DEVELOPMENTAL ASPECTS OF PHENOLIC COMPOUNDS IN LENTILS

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DEDICATION

This thesis is dedicated to my parents Sham Rani and Triloki Nath Kachroo. Their love and understanding were a constant source of encouragement.

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ABSTRACT

A comparative developmental study of the flavonoid pattern, histochemical localization and quantitative estimations of phenolic compounds in two lentil lines, cultivar Eston and plant introduction (P.I.) 345635 was carried out. Seeds of the former contain proanthocyanidins, whereas seeds of the latter do not. The flavonoid patterns in the vegetative parts of both lentil lines were similar. 2-D chromatograms of extracts of their leaves and stems showed a complex mixture of flavonol glycosides, with kaempferol glycosides constituting the majority. Roots of both showed the presence of flavones. However, the flavonoid pattern in the reproductive parts of both lentil lines showed a considerable difference with quercetin glycosides being predominant in both lentil lines. The flavonol quercetin-3-rhamnoside was identified in the petal and pericarp of cultivar Eston. This glycoside was not detectable in the pericarp of P.I.345635 and was found in relatively smaller amounts in its petals. Only quercetin was present in the petals and pericarp of cultivar Eston whereas both quercetin and kaempferol were present in the petal of P.I.345635. The testa and embryo of both lentil lines did not show any presence of quercetin. Quantitative analysis of total phenols in the vegetative and reproductive parts of both lentil lines showed that the amounts of total phenols were always lower in P.I.345635 at comparable stages of development. Proanthocyanidins were present only in the reproductive parts of cultivar Eston with the bulk confined to its testa. No proanthocyanidins were detected in any part of P.I.345635.

The possible antifungal nature of the seed exudates of two proanthocyanidin-containing lines, cultivar Eston and TB406M was indicated by an initial 14-22 hour lag phase of the growth of *Fusarium oxysporum* isolate 82A and *F. oxysporum* var. *callistephi*. The growth of

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RÉSUMÉ

Une étude comparative du développement du patron des flavonoides, de la localisation histochimique et estimations quantitatives des composés phénoliques, fut faite chez deux lignées de lentille, cultival Eston et Introduction de plante (P.I.)345635. Les graines de la première lignée contiennent des proanthocyanidines, celles de la deuxième n'en contiennent pas. Les patrons de flavonoides dans les tissus végétatifs étaient similaires chez les deux lignées. Les chromatogrammes à deux dimensions d'extraits de feuilles et de tiges ont montré un mélange complexe de glycosides de flavonol, les glycosides de kaempferol constituaient la majeure partie. Les flavones étaient présents dans les racines des deux lignées. Cependant, dans les tissus reproducteurs, le patron de flavonoides différèrent considérablement chex les deux lignées, les glycosides de quercétine étant prédominant. Le flavonol, quercétine-3-rhamnoside fut identifié dans les pétales et le péricarpe du cultival Eston. Ce glycoside ne fut pas détecté dans le péricarpe de P.I.345635 et fut trouvé en quantité relativement plus petites dans les pétales. Seul quercétine fut présent dans les pétales et péricarpe du cultival Eston alors que quercétine et kaempferol étaient présents dans les pétales de P.I.345635. Le testa et l'embryon des deux lignées ne montrèrent pas la présence de quercétine. L'analyse quantitative de la totalité des phénols dans les tissus végétatifs et reproducteurs des deux lignées ont montré que les montants de phénols étaient toujours plus bas chez P.I.345635 à des stades de développement comparables. Les proanthocyanidines étaient présents seulement dans les tissus reproducteurs du cultival Eston, la plus grande quantité étant confinée au testa. Aucuns proanthocyanidines ne furent détectés dans les tissus de P.I.345635.

La possibilité de la nature antifongique des exudats de graines de deux lignées contenant des proanthocyanidines, cultival Eston and TB406M fut indiqué par une phase de latence de

14-22 heures dans la croissance de *Fusarium oxysporum* isolat 82A et de *F. oxysporum* var. *callistephi.* La croissance de ces champignons fut suivie à 14, 22, 30, 48 et 66 heures. Plus de recherche est nécéssaire pour établir si les composés phénoliques exudés par les graines durant l'imbibition ont un rôle fungistatique.

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CHAPTER 1

INTRODUCTION

1.1 LENTILS:

The latin name of the species, *Lens culinaris*, was first published by Medikus in 1787 and predates the other commonly used, but incorrect, name *Lens esculenta* published by Moench in 1794 (Webb & Hawtin 1980). *L. culinaris* Medik. is the only cultivated species of the genus and is thought to have originated from the wild lentil *L. orientalis* (Boiss) M. Popov. (Barulina 1930) although the possibility of gene flow from *L. nigricans* (Bieb) Godn. cannot be overlooked (Smartt 1976).

There is evidence to indicate that lentils were one of the first plant species brought under cultivation by man (Ladizinsky 1979b). The remains of lentil seeds have been found in archeological digs throughout the Middle East suggesting that neolithic man was already familiar with this plant as early as 8000 B.C. The place of domestication of lentils is uncertain. Renfrew (1973) on the assumption that *L. nigricans* is the progenitor of the cultivated lentil concludes that Southern Europe is the place where lentils evolved whereas other authors have suggested Northern India (Barulina 1930), the Near East (Zohary 1972) and the Mediterranean (Vavilov 1949).

1.2 THE LENTIL PLANT:

The lentil is a short, slender many branched annual legume and generally has a bushy growth which may range from fairly erect to more spreading in habit. The flowers are small, white, pink or pale blue and are borne on axillary inflorescences (Szucs 1972). The pods are flattened, and usually contain one or two seeds which are round, bi-convex, and may range from about 2-8 mm in diameter (Muehlbauer 1974).

The species is usually divided into two subspecies: (i) *microsperma* with small rounded seeds, 2-6 mm in diameter, yellow or orange cotyledons, and a testa of various colours from pale yellow to black and (ii) *macrosperma* with larger more flattened seeds which normally have yellow cotyledons and a pale green testa, which may be speckled (Papp, 1980). The microsperma are generally considered to be the older of the two subspecies. It has been suggested that *macrosperma* arose by selection from *microsperma* (Muehlbauer & Slinkard 1980).

1.3 LENTIL CULTIVATION:

The subspecies *microsperma* is the main type cultivated in the Indian subcontinent, Afghanistan, Ethiopia and Egypt, whereas *macrosperma* predominates in Southern Europe, North Africa and North and Latin America (Webb & Hawtin 1980). Lentils are best adapted to temperate environments. However, in certain tropical countries e.g. Ethiopia, Kenya, Columbia and Eucador, they are successfully grown at higher altitudes. Lentils are cultivated on about 1.9 million hectares annually and the total production is greater than 1.2 million tonnes (Bhalla 1982). India and Turkey are the largest producers of lentils followed by Syria,

Spain and then the United States of America, the largest lentil producers in the Western Hemisphere. Ninety-nine percent of the U.S.A. production is centered in the Palouse area of Eastern Washington State and Northern Idaho (Youngman 1968) where over 50,000 hectares are sown to this crop annually.

Commercial production of lentils in Canada was initiated in 1970 when 600 hectares were grown in the Eatonia district of West-Central Saskatchewan (Slinkard & Drew 1981). In 1980, production increased to 44,000 hectares with most of this acreage in Saskatchewan but with some acreage contributed by Manitoba and Alberta (Slinkard & Drew 1981).

1.4 IMPORTANCE OF LENTIL AS FOOD:

Lentil is a high protein crop that is used in human food in the form of soup in America and Europe and in other dishes in the Middle-East and the Indian subcontinent (Bhatty et al. 1983). On average, grain legumes, such as lentils, provide more than twice the amount of dietary protein as cereals (Majeed 1977). The protein content of lentils is comparable to that of faba bean; higher than chickpeas, and more than double that of wheat. Lentils are rich in iron and other minerals (Wassimi et al. 1978). Lentils, like chickpeas and faba beans, are good sources of thiamine and niacin, but are poor in carotene and vitamin C (Aykroyd & Doughty 1964).

In addition to the total level of protein in lentil seeds, the amino acid content of the protein is very important to its nutritional quality. Lentil seeds along with other legumes have low amounts of sulphur amino acids and tryptophan. However the level of lysine is high, as in other legume seeds, compared with wheat which is low in lysine but high in total sulphur amino acids, methionine and cystine (Bhatty & Slinkard 1979).

Lentil seed coats have also been reported to contain proanthocyanidins (D'Arcy & Jay 1978), also known as condensed tannins. In the following text, these compounds will be referred to simply as tannins. Tannins form cross links with proteins, and result in astringency (Roux 1972). These tannin-protein complexes are believed to be responsible for growth depression, low protein digestibility, and increased fecal nitrogen. Thus, once consumed, the deleterious effects of tannins in the diet seem to be related to their binding of the dietary protein (Sathe & Salunkhe 1982). The tannin-protein complexes may resist *in vivo* enzymatic digestion. Thus, many grain legumes, e.g. peas and beans, have been selected for the absence of tannins. Breeding programmes have also been initiated to eliminate tannins from lentil seed coat (Slinkard, personal communications).

1.5 DISEASES OF LENTIL:

Lentil is a valuable pulse crop and suffers from a number of diseases (Bhalla 1982). Lentils are susceptible to mildew caused by *Erysiphe communes* Grev. and to a rust produced by *Uromyces ervi* Winter (Bhalla 1982). The most common soil-borne disease of lentils is incited by *Fusarium oxysporum* Schlecht f. sp. *lentis* resulting in root rot (Vasudeva & Srinivasan 1952). The genus *Fusarium* is world-wide in distribution and comprises a number of species that are soil-borne pathogens of many agriculturally important crops. *F. oxysporum* is considered to be the most economically important species, inciting severe wilts in many host plants (Booth, 1971).

Fusaria in general and *F. oxysporum* in particular are quite variable morphologically as well as physiologically (Bhalla 1982). In their description of the pathogen, Vasudeva and Srinivasan (1952) noted that it produces microconidia, macroconidia and chlamydospores in culture. Microconidia have been reported to be single-celled, hyaline, ovoid and measure

6.7 x 3.7 microns (Bhalla 1982). Macroconidia are pointed at the tip and notched at the base, fusaroid, with 1-6 septa and 10 x 50 x 5-6.7 microns in size. Chlamydospores are unicellular and terminal or intercalary with thick walls (Bhalla 1982). The pathogen grows best in the range of 27^{o} to 30 ^{o}C and prefers a pH of 5 in the medium although it is capable of tolerating a wide range (Vasudeva & Srinivasan 1952).

The severity of *Fusarium* incited disease has been variable, with the extent of mortality of plants ranging from 25 percent in the seedling stage (Kannaiyan & Nene 1972) to 50 percent at the flowering and fruiting stage (Bhalla 1982). Certain factors such as soil moisture, soil type and soil temperature have an influence on disease incidence as evidenced by the work of Khare et al. (1971).

1.6 LENTIL PHENOLICS AND THEIR IMPORTANCE:

Information regarding lentil phenolics is not extensively available in the literature and most of the studies are confined to the presence of phenolics in seeds and roots. For example, Gaspar et al. (1969) studied the effects of water-soluble diffusates from seeds of *Lens culinaris* Med. on seedling growth and tried to determine whether these effects were due to the seedcoat or diffusates from the embryo. Substances of phenolic nature which retard and promote seedling growth were suspected to be present in both seedcoat and embryo. D'Arcy and Jay (1978) identified the flavonoids present in the lentil seedcoat as proanthocyanidins and flavones and as flavonols in the embryo (cotyledons). More recently D'Arcy-Lemeta (1986) also identified three desoxy-5 flavones in the root exudates of lentils. The ability of *Lens culinaris* seeds to synthesize the furanoacetylenes wyerone and wyerone epoxide as well as the pterocarpan variabilin, has been demonstrated by Robeson (1978). He attributed the dynamic resistance of lentil to its ability to produce these phytoalexins, when exposed to a dense spore

suspension of *Botrytis cinerea* Pers.. De Bezada (1981) reported the presence of proanthocyanidins of different molecular weights in the lentil seed coat. Vaillancourt (1984) studied the inheritance of tannin content in lentil. He estimated the average tannin content to be in the order of 23 mg g^{-1} of dry seed coat. No systematic qualitative or quantitative study of flavonoids in lentil has ever been conducted. A study of this nature would be of interest because of implications to disease resistance and also, in the case of antinutritional properties. According to Zucker (1983), tannins are specialized in their functions. Thus, it may be possible to increase disease resistance by breeding for tannins with a different structure (different function) rather than by breeding for higher tannin content.

1.6.1 THE COMMON FLAVONOIDS:

A large number of naturally occurring flavonoids have been described with about 2000 known structures (Harborne & Williams 1975). All are based on the same C15 skeleton of flavone. Flavonoids are conveniently divided into twelve classes (Table 1) according to the oxidation level of the central pyran ring. Of these, flavones and flavonols are the most abundant and most widespread classes. These will be described further, along with the important polymeric proanthocyanidins in the following sections.

1.6.1.1 FLAVONOLS:

Flavonols occur almost universally in leaves (Swain & Bate-Smith 1962). They usually accompany anthocyanins in flowers where they aid in UV absorption, functioning as a nectar guide (Thompson et al. 1972). For quercetin, (Fig.1A) the most common flavonol aglycone,

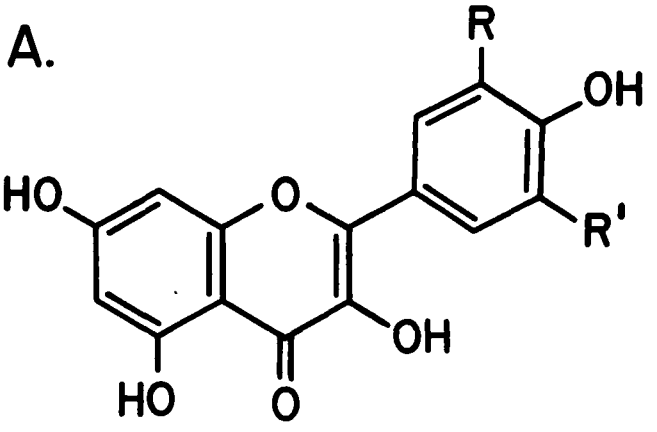
Table 1. The major known classes of flavonoid

Class	No. of known structures	Biological properties
Anthocyanins	250	Red to blue pigments
Chalcones	60	Yellow pigments
Aurones	20	
Flavones	350	Cream pigments in flowers; feeding repellents (?) in leaves
Flavonols	350	
Flavanones	150	Some have bitter tastes
Dihydrochalcones	10	
Proanthocyanidins	50	Astringent substances, some with tanning ability
Catechins	20	
Flavan-3,4-diols	20	
Biflavonoids	65	None known
Isoflavonoids	150	Oestrogenic and fungitoxic

Harborne 1980

Figure 1. A- Structures showing major flavonols B- Structures showing major flavones

A.

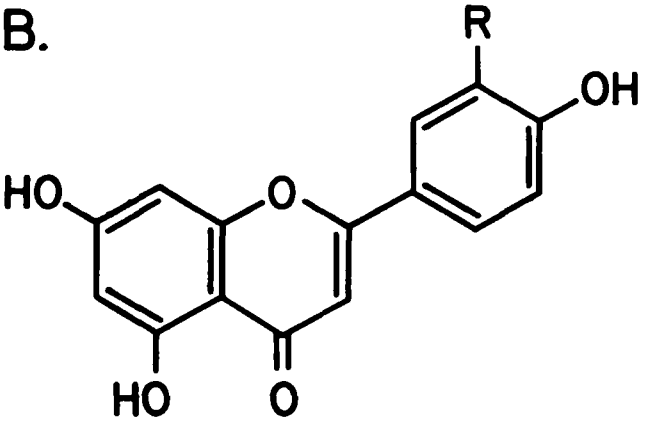


Kaempferol , R = R' = H

Quercetin , R = OH , R' = H

Myricetin , R = R' = OH

B.



