ^{0}C for 24 hours, in petri dishes (10 segments/dish) containing 10 ml of incubation solution (.5 M sodium potassium phosphate buffer, pH 6.8, 2% sucrose, growth regulator). Standard error bars included.

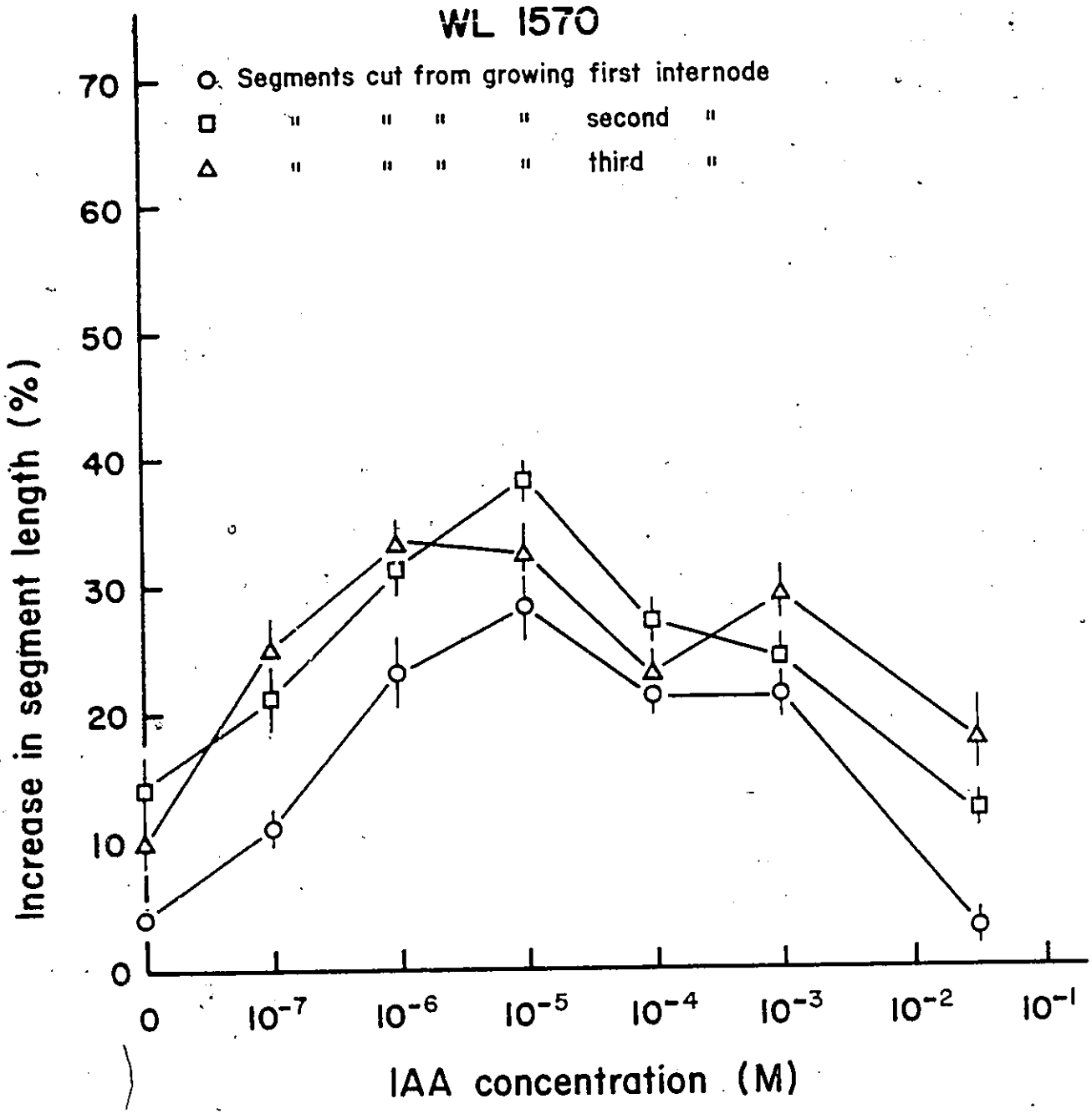

WL 1570
Segments cut from growing first internode
" " " " second "
" " " " third "
70
60
50
40
30
20
10
0
Increase in segment length (%)
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
IAA concentration (M)

Figure 5. Extension of etiolated pea stem segments of dwarf WL1570 in response to various concentrations of indole-3-acetic acid. The segments were excised at three stages of development. Growth conditions as described in Fig.4. Standard error bars included.

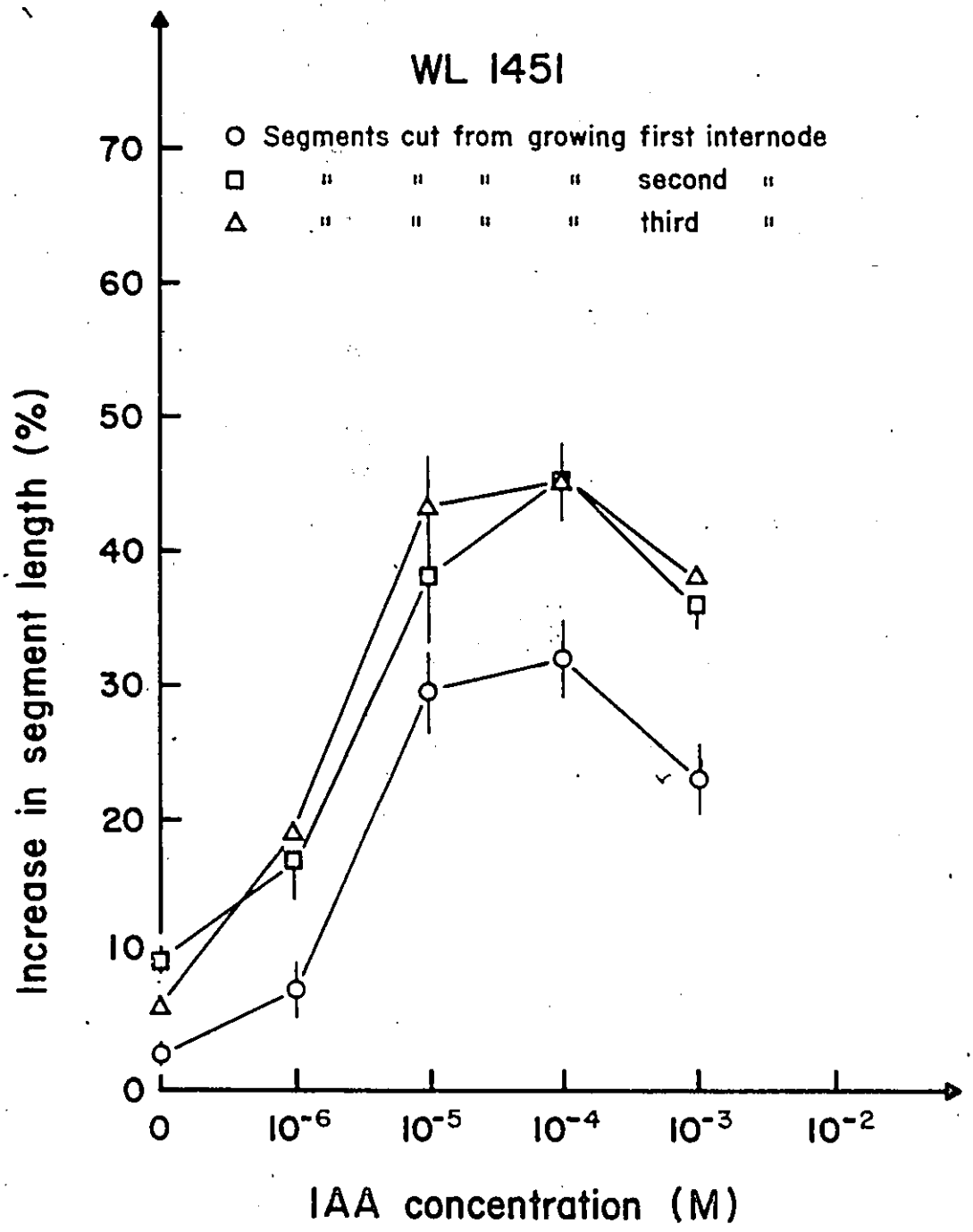

WL 1451
Segments cut from growing first internode
" " " " second "
" " " " third "
Increase in segment length (%)
70
60
50
40
30
20
10
0
0
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
IAA concentration (M)

Figure 6. Extension of etiolated pea stem segments of dwarf WL1415 in response to various concentrations of indole-3-acetic acid. The segments were excised at three stages of development. Growth conditions as described in Fig.4. Standard error bars included.

Segments from the growing third and second internodes of the dwarf line 1451, which also has long first (69mm) and second (76mm) internodes, responded similarly. They grew more than segments obtained from the first internode (Fig.7). The growth response was about 45% for both third and second internode segments and about 30% for segments from the first internode. The optimum concentration of IAA was 10^{-4}M.

3.4 ENZYME ACTIVITY

IAA oxidase and peroxidase activities were evaluated in two dwarf lines, one with a short first internode (WL1570) and the other (WL1415) with a longer first internode. Both lines have a long second internode.

Specific and total IAA oxidase activities for both lines are given in Table 15. The specific and total activities followed the same pattern, thus only total activity will be described.

3.4.1 IAA OXIDASE

In line WL1415, total IAA oxidase activity decreased toward the base of the stem during stage 1 (1A>1B>1C). The same pattern occurred in WL1570, although the activity was slightly lower and decreased more rapidly towards the base in WL1570.

During stage 2, total IAA oxidase increased in WL1415 and fluctuated along the stem. It was higher in the segments cut just below the apex (2A), decreased in the elongating region (2B), increased at the base of the growing second internode (2C) and

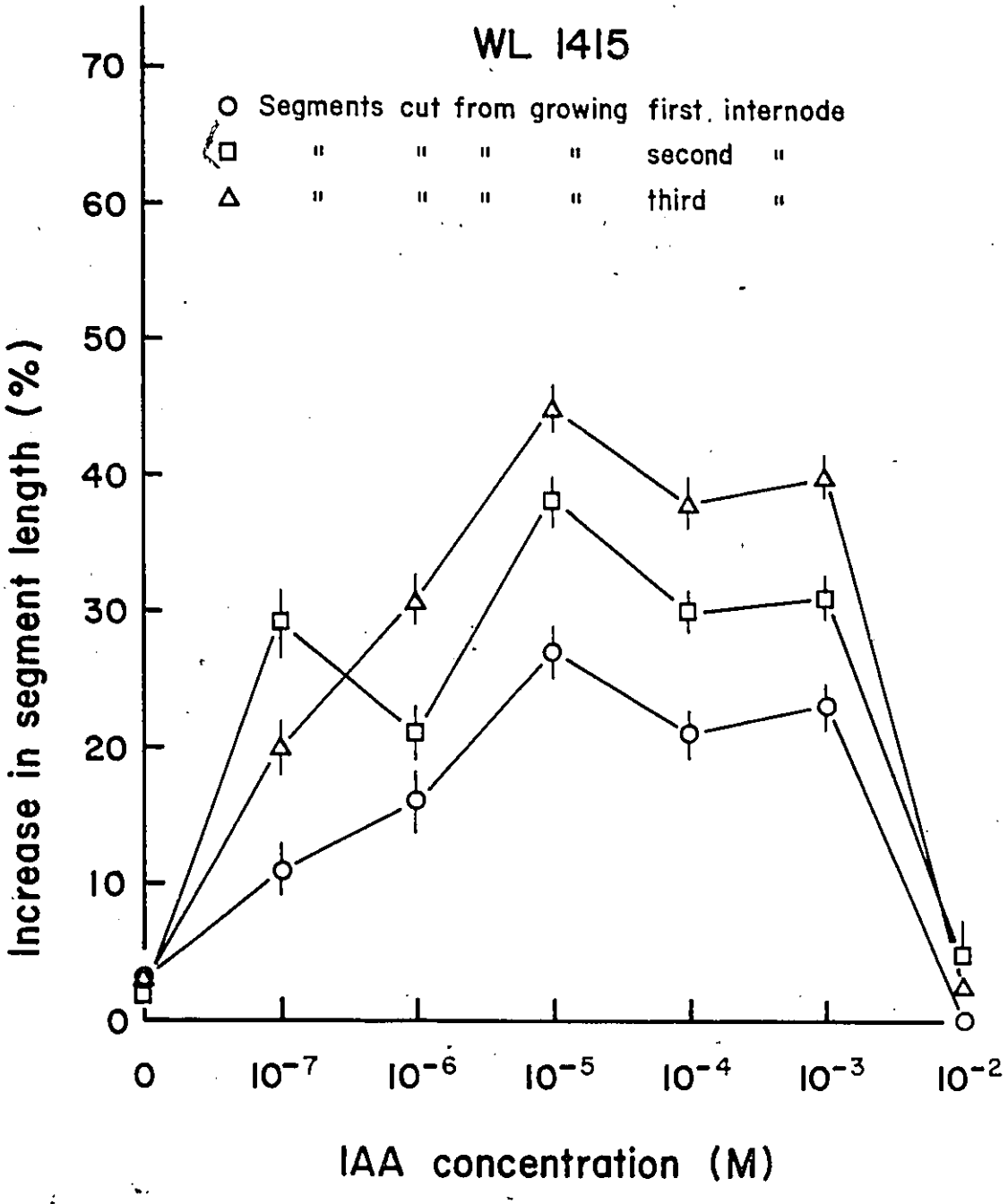

WL 1415
Segments cut from growing first. internode
" " " " second "
" " " " third "
Increase in segment length (%)
70
60
50
40
30
20
10
0
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
IAA concentration (M)

Figure 7. Extension of etiolated pea stem segments of dwarf WL1451 in response to various concentrations of indole-3-acetic acid. The segments were excised at three stages of development. Growth conditions as described in Fig.4. Standard error bars included.

TABLE 15

IAA oxidase activity in stems of etiolated pea seedlings at various stages of growth.

		IAA OXIDASE			
STAGE	POSITION	SPECIFIC ACTIVITY ($\times 10^{-3}$) ± S.D. μg IAA destroyed min^{-1} mg fresh $weight^{-1}$		TOTAL ACTIVITY μg IAA destroyed min^{-1} $segment^{-1}$ ($\times 10^{3}$)	
		WL 1415	WL 1570	WL 1415	WL 1570
1	A	3000 ± 2007	2000 ± 813	56.1 ± 37.4	54.0 ± 21.6
	B	2600 ±2193	1290 ± 262	55.4 ± 46.2	41.0 ± 8.4
	C	2100 ±1609	1020 ± 56	49.8 ± 38.3	36.1 ± 2.0
2	A	6700 ±4386	1360 ± 495	99.8 ± 66.5	30.2 ± 11.2
	B	4200 ±2227	1200 ± 361	68.0 ± 35.8	30.8 ± 9.3
	C	5200 ±4954	1070 ± 177	90.5 ± 86.2	29.2 ± 4.9
	D	4000 ±2831	980 ± 42	84.4 ± 60.3	29.6 ± 1.3
3	A	3500 ±2135	1140 ± 330	49.0 ± 30.6	28.9 ± 8.3
	B	3900 ±1434	910 ± 309	59.7 ± 22.1	24.8 ± 8.6
	C	2800 ± 663	880 ± 269	44.0 ± 10.5	24.1 ± 7.3
	D	2900 ±1827	810 ± 460	50.2 ± 31.4	23.1 ± 12.8
	E	2200 ±1005	490 ± 360	44.2 ± 20.1	14.7 ± 8.2

S.D. - standard deviation

decreased again in the mature first internode (2D). For WL1570, the total IAA oxidase activity did not increase during this stage. Along the stem it decreased slightly toward the base. There was about 3 to 4 times less IAA oxidase activity in WL1570 during this stage than in WL1415.

During stage 3, total IAA oxidase in WL1415 decreased to levels similar to those of stage 1. Along the stem the IAA oxidase activity still fluctuated: it increased in the elongating region (3B), decreased at the base of the growing internode (3C), increased in the mature second internode (3D) and decreased in the even more mature first internode (3E). In WL1570, the IAA oxidase activity decreased from the level found in stage 2 and decreased down the stem as it did in the previous stages. The activity in this line was still 2 to 4 times lower than in WL1415.

As the tissues aged (1A to 2D to 3E and 2A to 3D), total IAA oxidase increased, then decreased and finally increased again in WL1415. In WL1570, the activity decreased continuously as the tissues matured. As the seedling aged (1A to 2A to 3A), the activity near the apex increased during stage 2 and decreased during stage 3 in WL1415. In line WL1570, the activity near the apex decreased sharply between stage 1 and stage 2. It decreased slightly from stage 2 to stage 3.

3.4.2 PEROXIDASE

Specific and total peroxidase activities for both lines are found in Table 16. Here again, only total activity is described. During stage 1, total peroxidase activity for line WL1415 was low in the region below the apex (1A), increased slightly in the elongating region (1B) and increased more steeply in the maturing and mature tissues at the base of

TABLE 16

Peroxidase activity in stems of etiolated pea seedlings at various stages of growth.

STAGE	POSITION	PEROXIDASE			
		SPECIFIC ACTIVITY ± S.D. Δ O.D. sec^{-1} mg fresh $weight^{-1}$ (x 10^{-3})		TOTAL ACTIVITY ± S.D. Δ O.D. sec^{-1} $segment^{-1}$ (x 10^{3})	
		WL 1415	WL 1570	WL 1415	WL 1570
1	A	560 ± 138	2130 ± 233	10.5 ± 2.6	57.5 ± 6.3
	B	600 ± 45	1580 ± 156	12.8 ± 1.0	50.2 ± 5.0
	C	860 ± 135	2280 ± 389	20.4 ± 3.2	80.7 ± 13.7
2	A	680 ± 233	1610 ± 410	10.1 ± 3.2	35.7 ± 9.2
	B	430 ± 177	890 ± 283	7.0 ± 2.9	22.9 ± 10.9
	C	510 ± 193	1180 ± 382	8.9 ± 3.4	32.2 ± 10.4
	D	700 ± 250	1560 ± 332	14.8 ± 5.3	47.1 ± 10.0
3	A	650 ± 44	1020 ± 102	9.1 ± 0.6	25.9 ± 2.6
	B	390 ± 39	710 ± 106	6.0 ± 0.6	19.4 ± 2.9
	C	550 ± 79	870 ± 101	8.6 ± 1.2	23.8 ± 2.8
	D	630 ± 129	820 ± 410	10.9 ± 2.2	23.4 ± 11.7
	E	870 ± 195	1420 ± 283	17.5 ± 3.9	42.6 ± 8.5

S.D. - standard deviation

the stem (1C). For WL1570, the total activity decreased in the elongating region (1B) and increased in the maturing region (1C). The activity at the base in both line was higher than in the segments just below the apex. Total peroxidase activity in line WL1415 was 4 to 6 time lower than in line WL1570.

During stage 2, total peroxidase activity was low below the apex (2A), decreased further in the elongating region (2B) and increased slightly in the maturing regions in both lines (2C,2D). Total activity in the internode was still higher than the activity below the apex, although the difference was not as pronounced as in stage 1. A comparison of both lines shows more activity in WL1570 than in WL1415 (about 3 times more).

During stage 3, the same pattern in peroxidase activity as in stage 2 was found along the stem in both lines studied. Again, total peroxidase activity was low below the apex (3A), decreased further in the elongating region (3B) and increased in the maturing (3C) and mature regions (3D,3E) of the second and first internodes. The activity in the first internode (3E) was more than the activity just below the apex (3A). The peroxidase activity of WL1570 remained higher than in WL1415.

As the tissues aged, total peroxidase activity increased in WL1415 (1A to 2D to 3E) but decreased in WL1570. As the seedlings aged, total peroxidase activity below the apex (1A to 2A to 3A) decreased in both lines.

The relative proportions of IAA oxidase to peroxidase differed between the lines (Table 17). During stage 1, the proportion of IAA oxidase to peroxidase was 5.3 times more in the segment below the apex in WL1415. The proportion of IAA oxidase decreased in the elongating (4.3 times) and maturing region (2.4 times). In WL1570, the proportion of IAA oxidase to peroxidase was 0.94 times in the segment below the apex

TABLE 17

Ratio of IAA oxidase/peroxidase in stems of etiolated pea seedlings at various stages of growth.

STAGE	POSITION	IAA OXIDASE/PEROXIDASE	
		WL1415	WL1570
1	A	5.3	0.94
	B	4.3	0.82
	C	2.4	0.45
2	A	9.9	0.85
	B	9.7	1.34
	C	10.2	0.91
	D	5.7	0.63
3	A	5.3	1.12
	B	10.0	1.28
	C	5.1	1.01
	D	4.6	0.99
	E	2.5	0.35

FOR POSITION AND STAGE REFER TO FIG. 1

and it decreased in the elongating (0.82 times) and maturing region (0.45 times). Line WL1415 had about 5 times more IAA oxidase than peroxidase whereas WL1570 had about the same proportion of both enzymes.

During stage 2, the proportion of IAA oxidase to peroxidase, in WL1415, is high below the apex (2A), decreases in the elongating region of the internode (2B) and increased in the maturing regions (2C)(10.2 times) and decreased again in more mature tissue (2D)(5.7 times). In WL1570, the proportion of IAA oxidase to peroxidase was 0.85 times in the segment below the apex, the proportion increased in the elongating region (1.28 times) and subsequently decreased in the maturing (1.01 times) and matured tissue (0.99 to 0.35 times). The proportion of IAA oxidase remained higher (7-11X) in WL1415, whereas in WL1570, the proportion of both enzymes was about equal.

During stage 3, the proportion of IAA oxidase to peroxidase was 5.3 times for WL1415 and 1.12 times for WL1570 in the segment just below the apex (3A). It increased in the elongating region (3B) and decreased again in the maturing and mature tissues (3C,3D,3E). This occurred in both lines. Line WL1415 again had a higher proportion of IAA oxidase to peroxidase than line WL1570. As the stem segments matured (1A to 2D to 3E), the proportion of IAA oxidase to peroxidase decreased.

3.5 ENDOGENOUS GROWTH REGULATOR ANALYSIS

3.5.1 EXTRACTION, PURIFICATION AND QUANTIFICATION

SepPak C18 cartridges retained zeatin and zeatin riboside at pH 4.0 and 8.0. IAA and ABA were retained at pH 2.8 but passed through the SepPak C18 cartridge at pH 8.0. In the presence of traces of ether, the retention of the growth regulators was altered, 48% ZR and 44% Z were eluted through the cartridge with the aqueous sample (step 5) and thus were lost.

Different solvents were used for the elution of the Sep Pak C18 cartridges following the application of the aqueous extract (Table 18). Methanol and n-butanol eluted all of the zeatin and zeatin riboside, ethyl acetate gave 56% Z and 69% ZR when eluting with a volume limited to 5 ml. The growth regulators were not eluted by methylene chloride.

For IAA and ABA, ethyl acetate was a good eluant; 99% of both were recovered with a volume of 13 ml. Diethyl ether and methylene chloride did not elute these compounds substantially from the cartridge even when a larger volume (10 ml) was used.

Methanol was the best eluant for the SepPak C18 cartridges since only a small volume (1ml) was necessary to obtain total recovery. Unfortunately a broad range of other compounds was also eluted from the cartridge when plant extracts were applied. An HPLC analysis of such fractions showed large peaks overshadowing the region where the standard growth regulators would elute. Thus methanol was not used to elute the cartridge. Eluants, such as methylene chloride, which did not elute the growth regulators were tried in order to purify more the extract. The cartridge was then eluted with methanol but the fractions obtained did not give better HPLC chromatograms. Methylene chloride probably removed from the cartridge compounds which would be retained when methanol was used as an eluant. I thus tried a less polar eluant, ethyl acetate. Good recovery of the growth regulators was obtained and a better purification was also found. Ethyl acetate probably eluted a smaller range of compounds from the cartridge. Also, upon the addition of water to the ethyl acetate solution, a precipitation of some yellow

TABLE 18

Recovery of zeatin, zeatin riboside, IAA, and ABA from a SepPak C18 cartridge, using various organic solvents as eluents

SOLVENT	Recovery (%) pH 8.0 Z	pH 8.0 ZR	pH 2.8 IAA	pH 2.8 ABA
METHANOL	100 (1)	100 (1)	-	-
N-BUTANOL	100 (5)	100 (5)	-	-
ETHYL ACETATE	56 (5)	69 (5)	84 (3)	82 (3)
	-	-	99 (13)	99 (13)
DIETHYL ETHER	-	-	-	11 (1)
	-	-	39 (5)	13 (5)
	-	-	43 (10)	-
METHYLENE CHLORIDE	0 (5)	0 (5)	6 (3)	-

*Numbers in parentheses represent the volume used (ml)

coloured compounds (probably carotenoid), could be observed. These could be removed by centrifugation which helped to obtain cleaner fractions.

Fractionation with diethyl ether removed lipoidal substances from the sample. Filtration through celite did not purify more the extract and added to losses, thus this step was not incorporated. Polyvinylpyrrolidone did not give cleaner HPLC chromatograms even though it removed some flavonoids and phenolics. Losses of the growth regulators also occurred so this step was removed. It is possible to obtain the cytokinins in the organic phase by fractionation with n-butanol at pH 7.0 (Yokota et al., 1980). Here again the final fraction was not improved, this step is also time consuming since n-butanol evaporates very slowly. It was not utilized in the final procedure.

The plant extract chromatograms obtained from the final procedure did not permit the identification of zeatin since it was hidden under larger peaks. I tried to ameliorate the separation with linear gradients and various mobile phases. Only the region where ZR elutes could be better separated thus only ZR was quantified directly using HPLC. Zeatin was identified indirectly by bioassay of HPLC fractions.

In plant extracts, IAA and ABA elute in the tail region of large peaks. A better separation using different gradients and different mobile phase was attempted but I was never successful in obtaining the IAA and ABA peaks well separated from the impurities. However, peak area for IAA and ABA can be estimated, thus amounts were quantified. Spiking the chromatograms permitted verification of which peak was IAA and which was ABA since they become more visible.

Zeatin riboside eluted in a region of the chromatogram which was fairly clean. Thus the amount of ZR could be estimated. Z eluted in a region containing large peaks, thus it was hidden and the amount could not be estimated.

Percent recovery from the final extraction procedure of ^{14}C-IAA and ^{3}H-ABA is shown in Table 19. During ether fractionation, 2 to 3.5% of ABA and 5 to 11% of IAA were lost in the ether phase which was discarded (step 3). The growth regulators IAA and ABA were minimally retained by the first SepPak C18 cartridge (pH 2.8) since 86 to 97% ABA and 74 to 78% IAA were eluted (step 5). IAA and ABA were mostly retained by the second SepPak C18 cartridge (pH 8.0), since only 7 to 12% ABA and 1 to 2% IAA were lost with the discarded aqueous eluate (step 7). From the SepPak C18 cartridge, 63 to 89% of ABA and 71 to 75% of IAA were recovered (step 8). Losses were more extensive in plant extracts.

The recovery of zeatin and zeatin riboside is given in Table 20. Zeatin and zeatin riboside were minimally lost in the discarded ether phase (2% ZR and 5 to 9% Z)(step 3). These growth regulators were retained by the first SepPak C18 cartridge, since no zeatin and zeatin riboside could be detected in the aqueous sample (step 5), 97% ZR and 91% Z could be recovered from the cartridge at pH 8.0 and 100% at pH 4.0 (step 6). The precursors, adenine and adenosine, were also tested. They were not retained on the first SepPak C18 cartridge, neither at pH 8.0 nor pH 2.8, but stayed in the aqueous solution at all times. Losses with plant tissues were not measured since labeled zeatin and labeled zeatin riboside were not available and the HPLC traces did not permit the estimation of zeatin. Amounts were corrected for losses by using the percent recovery of blank samples spiked with growth regulator standards. Values thus obtained are probably overestimated since less growth regulator was usually lost with the spiked blank.

A typical HPLC chromatogram for IAA and ABA is shown in Fig.8. IAA eluted after about 10 minutes and was followed by ABA at about 12 minutes. A typical HPLC trace for zeatin and zeatin riboside is shown in Fig.9. Zeatin riboside eluted at about 19 minutes and zeatin eluted after 25 minutes. A histogram of fractions collected from an

TABLE 19

Recovery from plant tissue extracts and from solvent (without plant tissue) of ^{14}C-IAA and ^{3}H-ABA through various steps of the purification procedure.

Steps	^{3}H-ABA (% recovered)		^{14}C-IAA (% recovered)	
	Solvent alone	Plant tissue extract	Solvent alone	Plant tissue extract
2	100	89	100	100
3	2	3.5	4	11
4	98	91	95	89
5	97	86	78	74
6	1	5	2	1
7	7	12	1	2
8	89	63	75	71

For steps, refer to Fig. 2

TABLE 20

Recovery from solvents of zeatin and zeatin riboside at pH 4.0 and 8.0. The effect of the presence of traces of diethyl ether upon the elution from the SepPak C18 cartridge (step 4) is shown for pH 8.0. No plant tissue was present.

Steps	Potassium phosphate buffer, .005 M, pH 4.0	Water, pH 8.0			
		Ether traces		No ether traces	
	ZR (% recovered)	ZR (% recovered)	Z (% recovered)	ZR (% recovered)	Z (% recovered)
2	-	100	100	100	100
3	-	2	5	2	9
4	100	92	87	97	91
5	0	48	44	0	0
6*	100	17	5	97	91

* Methanol was used to elute the cartridge instead of ethyl acetate

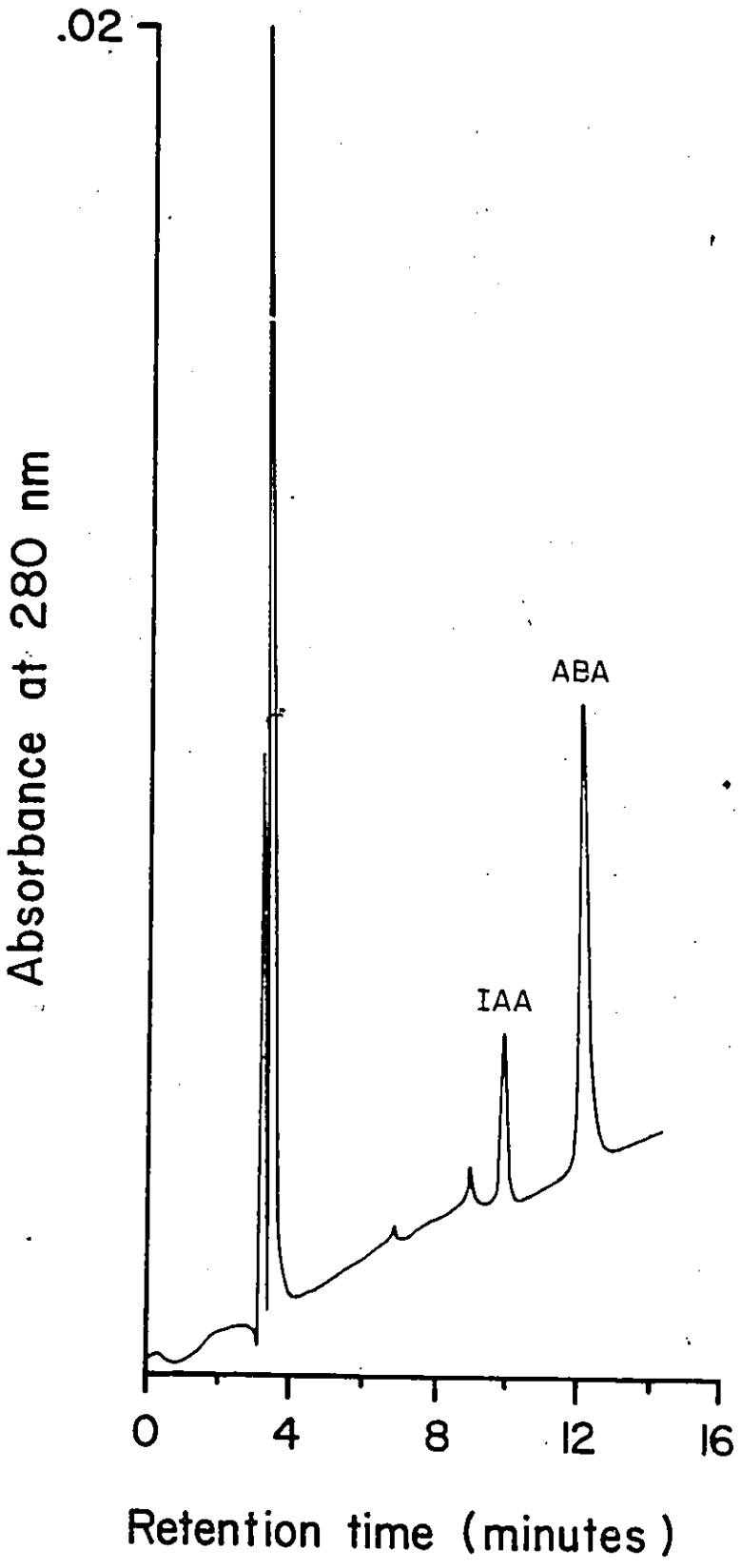

.02
Absorbance at 280 nm
ABA
IAA
0
4
8
12
16
Retention time (minutes)

Figure 8. Chromatogram of IAA and ABA standards on a 25 X 4.6 mm I.D. Supelcosil LC-18 column (5 um). Amounts injected, 100 ng; volume injected, 100 ul; solvent A, 0.1% glacial acetic acid in water; solvent B, acetonitrile; Elution conditions: flow rate, 1 ml/min; linear gradient, 75%-60%A in 15 minutes. Detection by UV absorption at 280 nm.

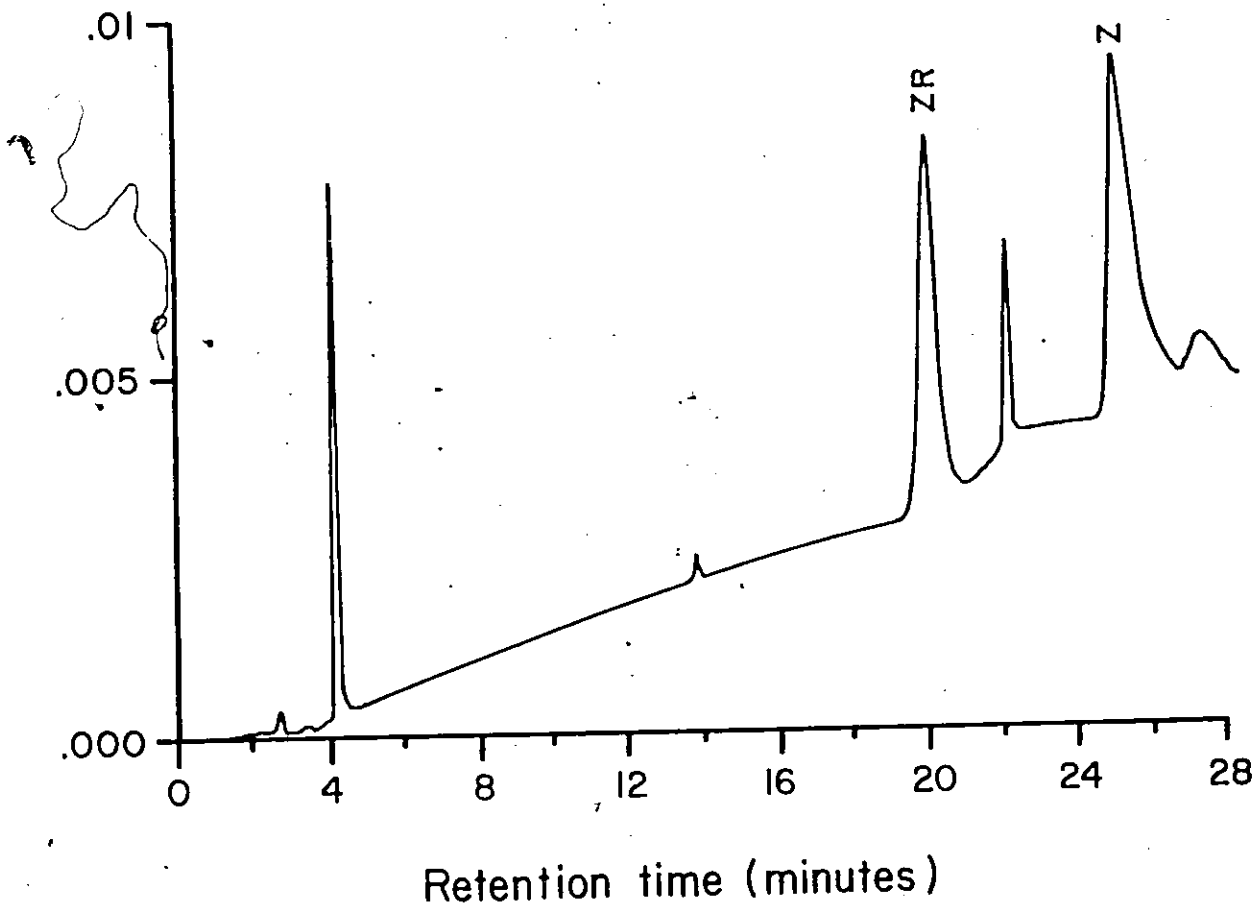

.01
.005
.000
ZR
Z
0
4
8
12
16
20
24
28
Retention time (minutes)

Figure 9. Chromatogram of zeatin and zeatin riboside standards on a 25 X 4.6 mm I.D. Supelcosil LC-18 column (5 um). Amounts injected, 100 ng; volume injected, 100 ul; solvent A, 00.1% glacial acetic acid in water; solvent B, methanol; Elution conditions: flow rate, 1 ml/min; linear gradient, 70%-55%A in 30 minutes. Detection by UV absorption at 270 nm.

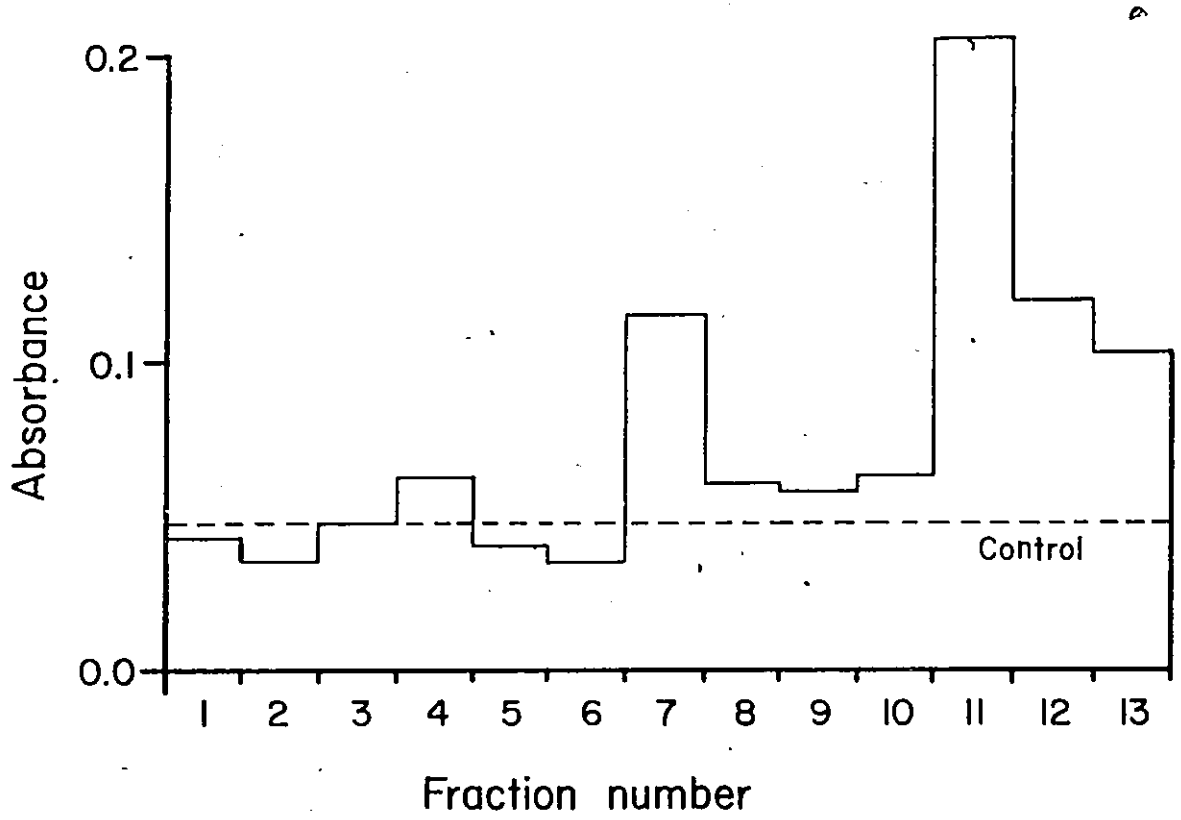

0.2
0.1
0.0
Absorbance
Control
1
2
3
4
5
6
7
8
9
10
11
12
13
Fraction number

Figure 10. *Amaranthus* bioassay as described in Fig.3, of 100 ng of zeatin and zeatin riboside after their separation (as described in Fig.9). Fractions of 2 ml were collected.

HPLC run of standard zeatin and zeatin riboside is shown in Fig.10. Fraction 7 and fractions 11,12 and 13 induced measurable betacyanin synthesis. Fraction 7 corresponds to the elution of ZR and fractions 11, 12 and 13 correspond to Z. By using this bioassay, the presence of cytokinin within the fraction was thus verified.

HPLC chromatograms of IAA and ABA containing fractions obtained from line WL1415 and WL1570, are found in Figs. 11 to 14. Both lines had similar chromatograms and they did not differ between the two stages of development.

Fig. 11 shows that ABA eluted after 12 minutes and could be detected in both the spiked and unspiked chromatograms of 8 day-old seedlings of WL1570. IAA was visible in the spiked chromatogram at 10 minutes. A small peak was visible in the same area of the unspiked chromatogram and may be IAA.

Fig. 12 shows a peak for ABA just before 12 minutes in both the spiked and unspiked chromatograms of 11 day-old seedlings. IAA gives a detectable peak before 10 minutes.

In the spiked chromatogram of 8 day-old pea seedlings of line WL1415 (Fig. 13), the peaks for ABA (12 minutes) and for IAA (10 minutes) are clearly visible. In the unspiked chromatogram, only ABA can be detected. These chromatogram were run at a smaller attenuation than the previous ones and thus reached full scale at a lower absorbance value.

IAA and ABA are detected in the spiked chromatogram of 11 day-old pea seedlings of line WL1415 (Fig. 14), at 10 and 12 minutes, respectively. ABA is present in the unspiked chromatogram and a very small peak is visible in the region where IAA elutes.