Apigenin , R = H

Luteolin , R = OH

over seventy glycosidic combinations have been fully characterized and many more have been partly analyzed. A few quercetin glycosides e.g. 3-rutinoside, 3-glucoside, 3-rhamnoside and 3-galactoside are very commonly found in plants. Other fairly common glycosides are the 3-glucoronide, 3-arabinoside, 3-sophoroside and 7-glucoside. Almost as many glycosides have been described in the case of two other common flavonols, kaempferol and myricetin. D'Arcy & Jay (1978) reported that lentil cotyledons contain a kaempferol glycoside. There are many other flavonol methyl ethers of more restricted natural occurrence.

Structural variation among flavonol glycosides is considerable, both in the nature of the sugar residues and the position of attachment through hydroxyl groups to the flavonol nucleus. The complications of studying these glycosides can be considerable particularly since in many plant species, complex mixtures of related glycosides are often encountered, the particular mixture varying according to the plant organ examined (Harborne & Williams 1975). These glycosides occur in vastly differing concentrations from as much as 15-20% of the plant dry weight to as little as 0.001%. While the major components may be easily characterized, the minor constituents are more difficult to identify.

1.6.1.2 FLAVONES:

The number of naturally occurring flavones is 128 excluding their glycosides (Venkataraman 1975). Flavones lack the 3-hydroxyl group present in flavonols (Fig.1B). Only two structures are common: apigenin and luteolin. Luteolin, free and as glycosides, is the most widely occurring flavone. Luteolin 7-glucoside is the commonest of all flavone glycosides, being generally widespread in herbaceous angiosperms. Among families in which it is characteristically present are the Acanthaceae, Compositae (Harborne 1967), Juncaceae, Umbelliferae and Leguminosae (Crowden et el. 1969, Harborne 1969). D'Arcy and Jay (1978)

noted its presence in lentil seed coats.

Tricin glycosides are relatively of recent discovery (Venkataraman 1975). Some eight derivatives of tricin are now known. One of these, tricin 5-glucoside, is quite widespread as a trace constituent, occurring regularly in members of the Cyperaceae, Palmae and Gramineae. Some of the other glycosides of tricin (e.g. the 7-glucoside) are probably also widespread in these families, including the Leguminosae (Harborne). D'Arcy and Jay also noted its presence in lentil seed coats.

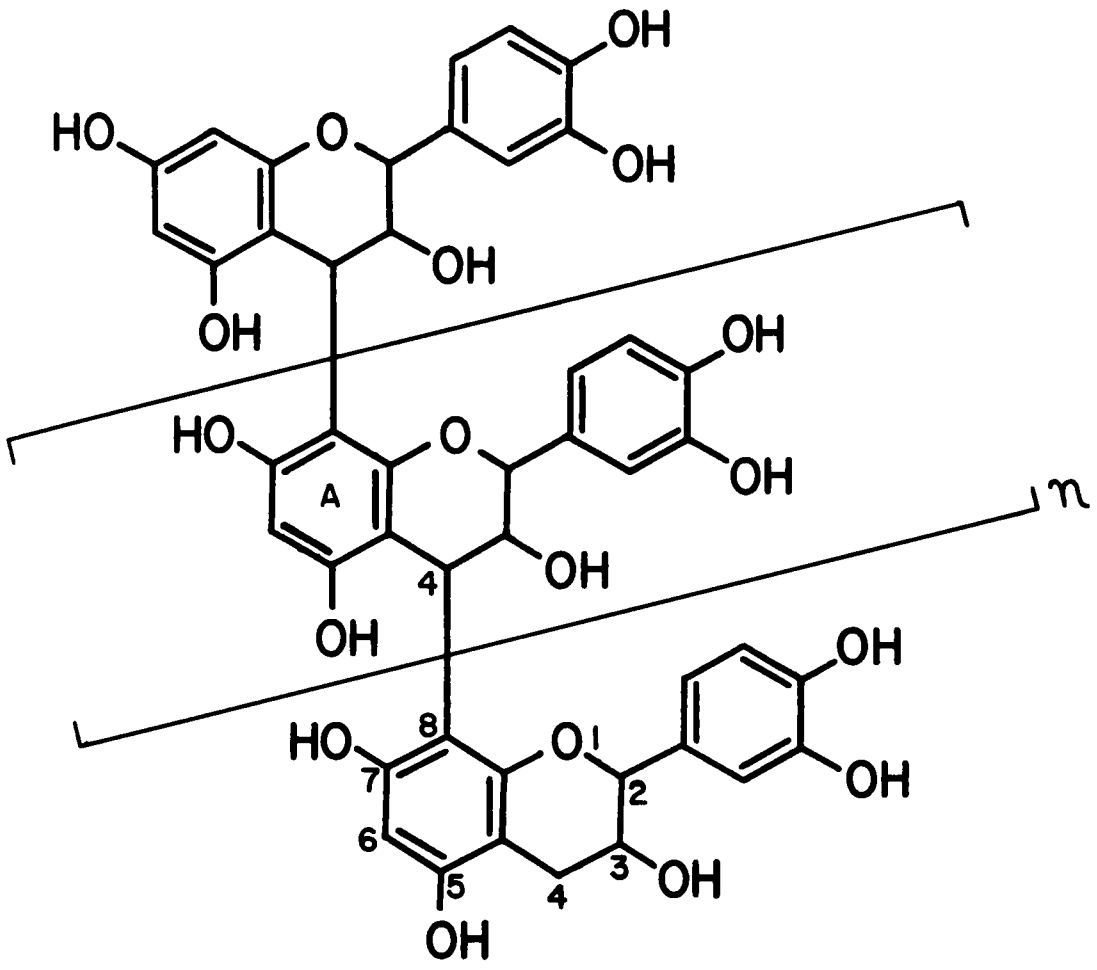
With the flavones, glycosidic complexity is compounded by the fact that sugars can be bound to phenolic hydroxyl groups and to the carbon nucleus of the aromatic ring at the 6- and/or 8 positions. Such C-glycosides are called C-glycosylflavones and are capable of having O-sugars attached as well, either to one of the carbon-bound sugars or to a phenolic group.

1.6.1.3 PROANTHOCYANIDINS:

A most important group of secondary metabolites is the proanthocyanidins (Bate-Smith 1975; Swain 1965; Synge 1975). Proanthocyanidins are defined by Bate-Smith (1962) as "water-soluble phenolic compounds having molecular weights between 500 and 3000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins".

Proanthocyanidins have a polymeric flavan-3-ol structure in which the interflavan bonds are commonly C-4 to C-8 (Haslam 1981) (Fig.2). A wide range of proanthocyanidin structures is possible. Structure varies depending on 1) whether flavan-3-ol or flavan-3,4-diol or both comprise the monomeric units, 2) the type of bond between flavan units, 3) the hydroxylation pattern of the A and B ring and 4) the stereochemistry of the polymer (Zucker 1983).

Figure 2. Procyanidin: These substances posess di-, tri-, and tetra flavan structures in which the flavan monomer units are linked by C-C bond between the fourth position of one flavan unit and the A ring of the next unit (Harborne 1975).



Procyanidin

Proanthocyanidins are so named because they are chemically degradable to give red anthocyanidin pigments.

Of all the phenolic compounds, the tannins seem to be the most widely distributed and are also of nutritional concern since these are known to complex with proteins which results in low protein digestibility (Van Sumere et al. 1975). Most of the tannins appear to be found in a few families of dicotyledons such as the Leguminosae (White 1957). Tannins have been reported in a variety of plants utilized for food and feed purposes (Van Buren 1970; Reichert et al. 1980; Rooney et al. 1980; Price et al. 1980; Salthe & Salunkhe 1982; Deshpande et al. 1982).

1.6.2 FUNCTIONS OF FLAVONOIDS:

The physiological importance of flavonoids as light screens in leaves and as regulators of metabolism has been recognized (McClure 1975). The presence of flavonoids and other polyphenolic compounds in the root exudates of lentils and other legumes have been shown to be of much importance in the Leguminosae-*Rhizobium* symbiosis (D'Arcy-Lameta 1986). The influence of root exudates on the rhizosphere has agronomic implications because it can affect nodule formation (Pankhurst & Jones 1980).

Proanthocyanidins are associated with resistance of many crops to diverse diseases. Extracts of proanthocyanidins from peanuts inhibit the growth of *Aspergillus parasiticus* (Sanders & Mixon 1978). Harris and Burns (1973) found a high correlation between the proanthocyanidin content of sorghum cultivars and resistance to seed mold caused by *Fusarium* spp. Proanthocyanidins leach out of the seeds of certain legumes and are highly toxic to certain bacteria (Young & Paterson 1979; Rangaswamy et al. 1974; Pankhurst & Jones 1980).

The presence of proanthocyanidins in lentil seed coats (D'Arcy & Jay 1978; Vaillancourt 1984) can be a factor responsible for resistance to certain soil-borne diseases. Black-seeded bean cultivars containing tannins are more resistant to root rot than white-seeded bean cultivars lacking them (Statler 1970; Deakin & Dukes 1975). Griffith and Jones (1977) reported that the enzyme inhibition exhibited by extracts of seed coat of coloured varieties of *Vicia faba* was due to the presence of proanthocyanidins. The inhibitory effect of seed diffusates of pea, Bengal gram, green-gram, soybean, lucerne, cowpea etc. toward different sps. of Rhizobia and other bacteria has been reported by Dadarwal and Sen (1973). Furthermore, Young and Paterson (1979) found that tannins leaching out of white clover seeds had antibiotic activity. Thus, tannins might have a role in disease resistance of seeds. Crofts (1979) found that tannin-free seeds of faba bean were more susceptible to mechanical damage than tannin-containing seeds. The tendency of the tannin-free seed coats to split or crack could have a deleterious effect on field germination and emergence. Seeds with damaged seed coats often have a higher incidence of seed rot than other diseases (Prasad & Weingle 1976).

Proanthocyanidins may act directly as a barrier against absorption of water (Halloin 1982). The localization of tannins in seeds of cotton, legumes, sorghum and watercress is consistent with this proposed function. Marback and Mayer (1974) observed that the impermeability of *Pisum* seeds was associated with seed darkening and an increase in phenol content.

Darkening of seed coats in lentils after ripening or during storage is apparently caused by the oxidative polymerization of condensed tannins (de Bezada 1981). It is associated with reduced germination in pea (Nozzolillo 1978), bean (Hughes & Sandsted 1974), lentil (de Bezada 1981) and watercress (Biddington et al. 1983). This association is not absolute since some pea genotypes do not show decreased germination with natural darkening (Nozzolillo 1978).

1.7 OBJECTIVE OF THE PRESENT STUDY:

In view of these findings regarding the role of phenolics in legumes, it is important to generate the necessary data concerning the types of phenolics present, their location and distribution at various stages of plant growth right through to final seed formation. Information of this nature would improve the basic understanding of plant behaviour, and lead to possible avenues of utilizing the optimum protein content from a healthy variety of legume. The objectives of the present investigation was to study the developmental aspects of phenolic compounds in lentils. Two types of lentils were used for this purpose; Eston a small-seeded cultivar developed by Slinkard (1980) for Canadian conditions, containing tannins in the seed coat and P.I.345635 a small seeded line devoid of tannins. The flavonoid 'fingerprint' of the various plant organs of the tannin and non-tannin lines was compared at various stages of growth. Histochemical analysis was used to study the localization of phenolic substances. Total concentration of phenols especially proanthocyanidins in the various plant organs of both lentil lines at various stages of development was also measured.

A second objective was to examine a possible role of tannins in the testa as defence chemicals against attack by *Fusarium* spp. during germination. *Fusarium oxysporum* was chosen since Bhalla (1982) found that this fungus is a pathogen of lentils in Canada.

CHAPTER 2

MATERIALS AND METHODS

2.1 PLANT MATERIAL:

Lentil seeds (*Lens culinaris* Medic), were provided by A.E. Slinkard, Crop Development Centre, University of Saskatchewan, Saskatoon.

Cultivar Eston and TB406M are tannin-containing lines whereas P.I.345635 is not. The lentil line P.I.345635 was found by A.E. Slinkard to not discolour during prolonged storage at room temperature (Vaillancourt 1984). It was discovered when some old seed increases were being discarded after a sample had been regrown to maintain viability. Seed coats of all lines were dark brown except for this entry which still had bright, light coloured seed coats. Seed of this line was supplied to de Bezada (1981) who found that P.I.345635 was a non-tannin line. Cultivar Eston is a small-seeded lentil line which is shorter than commercial chilean but produces more seed (Slinkard 1980) and is well adapted to the growing conditions in certain area of Canada. TB406M was selected as a second tannin line readily available at the time.

Seeds from each variety were sown 2 to 3 cm deep in soil in about 15 cm diameter pots. Some 10 to 12 pots, each containing the same line, were placed in a Conviron growth chamber under cool white fluorescent and incandescent lamps for a 14 hr photoperiod, at an intensity of about 20 w m^{-2}. The temperature in the growth chamber was maintained at 15 ^{o}C. Each lentil line was grown in sequence.

Lentil seeds were also grown in 20 to 25 pots in a greenhouse where temperature ranged

from 30-34 ^{o}C during the day and 16-19 ^{o}C at night.

The seedlings were thinned 10-12 days after sowing to reduce the number to 2 to 3 per pot. The seedlings/plants/reproductive parts were harvested at various stages of plant growth (Tables 2 & 3). The number of seedlings required for extraction was more (~6 to 7), when the seedlings were 20 days old, but at later stages when the seedlings were 45, 65 and 80 days old, material from 2 to 3 plants sufficed for the extraction. Some of the plants were left to grow beyond 80 days for the seeds to mature (about 68 days after anthesis).

2.2 SAMPLING:

2.2.1 OF PLANT PARTS FOR PREPARATION OF CRUDE EXTRACTS:

Entire plants were pulled out from the soil in pots and cut into leaves, stem and root (Table 2). Stage 1 was harvested 20 days after sowing, when the seedlings were about 10 to 12 cm long with no side branches yet formed (Fig.3). Stage 2 was selected at the time when the seedlings were profusely branched and 45 days old. The plant was arbitrarily divided into two halves. The younger plant parts from the upper half (from apex to the middle) and the older parts from the lower half (from middle to the base) were collected separately. Stage 3 was assigned to plants beginning to flower. This occurred by 65 days after sowing. Younger and older plant parts were collected as in stage 2, as well as flowers which were dissected into petals and pistils. Stage 4 was assigned to plants beginning to form fruits. This occurred by 80 days after sowing. Leaves, stem and roots were collected as for stages 2 and 3 as well as fruits which were dissected into pericarp and ovules.

Table 2 : Stages at which the plant parts of Eston and P.I. 345635 were harvested for preparation of crude extracts for 2D chromatography and quantitative estimation of total phenols and proanthocyanidins. These plant parts were also used for histochemical analysis, and the test for proanthocyanidins.