The amounts of ABA were estimated using the peak area method (Table 21). At 8

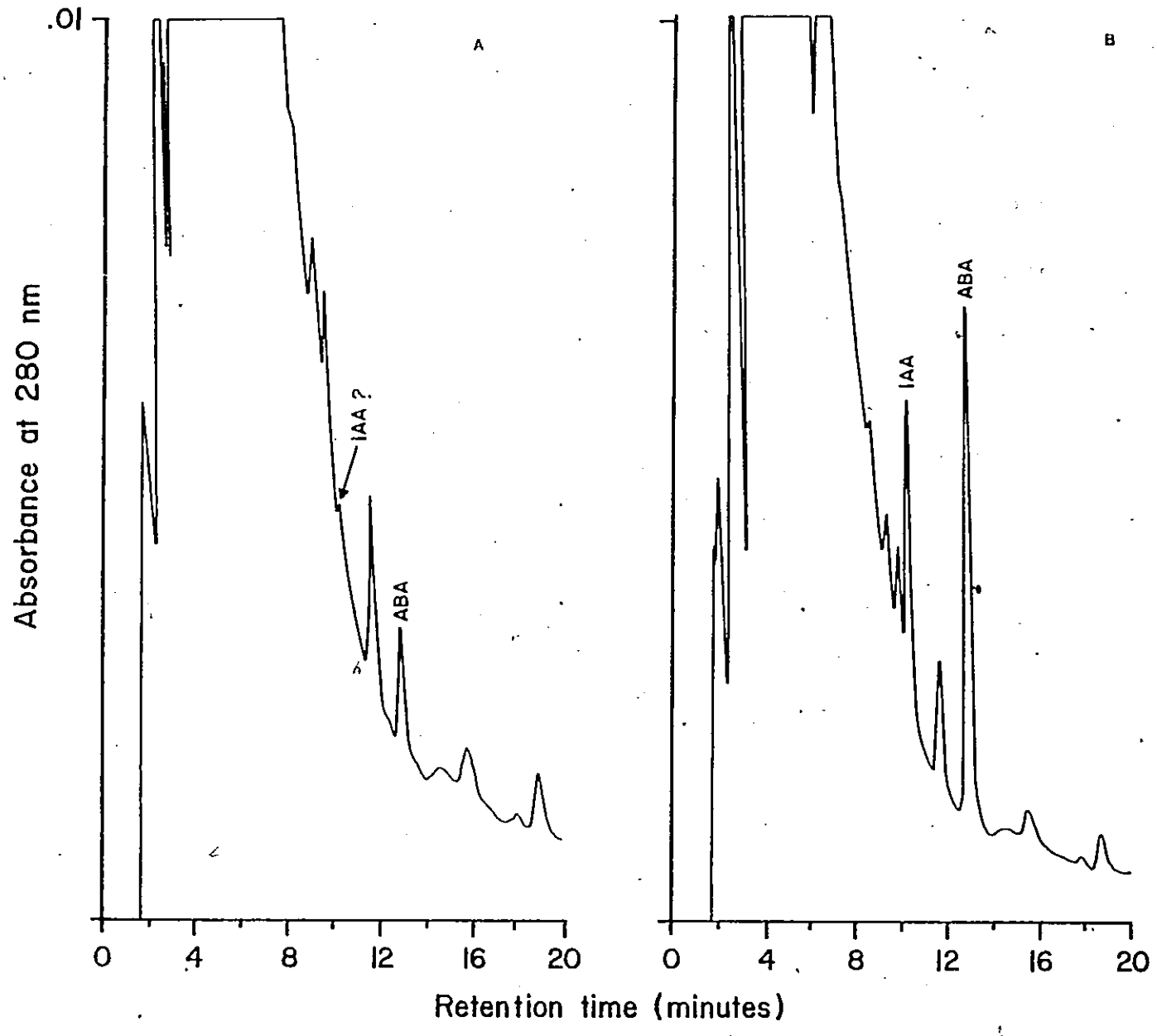

.01
Absorbance at 280 nm
A
IAA ?
ABA
B
IAA
ABA
0
4
8
12
16
20
Retention time (minutes)

Figure 11. Spiked and unspiked chromatograms of the IAA and ABA fraction extracted from the apical tissue of etiolated 8 day-old seedlings of WL1570 (dwarf pea).

a) 90 ul fraction, 10 ul water injected

b) 50 ul fraction, 50 ul IAA, ABA (100 ng of each) injected

HPLC conditions as described in Fig.8.

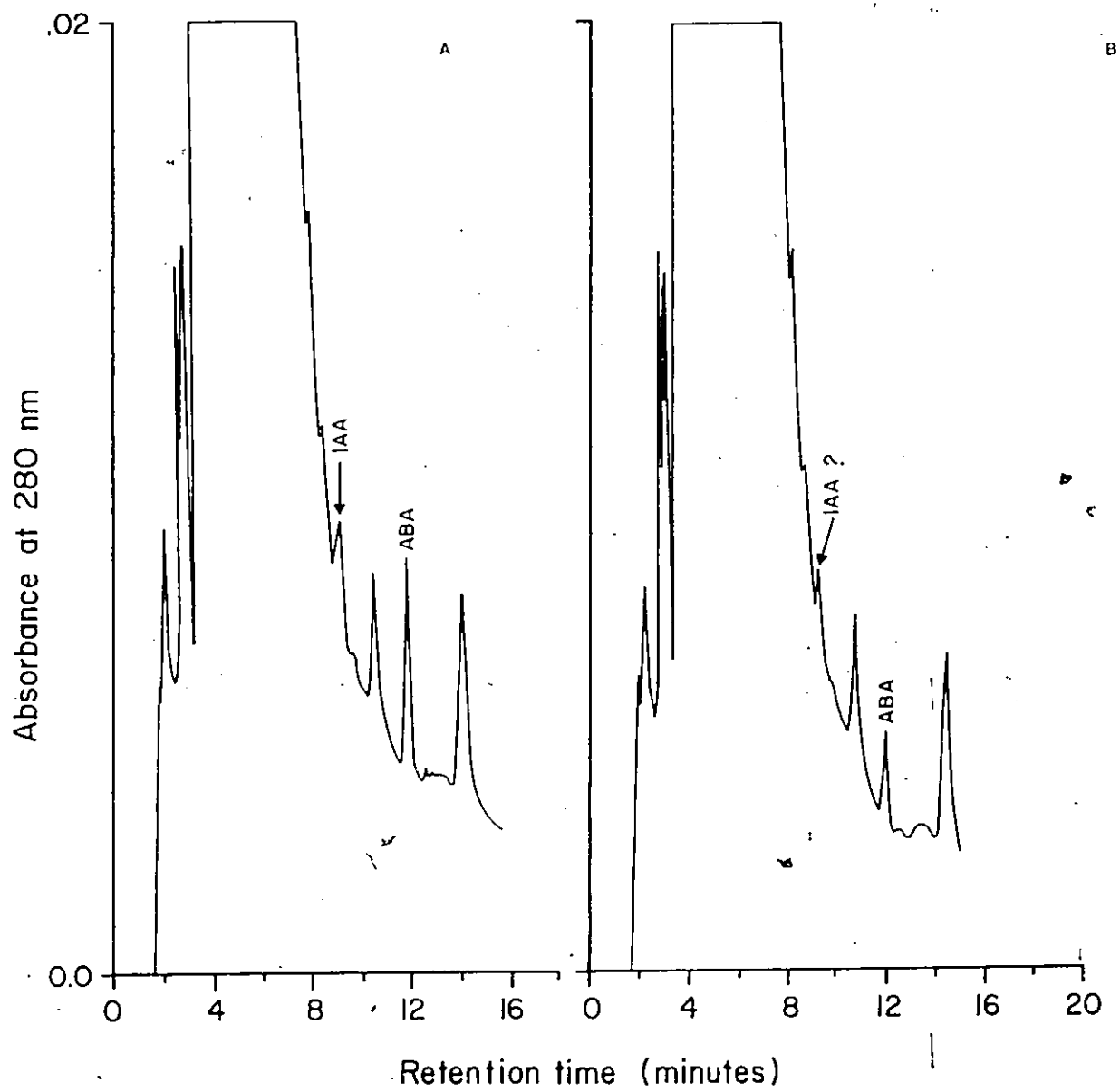

.02
0.0
A
B
IAA
ABA
IAA ?
ABA
Absorbance at 280 nm
0
4
8
12
16
0
4
8
12
16
20
Retention time (minutes)

Figure 12. Spiked and unspiked chromatogram of the IAA and ABA fraction extracted from the apical tissue of etiolated 11 day-old seedlings of WL1570 (dwarf pea).

a) 90 ul fraction, 10 ul IAA, ABA (100 ng)

b) 90 ul fraction, 10 ul water

Conditions as in Fig.11.

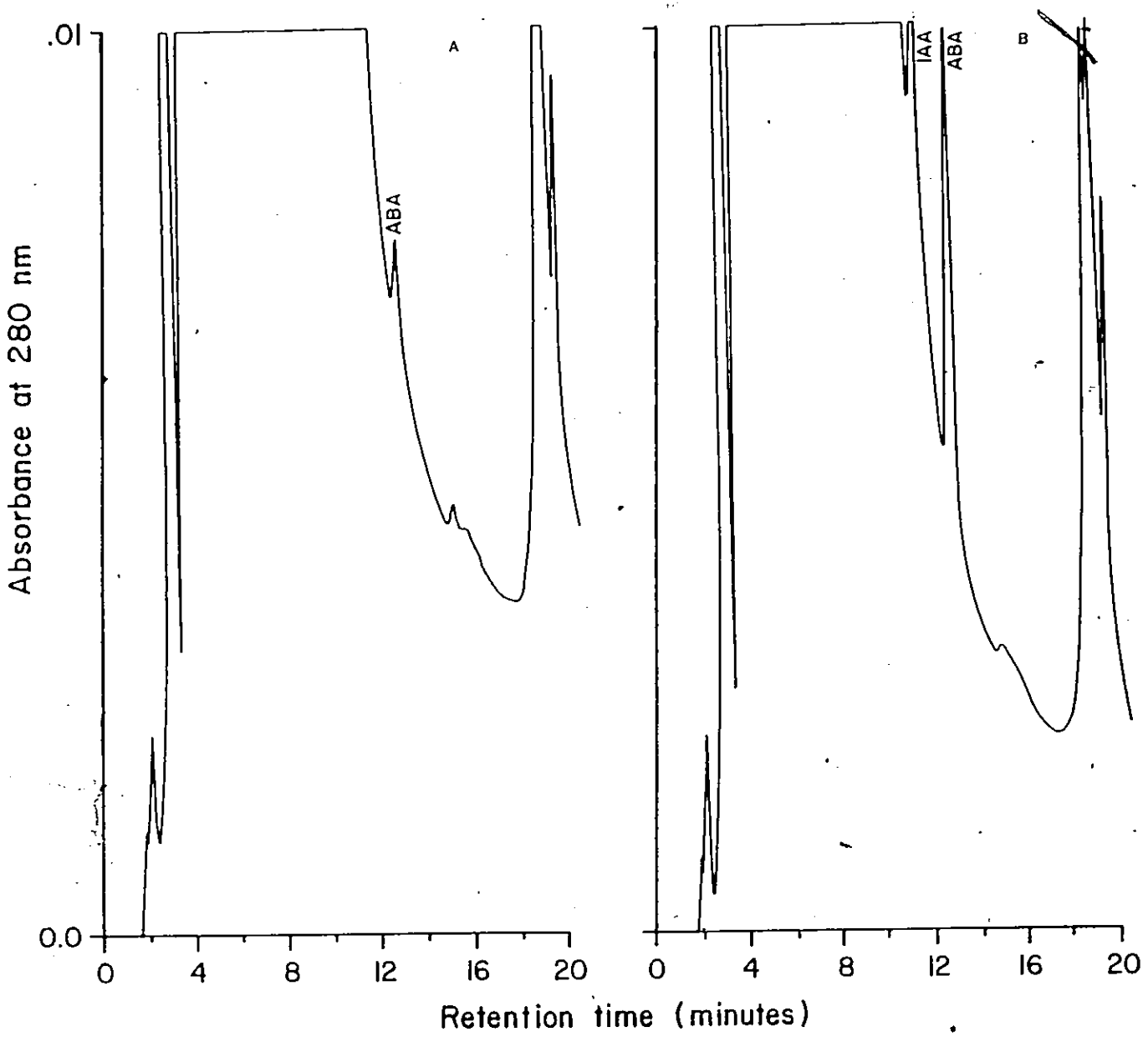

.01
0.0
Absorbance at 280 nm
A
ABA
B
IAA
ABA
0
4
8
12
16
20
Retention time (minutes)

Figure 13. Spiked and unspiked chromatogram of the IAA and ABA fraction extracted from the apical tissue of etiolated 8 day-old seedlings of dwarf WL1415.

a) 90 ul fraction, 10 ul water

b) 90 ul fraction, 10 ul IAA, ABA (100 ng)

Conditions as in Fig.11.

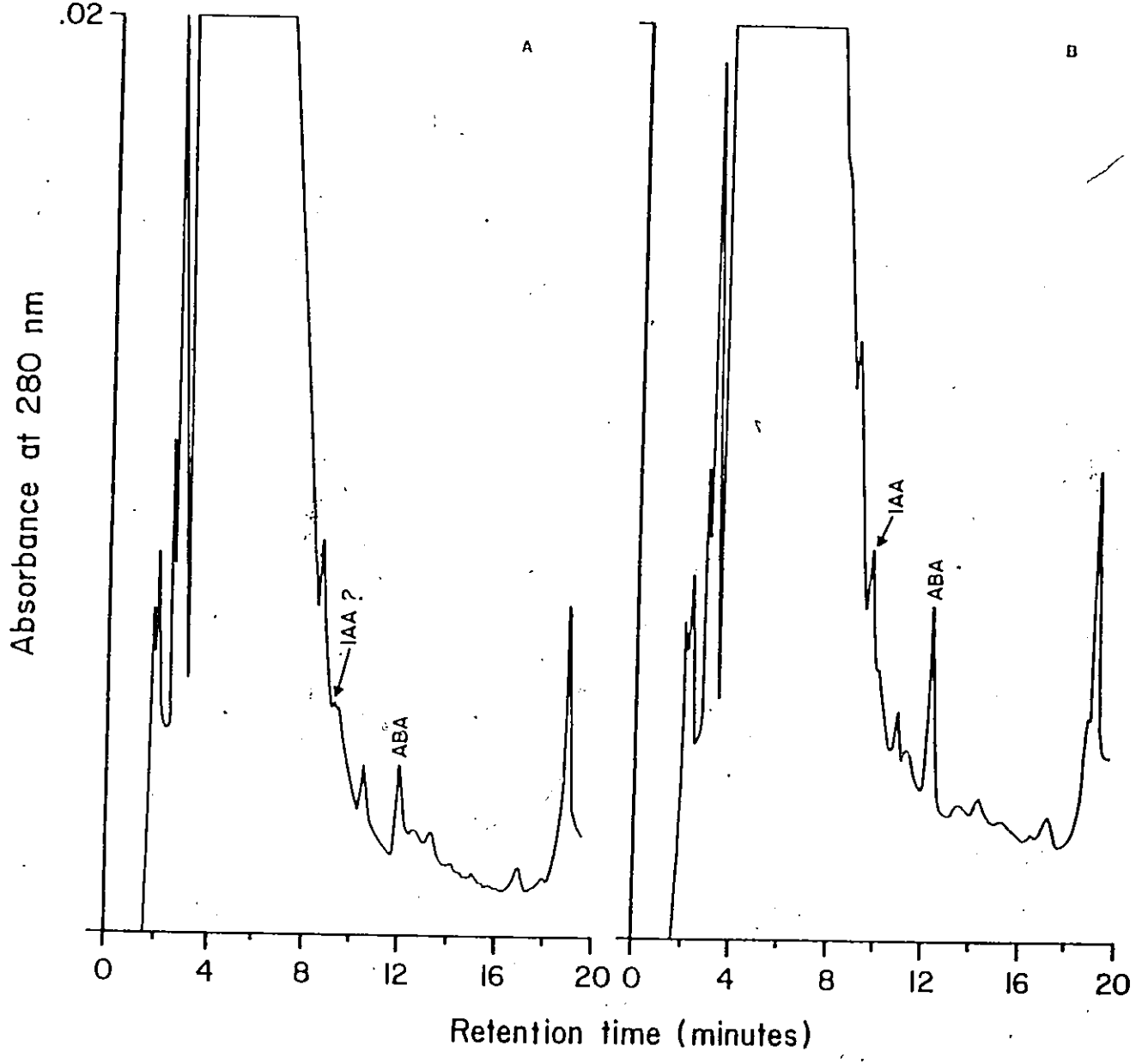

.02
Absorbance at 280 nm
A
B
IAA ?
ABA
IAA
ABA
0
4
8
12
16
20
0
4
8
12
16
20
Retention time (minutes)

Figure 14. Spiked and unspiked chromatogram of the IAA and ABA fraction extracted from the apical tissue of etiolated 11 day-old seedlings of dwarf WL1415.

a) 90 ul fraction, 10 ul water

b) 90 ul fraction, 10 ul IAA, ABA (100 ng)

Conditions as in Fig.11.

TABLE 21

Estimated amounts of ABA in lines WL1415 and WL1570 at two stages of development.

PEA LINE	STAGE	ESTIMATED AMOUNT (NG/G FRESH WEIGHT)
WL1570	8 DAYS	18.7
	11 DAYS	25.2
WL1415	8 DAYS	15.3
	11 DAYS	24.0

days, the apex of line WL1415 contained about 15.3 ng/g fr wt of ABA, and that of line WL1570 had 18.7 ng/g fr wt. At 11 days, WL1415 contained 24 ng/g fr wt and WL1570 had 25.2 ng/g fr wt.

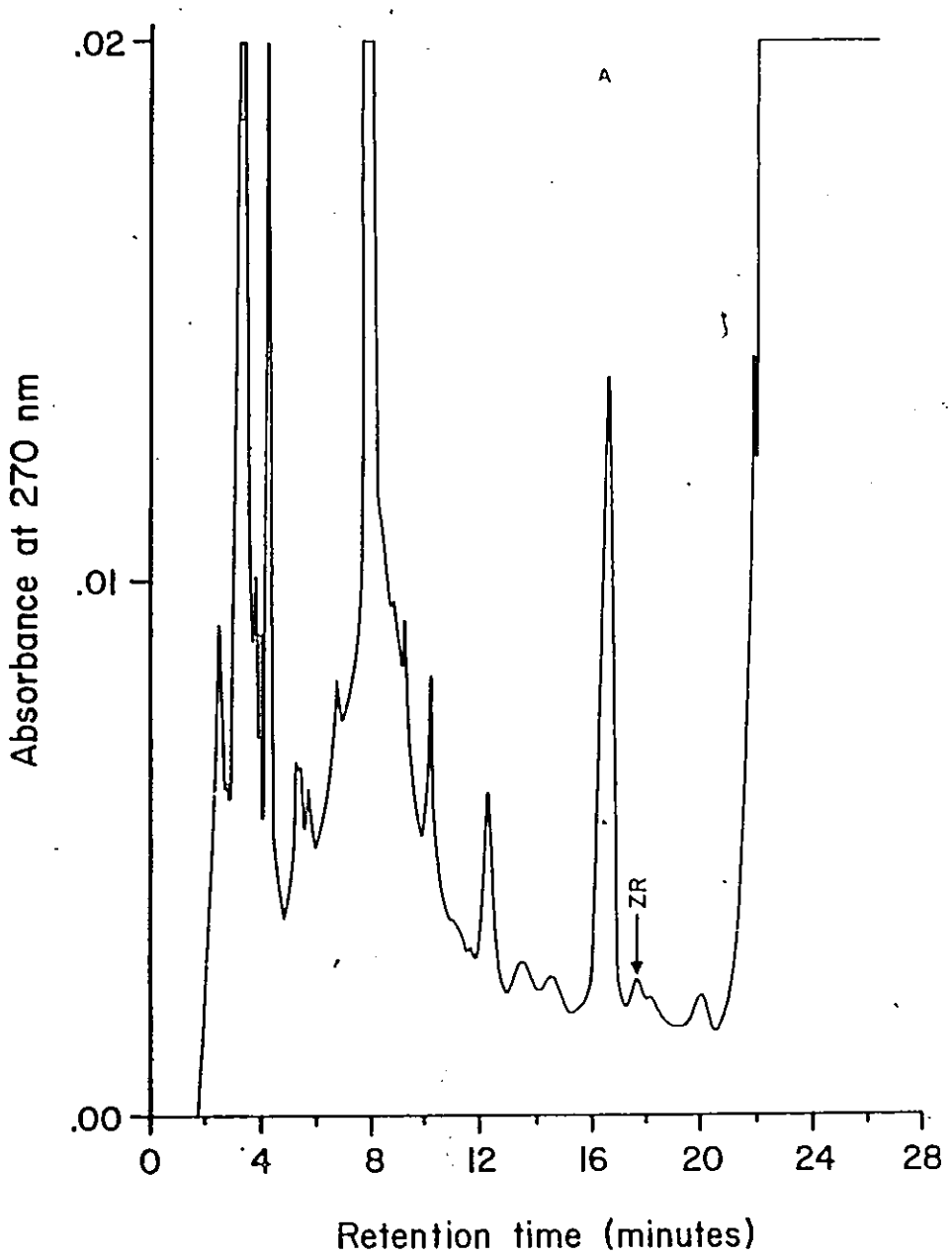
The HPLC chromatograms of the zeatin and zeatin riboside fraction are found in Figs. 15 to 18. The chromatograms from WL1415 differed slightly from those of WL1570 in that line WL1570 had more absorbing material eluting before zeatin riboside.

Zeatin riboside eluted after a fairly large peak at 18 minutes in the chromatogram of the cytokinin containing fraction of 8 day-old seedlings of line WL1570 (Fig. 15). At day 8, the chromatogram shown is from an extract which had been spiked prior to extraction, this had to be taken into account when calculating estimated amounts. It is also why the bioassay gave a large cytokinin activity. Upon subtracting the added amount, the cytokinin activity remaining was low.

Chromatograms of 11 day-old pea seedlings of line WL1570 showed a similar peak for zeatin riboside between 18 and 19 minutes (Fig. 16). At day 11, the ZR peak was hidden under another peak thus amounts were not estimated by HPLC.

Zeatin riboside elutes after almost 16 minutes in the spiked and unspiked chromatograms of the Z and ZR containing fraction of WL1415 (8 days and 11 days) (Figs. 17,18).

The *Amaranthus* betacyanin bioassay showed the presence of cytokinin activity in all of the extracts. For WL1570, cytokinin activity was detected in fractions 7,8,11 and 12 (Fig. 19) in 8 day-old pea seedlings. In 11 day-old pea seedlings, activity was found in fractions 7, 11, 12, 13. (Fig. 20). For WL1415, cytokinin activity was detected for 8 day-old seedlings in fractions 7, 11, 12 (Fig. 21). and for 11 day-old seedlings, in fractions 8 and 12 (Fig. 22). The active fractions corresponded to those obtained with


A
.02
.01
.00
Absorbance at 270 nm
ZR
0
4
8
12
16
20
24
28
Retention time (minutes)

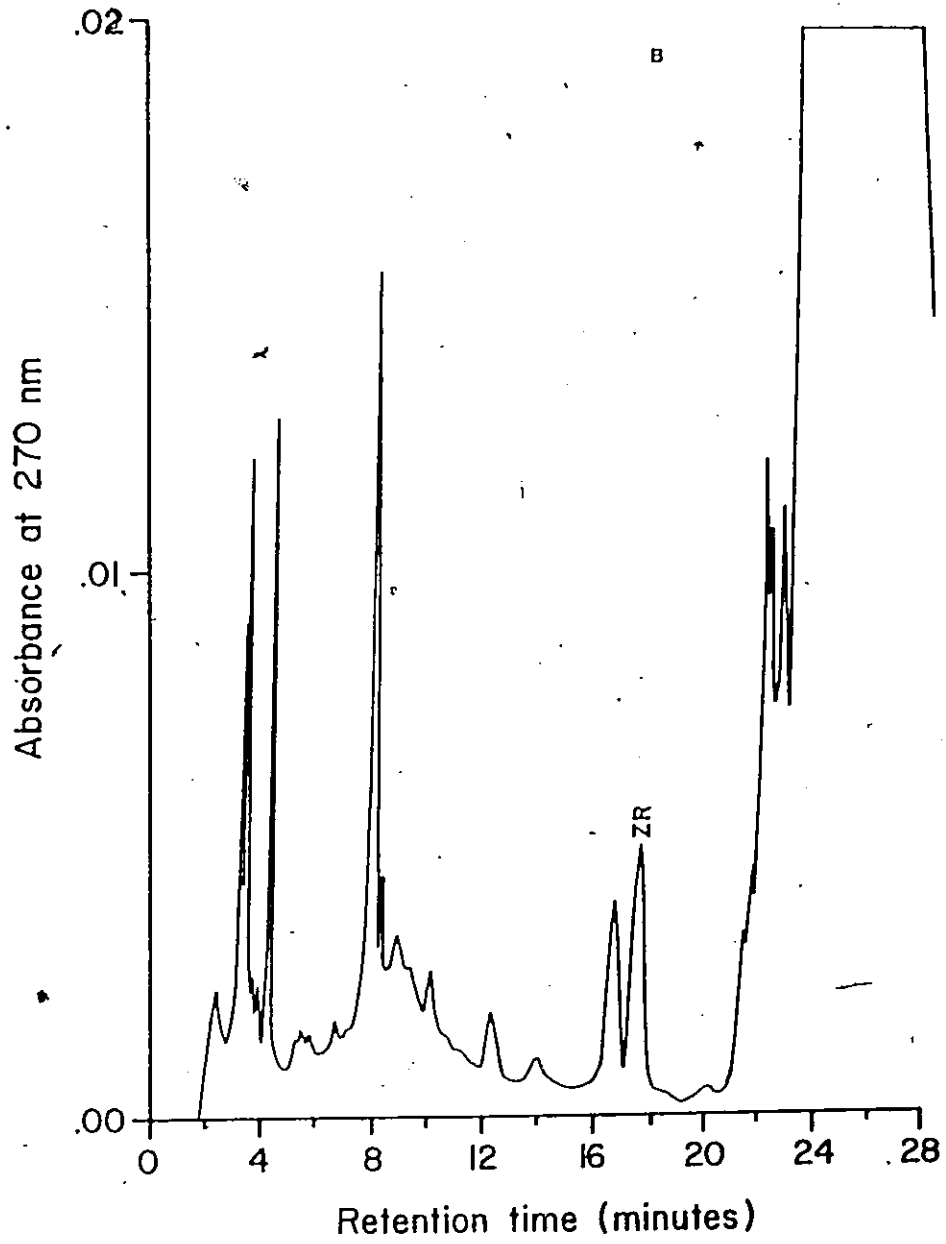
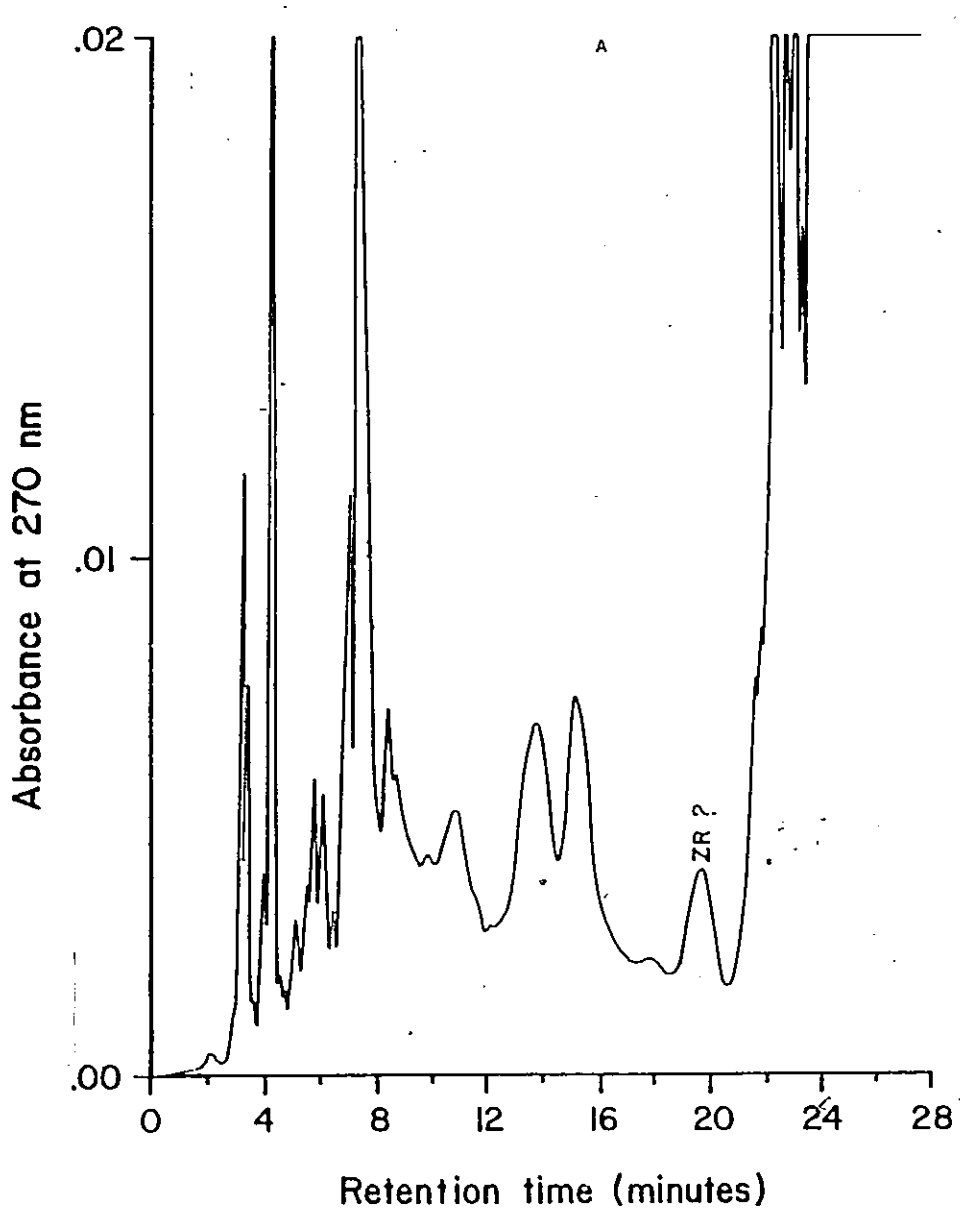

B
.02
.01
.00
Absorbance at 270 nm
ZR
0
4
8
12
16
20
24
28
Retention time (minutes)

Figure 15. Spiked and unspiked chromatograms of the zeatin and zeatin riboside fraction extracted from the apical tissue of etiolated 8 day-old seedlings of dwarf WL1570.

a) 90 ul fraction, 10 ul water

b) 25 ul fraction, 75 ul Z, ZR (75 ng)

HPLC conditions as described in Fig.9.


A
.02
.01
.00
Absorbance at 270 nm
ZR ?
0
4
8
12
16
20
24
28
Retention time (minutes)

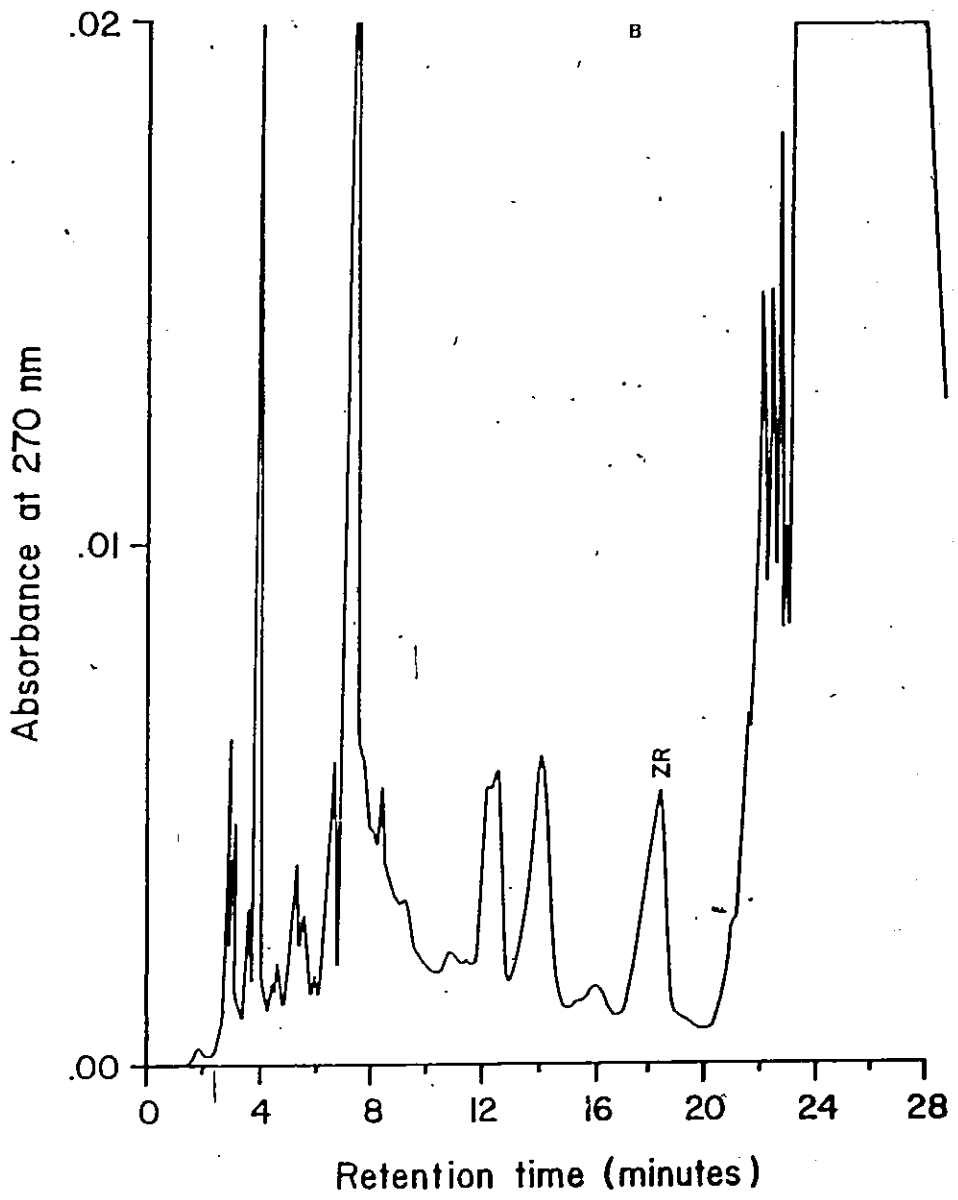

B
.02
.01
.00
Absorbance at 270 nm
ZR
0
4
8
12
16
20
24
28
Retention time (minutes)

Figure 16. Spiked and unspiked chromatograms of the zeatin and zeatin riboside fraction extracted from the apical tissue of etiolated 11 day old seedlings of dwarf WL1570.

a) 90 ul fraction, 10 ul water

b) 50 ul fraction, 50 ul Z, ZR (50 ng)

Conditions as in Fig.16.

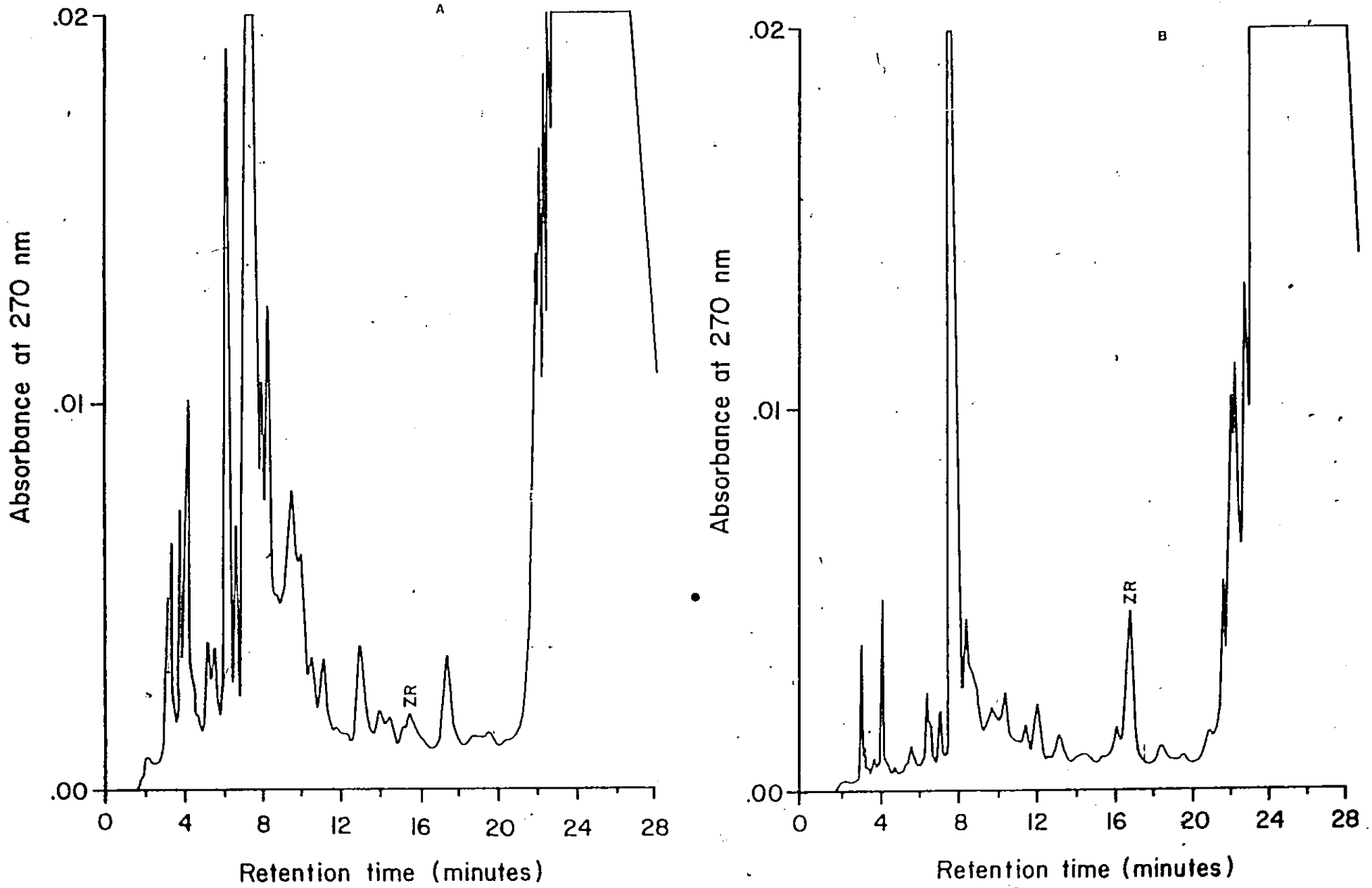

A
.02
.01
.00
Absorbance at 270 nm
ZR
0
4
8
12
16
20
24
28
Retention time (minutes)
B
.02
.01
.00
Absorbance at 270 nm
ZR
0
4
8
12
16
20
24
28
Retention time (minutes)

Figure 17. Spiked and unspiked chromatograms of the zeatin and zeatin riboside fraction extracted from the apical tissue of etiolated 8 day-old seedlings of dwarf WL1415.

a) 90 ul fraction, 10 ul water

b) 50 ul fraction, 50 ul Z, ZR (50 ng)

Conditions as in Fig.16.

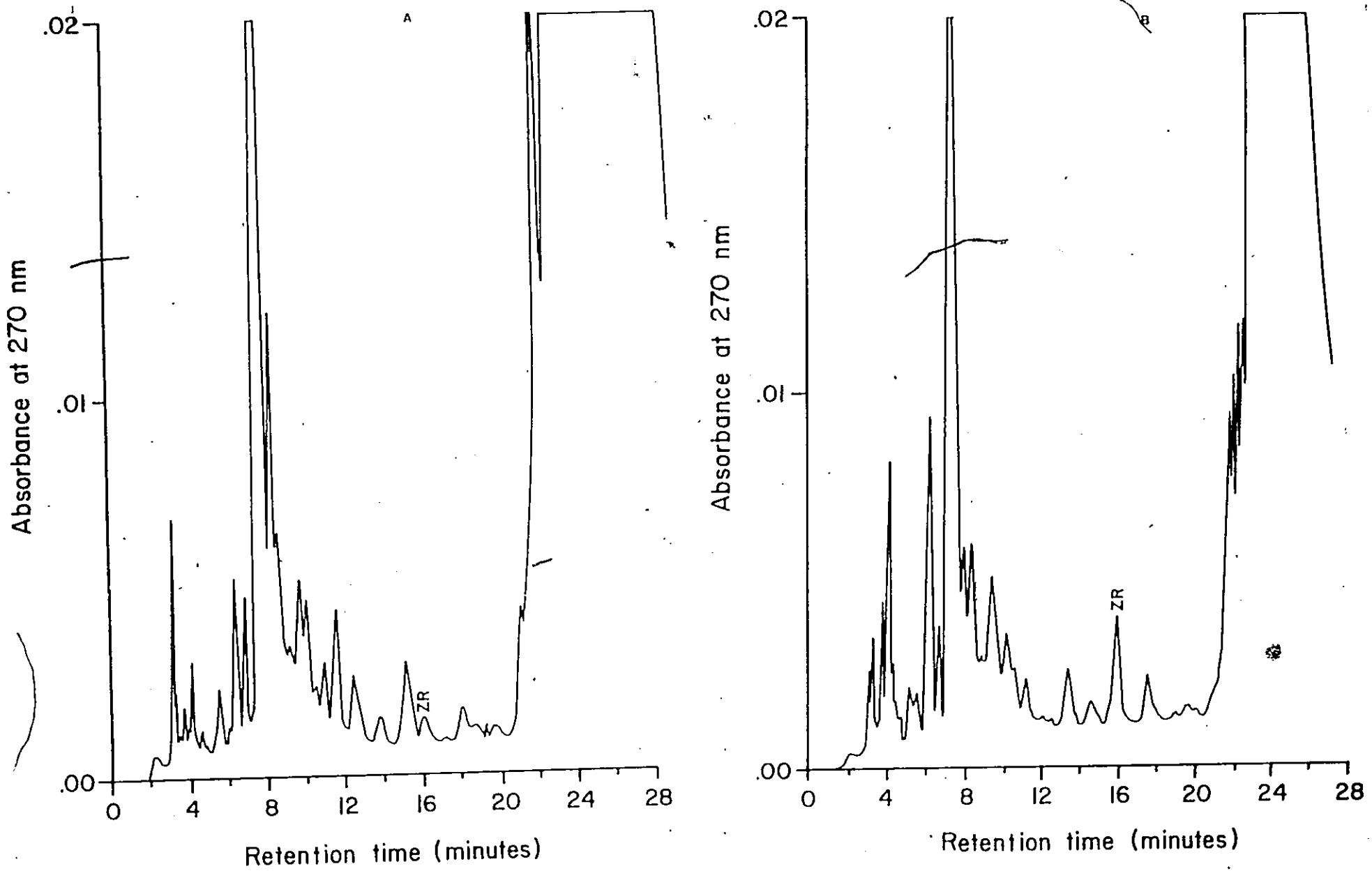

A
.02
.01
.00
Absorbance at 270 nm
0
4
8
12
16
20
24
28
Retention time (minutes)
ZR
B
.02
.01
.00
Absorbance at 270 nm
0
4
8
12
16
20
24
28
Retention time (minutes)
ZR

Figure 18. Spiked and unspiked chromatograms of the zeatin and zeatin riboside fraction extracted from the apical tissue of etiolated 11 day-old seedlings of dwarf WL1415.

a) 90 ul fraction, 10 ul water

b) 50 ul fraction, 50 ul Z, ZR (50 ng)

Conditions as in Fig.16.

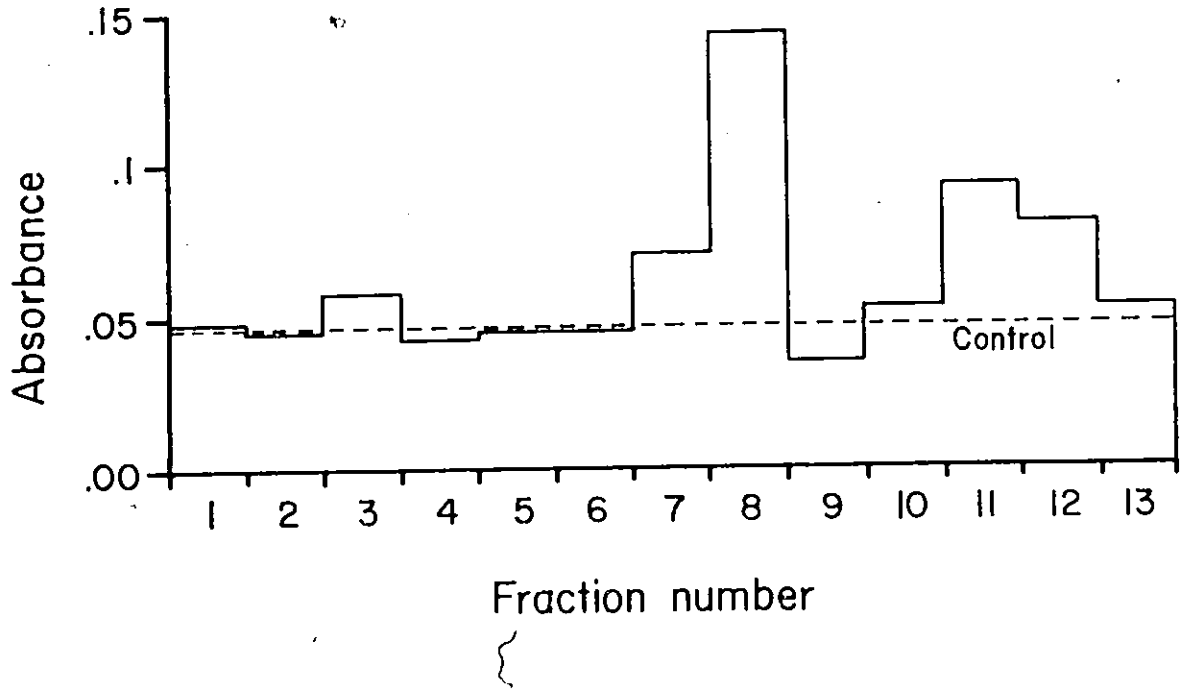

.15
.1
.05
.00
Absorbance
Control
1 2 3 4 5 6 7 8 9 10 11 12 13
Fraction number

Figure 19. *Amaranthus* bioassay of 180 ul of the zeatin and zeatin riboside fraction from WL1570 (8 days) after separation by reversed-phase HPLC. Conditions as in Fig.10.