Growth stage of plant	Age (days after sowing the seeds)	Plant parts collected *								
		YL	OL	YS	OS	R	Pe	Pi	P	O
1. Monopodial seedlings	20	x	-	x	-	x	-	-	-	-
2. Bushy young plant (no flowers)	45	x	x	x	x	x	-	-	-	-
3. Flowering plant	65	x	x	x	x	x	x	x	-	-
4. Fruiting plant	80	x	x	x	x	x	-	-	x	x

* Abbreviations used: YL, young leaves; OL, old leaves; YS, young stem; OS, old stem; R, root; Pe, petal; Pi, pistil; P, pericarp; O, ovule; x- collected; - not collected

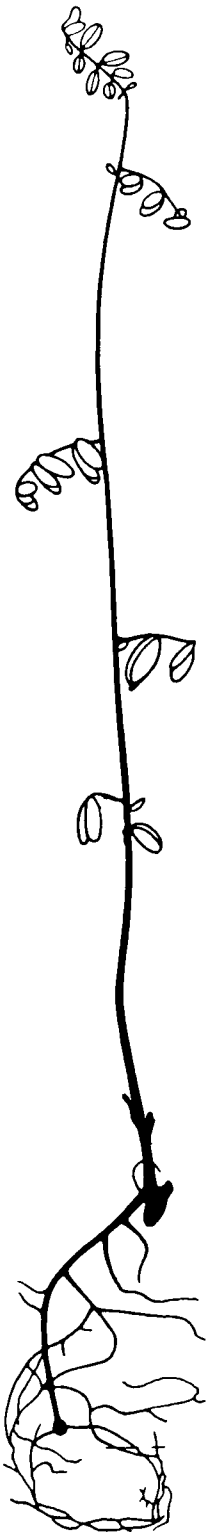
The fruits were 2nd or 3rd stage in relation to Table 3.

Table 3 : Stages at which the fruit parts of Eston and P.I. 345635 were harvested for preparation of crude extracts. These extracts were used for estimation of total phenols and proanthocyanidins. The ovules/seeds from 1st, 2nd and 3rd stage were also used for microtome sectioning.

Growth stage of fruit	Days after anthesis	Fruit parts collected *			
		O	P	Sc	E
1	11	x	x	-	-
2	18		x	x	x
3	30		x	x	x
4	68		x	x	x

* Abbreviations used: O - entire ovule
P - pericarp
Sc - seed coat (testa)
E - embryo
x - collected
– - not collected

Figure 3. Drawing of a 20 day old lentil seedling.



Developmental studies on fruits were similarly conducted. The flowers were tagged at anthesis, that is when the flowers were newly opened, fresh and turgid. The tags were made from small pieces of white tape, labelled and attached with ordinary cotton thread rings. These labels were then put around the pedicels. Fruits were collected at 11, 18, 30 and 68 days after anthesis (Stages 1, 2, 3 and 4). Pericarp (ovary-wall), seeds (ovules), testa (seed coat) and embryo (cotyledons) were separated from the collected fruits as shown in Table 3.

For preparation of extracts for 2-D chromatography, plant parts were air dried at room temperature for 4 to 5 days and stored in ~3" by 4" paper envelopes, in a freezer. For analysis of total phenols and proanthocyanidins, fresh tissue not separated into younger and older parts was extracted.

2.3 EXTRACTION METHODS:

2.3.1 EXTRACTION OF FRESH MATERIAL FOR TOTAL PHENOLS AND PROANTHOCYANIDINS:

Samples were weighed under absolute isopropanol (modified from the method of Hillis & Swain 1959) in tared screw-cap flasks to determine their fresh weights. The weight of a screw-cap flask containing isopropanol (volume undertermined) was recorded. A portion of the fresh tissue was added to the flask and the weight noted down again. The difference between the two weights gave the fresh weight of the tissue. Percent dry weight of each tissue was calculated by first determining the fresh weight of a portion of tissue; the fresh tissue was then air dried at room temperature until it reached a constant weight and the dry wt. of the tissue was also recorded. Such data were used to calculate the dry wt. of the fresh tissue used

for extraction. The extraction of the fresh tissue was carried out as described below (Classen & Nozzolillo 1981).

Repeated isopropanol extractions were made in a 60 °C water bath until no further colour went into the solution. The isopropanol extracts were combined, dried in vacuo on a rotovapor and washed with several small aliquots of petroleum ether to remove the chlorophyll. The residual plant material was re-extracted with absolute methanol using the same procedure as for the isopropanol extractions. These extracts were combined with the dried isopropanol extract and the entire solution was dried in vacuo and washed again with petroleum ether. The dried extract in the flask was finally taken up in 5 ml of 80% methanol and stored in small screw-cap vials of 10 ml capacity in a freezer. The residue was used for further extraction (Fig.4).

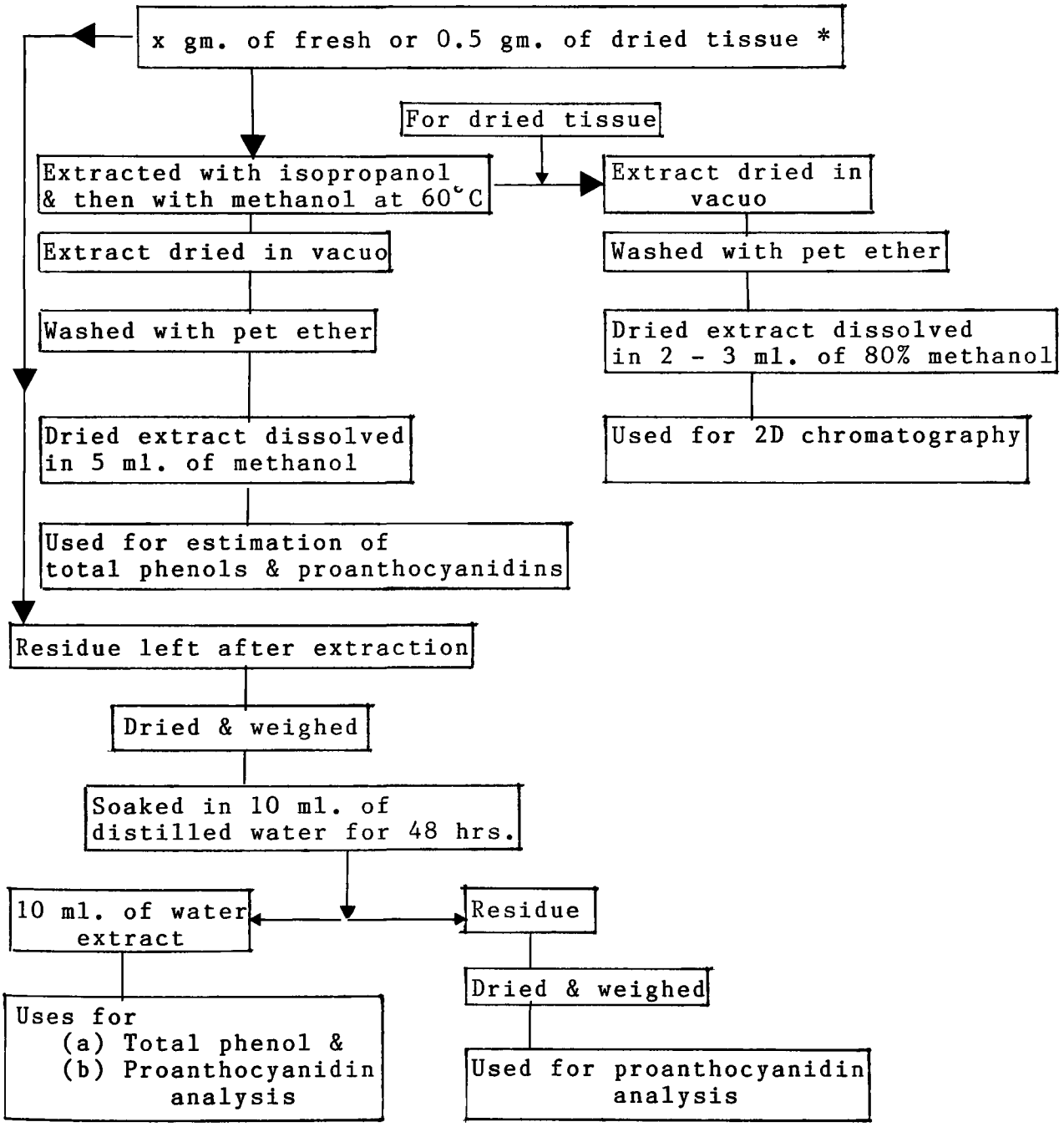
2.3.2 EXTRACTION OF DRIED MATERIAL FOR CHROMATOGRAPHY:

Dried plant material (0.5gm) was powdered and placed in a screw-cap test tube of 20 ml capacity for extraction as described in section 2.3.1. The residue left after extraction was discarded. The dried extract in the flask was finally taken up in 2 ml of 80% methanol and stored in screw-cap vials of 5 ml capacity in a freezer.

2.3.3 EXTRACTION OF RESIDUE WITH WATER:

The residue of the fresh plant material left after extraction with isopropanol and methanol was air-dried and weighed. Ten ml of distilled water were added to the residue in a 50 ml screw-cap culture tube. The contents of the tube were mixed occasionally on a vortex-genie stirrer over a period of 48 hrs., after which the water was decanted, centrifuged and the

Figure 4. Procedure used for the extractions of various plant parts, modified from Classen & Nozzolillo, (1981).



* This procedure was performed on both dried and fresh material.

supernatant stored in screw-cap vials in a freezer until tested for total phenols.

2.4 ANALYTICAL METHODS:

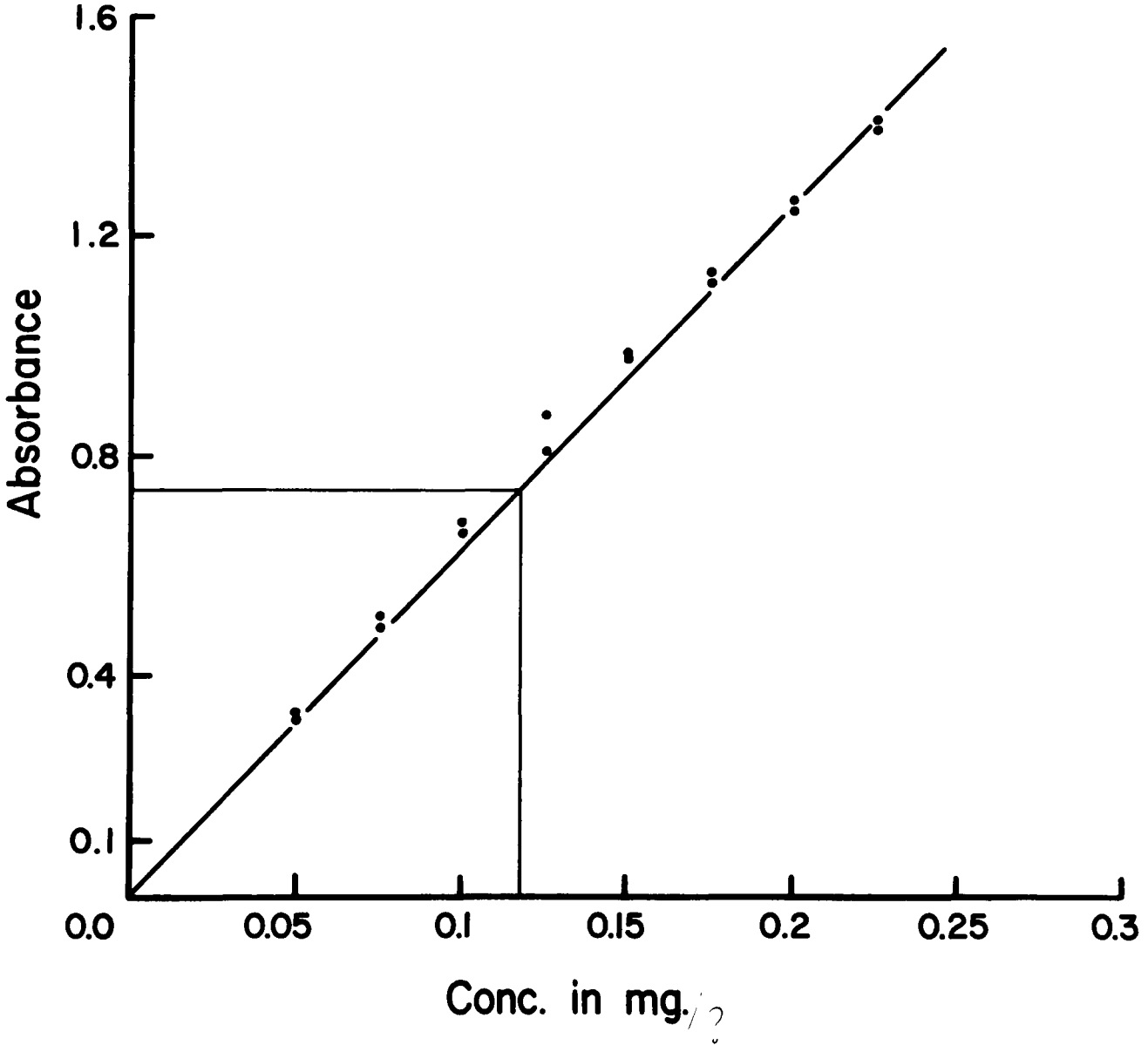
2.4.1 ESTIMATION OF TOTAL PHENOLS:

The method of Swain and Hillis (1959) was followed. An aliquot of solution under test (0.1 ml of alcohol extract and 0.5 ml of water extract) was diluted with water to 7 ml in a 15 ml graduated screw-cap tube. The contents were well mixed, 0.5 ml of Folin-Ciocalteau reagent (Fisher Scientific Co.) was added and the tubes were again thoroughly shaken. Exactly 3 minutes later 1 ml of saturated sodium carbonate was added and the mixture made up to 10 ml with water and shaken well. After 1 hr., the absorbance was determined at 725 nm using as a blank water and reagents only. The total phenol content in different plant organs of both lines of lentils was determined as mg chlorogenic acid equivalent per gm dry wt. A standard curve was prepared for known concentrations of chlorogenic acid in water against visible light absorbance at 725 nm wavelength (Fig.5). Amounts as low as 0.04 mg could be detected with reasonable certainty and the absorbance was noted to increase linearly with increasing chlorogenic concentration.

2.4.2 ESTIMATION OF PROANTHOCYANIDINS:

2.4.2.1 IN ALCOHOL AND WATER EXTRACTS:

Figure 5. A standard curve for quantitative estimation of total phenols, using chlorogenic acid and the method of Swain & Hillis (1959).


1.6
1.2
0.8
0.4
0.1
0.0
0.05
0.1
0.15
0.2
0.25
0.3
Absorbance
Conc. in mg.

The method of Swain and Hillis (1959) was followed. An aliquot of alcohol/water extract (0.5 ml) was placed in each of two separate screw-cap test tubes of uniform bore and thickness and 10 ml of a solution containing conc. hydrochloric acid (25 ml of 36% w/w HCl diluted with n-butanol to 500 ml) was added to each tube. The tubes were shaken until the solution was homogeneous. One of the tubes was placed in an all-metal water bath maintained at 97 ± 1 °C and heated for a total of 40 min. After that period of heat treatment, this tube was cooled under running tap water for 5 minutes and absorbance of the solution was determined at 550 nm using the contents of the unheated tube as a blank.

2.4.2.2 IN RESIDUE:

The residue left after extraction with water (section 2.3.3) was dried in the oven at 60 °C. The dried residue was weighed and suspended in n-butanol-HCl solution in a 15 ml screw-cap test tube. The rest of the procedure followed was the same as described in section 2.4.2.1. The colour of the residue, after boiling with the HCl-n-butanol reagent, was also recorded using an ISCC-NBS centroid colour chart.

The approximate total proanthocyanidin content of the samples was calculated using the appropriate weight, volume, and dilution factor and the average extinction coefficient at 1% at 535 nm (E) for anthocyanidins produced by acid treatment (Lee & Francis 1972).

Total anthocyanidin (mg/100g) = Absorbance x dilution x 10/E.