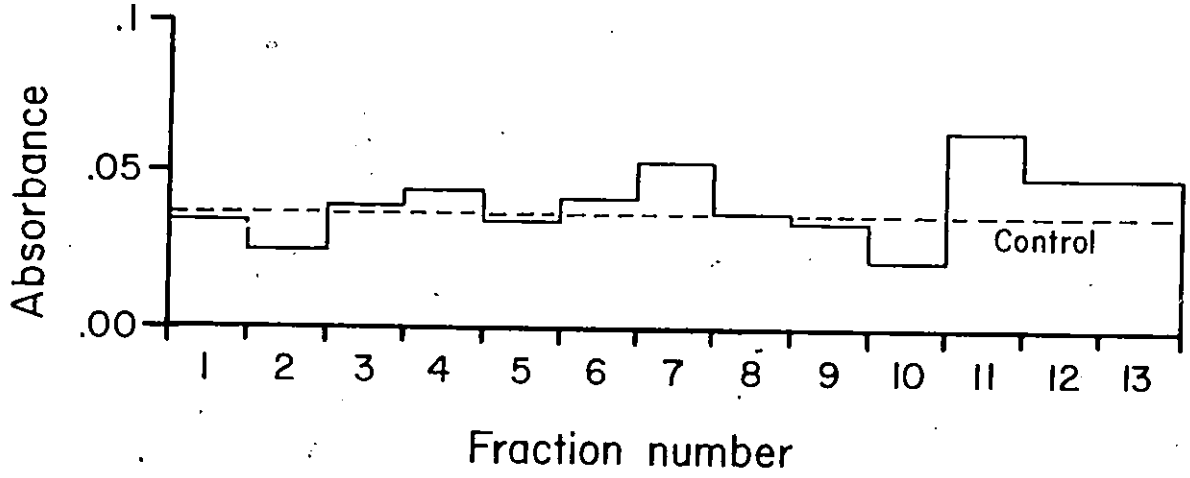

.1
.05
.00
Absorbance
Control
1
2
3
4
5
6
7
8
9
10
11
12
13
Fraction number

Figure 20. *Amaranthus* bioassay of 190 ul of the zeatin and zeatin riboside fraction from WL1570 (11 days) after separation by reversed-phase HPLC. Conditions as in Fig.10.

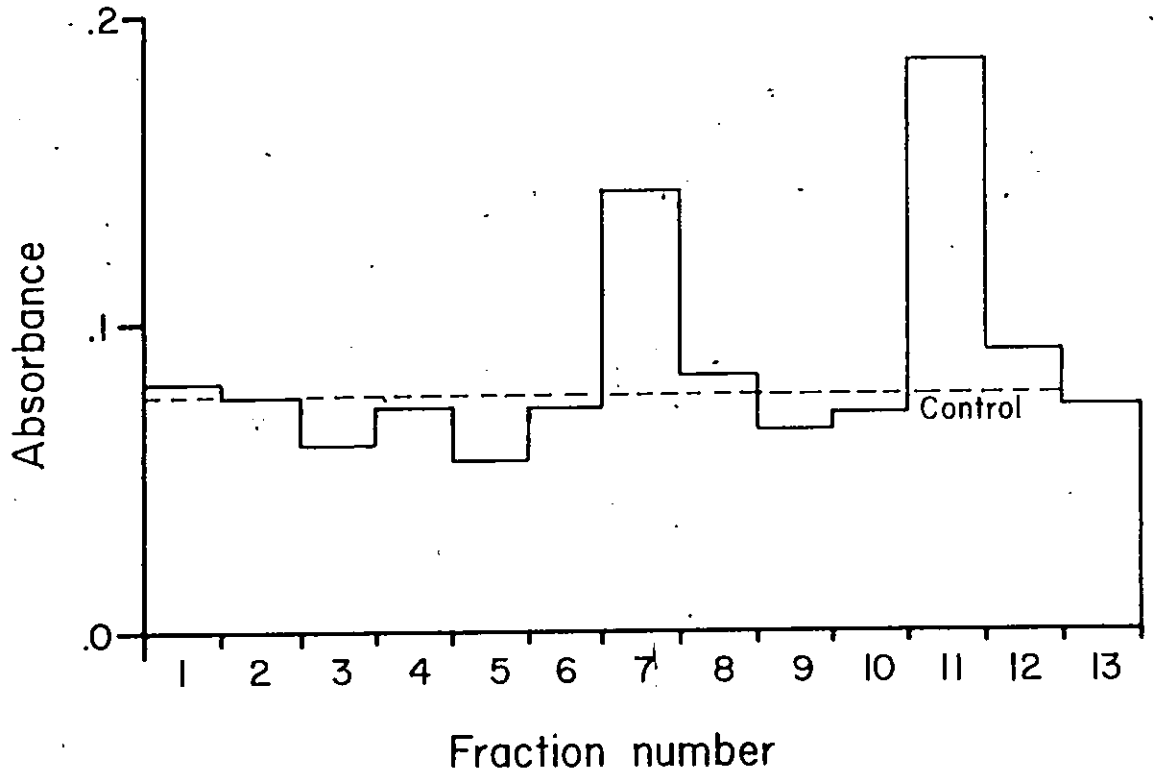

.2
.1
.0
Absorbance
Control
1
2
3
4
5
6
7
8
9
10
11
12
13
Fraction number

Figure 21. *Amaranthus* bioassay of 190 ul of the zeatin and zeatin riboside fraction from WL1415 (8 days), after separation by reverse-phase HPLC. Conditions as in Fig.10.

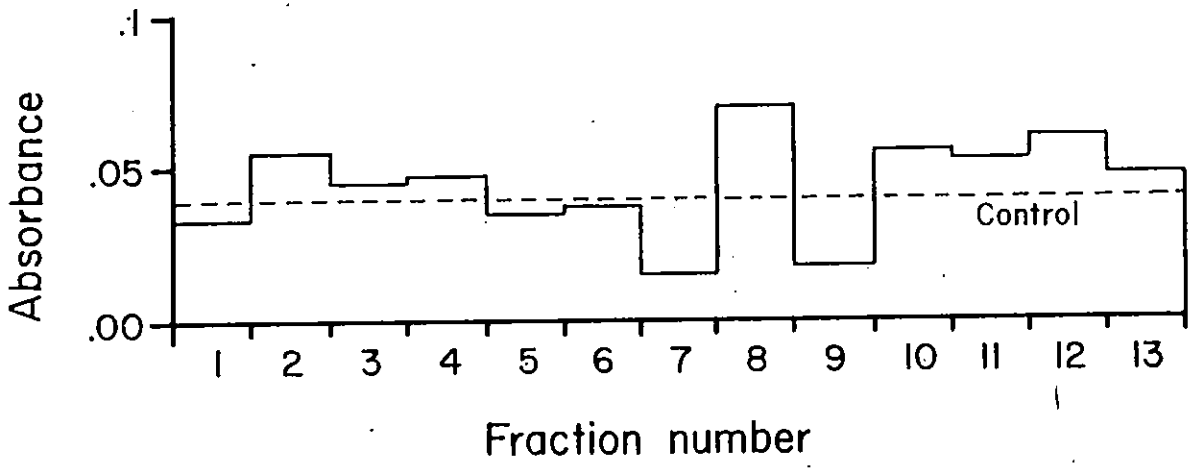

.1
.05
.00
Absorbance
Control
1 2 3 4 5 6 7 8 9 10 11 12 13
Fraction number

Figure 22. *Amaranthus* bioassay of 190 ul of the zeatin and zeatin riboside fraction from WL1415 (11 days), after separation by reversed-phase HPLC. Conditions as in Fig.10.

standard Z and ZR.

The amounts estimated by the bioassay were higher than those estimated with the peak area method. (Table 22). In apices of WL1570 seedlings a small amount (8.3 ng/g fr wt) of zeatin riboside was present at 8 days and none could be detected by HPLC analysis in 11 day-old seedlings. In seedlings of line WL1415, 18.8 ng/g fr wt of ZR was estimated at 8 days and at 11 days, the amount dropped to 6.7 ng/g fr wt. Zeatin was not detected by HPLC, however both cytokinins were detected by bioassay, using fractions from HPLC. Z is found in lesser amounts than its riboside, however the amounts changed similarly. Estimated amounts of 10.7 ng of ZR and 0 ng and Z were found in WL1570 seedlings at 8 days. At 11 days, 8.7 ng of ZR and 7.1 ng of Z were estimated. In WL1415 seedlings (8 days), larger amounts of Z were found (54.5 ng ZR, 36.8 ng Z) whereas at 11 days the amounts were lower (8.1 ng ZR. 4.2 ng Z).

TABLE 22

Estimated amounts of zeatin and zeatin riboside in lines WL1415 and WL1570 at two stages of development. The amounts were estimated from HPLC peak areas and from the *Amaranthus* bioassay.

PEA LINE	STAGE	ESTIMATED AMOUNT (NG/G FRESH WEIGHT)		
		PEAK AREA METHOD	AMARANTHUS BIOASSAY	
		ZR	ZR	Z
WL1570	8 DAYS	8.3	10.7	0
	11 DAYS	-	8.7	7.1
WL1415	8 DAYS	18.8	54.5	36.8
	11 DAYS	6.7	8.1	4.2

CHAPTER IV

DISCUSSION

4.1 INTERNODE AND CELL MEASUREMENTS

As shown in the survey, there is a wide variation in final internode lengths among different pea lines (Nozzolillo & Seguin, 1984). Basal internode development does not appear to be under the control of the internode length gene locus, Le, since both dwarf and tall lines showed similar early internode length variability.

At seed maturity, the embryo contains up to six leaf primordia (Lyndon, 1977). The basal internodes are the most developed, and their potential for elongation may already be defined (Reid, 1983). Reid (1983) observed that the first internode of etiolated nana plants elongate more during germination as compared to succeeding internodes, thus suggesting that the Na locus was ineffective during early seedling development or that the potential for elongation of this internode was already determined in the embryo. The length of the first internode cannot be entirely predetermined; new material must be laid down since cell division occurs throughout the first internode during germination (Nougarede & Rembur, 1980).

Longer first and second internodes have a greater number of cortical cells than do shorter internodes. The cell lengths are very similar in all first internodes except for a tendency toward longer cells in the second internodes. It can be concluded from these

results that cell division is the major component determining the difference in internode lengths of the three (1 tall, 2 dwarf) lines under study. Reid (1983) also found no significant differences in cortical cell lengths in stems of dark-grown tall and dwarf pea segregates. Cytokinins may play a direct role in the regulation of cell division in living plant tissue since they promote cell division in cultured plant tissue (Evan, 1984). Cytokinins can interact with auxins and affect growth. There is some evidence that IAA from the apex may control the transport and accumulation of cytokinins within the intact seedling. In intact plants, ^{14}C-benzyladenine, applied through the roots, accumulates in the apical part of the epicotyl (Prochazka, 1981), where the meristematic region of the stem is found.

4.2 SEEDLING GROWTH IN RESPONSE TO EXCISION

At the imbibition stage, neither the first nor the second internode has elongated, thus any effect from the treatment should appear in both internodes. At the first internode stage, the first internode is elongating, thus organ excision may not affect extensively its growth. However, the second internode should be affected. At the second internode stage, the first internode is maturing and the second internode is actively elongating. Thus major effects on the second internode length are not expected, and no effect should be seen on the first internode length.

In the present study the removal of the roots tended to reduce epicotyl growth, especially in the tall line WL1267. This was also observed in etiolated Alaska pea seedlings by Lockhart (1957). Plant growth regulators are produced in the root tip region and can be transported to the growing apical shoot. Gibberellin-like activity has been detected in the sap of tall pea plants (Carr et al., 1964) and in the bleeding sap of

Helianthus annuus (Phillips & Jones, 1964). Cytokinins are transported from the roots to the growing apex in intact etiolated pea seedlings (Prochazka, 1981). Indole-3-acetic acid is also produced by roots (Pengelly & Torrey, 1982) and transported basipetally through the cortex (Kaldewey, 1984). When applied exogenously through the roots, it inhibits the elongation of the first and second internodes (Nougarède & Rondet, 1983). Removing the root can therefore alter the distribution of growth substances throughout the plant and thus affect growth.

The testa is a barrier to the release of solutes from seeds. Diffusates from seed without testa contain more solutes than the diffusate from seed with testa (Larson, 1968). The solutes are comprised of sugars, nitrogen-containing compounds and amino acids (Larson, 1968). Leachates have also been found to contain nucleotides (Pollock & Tode, 1966), gibberellins (Wheeler, 1965), glycine betaine, trigonelline (Tramontano et al., 1982), malic acid and citric acid (Morohashi & Shimokoriyama, 1972). Removal of the testa resulted in no significant difference in internode growth in line WL1570, which implies that compounds necessary for growth were not leached out in sufficient amount to have an effect on seedling growth. In WL1267, the removal of the testa had some significant effects. Growth was enhanced when the testa was removed at the imbibition stage suggesting the presence of inhibitory substance(s) such as condensed tannins and phenolics, in the seedcoat. At the first internode stage, the second internode had a reduced growth, which suggests that the enhancement of growth is dependent upon early removal of the testa.

In the present study, wild pea lines are used. WL1570 is a wild type from Afghanistan and WL1267 is a cross-derivative. Such lines may contain more compounds in their seedcoats than the white flowered pea lines (e.g. Alaska) commonly utilized for research. It is also possible that inhibitory compounds are present in the cotyledons as

well as in the testa. Removing the testa will allow the leaching of these compounds from the cotyledons and enhance growth.

Larson (1968) observed a reduction in the stem length of light-grown Alaska pea when the seedcoat had been removed. He removed the testa prior to imbibition whereas in this study the testa, was removed after imbibition. With no testa, the seed takes up water more rapidly, cell injury occurs, and more solutes are leached. As the seed hydrates, leakage slows down (Simon, 1974) thus if the testa is removed after imbibition less material will be lost and growth may not be affected extensively as was the case for WL1570.

The removal of one cotyledon at the first and second internode stages enhanced growth in WL1267, suggesting again that an inhibitor may be produced by the cotyledons. Line WL1570 showed no enhancement of growth and lengths did not differ significantly from the control at any time.

Factors from the cotyledons can influence stem development. In some soybean cultivars, the removal of one cotyledon causes an increase in hypocotyl elongation at 25 ^{0}C. This was shown to be caused by a factor from the cotyledon that influenced ethylene production and thus regulated hypocotyl growth (Seyedin et al., 1982).

It is possible that in WL1267, a factor from the cotyledon also increased ethylene production. Ethylene can alter stem growth of etiolated pea seedlings (Stewart et al., 1974). In peas, ethylene arrests cell division in the apical hook region (Apelbaum & Burg, 1972, Stewart et al., 1974) by inhibiting DNA synthesis (Apelbaum & Burg, 1972). It also inhibits cell division in lateral buds, an effect which is partially reversed by benzyladenine (Apelbaum & Burg, 1972). The ethylene produced could inhibit cell division during the growth of the first internode and a short first internode would

result.

Trigonelline, an intermediate of the pyridine nucleotide metabolic pathway (Godavari & Waygood, 1970) is also present in cotyledons and can be transported to the root and shoot during germination (Tramontano et al., 1982). It promotes cell arrest in the G2 phase of the cell cycle in 40% of root cells and it may act similarly in the shoot (Evans & Tramontano, 1981).

Seedling growth was inhibited by the excision of both cotyledons as was also found by other workers (Lockhart, 1957, Shininger, 1972). Growth depends upon stored reserves from these organs. The transport of soluble carbohydrates and nitrogen from the cotyledons to the axis begins when the shoot system becomes free of the cotyledons and testa and expand to reach the surface where the leaves become green. The cotyledons start to deteriorate 10-12 days after planting (Bain & Mercer, 1966a), but the young plant is dependent upon the seed reserves even when the root and shoot system are completely developed (up to 3 weeks after planting) (Bain & Mercer, 1966b). The transport of soluble material increases during this phase and removal of the cotyledons at that late stage inhibits further growth (Bain & Mercer, 1966b).

Amino acids from the cotyledons are precursors of polyamines (Young & Galston, 1983) which are thought to be involved in cell proliferation (Smith et al., 1985, Smith, 1985). The amino acids are translocated through the stem to the epicotyl where polyamines are synthesized (Young & Galston, 1983). Highest levels of polyamines are found in the young internodes, the levels decreasing in the regions of cell expansion (Smith et al., 1985). The removal of both cotyledons would remove the amino acid source and thus lower the levels of polyamines and hence affect growth.

The excision of the apex removes the meristematic region of the stem, thus bringing

an end to primary stem growth. It also removes the source of auxin needed for cell elongation and causes a reduction in the gradient of auxin within the stem tissue (Scott & Briggs, 1960). These changes initiate the release from dormancy of the closest buds which can be reversed by the application of auxin to decapitated plants (Nagao & Rubinstein, 1976). Cytokinins may also be involved in stimulatory lateral bud development. Application of cytokinins to rootless plants induces the growth of lateral buds in a fashion similar to decapitation (Nagao & Rubinstein, 1976), ^{14}C-BA accumulates in the buds (Prochazka & Jacobs, 1984) and stimulates their growth (Burg & Burg, 1968, Nagao & Rubinstein, 1976).

4.3 RESPONSE OF STEM SEGMENTS TO GROWTH FACTORS

In the present study, IAA stimulated stem extension when applied exogenously to excised stem segments, whereas ABA, kinetin and ethylene did not and only the third internode of the dwarf line WL1570 showed a response to GA3. Such results are in accord with observations reported in the literature for auxin (Purves & Galston, 1960), ethylene (Ridge & Osborne, 1969). and kinetin (Katsumi, 1963). Gibberellic acid usually promotes segment extension; however, Purves and Hillman (1958) have shown that the response to GA of segments cut from the third internode decreases with increasing distance from the apex. The sections in the present study were taken 3 mm below the apex and contain some tissue which is not very responsive to GA3 and thus growth would not be extensively promoted. There is no well-defined GA optimum for the effect of GA3 on stem sections (Purves & Hillman, 1959), as is also shown by my results. There is evidence that auxins and GA interact (Galston & Warburg, 1959). A synergism between IAA and GA occurs in etiolated dwarf pea stem sections but not in tall Alaska

pea sections (Ockerse & Galston, 1967) thus the response of the dwarf line would be greater. The tissues of the third internodes of dwarf WL1570 may contain more auxin than those of tall WL1267 and thus an interaction between GA and IAA affected the growth of the segments of WL1570. The fact that the tall line WL1267 did not show such a response implies that these dwarf and tall lines differ in their sensitivity to GA3 and/or in their levels of endogenous auxins.

The failure of WL1570 segments to respond to ethylene or kinetin suggests that their response to GA was to GA alone and not to endogenous GA + IAA, since it has been shown that in the presence of auxin, ethylene and kinetin inhibit pea segment elongation and induce thickening (Mondal & Nance, 1975, Katsumi, 1963).

In WL1570, segments from the growing first and second internodes are insensitive to GA3 which suggests a difference in the control of growth in these internodes compared to third internodes. The data of Reid (1983) showed that etiolated pea plants responded to GA application by increasing the internode length. The first and second internodes increased about the same length but the third internode increased much more in both tall and dwarf.

IAA causes the elongation of cells within an excised stem segment, by coordinating wall loosening and the supply of wall material needed for elongation (Vanderhoef & Dute, 1981). Wall loosening is thought to result from proton extrusion (Vanderhoef, 1979), and auxin has been shown to induce a decrease in free space pH within the segments (Jacobs & Ray, 1976). IAA also increases the levels of translatable mRNA (Zurfluh & Guilfoyle, 1982), RNA synthesis (Fan & MacLachlan, 1967), cellulase synthesis (Fan & MacLachlan, 1967, Sargent et al., 1973) and stimulates xyloglucan and glucomannan turnover (Gilkes & Hall, 1977). The epidermis may be the auxin responsive tissue in stems since peeling the epidermis inhibits segment extension (Brummell & Hall,

1981).

Supraoptimal levels of auxins induce the production of ethylene (Burg & Burg, 1966). Ethylene can affect growth by inhibiting lateral auxin transport and by reducing polar auxin transport in pea stem sections supplied with auxin (Burg & Burg, 1966). Supraoptimal IAA levels can also act by an inhibitory effect on the electron-transport chain and mitochondrial ATPase activity (Columbo & Ferrari-Bravo, 1982).

4.4 ENZYME ACTIVITY

IAA oxidase and peroxidase enzymes have been implicated in a number of physiological processes (MacNicol, 1966). Two important aspects are 1) their involvement in the breakdown of indole-3-acetic acid (Hinman & Lang, 1965, Ricard & Job, 1974), and 2) the peroxidase-mediated polymerization of phenolic alcohols during cell wall lignification (Siegel, 1955, Siegel, 1956). The breakdown products, 3-methyleneoxindole and indole-3-acetaldehyde, are not active in the pea epicotyl section test (Galston, 1956). It has been suggested that both IAA oxidase and peroxidase activities reside on the same molecule (Galston et al., 1968). In etiolated Alaska pea, enzymes with both activities have indeed been found, however enzymes with only peroxidase or IAA oxidase activity also exist (Bryant & Lane, 1979). Tissues with high peroxidase and/or IAA oxidase activity presumably oxidize IAA readily, thus reducing endogenous levels and altering growth patterns (Galston & Dalberg, 1954).

4.4.1 IAA OXIDASE

Differences in total IAA oxidase and peroxidase activities were found between the two dwarf lines under study. In general, there is a decrease in total IAA oxidase activity from the apex toward the base of the stem. High IAA oxidase activity would be expected to cause an increase in the degradation of IAA molecules in the tissue just under the apex. I was unable to detect any IAA in the apical tissues of lines WL1570 and WL1415. This suggests that low endogenous IAA levels are present. Low levels of IAA (8 ng/g fr wt) have also been reported in the apex of etiolated Alaska pea (Schneider et al. 1985). In the region below the apex, the cells are only beginning to expand. As the IAA is translocated down the stem, it is degraded. The levels of auxin produced by the apex must be supraoptimal so that as the levels are reduced a sufficient amount remains for cell elongation to occur in the elongating zone of the internode. IAA is found in smaller quantities in etiolated tissues (Scott & Briggs, 1960, Tillberg, 1974). Etiolated tissues are more sensitive to IAA, stem segments from etiolated plants respond to lower concentration of IAA than segments exposed to red light (Gālston 1977). As more IAA is metabolized along the stem and passes the elongating zone, levels may become suboptimal and cell elongation will stop. Line WL1415 with its higher IAA oxidase activity would be expected to have low levels of IAA and shorter internodes.

I already have shown that the length differences are not due to cell elongation but to difference in cell number, thus it is not surprising that IAA oxidase levels do not correlate with the first internode length. I have shown that segments from the growing second and third internodes are able to grow more in response to auxin. They have less IAA oxidase activity and do not metabolize IAA as fast, thus the elongation region may elongate during a longer period of time. Cells would be longer than in the first internode. There was a tendency toward longer cells in the second internode, however it was not significant. It still may explain why succeeding internodes are longer than the previous one. In WL1415, total IAA oxidase fluctuates along the stem during stage 2

and 3. Such a pattern is difficult to correlate with growth.

The decrease in IAA oxidase activity along the stem observed in my study is contradictory to the results of Galston and Dalberg (1954) who observed an increase toward the base of the stem. They expressed IAA oxidase activity on a mg protein N basis whereas the activity in the present study is expressed on a mg fr wt or per segment basis. Since they found that the amount of protein was highest in the apical bud and decreased down the growing third internode, the total activity in the apex would decrease when expressed on a protein basis, but should not have changed much in the rest of the stem.

4.4.2 PEROXIDASE

The distribution of total peroxidase activity along the stem was similar in both lines. It was high below the apex, decreased in the elongating zone, and then increased in the maturing tissue. Such a distribution has also been observed in light-grown dwarf pea stem (Birecka & Galston, 1970). The only difference between the two lines lies in the level of activity, line WL1415 had less peroxidase activity than WL1570. As tissue is maturing, extension growth stops, cell walls thicken and lignification occurs even in dark-grown plants (Cheng & Marsh, 1968). The increase in peroxidases in the maturing region may be linked to their involvement in this process.

In the region of the stem below the apex, peroxidase activity is higher than in the elongating region. Ethylene is known to cause an increase in peroxidase activity in all parts of the etiolated Alaska pea plant (Ridge & Osborne, 1970). Ethylene induces lateral cell expansion and inhibits cell division in the apical tissue (Apelbaum & Burg, 1972).

The first internode of WL1570 is thicker and shorter than the internode of WL1415. WL1570 also has more peroxidase activity. This is not due to an increase in cell diameter. There is some evidence that the effect of ethylene on cell expansion can be separated from the inhibition of cell division. Stewart et al. (1974) have shown that the effect of ethylene on the cessation of cell division is not reversed by gibberellic acid whereas it counteracted the effect of ethylene on cell elongation in the subhook region. Furthermore, gibberellins have been shown to have no effect on total peroxidase activity (Catalfamo et al., 1978). This implies that ethylene affects these two processes in different ways.

Supraoptimal levels of IAA produce an increase in ethylene production (Burg & Burg, 1968) and pretreatment with IAA causes a rise in peroxidase levels (Galston, 1956). If the effect of ethylene on cell division is related to peroxidase, cell division would be decreased in tissues with high enzyme activity. The second internode of WL1570 is long and the third is even longer, cell division is high and as expected peroxidase activity decreases. Line WL1415 has a long first internode and its level of peroxidase is low. Succeeding internodes are also long and levels of peroxidases are slightly decreased.

4.5 ENDOGENOUS GROWTH REGULATOR ANALYSIS

4.5.1 EXTRACTION, SEPARATION AND QUANTIFICATION

The extraction procedure finally developed permitted the detection of ABA, and zeatin in less than 10 g of plant tissue. The limits of detection by HPLC analysis were

5 ng of ABA, 25 ng of IAA, 15 ng for both zeatin and zeatin riboside. Thus IAA was the hardest to detect in a chromatogram. Homogenization of the tissue with acetone provides a thorough extraction of the growth regulators from plant tissue (Yokota et al., 1980). Fractionation to remove lipoidal substances from the extract leaves most of the growth regulators in the aqueous phase as is expected from their partition coefficients (Ciha et al., 1977, Letham, 1974).

SepPak C18 cartridges permit separation of the cytokinins from IAA and ABA. The present study is the first report of the use of SepPak cartridges for this purpose. SepPak C18 cartridges have been used for the purification of cytokinins (Ernst et al., 1984, McGaw et al., 1985).

IAA and ABA are retained on the SepPak C18 cartridges upon acidification of the extract since compounds with a low polarity (like ABA at low pH) have a high affinity for C18 (Guinn, 1985). SepPak C18 cartridges have been used for the purification of ABA (Guinn, 1985, Oden & Dunberg, 1984, Creelman & Zeevaart, 1985), IAA and ABA (Guinn et al., 1986, Van Onckelen et al., 1984) and IAA (Law & Hamilton, 1982).

Fairly good recovery was obtained with this extraction method. The utilization of SepPak C18 cartridges permits purification of the extract (by using eluants of different polarity) and it concentrates the extract in a smaller volume thus less plant tissue is required. The cartridges removed some phenolics, flavonoids, and carotenoids from the extract as determined from the absorbance spectra. However contaminants are still present within the final fraction.

ABA was present in enough quantity to be detected in the unspiked chromatograms. IAA was not present in large quantity and in many instances the spiked IAA eluted very near another small peak, thus the presence of IAA could not be verified. IAA is

either too low to be detected or it was not present in the pea tissue. The first is probably true, IAA has been detected in Alaska pea tissue, but in very low quantities (Schneider et al., 1985). IAA was more difficult to separate from contaminants and it had a high detection limit (25 ng). The two lines did not show any differences in the appearance of the chromatograms of the IAA and ABA containing fraction.

Zeatin and zeatin riboside were both present in the plant extract fraction, but only zeatin riboside was detected by HPLC. The bioassay permits the detection of the presence of cytokinins even when other compounds are present. A certain caution must be exercised though when interpreting the data of the bioassay since compounds with the extract can affect its response. For example, it is well known that phenolic compounds within plant extracts have an inhibitory effect upon the cytokinin-stimulated betacyanin synthesis (Challice, 1977).

In plant extracts, levels of IAA were low in both lines since none was detected by HPLC, despite their difference in internode length. Low IAA levels coupled with high IAA oxidase activity will not leave much IAA for elongation. Other analogues of IAA were not measured, these could be involved in growth. IAA and its effect on cell elongation are not directly involved since cell division is the important factor.

Cytokinin levels (zeatin and zeatin riboside) differed between the lines and between the stages of development. The level of cytokinin in WL1570 was low and did not change at the two stages of development. However, the internode length does. In line WL1415, the cytokinin level was high at the first stage (day 8) and decreased at stage 2 (11 days). High levels of cytokinins imply that cell division is increased. Internode length would be greater in WL1415 thus at day 8 there is some correlation between cytokinin levels and internode length. At the second internode stage, cytokinin levels were low in both lines, and both lines had longer second internodes. There does not

seem to be a correlation between second internode growth and the quantity of Z, and ZR. Other cytokinins are found in peas and these may affect cell division. ABA levels were similar and tended to increase slightly at 11 days. The levels correspond well with those calculated by Simpson and Saunders (1972) in light-grown dwarf pea plants.

CHAPTER V

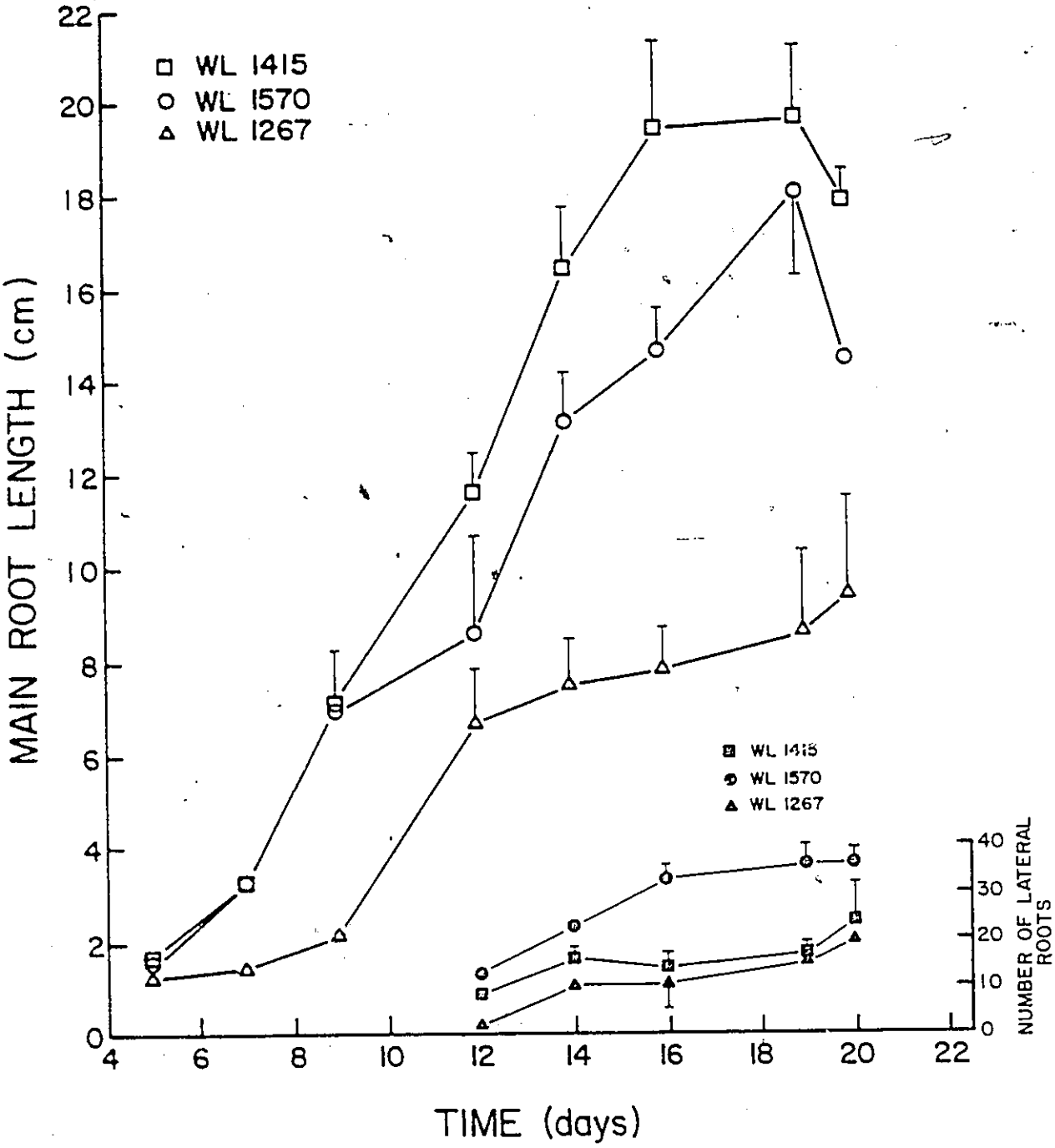
CONCLUSION

The growth of pea stems in darkness is controlled by various factors, both genetic and environmental. The objective of this thesis was to determine factors which were involved in the control of early basal internode growth. A variety of wild pea lines were screened for their internode length and lines with differences in their etiolation response were further studied.

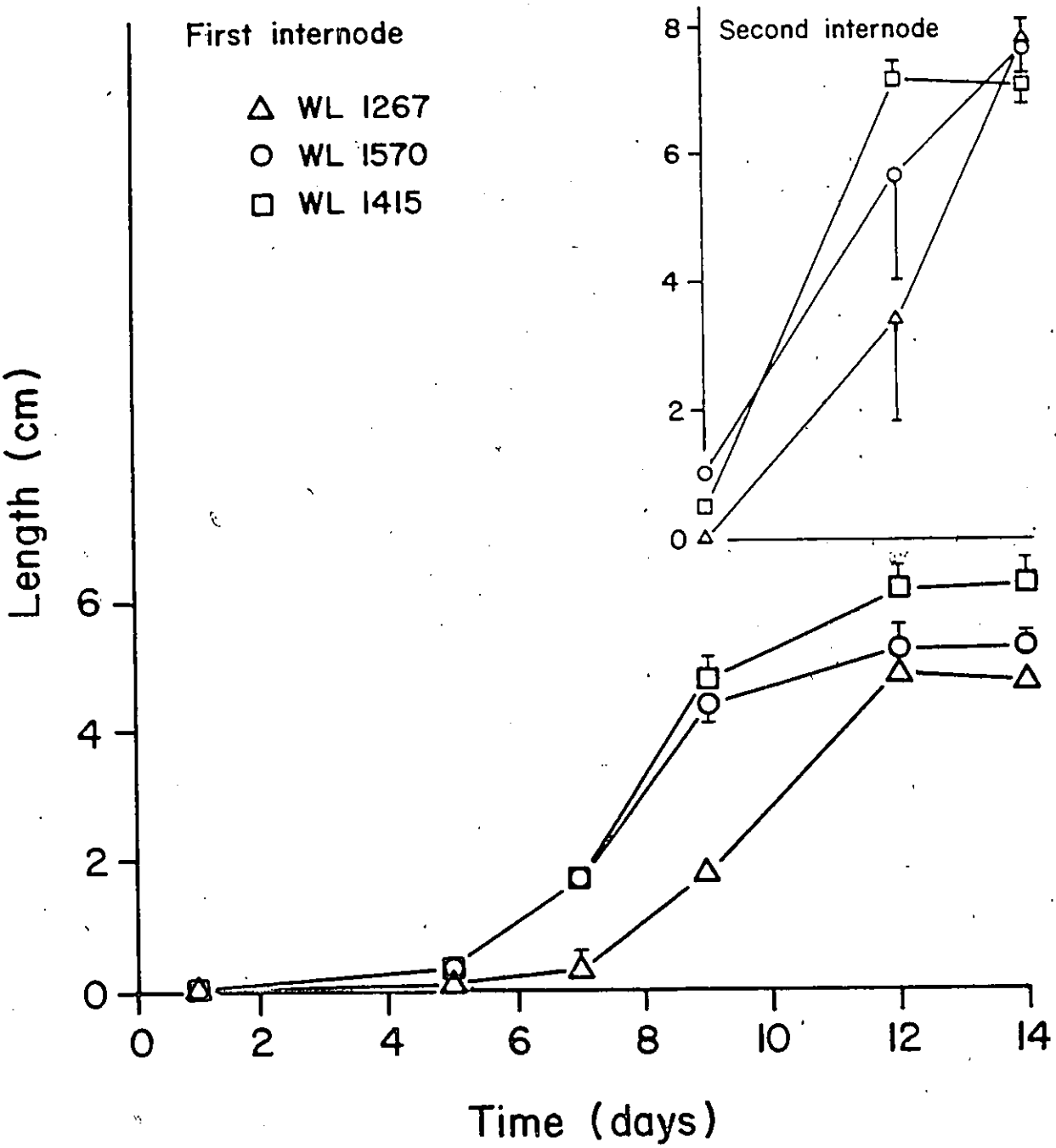
The characterization of the cell dimension within mature internode was necessary to pinpoint whether the length was influenced by cell division and/or by cell elongation. Studies such as cell size determination, growth of excised segments in the presence of exogenous growth regulators and determination of IAA oxidase and peroxidase activities point out that cell elongation during internode growth is not the major factor controlling the final internode length and that cell division has an important influence.

Excision data suggests that an inhibitor is present in the testa or cotyledons and influences growth in the tall WL1267.

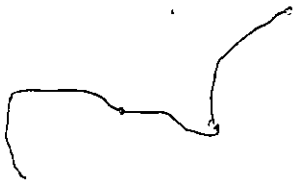
An extraction method for cytokinins, IAA and ABA was developed in order to determine if endogenous levels varied between lines and during internode development. Cytokinins may be involved since their level varied between lines and between stages of development in WL415.

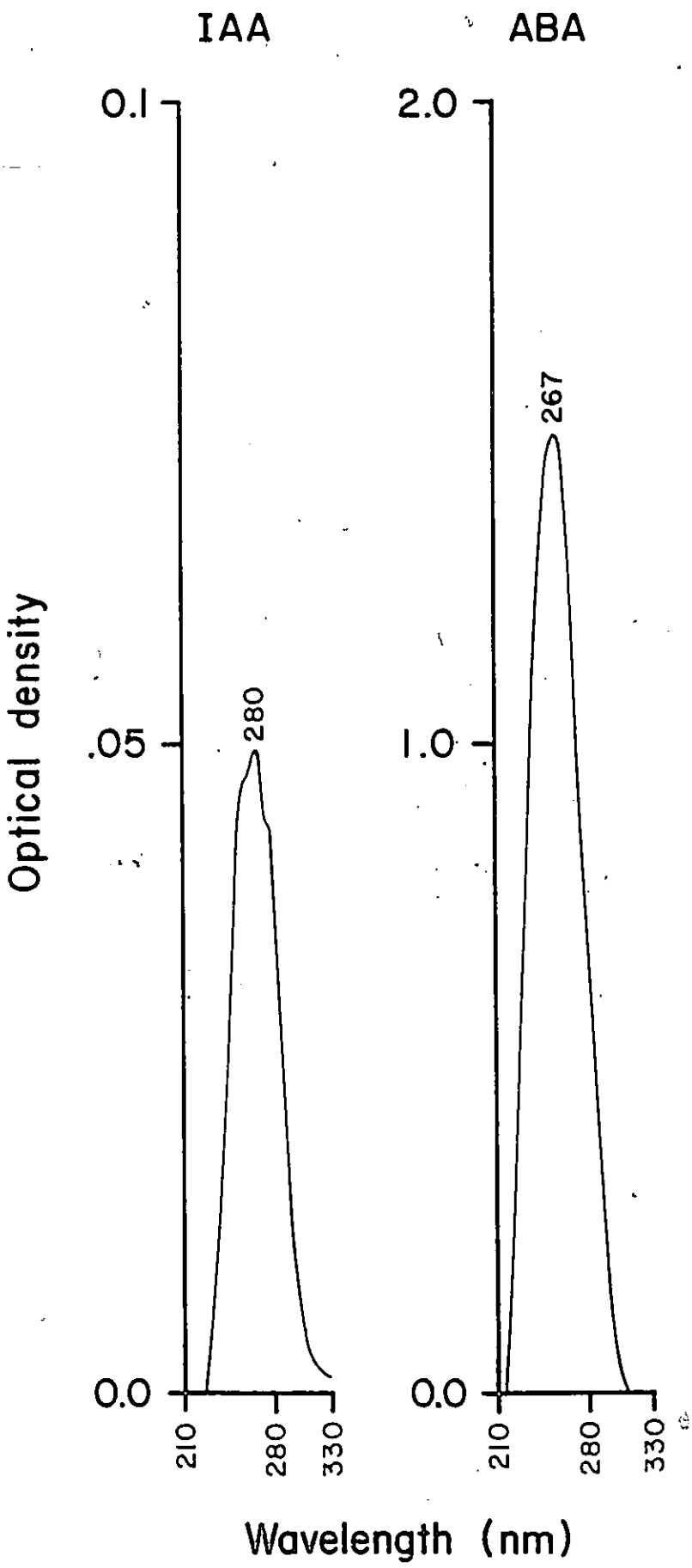

WL 1415
WL 1570
WL 1267
MAIN ROOT LENGTH (cm)
TIME (days)
NUMBER OF LATERAL ROOTS
0
2
4
6
8
10
12
14
16
18
20
22
4
6
8
10
12
14
16
18
20
22
0
10
20
30
40

Appendix 1. Growth of the first and second internode of etiolated seedlings of 3 lines of peas during 14 days at 15 ^{0}C. Standard deviation shown.

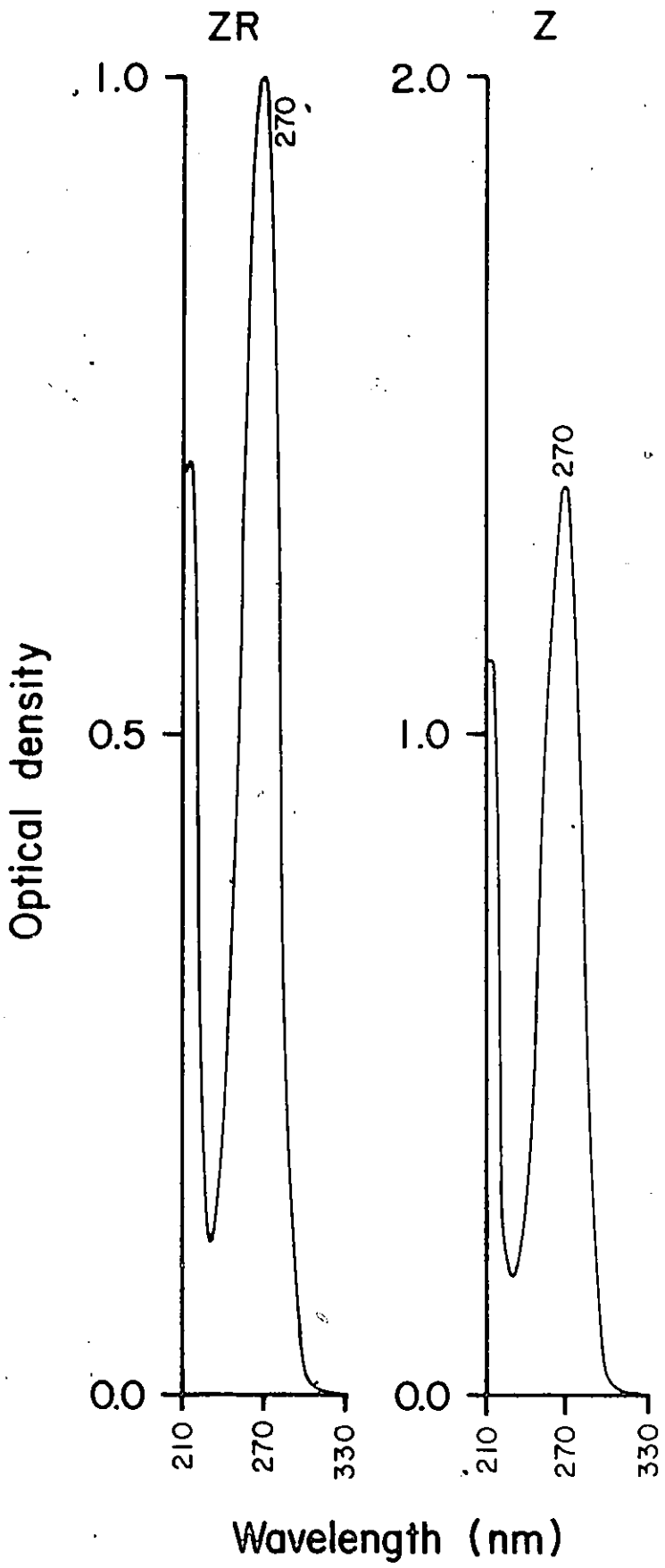

First internode
WL 1267
WL 1570
WL 1415
Second internode
Length (cm)
Time (days)
0
2
4
6
8
10
12
14

Appendix 2. Growth of the root and number of lateral roots of 3 lines of etiolated pea seedlings during 22 days at 15 ^{0}C Standard deviation shown.