= (Absorbance x dilution factor)/98.2

2.4.3 TEST FOR THE PRESENCE OF PROANTHOCYANIDINS:

Dried plant material (0.5gm) from all samples collected (as described in section 2.2.1) was boiled with 5 ml of 2*N* HCl in 15 ml test tubes in a water bath at 100 °C for 20 minutes (Gibbs, 1975). The contents of the tube were then cooled and the supernatant decanted into a separatory funnel. The supernatant was extracted with 2 ml of isoamyl alcohol. The isoamyl alcohol solution was stored in screw-cap vials in a freezer. The colour of the residue and the supernatant was recorded using an ISCC-NBS centroid colour chart.

The pigmented isoamyl alcohol extracts were spotted on Whatman No.1 paper with cyanidin, pelargonidin and delphinidin as standards. The chromatogram was run descendingly in Forestal (glacial acetic acid: water:hydrochloric acid, 30:10:3) in an air-tight glass chamber and after drying, the paper was observed under visible light for detection and identification of anthocyanidins.

2.4.4 CHROMATOGRAPHY OF CRUDE EXTRACTS:

2.4.4.1 2-D THIN-LAYER CHROMATOGRAPHY OF UNHYDROLYZED EXTRACTS:

Extracts as prepared in section 2.3.2 were chromatographed on 75 mm x 75 mm sheets of F 1700 polyamide microlayer (Mandel Scientific Co. Ltd.) (Classen & Nozzolillo 1981). An aliquot of extract corresponding to approximately 125 ug of dried plant material (0.5 ul) was spotted along with quercetrin (quercetin 3-rhamnoside) rutin and caffeic acid as standards.

The chromatograms were run ascendingly in the first dimension in

water:pyridine:cyclohexanone (90:5:5 v/v), dried and then run in the 2nd dimension in n-butyl acetate:methanol:formic acid:water (60:30:5:5). The plates were then dried and sprayed with 1% diphenylboric acid ethanolamine complex in water and observed under visible and ultraviolet (UV) light.

The overall flavonoid pattern for each organ in both tannin and non-tannin lentil lines was constructed by examining several chromatograms of that particular organ and evaluating the number of glycosides present at all stages of plant growth. The glycosides were primarily identified on the basis of colour and Rf values. Those glycosides that were not consistently present at all stages of organ development were not included in formulating the basic flavonoid pattern of a particular organ.

Some of the duplicate chromatograms were sprayed with a solution of 1% $FeCl_3$-1% $K_3Fe(CN)_6$ in water. This spray is also known as Turnbull's blue reagent (Dass & Weaver 1972). The compounds were allowed to react for 5 minutes and then the chromatograms were rinsed under a gentle stream of distilled water until no more colour was washed off. Most phenolic compounds stain dark blue on a white background.

2.4.4.2 HYDROLYSIS OF CRUDE EXTRACTS AND 2-D THIN-LAYER CHROMATOGRAPHY OF AGLYCONES:

The alcohol extracts (0.5 ml), prepared as in section 2.3.2, were combined with 3 ml of 2*N* HCl and 3 ml of 95% ethanol in 50 ml screw-cap culture tubes and placed in a 100 °C water bath for 4 hrs. At the end of this time all O-glycosides should have been completely hydrolysed (Harborne 1967). The tubes were allowed to cool under running tap water and 6 ml of distilled water were added to render any traces of chlorophyll insoluble. The

chlorophyll was removed by centrifugation and discarded. The flavonoid aglycones were extracted twice into 1 ml of ethyl acetate and stored in small screw-cap vials of 5 ml capacity in a freezer.

An aliquot of extract (0.5 ul) corresponding to approximatively 31 ug of dried plant material was similarly chromatographed as described in sec. 2.4.4.1. The standards, quercetin, kaempferol and caffeic acid were used. The chromatogram was run ascendingly in the first dimension in chloroform:methanol:butanone (12:2:1 v/v) dried and run in methanol-:water:acetic acid (18:1:1 v/v) in the 2nd dimension (Mabry et al. 1970). The chromatogram was sprayed as described in 2.4.4.1.

2.4.4.3 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC):

Suitably diluted samples (25 ul) were injected into a C18-uBondapak reversed-phase column of a Beckman HPLC. The eluting solvent, water:acetic acid:methanol (65:5:30) (Wulf and Nagel 1976) was pumped at a rate of 0.5 ml/min, (Beckman 110A pump and 420 controller). The eluate was scanned (220-400 nm) spectrophotometer. Compounds were identified by comparing their retention time and UV spectrum with those of standards. After 1 hr. the delivery system was programmed to change linearly to absolute methanol over a period of 15 minutes. Absolute methanol was allowed to run for ~2 hrs. to clean the column of any impurities.

2.4.5 PURIFICATION OF COMPOUNDS:

2.4.5.1 PAPER CHROMATOGRAPHY:

Glycoside and aglycone extracts were banded 10 cm from the short edge and 8 cm on each side from the long edges of 24 cm x 58 cm sheets of Whatman paper No. 1. Glycosides were run decendingly in 6% acetic acid (Harborne 1967) whereas aglycones were run in glacial acetic acid:water:chloroform (9:1:10 v/v) (Mabry et al. 1970) using quercetin and kaempferol as standards.

2.4.5.2 ELUTION OF COMPOUNDS FROM PAPER:

The bands separated on Whatman paper were marked with a pencil under UV light. The marked strips of paper were cut out, with one end narrowed to facilitate the dripping of the solvent. The square end of the paper was dipped in a petri dish containing the solvent and the compound that washed down from the paper was collected at the narrower end in a flask. Absolute methanol was used for eluting glycosides whereas chloroform :acetic acid:water (10:9:1 v/v) was used for eluting aglycones. The whole set up was placed in a closed chamber to keep the atmosphere saturated and thus facilitate the movement of the solvent front down the paper. The solvent containing the compound was dried *in vacuo* and the residue was dissolved in 0.5 ml of absolute methanol.

2.4.5.3 COLUMN CHROMATOGRAPHY:

DC6 polyamide columns were prepared in disposabe pasteur pipettes as follows. DC6 polyamide powder was slurried in tap water. After 10 minutes of settling, the supernatant water was decanted. This process was repeated 19 times to ensure removal of suspended fine particles (de Bezada 1981). Glass wool was placed at the narrower end of the pipette and the

final DC6 suspension in water was pipetted into the column until the bed height was approximately 3 cm. Each of the compounds eluted from paper and dissolved in 0.5 ml of methanol, was poured onto a column. The loaded column was washed with 10-12 ml of distilled water to remove any sugars that had come from the paper chromatogram and then the flavonoids were eluted with 100% methanol. The progress of the compound through the column was monitored with a UV light. The eluate was concentrated to a volume of 1 ml and stored in a screw-cap vial. The compounds collected from each column were subjected to further tests.

2.4.6 IDENTIFICATION PROCEDURES OF COMPOUNDS PURIFIED BY PAPER AND COLUMN CHROMATOGRAPHY:

2.4.6.1 SPECTRAL ANALYSIS:

The absorption spectra of purified compounds were determined on a Unicam spectrophotometer model 1800. Diagnostic reagents NaOAc, NaOMe and NaOAc+H_3BO_3 were used as described by Mabry et al. (1970).

2.4.6.2 HYDROLYSIS OF GLYCOSIDIC COMPOUNDS:

Each isolated glycoside was mixed with an equal volume of 2*N* HCl in a screw-cap test tube and placed in a water bath at 100 °C for 10 minutes. The aglycone was separated from free sugar in ethyl acetate. Both the sugar fraction and the ethyl acetate fraction were stored separately in vials.

2.4.6.3 CHROMATOGRAPHY:

Glycosides and aglycones of the isolated compounds were spotted on 5 cm x 8 cm polyamide chromatograms along with quercetin and quercetrin as standards and run ascendingly in water:pyridine:cyclohexanone, (90:5:5 v/v) and n-butyl acetate:methanol:formic acid:water (60:30:5:5) (Classen & Nozzolillo 1981). These were also run on cellulose in n-butanol:acetic acid:water (4:1:5), water and 15% acetic acid (Ribereau-Gayon 1972).

The sugar fractions obtained by hydrolysing purified glycosides were co-chromatographed on 10 x 5 cm cellulose thin layer chromatogram with glucose, arabinose and rhamnose as standards. The chromatogram was run twice in ethyl acetate:pyridine:water (10:3.2:2) (Long 1961), dried, sprayed with aniline hydrogen phthalate and heated in an oven at 105 °C for 10 minutes.

2.5 HISTOCHEMICAL LOCALIZATION OF PHENOLS IN SEEDS:

Many plant tissues contain a variety of substances which are naturally fluorescent under the appropriate excitation wavelength and may be detected microscopically without further manipulations or staining procedures. Phenolic compounds are the most common autofluorescent materials and generally occur in the cell wall of many tissues (Fulcher et al. 1972).

2.5.1 SECTIONING:

The method of Yiu et al. (1983) was followed. The seeds from first, second and third stages of growth were harvested and immersed in a drop of embedding medium for freeze sectioning (Tissue-Tek II C.T. Compound) on a specimen holder. The holder was immediately frozen in dry ice (solid CO_2) and transferred to a cryostat microtome. Sections 8-10 um in thickness were cut at -15 °C to -20 °C. Sections of leaf, stem and root were cut by hand and mounted in a drop of water on a clean microscope slide. The microtome sections were placed on glass slides pre-coated with 1% gelatin.

2.5.2 MICROSCOPIC EXAMINATION:

Sections were examined with a fluorescence microscope which is a bright-field microscope fitted with a high intensity, broad-spectrum illuminator and two filter systems for providing excitation or illumination of the specimen called exciter filters and eliminating unwanted illumination from the fluorescent image called barrier filters. The exciter/barrier filter set have maximum transmission at 365 nm/>418 (FCI), 450-490/>520(FCII) and 546/>590(FCIII) (Fulcher 1972). Specimens were photographed after staining as described below (2.5.3) using 35 mm Kodak Ektochrome 400 daylight film.

2.5.3 STAINING PROCEDURES FOR PHENOLS:

Hand-cut sections of leaf, stem and root and microtome sections of frozen seeds prepared as described above (2.5.1) were treated dropwise with equal parts of 10% $NaNO_2$, 20% urea and 10% acetic acid for 3 minutes and later 2 parts of 2*N* NaOH were added (Reeve 1951). The colour reaction is immediate. A positive test for phenols is either a yellow product, or in the

case of catechols and other dihydroxy phenols a cherry red. Sections were oil mounted prior to microscopic examination.

2.6 SEED EXUDATE AND FUNGAL GROWTH:

2.6.1 GROWTH OF FUNGI:

Fusarium oxysporum isolate 82A and *F. oxysporum* var. *callistephi* were grown on potato dextrose agar (PDA) at 24 ± 2 °C. *F. oxysporum* isolate 82A was provided by Dr. E. Schneider, Agriculture Canada, Ottawa. It was the most virulent strain isolated from lentils affected by root rot (Bhalla, 1982). *F. oxysporum* var. *callistephi* was obtained from Dr. J.T. Arnason, University of Ottawa. Innocula, consisting of mycelia on 6 mm diameter agar discs were placed on PDA in the centre of the petri-dish. *F. oxysporum* isolate 82A mycelia usually covered the agar surface in 10 days of growth whereas var. *callistephi* took 5 to 8 days longer.

Macroconidia from PDA plates were obtained by surface agitation of the cultures with sterile glass beads in sterile distilled water (sterilization was done by wrapping the apparatus with aluminum foil and autoclaving at 125 °C for 20 minutes). The spore suspension was passed through a sterile nitex filter and centrifuged for 10 minutes at 1500 x g. The supernatant was discarded and the pellet resuspended in sterile water to give a concentrated spore solution (5.5×10^9/ml). Macroconidial density was estimated on a haemacytometer (Levy ultra plane clay-Adams New York U.S.A.). Spore concentration was kept constant in each innoculum used for seed exudates and control.

2.6.2 MEASUREMENT OF FUNGAL GROWTH IN SEED EXUDATES:

Triplicate lots of 30 seeds of Eston and P.I.345635 were weighed and soaked separately in 10 ml of sterile water in sterile beakers for 6 hrs. Seeds of a second tannin-containing line TB406M were also similarly included in this test as a replicate for tannin containing line Eston, but no non-tannin line other than P.I.345635 is presently available. Each of the 9 seed diffusates was decanted into a sterile beaker. PDA powder (0.39 gm) was mixed with each one and triplicates of 10 ml of sterile water containing 0.39 gm of PDA were used as control. After mixing well, the solutions were filtered through sterile glass wool and the filtrates collected in sterile 25 ml flasks. An innoculum containing 5.5 x 10^9/ml spore conc. was dispensed into the flasks under a laminar flow hood. The flasks were stoppered with sterile cotton plugs and covered with aluminum foil. The flasks were placed on a shaker at 50 revolutions/min. and the temperature was maintained at 24 ± 1°C. Fungal growth was monitored at time intervals of 14 hrs, 22 hrs, 30 hrs, 48 hrs and 66 hrs. A separate set up was used for each time period for each fungus. At these time periods, the mycelia were filtered onto tared filter paper discs and dried at room temperature to determine the dry wt. of fungus. The experiments were repeated for each time period with both fungi.

CHAPTER 3

RESULTS

3.1 TOTAL PHENOLS IN ESTON AND P.I.345635

3.1.1 IN THE VEGETATIVE PARTS:

Figure 6 shows the trends in total phenol accumulation in leaves of Eston and P.I.345635. The rate of accumulation in leaves of cultivar Eston showed a gradual increase from stage 1 (20 day-old seedlings) to stage 4 (80 day-old seedlings). The amount of total phenols had more than doubled from 2 mg/g dry wt. at stage 1 to 6 mg/g dry wt. at stage 4. The rate of phenol accumulation in P.I.345635 showed a different trend. There was an increase in the total phenols from stage 1 (20 day-old seedlings) to stage 2 (45 day-old seedlings) and then a gradual decrease in the rate of total phenol accumulation from the 2nd stage onwards with total phenol content reaching a plateau between the 3rd and 4th stages of development. The amount of total phenol in the non-tannin line was always less than half than that present in Eston at comparable stages of development.

The rate of accumulation of total phenols in the stem (Fig. 7) and and root (Fig. 8) showed similar trends in both kinds of lentil. Of all vegetative parts, the highest amount of total phenols was always present in the leaves. In vegetative parts of both lentil lines, the amount of phenols extractable in methanol was always much less than that present in the water extract as shown in Tables 4 & 5.

Figure 6. Total phenols in lentil leaves taken from plants of cv. Eston and P.I.345635 (a non-tannin line), grown in a growth chamber (G.C.) at 15 °C. Sampling as in Table 2, tissues extracted as in Fig. 5, Folin reagent for phenols (Hillis & Swain, 1959).

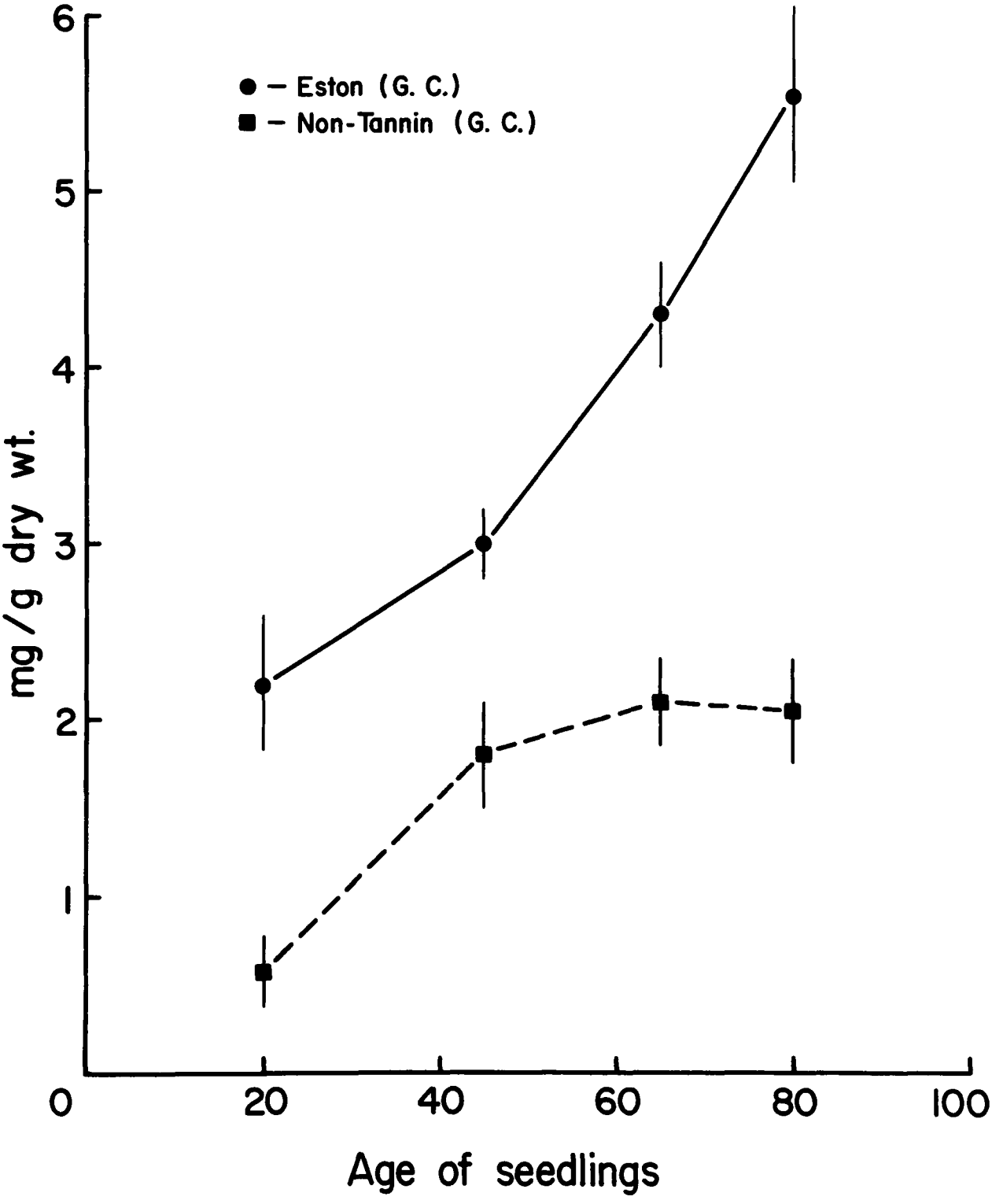

6
5
4
3
2
1
0
● — Eston (G. C.)
■ — Non-Tannin (G. C.)
mg/g dry wt.
20
40
60
80
100
Age of seedlings

Figure 7. Total phenols in the stem of Eston and P.I.345635. other conditions as in Fig. 6.

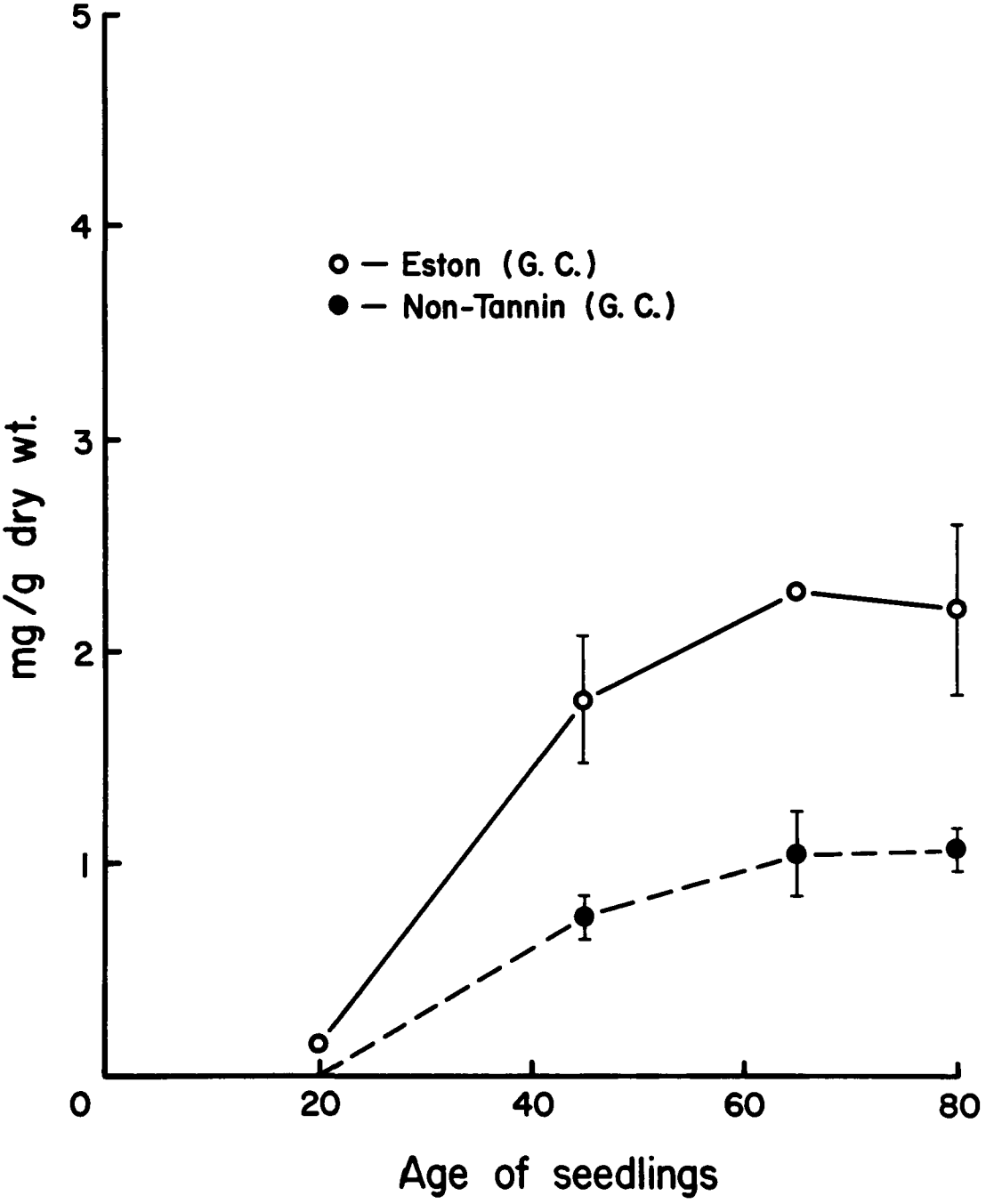

5
4
3
2
1
0
mg/g dry wt.
o — Eston (G. C.)
● — Non-Tannin (G. C.)
20
40
60
80
Age of seedlings

Figure 8. Total phenols in the roots of Eston and P.I.345635, other conditions as in Fig. 6.

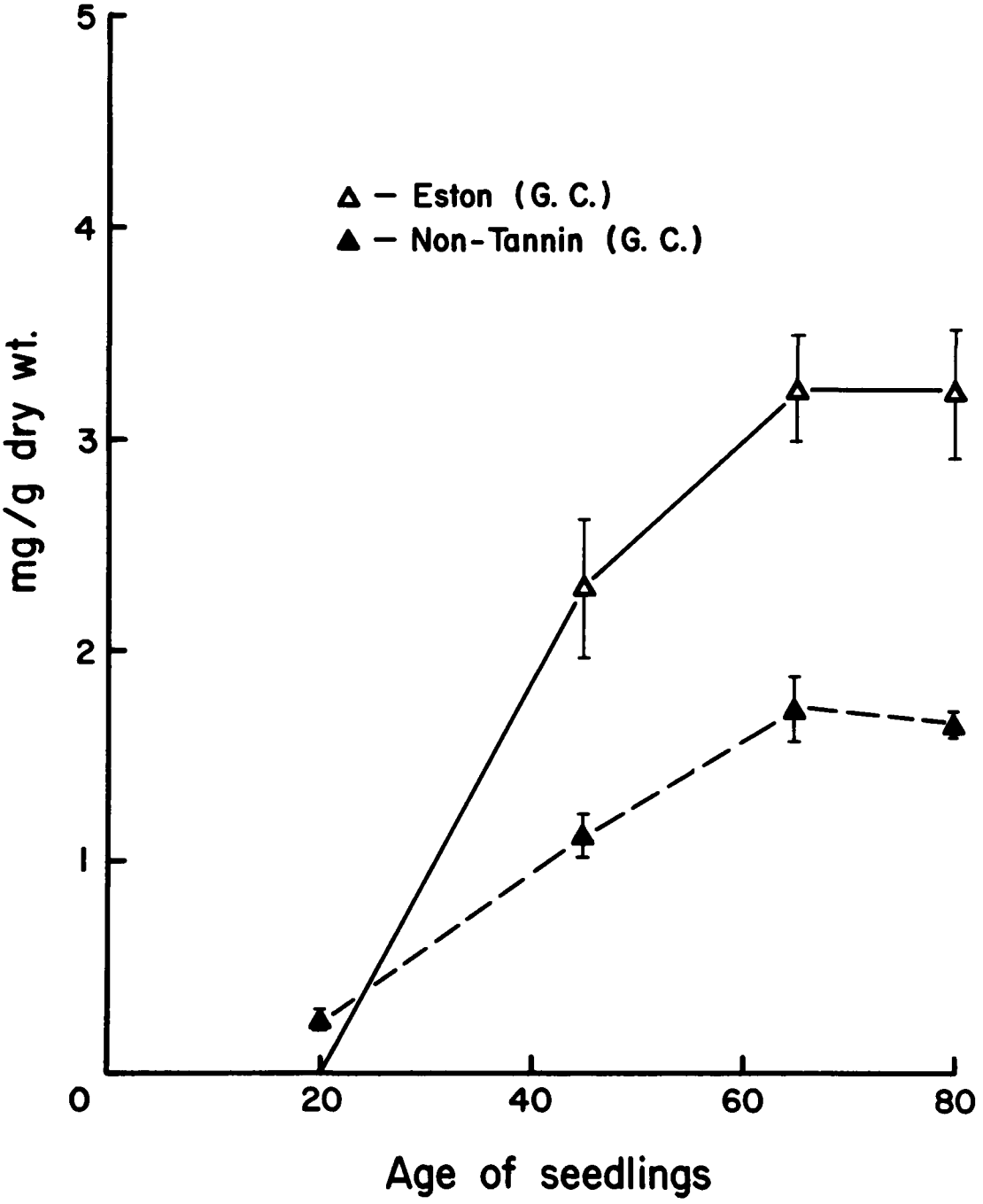

5
4
3
2
1
0
20
40
60
80
mg/g dry wt.
Age of seedlings
△ — Eston (G. C.)
▲ — Non-Tannin (G. C.)

Table 4 : The amount of phenols in alcohol and H_2O extracts of vegetative and reproductive parts of Eston.

Plant organ and stage of growth		Phenols in alcohol ext. mg /g dry wt. ±SD		Phenols in H_2O ext. mg /g dry wt. ±SD	
Leaf	1st (20-day old)	0.37	±.05	1.83	±.45
	2nd (45 ")	0.51	±.11	2.48	±.09
	3rd (65 ")	0.76	±.16	3.56	±.14
	4th (80 ")	0.61	±.21	4.92	±.29
Stem	1st (20-day old)	0.07	±.00	0.10	±.01
	2nd (45 ")	0.19	±.06	1.68	±.24
	3rd (65 ")	0.32	±.07	1.97	±.04
	4th (80 ")	0.43	±.12	1.77	±.28
Root	1st (20-day old)	0.02	±.00	N.D.	
	2nd (45 ")	0.17	±.03	2.13	±.29
	3rd (65 ")	0.21	±.04	3.05	±.21
	4th (80 ")	0.36	±.09	2.86	±.21
Peri-carp	1st (11 days post-anthesis)	19.36	±2.46	1.48	±.23
	2nd (18 ")	10.37	±1.14	2.14	±.34
	3rd (30 ")	9.78	± .64	2.01	±.31
	4th (68 ")	7.56	±2.32	1.26	±.17
Testa	1st (11 days post-anthesis)	16.26	±1.70	4.15	±.52
	2nd (18 ")	24.45	±3.49	3.81	±.47
	3rd (30 ")	55.0	±4.51	6.93	±.83
	4th (68 ")	69.46	±1.58	7.60	±1.12

ND - Not Detectable

Note: Plants grown in a growth chamber at 15°C and sampled as in Tables 2&3. Folin test for phenols (Millis and Swain 1959)

Table 5 : The amount of phenols in alcohol and H_2O extracts of vegetative and reproductive parts of P.I. 345635.

Plant organ and stage of growth		Phenols in alcohol ext. mg /g dry wt. ±SD	Phenols in H_2O ext. mg /g dry wt. ±SD
Leaf	1st	0.18 ± 0.03	0.40 ±0.23
	2nd	0.43 ± 0.09	1.37 ±0.32
	3rd	0.58 ± 0.07	1.52 ±0.18
	4th	0.44 ± 0.06	1.62 ±0.36
Stem	1st	N.D.	N.D.
	2nd	0.06 ±0.01	0.69 ±0.11
	3rd	0.12 ±0.01	0.93 ±0.21
	4th	0.17 ±0.02	0.93 ±0.12
Root	1st	N.D.	0.25 ±0.05
	2nd	0.18 ±0.02	0.94 ±0.13
	3rd	0.32 ±0.04	1.40 ±0.14
	4th	0.28 ±0.04	1.37 0.01
Pericarp	1st	3.20 ±0.88	1.10 ±0.38
	2nd	2.80 ±0.48	0.39 ±0.11
	3rd	2.33 ±0.41	0.85 ±0.21
	4th	2.39 ±0.36	0.77 ±0.12
Testa	All stages	<0.04	<0.04

ND - Not Detectable

Note: Plants grown as in Table4.

3.1.2 IN THE REPRODUCTIVE PARTS:

Fig. 9 shows the rate of accumulation of total phenols in the pericarp and testa of tannin and non-tannin lentil lines. The amount of total phenols, expressed in mg/g dry wt. in the pericarp of both tannin and non-tannin did not show any significant change from day 11 (stage 1) to day 68 (stage 4) post-anthesis. On the other hand, the amount of total phenols in the testa sharply increased from 21 mg/g dry wt. at 11 days after anthesis to 62 mg/g dry wt. at 30 days after anthesis, after that there was a gradual decrease in the rate of accumulation of total phenol with the amount calculated to be 77 mg/g dry wt. at the 4th stage (68 days after anthesis).

The amount of total phenols detected in the seed coat of the non-tannin line was less than 0.04 mg/g dry wt. The amount of total phenol content in the cotyledons of both lentil lines was usually less than 0.04 mg/g dry wt. It should be noted that out of all plant organs examined, the testa of the tannin variety contained the maximum amount of total phenols in mg/g of dry wt. at all stages of plant development. Total phenol content calculated on per organ basis did not show any significant change in the rate of total phenol accumulation of either pericarp or testa of tannin-containing Eston (Fig. 10).

The differences in total phenol of cultivar Eston, grown in the greenhouse and in the growth-chamber, were not significant although greenhouse-grown plants tended to have slightly higher contents in the testa (Fig. 11). The same was true for the non-tannin line grown at two different times in the growth chamber (data in Appendix I).

Figure 9. Total phenols in the reproductive parts of Eston and P.I.345635, other conditions as in Fig. 6.