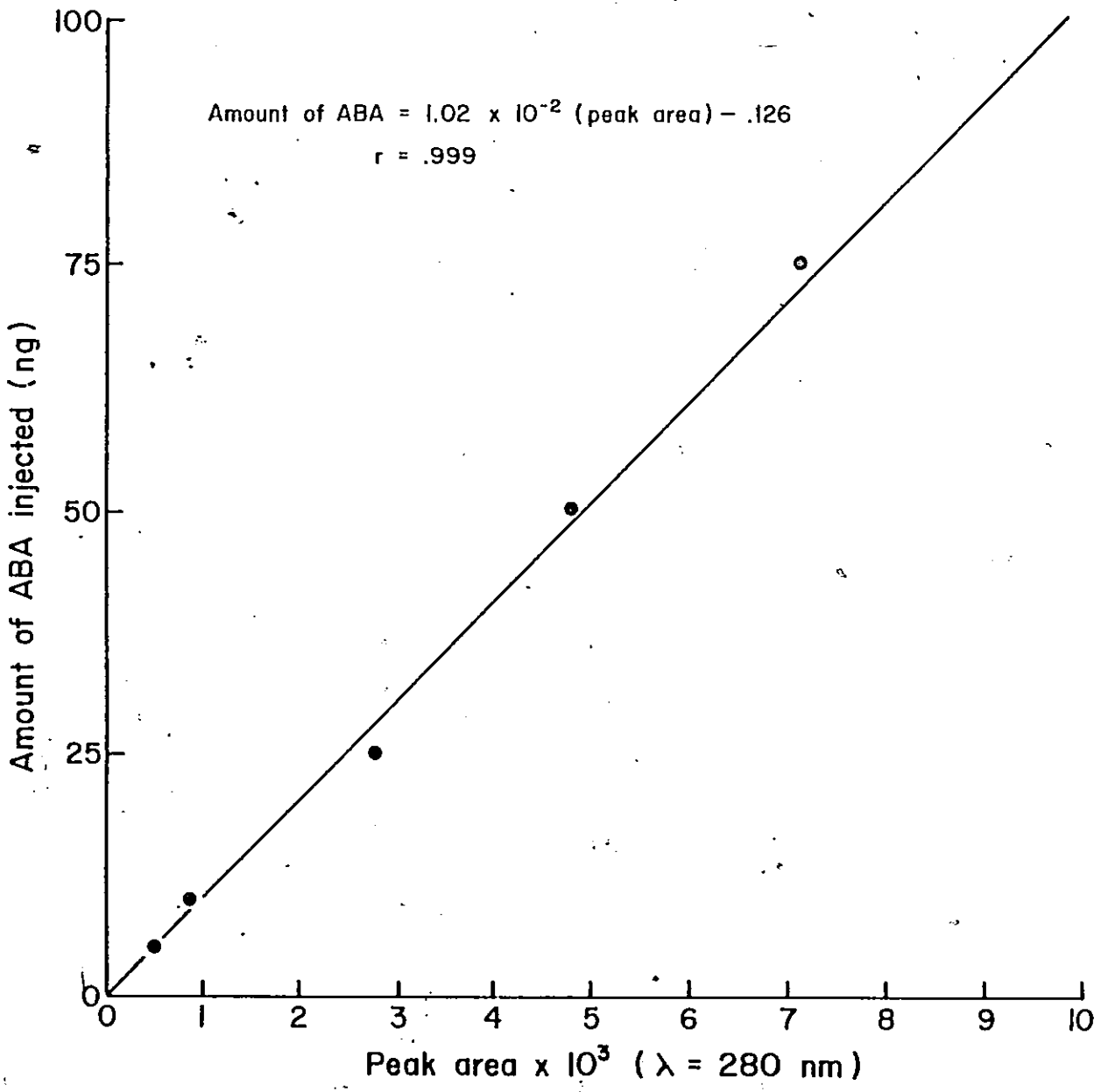



IAA
ABA
0.1
.05
0.0
2.0
1.0
0.0
280
267
210
280
330
210
280
330
Optical density
Wavelength (nm)

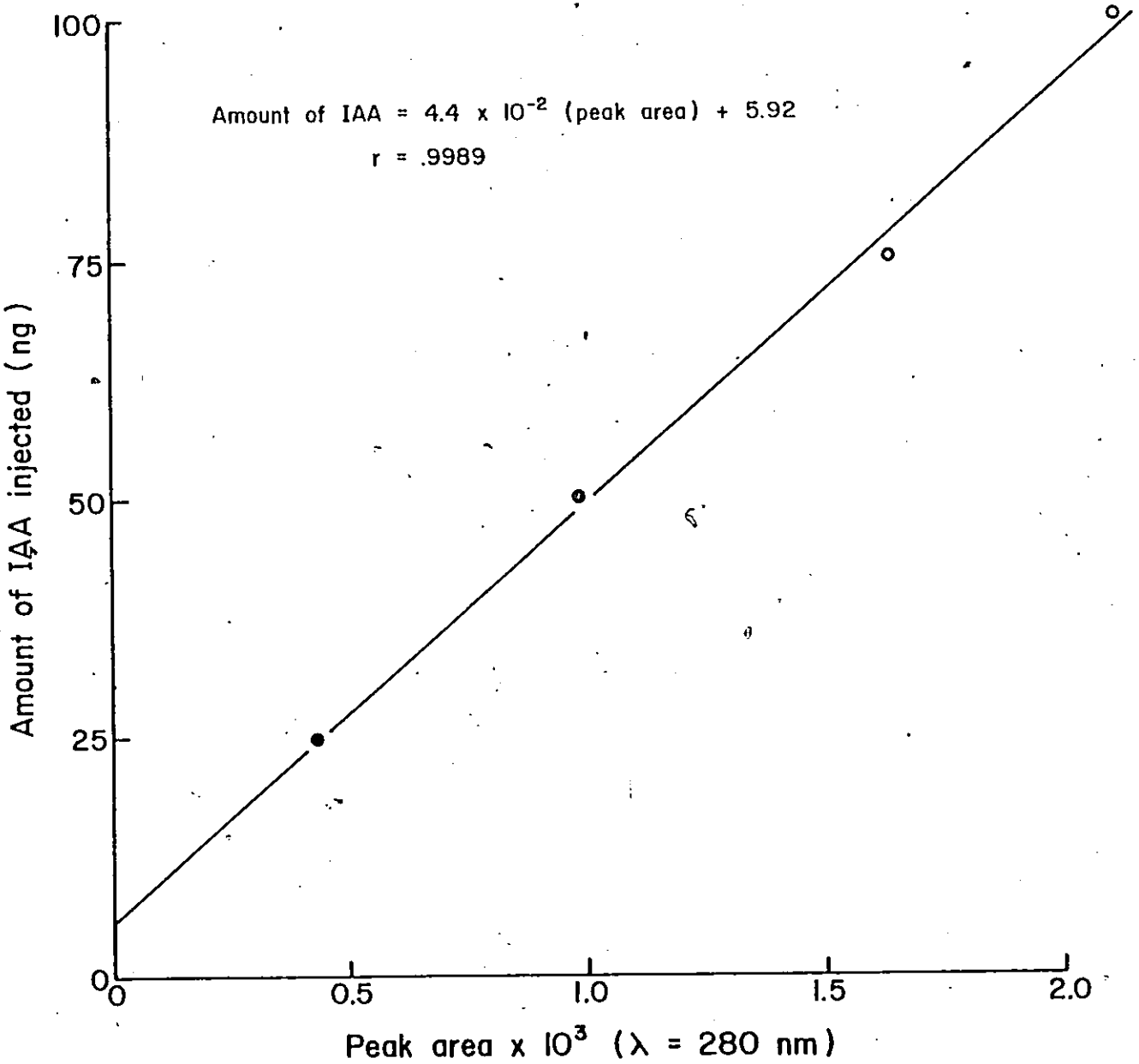
Appendix 3. Absorption spectra of IAA and ABA. IAA was dissolved in 65% of 0.1% glacial acetic acid in water and 35% acetonitrile at a concentration of 10 ng/ul. ABA was dissolved in 63% of 0.1% glacial acetic acid in water and 32% acetonitrile at a concentration of 3 ng/ul.


ZR
Z
1.0
2.0
270
270
0.5
1.0
Optical density
0.0
0.0
210
270
330
210
270
330
Wavelength (nm)

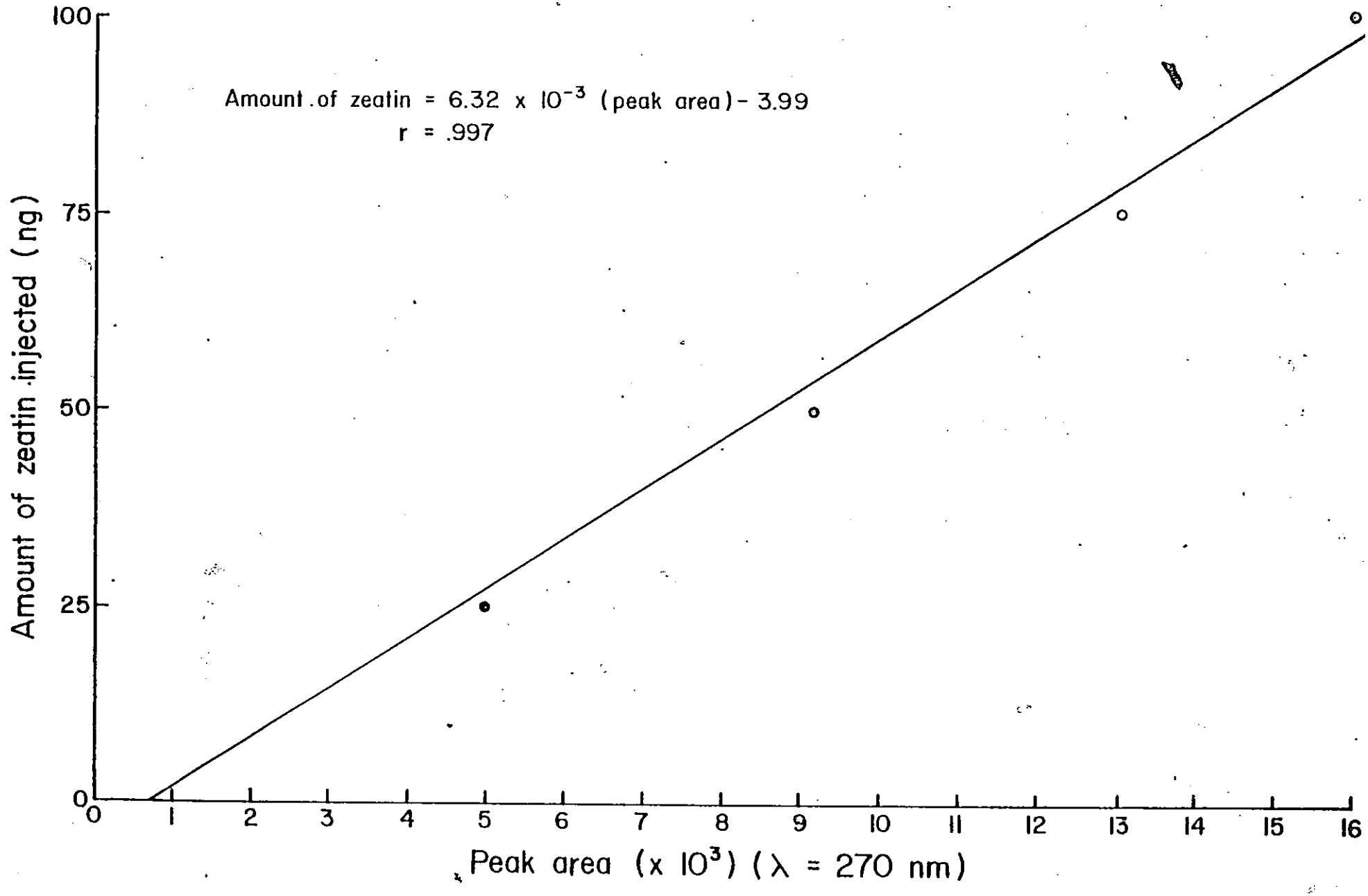
Appendix 4. Absorption spectra of zeatin and zeatin riboside. zeatin was dissolved in 50% of 0.1% glacial acetic acid in water and 50% methanol. Zeatin riboside was dissolved in 55% of 0.1% glacial acetic acid and 45% methanol. Both compounds were at a concentration of 10 ng/ul.


Amount of ABA = 1.02 x 10⁻² (peak area) – .126
r = .999
Amount of ABA injected (ng)
100
75
50
25
0
0
1
2
3
4
5
6
7
8
9
10
Peak area x 10³ (λ = 280 nm)

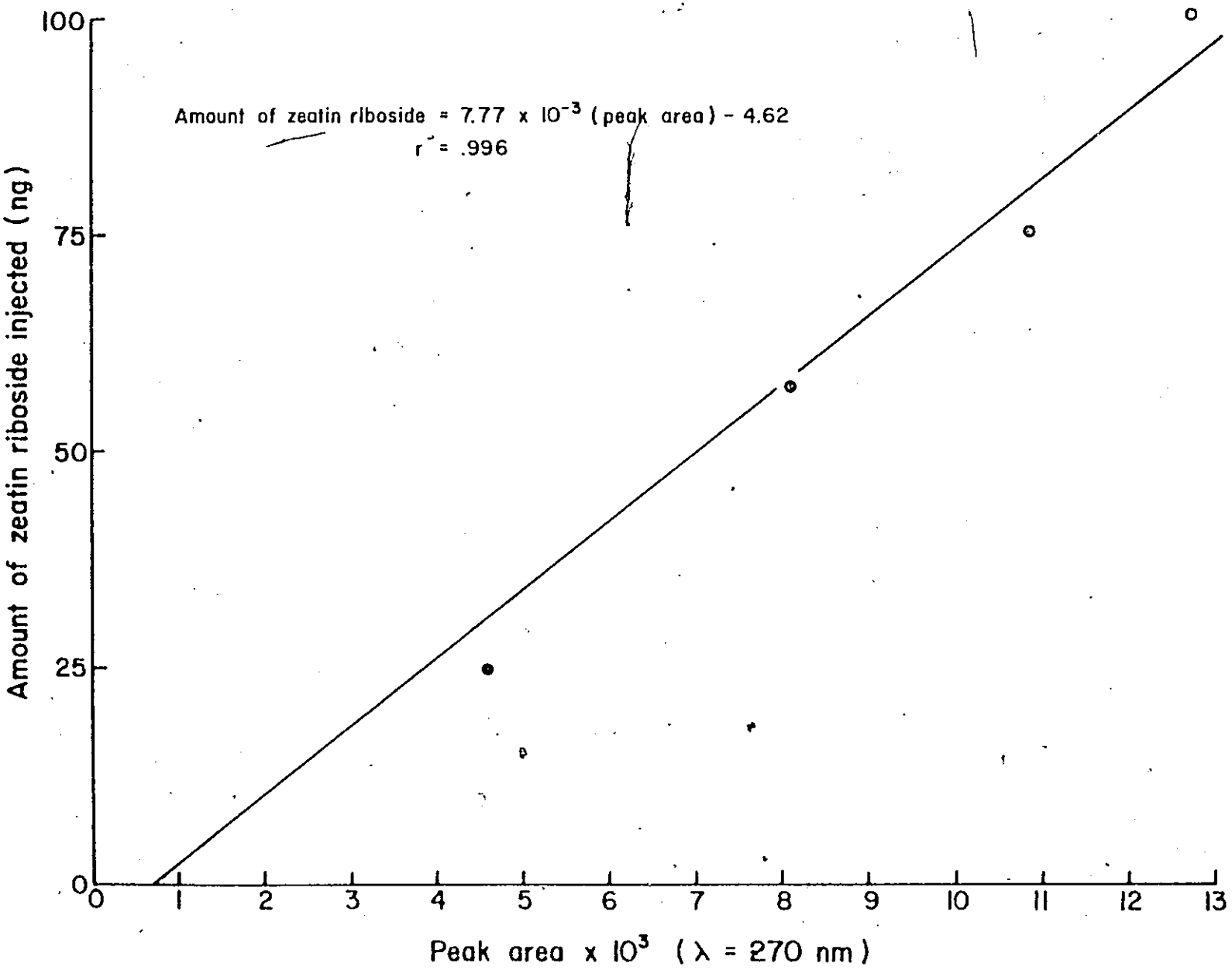
Appendix 5. UV detection at 280 nm of various amounts of ABA injected into a Supelcosil LC-18 column (5 um) attached to a Varian 5000 LC equipped with a Varian 4270 integrator.


Amount of IAA = 4.4 x 10^{-2} (peak area) + 5.92
r = .9989
100
75
50
25
0
0
0.5
1.0
1.5
2.0
Amount of IAA injected (ng)
Peak area x 10^3 (λ = 280 nm)

Appendix 6. UV detection at 280 nm of various amounts of IAA injected into a Supelcosil LC-18 column (5 um) attached to a Varian 5000 LC equipped with a Varian 4270 integrator.


Amount of zeatin = 6.32 x 10^{-3} (peak area) - 3.99
r = .997
Amount of zeatin injected (ng)
100
75
50
25
0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Peak area (x 10^3) (λ = 270 nm)

Appendix 7. UV detection at 270 nm of various amounts of zeatin injected into a Supelcosil LC-18 column (5 um) attached to a Varian 5000 LC equipped with a Varian 4270 integrator.


Amount of zeatin riboside = 7.77 x 10^{-3} (peak area) - 4.62
r^2 = .996
Amount of zeatin riboside injected (ng)
100
75
50
25
0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Peak area x 10^3 (λ = 270 nm)

Appendix 8. UV detection at 270 nm of various amounts of zeatin riboside injected into a Supelcosil LC-18 column (5 um) attached to a Varian 5000 LC equipped with a Varian 4270 integrator.

VARIABLE	N	MEAN	STANDARD DEVIATION	VARIANCE
VAR=1267 STAGE=1ST CONC=0				
LENGTH	53	1.11160377	0.05762758	0.00332094
DIAM	53	0.15094340	0.03137945	0.00098487
FRWT	53	0.02677358	0.01721096	0.00029622
DWT	53	0.00188226	0.00031999	0.00000010
VAR=1267 STAGE=1ST CONC=50				
LENGTH	50	1.14500000	0.05780862	0.00334184
DIAM	50	0.16100000	0.02773784	0.00076939
FRWT	50	0.02640000	0.00103018	0.00000106
DWT	50	0.00174000	0.00015119	0.00000002
VAR=1267 STAGE=2ND CONC=0				
LENGTH	21	1.12619048	0.04435383	0.00196726
DIAM	21	0.15833333	0.02886751	0.00083333
FRWT	21	0.02671429	0.00530229	0.00002811
DWT	21	0.00186190	0.00036121	0.00000013
VAR=1267 STAGE=2ND CONC=50				
LENGTH	16	1.10625000	0.05361903	0.00287500
DIAM	16	0.14375000	0.01443376	0.00020833
FRWT	16	0.02375000	0.00134164	0.00000180
DWT	16	0.00177500	0.00013416	0.00000002
VAR=1267 STAGE=3RD CONC=0				
LENGTH	6	1.14166667	0.08164966	0.00666667
DIAM	6	0.15416667	0.01020621	0.00010417
FRWT	6	0.02400000	0.00000000	0.00000000
DWT	6	0.00170000	0.00000000	0.00000000
VAR=1267 STAGE=3RD CONC=50				
LENGTH	7	1.08571429	0.03493191	0.00122024
DIAM	7	0.13214286	0.01889822	0.00035714
FRWT	7	0.02071429	0.00188982	0.00000357
DWT	7	0.00152857	0.00007559	0.00000001
VAR=1570 STAGE=1ST CONC=0				
LENGTH	15	1.12333333	0.04768448	0.00227381
DIAM	15	0.16500000	0.02803060	0.00078571
FRWT	15	0.02900000	0.00292770	0.00000857
DWT	15	0.00213333	0.00024398	0.00000006
VAR=1570 STAGE=1ST CONC=50				
LENGTH	29	1.10534483	0.05838498	0.00340881
DIAM	29	0.17758621	0.02240479	0.00050647
FRWT	29	0.03137931	0.00094165	0.00000089
DWT	29	0.00230000	0.00008452	0.00000001
VAR=1570 STAGE=2ND CONC=0				
LENGTH	20	1.10375000	0.07130134	0.00508388
DIAM	20	0.14000000	0.02206450	0.00048684
FRWT	20	0.02825000	0.00399835	0.00001599
DWT	20	0.00210000	0.00031623	0.00000010
VAR=1570 STAGE=2ND CONC=50				
LENGTH	22	1.17613636	0.11964165	0.01431412
DIAM	22	0.15227273	0.02662470	0.00070887
FRWT	22	0.02918182	0.00439303	0.00001930
DWT	22	0.00193182	0.00024955	0.00000006
VAR=1570 STAGE=3RD CONC=0				
LENGTH	31	1.14274194	0.08421044	0.00709140
DIAM	31	0.14354839	0.03025430	0.00091532
FRWT	31	0.02774194	0.00502638	0.00002526
DWT	31	0.00186129	0.00014984	0.00000002
VAR=1570 STAGE=3RD CONC=50				
LENGTH	27	1.19814815	0.10046190	0.01009259
DIAM	27	0.15740741	0.03007588	0.00090456
FRWT	27	0.03144444	0.00558386	0.00003118
DWT	27	0.00206296	0.00037226	0.00000014

Appendix 9. Statistics on length, diameter (diam), fresh weight (frwt) and dry weight (dwt) of stem segment (WL1267, WL1570) treated with ethylene.