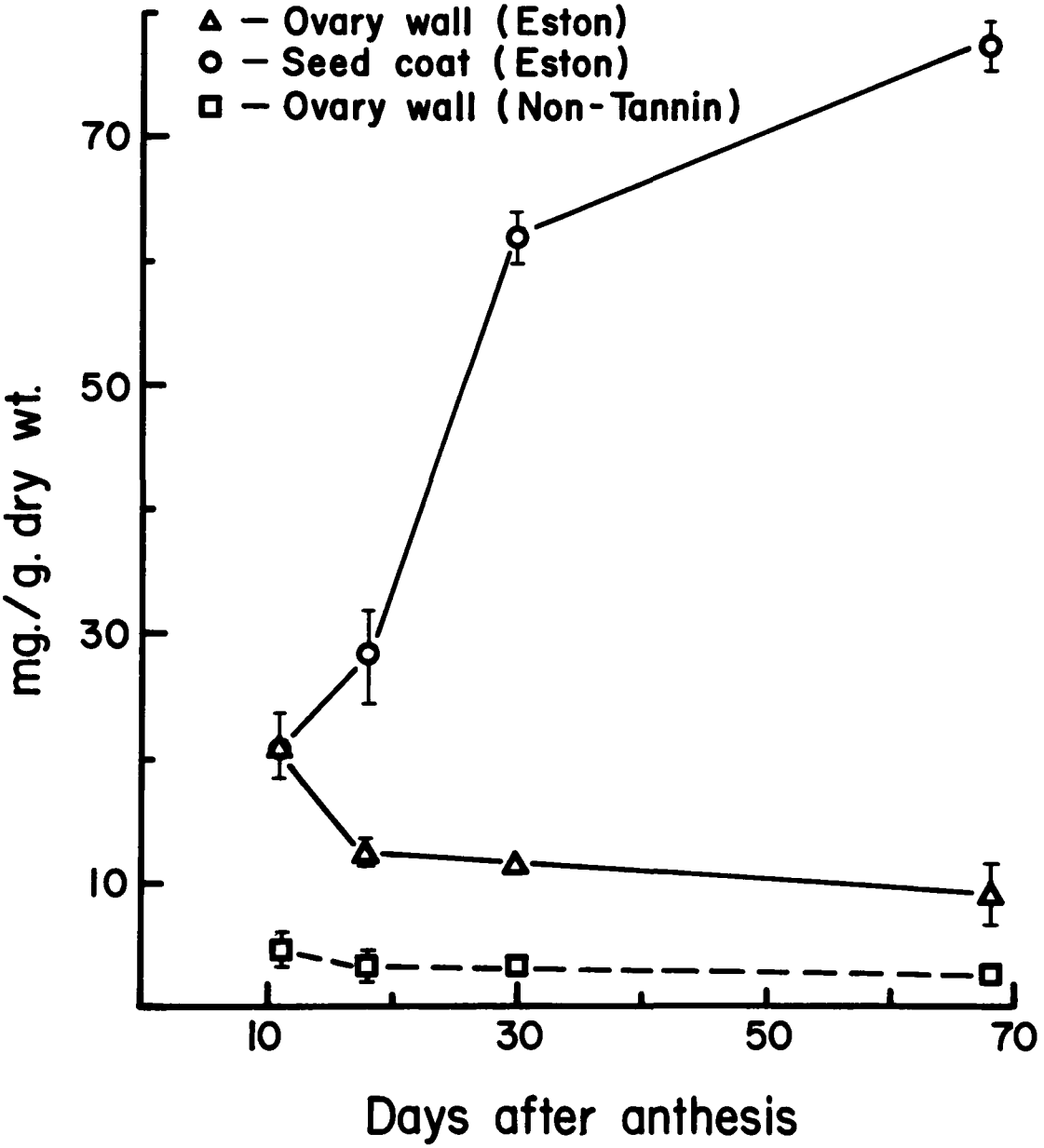

△ — Ovary wall (Eston)
○ — Seed coat (Eston)
□ — Ovary wall (Non-Tannin)
70
50
30
10
mg./g. dry wt.
10
30
50
70
Days after anthesis

Figure 10. Total phenols per organ in the reproductive parts of Eston, expressed on a dry wt. basis. Other conditions as in Fig. 6.

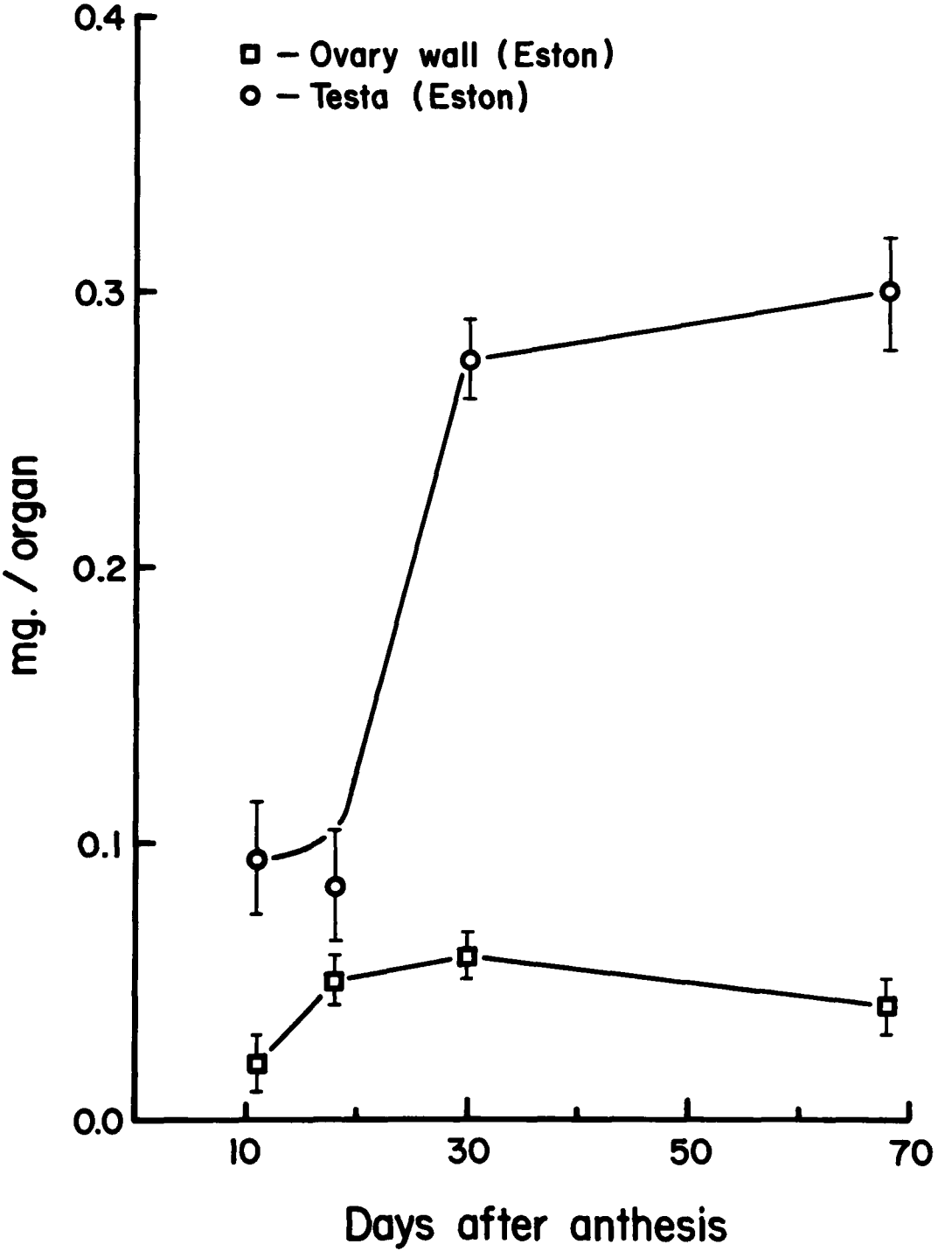
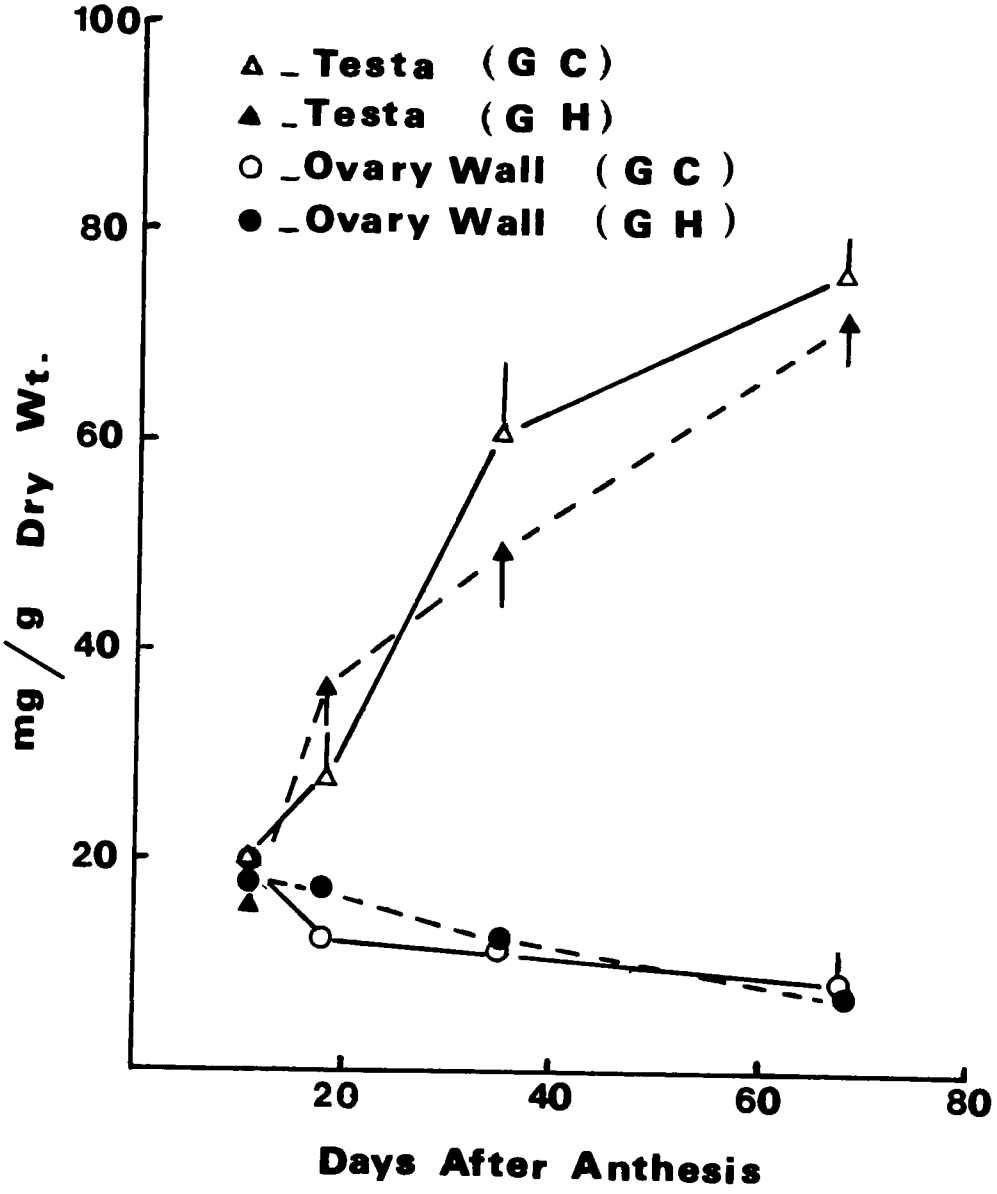

0.4
0.3
0.2
0.1
0.0
mg. / organ
□ – Ovary wall (Eston)
○ – Testa (Eston)
10
30
50
70
Days after anthesis

Figure 11. A comparison of total phenols in the reproductive parts of Eston grown in the greenhouse (G.H.) and in the growth chamber (G.C.). Other conditions as in Fig. 6.


100
80
60
40
20
mg / g Dry Wt.
Testa (G C)
Testa (G H)
Ovary Wall (G C)
Ovary Wall (G H)
20
40
60
80
Days After Anthesis

3.2 TOTAL PROANTHOCYANIDINS IN ESTON AND P.I.345635:

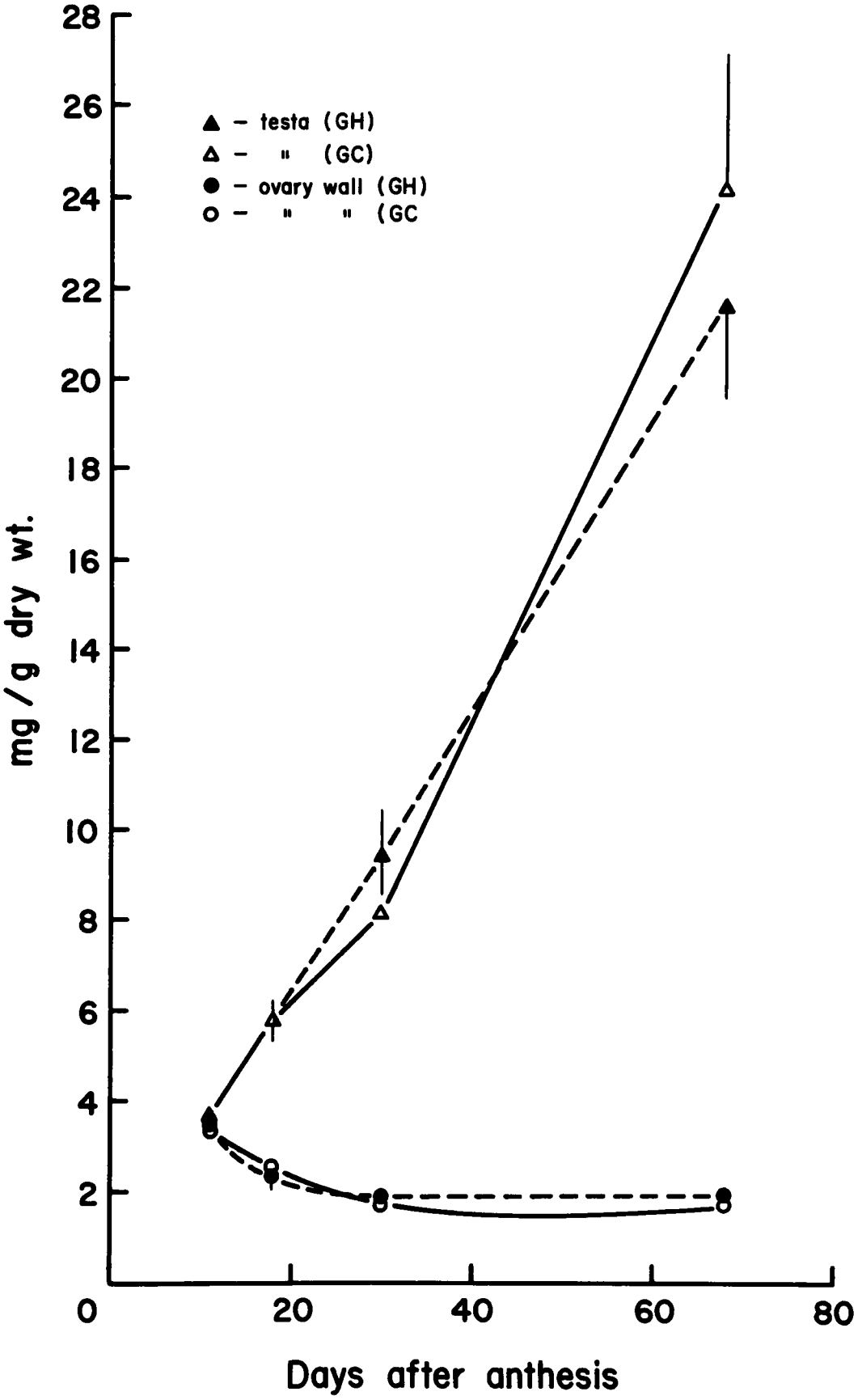
3.2.1 IN THE VEGETATIVE PARTS:

Proanthocyanidins were absent from all plant organs of the non-tannin line (P.I.345635) at all stages of development. In addition, no traces of proanthocyanidins were found in the vegetative parts of the tannin containing line Eston.

3.2.2 IN THE REPRODUCTIVE PARTS:

Proanthocyanidin accumulation in the Eston testa was linear with time (Fig. 12). The amount increased from 4 mg/g dry wt. to 24 mg/g dry wt. from 11 days after anthesis to 68 days after anthesis (Fig. 12). The extractable proanthocyanidin content in the pericarp however showed a gradual decrease from 11 days after anthesis to 68 days after anthesis (Table 6). The decrease in the yield of anthocyanidins (by boiling in acid solution) from pericarp residue (Table 6) could be due to the formation of an insoluble complex between tannin and some other cellular component or, as has been suggested (Haslam 1979) to an increase in degree of polymerization of proanthocyanidins. It is evident that the proanthocyanidins were primarily abundant in the testa of the tannin containing line. Again, the differences in total proanthocyanidin in cultivar Eston, grown in the greenhouse and in the growth-chamber, were not significant (Fig. 12).

Figure 12. Total proanthocyanidins in the testa (seed-coat) and pericarp (ovary-wall) of Eston, grown in the growth-chamber (G.C.) and in the greenhouse (G.H.). Sampling as in Table 3, and analysis as described in Methods, section 2.4.2.


▲ – testa (GH)
△ – " (GC)
● – ovary wall (GH)
○ – " " (GC
mg / g dry wt.
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
20
40
60
80
Days after anthesis

The alcohol extract of the testa contained little proanthocyanidin (2 mg/g dry wt.), 11 days after anthesis but the amount of alcohol soluble proanthocyanidins increased with maturity to 22 mg/g dry wt. 68 days after anthesis (Table 6). The intensity of the violet colour of the residue of both pericarp and testa left after boiling with acid solution also increased from 11 days after anthesis to 68 days after anthesis, thereby indicating the insoluble nature of much of the proanthocyanidins. The recovery of proanthocyanidins from the plant material is not absolute since the residue retains a varying intensity of deep violet colour depending on the stage of growth (Table 7).

The amount of proanthocyanidins in the methanol extract when calculated on a per organ basis did not show any change in the pericarp (ovary wall) from 11 days to 68 days of development (Fig. 13). The amount of proanthocyanidins in the testa when expressed in mg/organ showed a gradual increase from 11 days after anthesis to 30 days after anthesis and then there was a sharp increase to 68 days after anthesis indicating a significant rise in the production of proanthocyanidins in maturing seed coats (Fig. 13).

Table 8 shows the colour reaction of various dried plant parts (collected as shown in Table 2) of Eston and P.I.345635 after boiling with acid solution. None of the plant parts of P.I.345635 showed the presence of tannins whereas only reproductive parts of Eston contained tannins.

3.3 DISTRIBUTION OF FLAVONOIDS IN THE PLANT ORGANS AT VARIOUS STAGES OF GROWTH:

Few developmental changes were observed to occur in flavonoid distribution within a single organ. The following sub-sections individually describe the aglycone and glycoside

Table 6 : Proanthocyanidins in the alcoholic extract and residue of reproductive organs of Eston grown in the growth chamber. (None detected in vegetative tissues nor in the embryo.)

Plant organ and days after anthesis	Proanthocyanidins in alcohol extract mg /g dry wt ±SD	Proanthocyanidin in residue mg /g dry wt ±SD
Pericarp (11 days)	0.37 ±.07	3.10 ±.59
(18 ")	0.24 ±.1	2.25 ±.08
(30 ")	0.25 ±.04	1.60 ±.08
(68 ")	0.22 ±.05	1.60 ±.05
Testa (11 days)	1.53 ±.46	2.17 ±.32
(18 ")	0.86 ±.08	4.95 ±.43
(30 ")	2.81 ±1.08	5.35 ±1.22
(68 ")	21.68 ±2.88	2.58 ± .18

Table 7 : The colour of the residues of pericarp and testa of Eston after boiling with acid solution (10 ml. HCl + 490 ml. n-butanol).

Plant Part	Growth stage	Days after anthesis	Colour of the residue	No. on ISCC-NBS chart	Intensity of the colour *
Pericarp	1st	11	deep red	13	++
"	2nd, 3rd & 4th	18, 30 & 68	deep red	13	+++
Testa	1st	11	deep red	13	++
"	2nd	18	v. deep red	14	+++
"	3rd	30	"	14	+++
"	4th	68	"	14	++++

* More no. of "+" shows greater intensity of colour whereas lesser numbers mean less intensity of colour (tentative designation)

Figure 13. Proanthocyanidins per organ in the testa and pericarp (ovary-wall) of Eston. Other conditions as in Fig. 12.

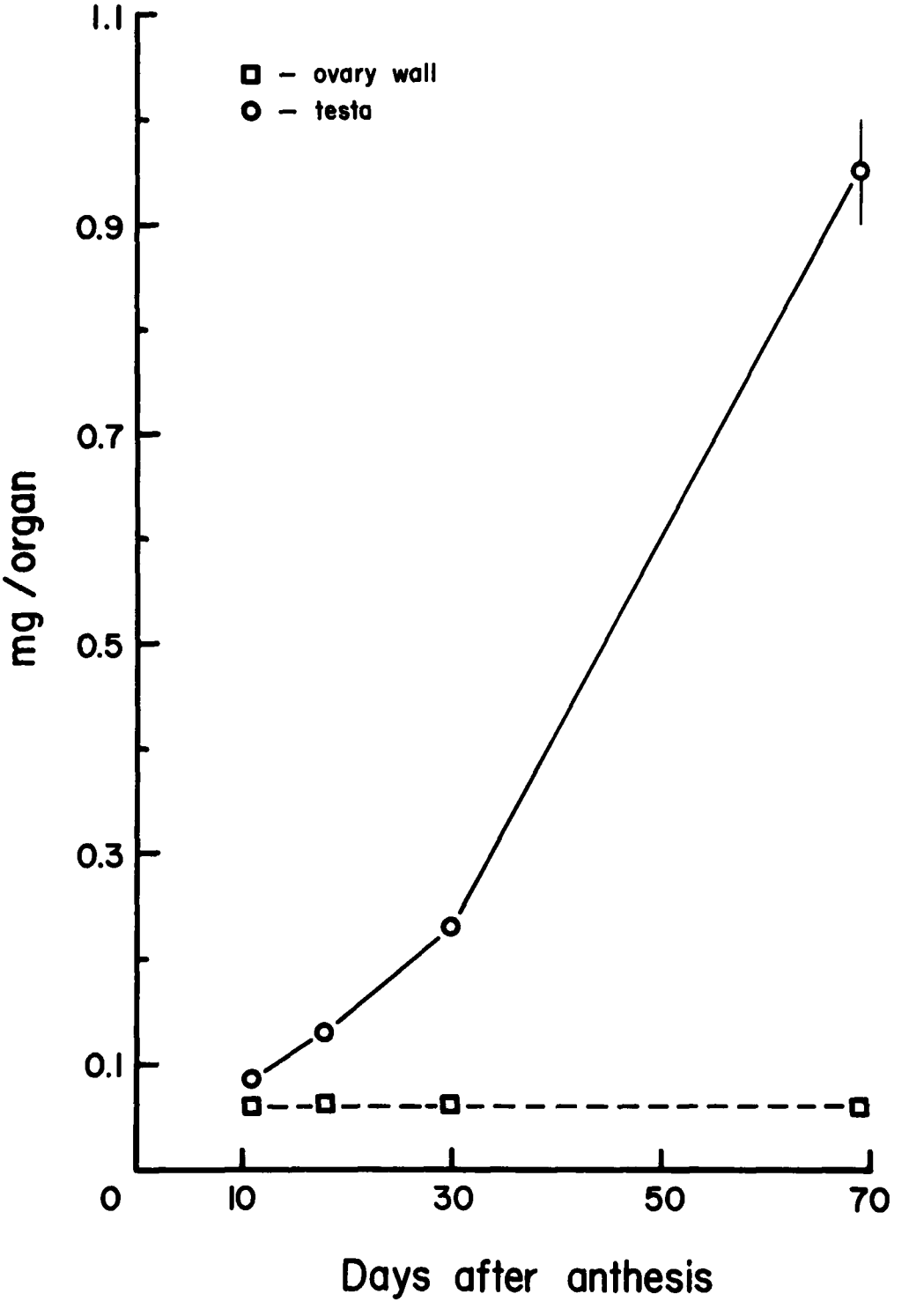

1.1
0.9
0.7
0.5
0.3
0.1
0
mg /organ
□ – ovary wall
○ – testa
10
30
50
70
Days after anthesis

Table 8 : The colour of the supernatant and residue after boiling various plant parts of Eston (dried tissue) with acid solution (10 ml. HCl + 490 ml. n-butanol).

Age of the plant (days)	Plant part	Colour of the supernatant	No. on ISCC-NBS chart	Colour of the residue	No. on ISCC-NBS chart
	Leaf	colourless	-	light yellow	77
20 - 80	Stem	"	-	deep yellow	75
	Root	"	-	deep brown	56
65	Petals	deep red	13	deep red	13
80	Pericarp	v. deep red	14	deep red	13
80	Testa	deep red	13	v. deep red	14

No red colour appeared in the supernatant of any P.I. 345635 plant parts and the colour of the residues varied from yellowish brown to deep brown.

distribution observed in different organs of both tannin (Eston) and non-tannin (P.I.345635) lines of lentil.

3.3.1 FLAVONOID AGLYCONE DISTRIBUTION IN THE VEGETATIVE PARTS OF ESTON AND P.I.345635:

Hydrolysed alcohol extracts of the leaf and stem of both Eston and P.I.345635 showed the predominance of a green spot at all the developmental stages of growth. This spot displayed the same chromatographic behaviour as standard kaempferol (Fig. 14a) suggesting that kaempferol is the major pigment. Trace amounts of a spot running similarly to quercetin point to presence of a second minor pigment, possibly quercetin. One major blue spot was evident in the hydrolysed extract of the root of both lentil lines. Several other minor blue and yellowish green spots were also visible (Fig. 14b). None of the spots co-chromatographed with the standard used.

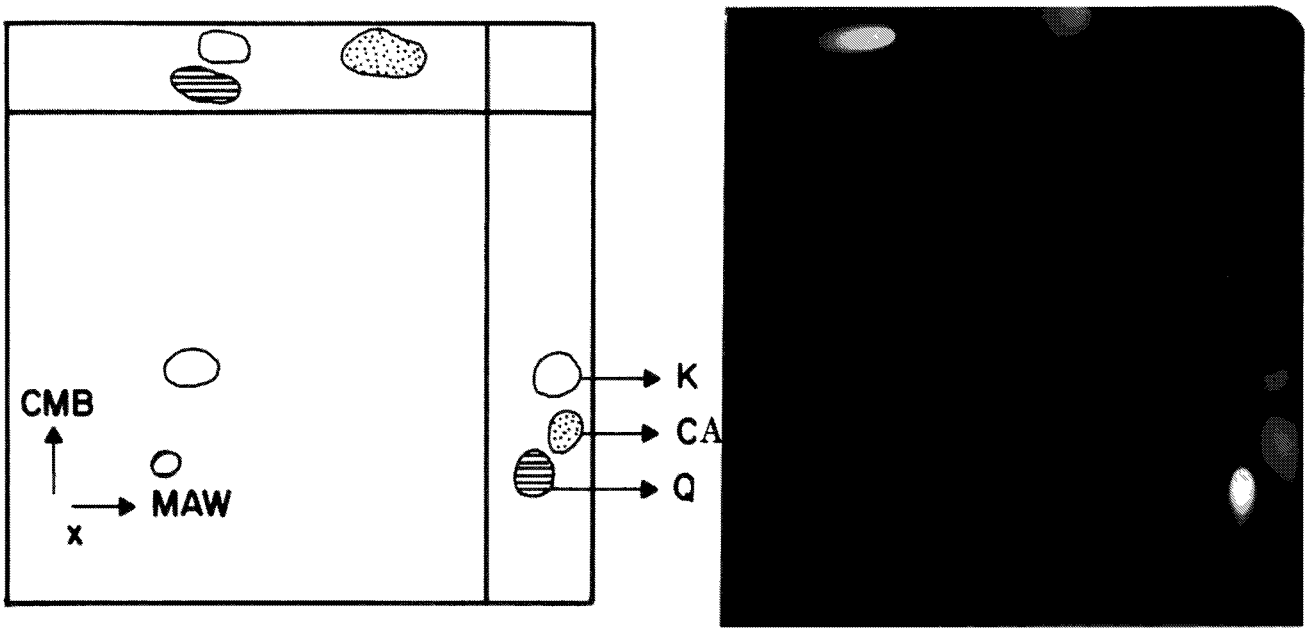
3.3.1.1 DISTRIBUTION OF FLAVONOID GLYCOSIDES IN THE VEGETATIVE PARTS OF ESTON AND P.I.345635:

Alcoholic extracts of each organ i.e. leaf, stem and root of Eston were found to have a characteristic flavonoid glycoside pattern (Figs. 15,16,17). Shoot parts, leaf and stem, showed an abundance of green spots, presumably kaempferol glycosides with a limited number of yellow spots, possibly quercetin glycosides interspersed with them (Figs. 15,16). The lesser amounts of flavonoid glycosides in leaf and especially the stem of P.I.345635 are apparent from the photographs (Figs. 15c, 16c). The distribution pattern of green and yellow spots in Eston

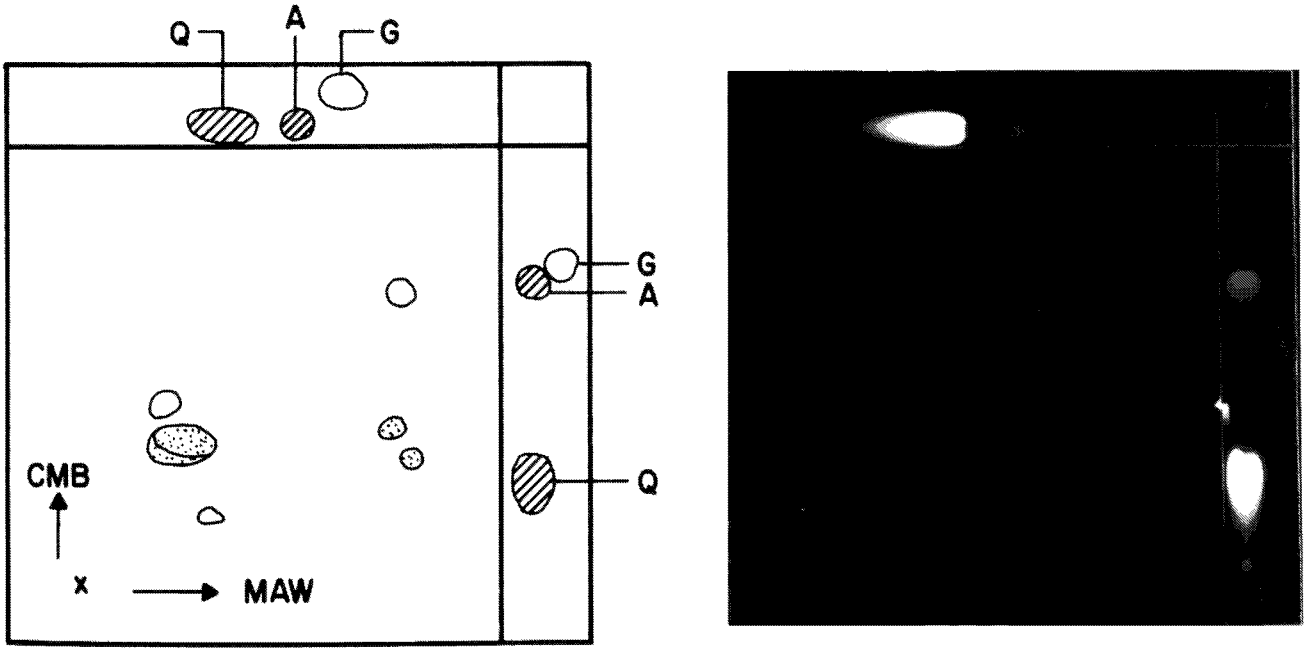
Figure 14. Drawings and photographs of chromatographic patterns of the aglycones of the vegetative parts of Eston and P.I.345635 on polyamide. Solvent systems used were chloroform:methanol:butanone (12:2:1 v/v) (CMB) in the vertical direction and methanol:acetic acid:water (18:1:1) (MAW) in the horizontal direction. Standard compounds were always spotted in the side lanes in two channels: usually quercetin (Q) and apigenin (A) in one and caffeic acid (CA) and kaempferol (K) or genistein (G) in the other. Under UV light and after spraying with flavone reagent, Q and QR appears yellow orange (hatched spot), K green, (blank spot), A dull green (barred spot), G dull yellow (blank spot), and C fluorescent blue (stippled spot).