VARIABLE	N	MEAN	STANDARD DEVIATION	VARIANCE
VAR=1267 STAGE=1ST CONC=0.001				
LENGTH	10	1.10300000	0.06972167	0.00486111
DIAM	10	0.13500000	0.01290994	0.00016667
FRWT	10	0.02500000	0.00000000	0.00000000
DWT	10	0.00220000	0.00000000	0.00000000
VAR=1267 STAGE=1ST CONC=0.01				
LENGTH	10	1.10500000	0.05966095	0.00358333
DIAM	10	0.14500000	0.01581139	0.00025000
FRWT	10	0.02300000	0.00000000	0.00000000
DWT	10	0.00220000	0.00000000	0.00000000
VAR=1267 STAGE=1ST CONC=1				
LENGTH	10	1.09250000	0.05277047	0.00278472
DIAM	10	0.14250000	0.02371708	0.00056250
FRWT	10	0.02600000	0.00000000	0.00000000
DWT	10	0.00210000	0.00000000	0.00000000
VAR=1267 STAGE=1ST CONC=10				
LENGTH	10	1.06750000	0.04866267	0.00236806
DIAM	10	0.15250000	0.02993047	0.00089583
FRWT	10	0.02600000	0.00000000	0.00000000
DWT	10	0.00220000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=0.001				
LENGTH	10	1.11250000	0.07569126	0.00572917
DIAM	10	0.14500000	0.02297341	0.00052778
FRWT	10	0.02400000	0.00000000	0.00000000
DWT	10	0.00140000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=0.01				
LENGTH	10	1.13500000	0.04594683	0.00211111
DIAM	10	0.13000000	0.01054093	0.00011111
FRWT	10	0.02100000	0.00000000	0.00000000
DWT	10	0.00180000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=1				
LENGTH	10	1.09250000	0.05277047	0.00278472
DIAM	10	0.14750000	0.02751762	0.00075694
FRWT	10	0.02400000	0.00000000	0.00000000
DWT	10	0.00180000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=10				
LENGTH	10	1.09250000	0.04779877	0.00228472
DIAM	10	0.13000000	0.01972027	0.00038889
FRWT	10	0.01900000	0.00000000	0.00000000
DWT	10	0.00120000	0.00000000	0.00000000
VAR=1267 STAGE=3RD CONC=0.001				
LENGTH	10	1.14250000	0.07269609	0.00528472
DIAM	10	0.12750000	0.00790569	0.00006250
FRWT	10	0.01800000	0.00000000	0.00000000
DWT	10	0.00150000	0.00000000	0.00000000
VAR=1267 STAGE=3RD CONC=1				
LENGTH	8	1.10000000	0.02672612	0.00071429
DIAM	8	0.14062500	0.02290313	0.00052455
FRWT	8	0.02000000	0.00000000	0.00000000
DWT	8	0.00150000	0.00000000	0.00000000
VAR=1267 STAGE=3RD CONC=10				
LENGTH	8	1.03437500	0.03994975	0.00159598
DIAM	8	0.14062500	0.01560060	0.00034598
FRWT	8	0.02000000	0.00000000	0.00000000
DWT	8	0.00150000	0.00000000	0.00000000

Appendix 10. Statistics on length, diameter (diam), fresh weight (frwt) and dry weight (dwt) of stem segment (WL1267) treated with abscisic acid.

VARIABLE	N	MEAN	STANDARD DEVIATION	VARIANCE
VAR=1570 STAGE=1ST CONC=0.001				
LENGTH	10	1.05750000	0.05277047	0.00278472
DIAM	10	0.18500000	0.02934469	0.00086111
FRWT	10	0.03700000	0.00000000	0.00000000
DWT	10	0.00230000	0.00000000	0.00000000
VAR=1570 STAGE=1ST CONC=0.01				
LENGTH	10	1.04000000	0.04216370	0.00177778
DIAM	10	0.16500000	0.02108185	0.00044444
FRWT	10	0.03200000	0.00000000	0.00000000
DWT	10	0.00240000	0.00000000	0.00000000
VAR=1570 STAGE=1ST CONC=1				
LENGTH	10	1.03750000	0.03385016	0.00114583
DIAM	10	0.15500000	0.01581139	0.00025000
FRWT	10	0.02900000	0.00000000	0.00000000
DWT	10	0.00210000	0.00000000	0.00000000
VAR=1570 STAGE=1ST CONC=10				
LENGTH	10	1.01750000	0.02648375	0.00070139
DIAM	10	0.13000000	0.02297341	0.00052778
FRWT	10	0.03300000	0.00000000	0.00000000
DWT	10	0.00180000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=0.001				
LENGTH	10	1.07500000	0.06009252	0.00361111
DIAM	10	0.14250000	0.01687371	0.00028472
FRWT	10	0.02400000	0.00000000	0.00000000
DWT	10	0.00210000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=0.01				
LENGTH	10	1.05250000	0.04632314	0.00214583
DIAM	10	0.16500000	0.02415229	0.00058333
FRWT	10	0.03000000	0.00000000	0.00000000
DWT	10	0.00230000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=1				
LENGTH	10	1.01500000	0.03374743	0.00113889
DIAM	10	0.16000000	0.02687419	0.00072222
FRWT	10	0.02500000	0.00000000	0.00000000
DWT	10	0.00210000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=10				
LENGTH	10	1.01250000	0.02429563	0.00059028
DIAM	10	0.15000000	0.01178511	0.00013889
FRWT	10	0.02600000	0.00000000	0.00000000
DWT	10	0.00260000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=0.001				
LENGTH	10	1.08500000	0.04594683	0.00211111
DIAM	10	0.14750000	0.01419118	0.00020139
FRWT	10	0.02700000	0.00000000	0.00000000
DWT	10	0.00170000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=0.01				
LENGTH	10	1.04250000	0.03343734	0.00111806
DIAM	10	0.15750000	0.01207615	0.00014583
FRWT	10	0.02500000	0.00000000	0.00000000
DWT	10	0.00210000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=1				
LENGTH	10	1.01500000	0.02415229	0.00058333
DIAM	10	0.14500000	0.01972027	0.00038889
FRWT	10	0.02600000	0.00000000	0.00000000
DWT	10	0.00230000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=10				
LENGTH	10	1.01250000	0.02124591	0.00045139
DIAM	10	0.16500000	0.01748015	0.00030556
FRWT	10	0.02900000	0.00000000	0.00000000
DWT	10	0.00240000	0.00000000	0.00000000

Appendix 11. Statistics on length, diameter (diam), fresh weight (frwt) and dry weight (dwt) of stem segment (WL1570) treated with abscisic acid.

VARIABLE	N	MEAN	STANDARD DEVIATION	VARIANCE
VAR=1267 STAGE=1ST CONC=0.001				
LENGTH	10	1.08750000	0.05779514	0.00334028
DIAM	10	0.13750000	0.02124491	0.00045139
FRWT	10	0.02600000	0.00000000	0.00000000
DWT	10	0.00190000	0.00000000	0.00000000
VAR=1267 STAGE=1ST CONC=0.01				
LENGTH	10	1.05000000	0.03333333	0.00111111
DIAM	10	0.14250000	0.01207615	0.00014583
FRWT	10	0.02600000	0.00000000	0.00000000
DWT	10	0.00160000	0.00000000	0.00000000
VAR=1267 STAGE=1ST CONC=0.1				
LENGTH	11	1.08409091	0.05944822	0.00353409
DIAM	11	0.14318182	0.01167748	0.00013636
FRWT	11	0.02600000	0.00000000	0.00000000
DWT	11	0.00140000	0.00000000	0.00000000
VAR=1267 STAGE=1ST CONC=1				
LENGTH	10	1.11500000	0.06146363	0.00377778
DIAM	10	0.15750000	0.01207615	0.00014583
FRWT	10	0.03400000	0.00000000	0.00000000
DWT	10	0.00220000	0.00000000	0.00000000
VAR=1267 STAGE=1ST CONC=10				
LENGTH	10	1.06000000	0.07378648	0.00544444
DIAM	10	0.14750000	0.02058182	0.00042361
FRWT	10	0.03400000	0.00000000	0.00000000
DWT	10	0.00220000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=0.001				
LENGTH	9	1.07222222	0.03410564	0.00116319
DIAM	9	0.13055556	0.01102396	0.00012153
FRWT	9	0.02300000	0.00000000	0.00000000
DWT	9	0.00160000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=0.01				
LENGTH	10	1.05250000	0.05329428	0.00284028
DIAM	10	0.12750000	0.01419116	0.00020139
FRWT	10	0.02200000	0.00000000	0.00000000
DWT	10	0.00160000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=0.1				
LENGTH	10	1.08250000	0.05894446	0.00347917
DIAM	10	0.13250000	0.01887371	0.00026472
FRWT	10	0.02400000	0.00000000	0.00000000
DWT	10	0.00160000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=1				
LENGTH	11	1.19181818	0.04865042	0.00236634
DIAM	11	0.13409091	0.01865300	0.00024409
FRWT	11	0.02500000	0.00000000	0.00000000
DWT	11	0.00140000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=10				
LENGTH	10	1.12000000	0.04933824	0.00205556
DIAM	10	0.13750000	0.01767767	0.00031250
FRWT	10	0.03000000	0.00000000	0.00000000
DWT	10	0.00190000	0.00000000	0.00000000
VAR=1267 STAGE=3RD CONC=0.001				
LENGTH	10	1.10000000	0.06346478	0.00402778
DIAM	10	0.13500000	0.01748015	0.00030556
FRWT	10	0.02300000	0.00000000	0.00000000
DWT	10	0.00170000	0.00000000	0.00000000
VAR=1267 STAGE=3RD CONC=0.01				
LENGTH	9	1.14444444	0.06094308	0.00371528
DIAM	9	0.13055556	0.01102396	0.00012153
FRWT	9	0.02200000	0.00000000	0.00000000
DWT	9	0.00140000	0.00000000	0.00000000
VAR=1267 STAGE=3RD CONC=1				
LENGTH	8	1.05000000	0.04818121	0.00232143
DIAM	8	0.13437500	0.01293875	0.00016741
FRWT	8	0.02500000	0.00000000	0.00000000
DWT	8	0.00140000	0.00000000	0.00000000
VAR=1267 STAGE=3RD CONC=10				
LENGTH	10	1.08000000	0.07149204	0.00511111
DIAM	10	0.13750000	0.02429563	0.00059028
FRWT	10	0.02500000	0.00000000	0.00000000
DWT	10	0.00170000	0.00000000	0.00000000

Appendix 12. Statistics on length, diameter (diam), fresh weight (frwt) and dry weight (dwt) of stem segment (WL1267) treated with kinetin.

VARIABLE	N	MEAN	STANDARD DEVIATION	VARIANCE
VAR=1570 STAGE=1ST CONC=0.001				
LENGTH	11	1.03636364	0.04522670	0.00204545
DIAM	11	0.15727273	0.02359700	0.00055682
FRWT	11	0.03200000	0.00000000	0.00000000
DWT	11	0.00220000	0.00000000	0.00000000
VAR=1570 STAGE=1ST CONC=0.01				
LENGTH	10	1.02750000	0.03987826	0.00159028
DIAM	10	0.16000000	0.02581989	0.00066667
FRWT	10	0.03600000	0.00000000	0.00000000
DWT	10	0.00240000	0.00000000	0.00000000
VAR=1570 STAGE=1ST CONC=0.1				
LENGTH	10	1.01000000	0.02415229	0.00058333
DIAM	10	0.16750000	0.01667371	0.00027812
FRWT	10	0.03800000	0.00000000	0.00000000
DWT	10	0.00260000	0.00000000	0.00000000
VAR=1570 STAGE=1ST CONC=1				
LENGTH	10	1.01750000	0.03822844	0.00131250
DIAM	10	0.16250000	0.01317616	0.00017361
FRWT	10	0.03500000	0.00000000	0.00000000
DWT	10	0.00220000	0.00000000	0.00000000
VAR=1570 STAGE=1ST CONC=10				
LENGTH	10	1.04750000	0.09583855	0.00311806
DIAM	10	0.18000000	0.01972027	0.00038889
FRWT	10	0.03600000	0.00000000	0.00000000
DWT	10	0.00230000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=0.001				
LENGTH	6	1.15833333	0.03763863	0.00141667
DIAM	6	0.14166667	0.02581989	0.00066667
FRWT	6	0.02800000	0.00000000	0.00000000
DWT	6	0.00170000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=0.01				
LENGTH	10	1.10250000	0.06503204	0.00422917
DIAM	10	0.15000000	0.01972027	0.00038889
FRWT	10	0.02500000	0.00000000	0.00000000
DWT	10	0.00200000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=0.1				
LENGTH	10	1.09750000	0.06266007	0.00392361
DIAM	10	0.14500000	0.01581139	0.00025000
FRWT	10	0.02900000	0.00000000	0.00000000
DWT	10	0.00220000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=1				
LENGTH	10	1.09500000	0.03689324	0.00136111
DIAM	10	0.16750000	0.01667371	0.00027812
FRWT	10	0.03700000	0.00000000	0.00000000
DWT	10	0.00220000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=10				
LENGTH	9	1.11388889	0.03560002	0.00126736
DIAM	9	0.17500000	0.02165064	0.00046875
FRWT	9	0.03400000	0.00000000	0.00000000
DWT	9	0.00220000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=0.001				
LENGTH	10	1.15500000	0.04972145	0.00247222
DIAM	10	0.14250000	0.01207615	0.00014583
FRWT	10	0.02800000	0.00000000	0.00000000
DWT	10	0.00190000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=0.01				
LENGTH	10	1.09500000	0.06546472	0.00427778
DIAM	10	0.13750000	0.01767767	0.00031250
FRWT	10	0.02400000	0.00000000	0.00000000
DWT	10	0.00180000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=0.1				
LENGTH	9	1.14166667	0.05153882	0.00265625
DIAM	9	0.14166667	0.01250000	0.00015625
FRWT	9	0.02700000	0.00000000	0.00000000
DWT	9	0.00180000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=1				
LENGTH	10	1.12750000	0.09827378	0.00339583
DIAM	10	0.15250000	0.00790569	0.00006250
FRWT	10	0.03200000	0.00000000	0.00000000
DWT	10	0.00200000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=10				
LENGTH	10	1.08000000	0.01972027	0.00038889
DIAM	10	0.14750000	0.02371708	0.00056250
FRWT	10	0.03800000	0.00000000	0.00000000
DWT	10	0.00240000	0.00000000	0.00000000

Appendix 13. Statistics on length, diameter (diam), fresh weight (frwt) and dry weight (dwt) of stem segment (WL1570) treated with kinetin.

VARIABLE	N	MEAN	STANDARD DEVIATION	VARIANCE
VAR=1267 STAGE=1ST CONC=0				
LENGTH	10	1.08000000	0.06213784	0.00386111
DIAM	10	0.15000000	0.01178511	0.00013889
FRWT	10	0.02800000	0.00000000	0.00000000
DWT	10	0.00230000	0.00000000	0.00000000
VAR=1267 STAGE=1ST CONC=0.001				
LENGTH	10	1.12000000	0.09413347	0.00886111
DIAM	10	0.14500000	0.01972027	0.00038889
FRWT	10	0.02600000	0.00000000	0.00000000
DWT	10	0.00210000	0.00000000	0.00000000
VAR=1267 STAGE=1ST CONC=0.01				
LENGTH	8	1.06875000	0.06911429	0.00477679
DIAM	8	0.14375000	0.02216013	0.00049107
FRWT	8	0.02600000	0.00000000	0.00000000
DWT	8	0.00190000	0.00000000	0.00000000
VAR=1267 STAGE=1ST CONC=1				
LENGTH	10	1.09000000	0.05676462	0.00322222
DIAM	10	0.14250000	0.02058182	0.00042361
FRWT	10	0.02500000	0.00000000	0.00000000
DWT	10	0.00190000	0.00000000	0.00000000
VAR=1267 STAGE=1ST CONC=10				
LENGTH	10	1.10750000	0.07364517	0.00542361
DIAM	10	0.13500000	0.02108185	0.00044444
FRWT	10	0.02200000	0.00000000	0.00000000
DWT	10	0.00200000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=0.001				
LENGTH	10	1.13000000	0.14710163	0.02163889
DIAM	10	0.15000000	0.01666667	0.00027778
FRWT	10	0.02600000	0.00000000	0.00000000
DWT	10	0.00170000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=0.01				
LENGTH	10	1.11500000	0.07090682	0.00502778
DIAM	10	0.13500000	0.01290994	0.00016667
FRWT	10	0.02400000	0.00000000	0.00000000
DWT	10	0.00200000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=1				
LENGTH	10	1.13250000	0.07458217	0.00556250
DIAM	10	0.13250000	0.01207615	0.00014583
FRWT	10	0.02300000	0.00000000	0.00000000
DWT	10	0.00160000	0.00000000	0.00000000
VAR=1267 STAGE=2ND CONC=10				
LENGTH	10	1.13750000	0.07569126	0.00572917
DIAM	10	0.12750000	0.00790569	0.00006250
FRWT	10	0.02300000	0.00000000	0.00000000
DWT	10	0.00180000	0.00000000	0.00000000
VAR=1267 STAGE=3RD CONC=0.001				
LENGTH	8	1.07187500	0.05077524	0.00257812
DIAM	8	0.15312500	0.00883883	0.00007812
FRWT	8	0.02400000	0.00000000	0.00000000
DWT	8	0.00170000	0.00000000	0.00000000
VAR=1267 STAGE=3RD CONC=0.01				
LENGTH	7	1.14285714	0.06244045	0.00389881
DIAM	7	0.13928571	0.01966989	0.00038690
FRWT	7	0.02700000	0.00000000	0.00000000
DWT	7	0.00210000	0.00000000	0.00000000
VAR=1267 STAGE=3RD CONC=1				
LENGTH	7	1.11428571	0.05175492	0.00267857
DIAM	7	0.13571429	0.01336306	0.00017857
FRWT	7	0.02400000	0.00000000	0.00000000
DWT	7	0.00170000	0.00000000	0.00000000
VAR=1267 STAGE=3RD CONC=10				
LENGTH	9	1.10833333	0.05000000	0.00250000
DIAM	9	0.13888889	0.01814208	0.00032986
FRWT	9	0.02500000	0.00000000	0.00000000
DWT	9	0.00210000	0.00000000	0.00000000

Appendix 14. Statistics on length, diameter (diam), fresh weight (frwt) and dry weight (dwt) of stem segment (WL1267) treated with gibberellic acid.

VARIABLE	N	MEAN	STANDARD DEVIATION	VARIANCE
VAR=1570 STAGE=1ST CONC=0.001				
LENGTH	12	1.10208333	0.12175718	0.01482461
DIAM	12	0.14791667	0.02709062	0.00073390
FRWT	12	0.03400000	0.00000000	0.00000000
DWT	12	0.00260000	0.00000000	0.00000000
VAR=1570 STAGE=1ST CONC=0.01				
LENGTH	10	1.09000000	0.11972180	0.01433333
DIAM	10	0.14750000	0.02188988	0.00047917
FRWT	10	0.03200000	0.00000000	0.00000000
DWT	10	0.00210000	0.00000000	0.00000000
VAR=1570 STAGE=1ST CONC=1				
LENGTH	10	1.07250000	0.06395528	0.00409028
DIAM	10	0.15250000	0.01419116	0.00020139
FRWT	10	0.03300000	0.00000000	0.00000000
DWT	10	0.00210000	0.00000000	0.00000000
VAR=1570 STAGE=1ST CONC=10				
LENGTH	10	1.00750000	0.01687371	0.00028472
DIAM	10	0.15000000	0.02357023	0.00055556
FRWT	10	0.02900000	0.00000000	0.00000000
DWT	10	0.00250000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=0.001				
LENGTH	10	1.08000000	0.05868939	0.00344444
DIAM	10	0.14250000	0.01207615	0.00014583
FRWT	10	0.02400000	0.00000000	0.00000000
DWT	10	0.00170000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=0.01				
LENGTH	10	1.09000000	0.05296750	0.00280556
DIAM	10	0.15000000	0.01178511	0.00013889
FRWT	10	0.03100000	0.00000000	0.00000000
DWT	10	0.00230000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=1				
LENGTH	10	1.14750000	0.06503204	0.00422917
DIAM	10	0.15750000	0.01207615	0.00014583
FRWT	10	0.03000000	0.00000000	0.00000000
DWT	10	0.00190000	0.00000000	0.00000000
VAR=1570 STAGE=2ND CONC=10				
LENGTH	10	1.12750000	0.07402139	0.00547917
DIAM	10	0.15250000	0.01844662	0.00034028
FRWT	10	0.02700000	0.00000000	0.00000000
DWT	10	0.00200000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=0.001				
LENGTH	13	1.27500000	0.08291579	0.00395833
DIAM	13	0.12307692	0.00693375	0.00004808
FRWT	13	0.02300000	0.00000000	0.00000000
DWT	13	0.00150000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=0.01				
LENGTH	10	1.26250000	0.05034602	0.00253472
DIAM	10	0.13750000	0.01317616	0.00017361
FRWT	10	0.02400000	0.00000000	0.00000000
DWT	10	0.00170000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=1				
LENGTH	10	1.21750000	0.07732507	0.00597917
DIAM	10	0.13750000	0.01317616	0.00017361
FRWT	10	0.02400000	0.00000000	0.00000000
DWT	10	0.00150000	0.00000000	0.00000000
VAR=1570 STAGE=3RD CONC=10				
LENGTH	13	1.23750000	0.06373774	0.00406250
DIAM	13	0.13000000	0.01054093	0.00011111
FRWT	13	0.02100000	0.00000000	0.00000000
DWT	13	0.00150000	0.00000000	0.00000000

Appendix 15. Statistics on length, diameter (diam), fresh weight (frwt) and dry weight (dwt) of stem segment (WL1570) treated with gibberellic acid.

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