(a) Aglycones present in a leaf extract of 80 day-old Eston plants. A similar pattern was obtained for stems of Eston and leaf and stems of P.I.345635 at all stages of growth.

(b) Aglycones of root extract of 80 day-old Eston plants. A similar pattern was obtained at all stages of root of Eston and of P.I.345635.



a



b

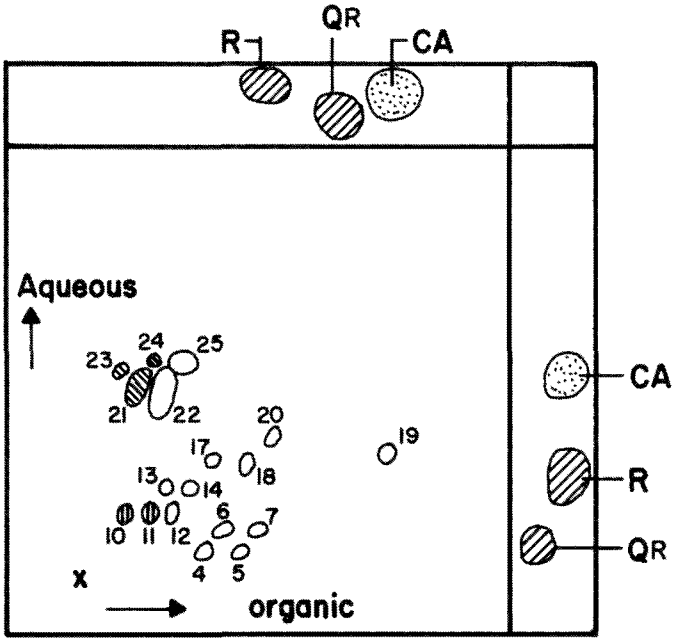
Figure 15. Photographs and drawing of typical 2-D chromatograms of flavonoid glycosides of leaves of Eston and P.I.345635. The crude extracts were chromatographed in an amount equivalent to 125 ug of dry tissue on polyamide in an aqueous solvent mixture (water:pyridine:cyclohexanone 90:5:5 v/v) in vertical direction and in an organic solvent mixture (n-butyl acetate:methanol:formic acid:water 60:30:5:5 v/v) in horizontal direction, sprayed with flavone reagent and illuminated by ultraviolet light. Standards were rutin (R), quercetrin (QR) and caffeic acid (CA) run in side lanes in 2 channels, QR (hatched spot) and R (hatched spot) in one and CA (stippled spot) in the other. Occasionally overloading of standards or overwetting of the plates during spraying resulted in smearing of the spot but did not affect determination of mobility.

(a) Extract of 80 day old Eston plant (tissue extracted fresh).

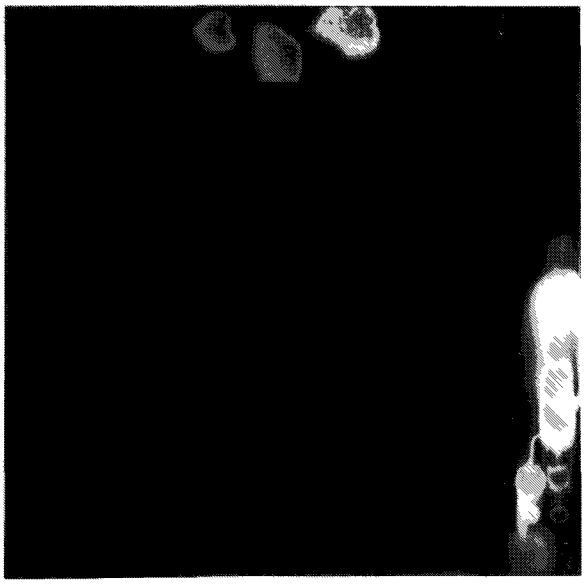
(b) Drawing of typical leaf flavonoid pattern. Individual spots are numbered in order of increasing mobility in aqueous solvent, then in order of increasing mobility in organic solvent. Yellow spots (putative quercetin) are hatched, blue spots are stippled and green spots (putative kaempferol) are clear.

(c) Extract of 80 day-old P.I.345635 plant (extract from dried tissue).

a


R
QR
CA
Aqueous
organic
x
24
25
23
21
22
20
19
17
18
13
14
6
7
10
11
12
4
5
CA
R
QR

b



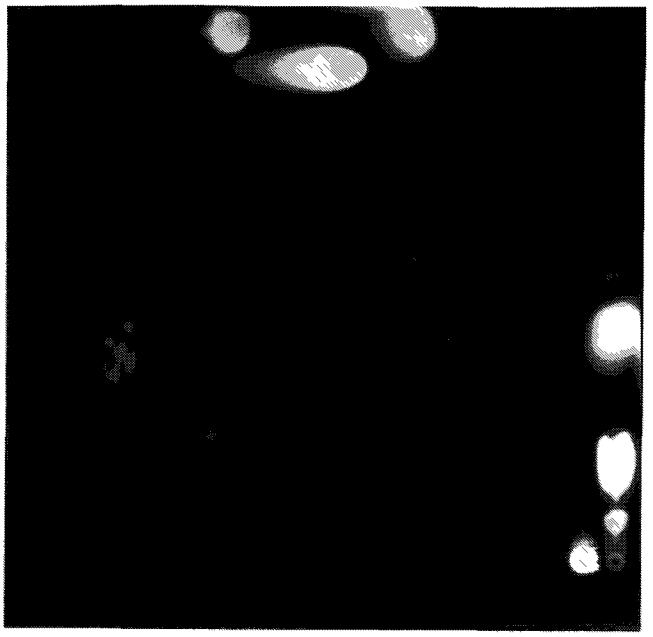
c

Figure 16. Photographs and drawings of typical 2-D chromatogram of flavonoid glycosides of stems of Eston and P.I.345635. Chromatographic conditions as for Fig. 15.

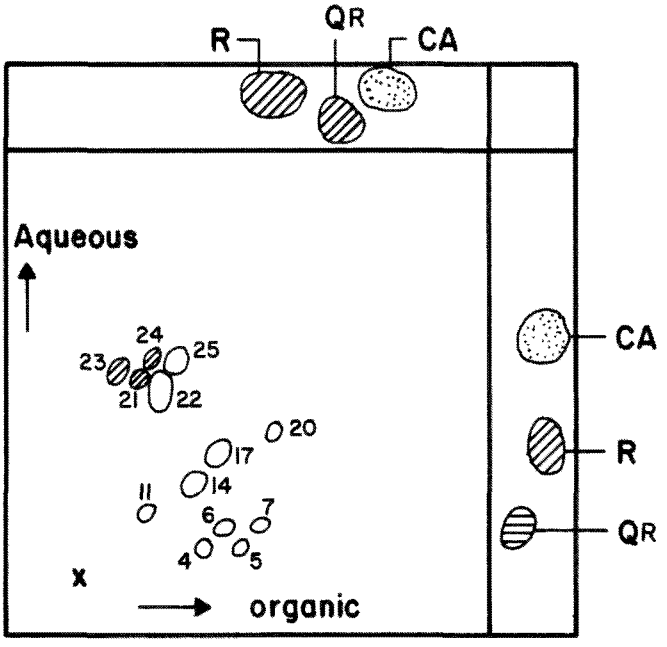
(a) Extract of 45 day-old Eston.

(b) Drawing of typical stem flavonoid pattern. Individual spots are numbered as in Fig. 15. Spots with the same number are presumed to be similar compounds. Yellow spots are hatched, green spots are clear.

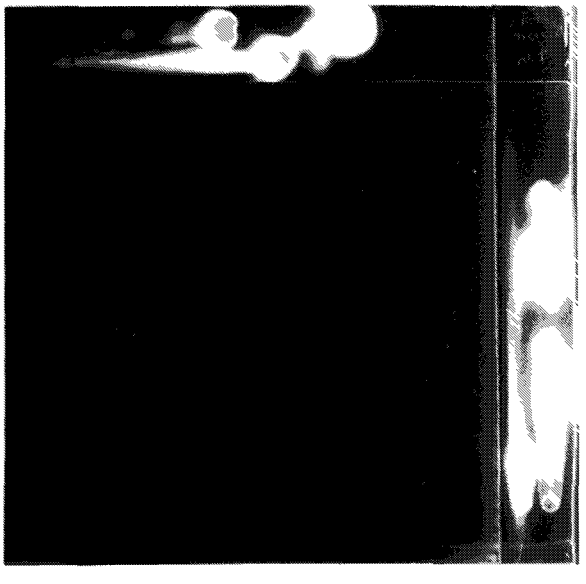
(c) Extract of 45 day-old P.I.345635 plant.



a



b



c

leaf and stem is very similar to that of leaf and stem of P.I.345635 as summarized in Table 9 and shows some change with age. The number of green spots (abbreviated as K in the following text) in leaf and stem of both lentil lines is more than yellow ones (abbreviated as Q in the following text). K4,5,6,7,14,17,20,22 and 25 are common to both leaf and stem (Table 10). Leaf has K12 and 18 only in older leaves that does not show up in the stem whereas K11 exists only in the stems at all stages. Similarly, Q21,23,24 are present at all stages of both leaf and stem but the leaf has in addition Q10 and Q11, the former being absent from young leaves. Only older leaf tissues have blue spot #19.

The root had a totally different glycoside pattern which showed the predominance of a few blue spots (Fig. 17) with no significant mobility in the aqueous solvent. The major compounds in lentil roots have been recently identified by D'Arcy-Lameta (1986) to be three desoxy-5 flavones (4',7-dihydroxy, 3',4',7-trihydroxy, 4',7-dihydroxy, 3'-methoxyflavones. The mobility of the latter would be highest in organic solvent (Wollenweber 1982) and it should therfore correspond to #3 on the chromatogram shown in Fig. 17b. The trihydroxy would have the lowest mobility and probably is spot #1, thus leaving spot #2 as the 4',7-dihydroxyflavone. No obvious change in flavonoid pattern of roots was observed from 20 days until 80 days after sowing the seeds.

3.3.2 AGLYCONE DISTRIBUTION IN THE REPRODUCTIVE PARTS (PERICARP AND PETALS) OF ESTON AND P.I. 345635:

Hydrolysed alcohol extracts of the petals of Eston showed only the presence of a bright yellow spot which had the same colour and Rf value as that of standard quercetin (Fig. 18a). Hydrolyzed alcohol extracts of the petal of P.I.345635 showed the presence of both green and yellow spot (Fig. 18b). The green spot had the same Rf value as that of standard kaempferol

Table 9 : Flavonoid glycosides present in the aerial parts of two lentil lines, Eston and P.I. 345635, at various stages of growth.

Plant organ and age	Green spots *													Yellow spots *				
	4	5	6	7	11	12	13	14	17	18	20	22	25	10	11	21	23	24
Leaf																		
(20 day old)	x	x	x	x	-	x	-	x	x	x	-	x	x	-	x	x	x	x
(45 " ")	x	x	x	x	-	x	-	x	x	x	-	x	x	-	x	x	x	x
(65 " ")	x	x	x	x	-	x	x	x	x	x	x	x	x	x	x	x	x	x
(80 " ")	x	x	x	x	-	x	x	x	x	x	x	x	x	x	x	x	x	x
Stem																		
(20 day old)	-	x	x	x	x	-	-	x	x	-	x	x	x	-	-	x	x	x
(45 " ")	-	x	x	x	x	-	-	x	x	-	x	x	x	-	-	x	x	x
(65 " ")	x	x	x	x	x	-	-	x	x	-	x	x	x	-	-	x	x	x
(80 " ")	x	x	x	x	x	-	-	x	x	-	x	x	x	-	-	x	x	x

* A low number (e.g. "green" 4) indicates that few sugars are attached to the flavonol and a high number (e.g. "green" 25) indicates a high degree of glycosylation. Similar nos. in the "yellow" series indicate a similar degree of glycosylation as determined by chromatographic behaviour (Wollenweber 1982).

Table 10 : Flavonoid glycosides present in the reproductive parts of two lentil lines, Eston (tannin) and P.I. 345635 (non-tannin). The same compounds were present at all stages of growth.

Plant organ	Lentil line	Green spots						Yellow Spots											Blue spots	
		9	16	18	22	24	26	7	8	10	11	12	15	21	24	25	27	28	30	20	29
Petal	Eston	x						x	x				x								
	P.I. 345635	x	x	x	x	x		x	x	x	x	x	x						x		
Peri-carp	Eston	x						x	x				x				x				
	P.I. 345635			x	x		x							x	x	x		x		x	x

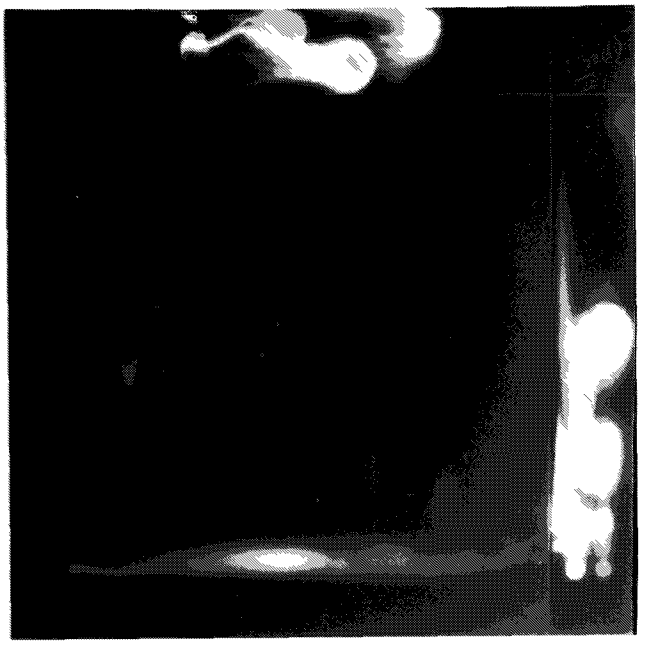
(NB) Attempt was made to assure that numbers used for reproductive parts were similar to those for vegetative parts.

Figure 17. Photographs and drawings of typical 2-D chromatogram of flavonoid glycosides of roots of Eston and P.I.345635. Chromatographic conditions as in Fig.15.

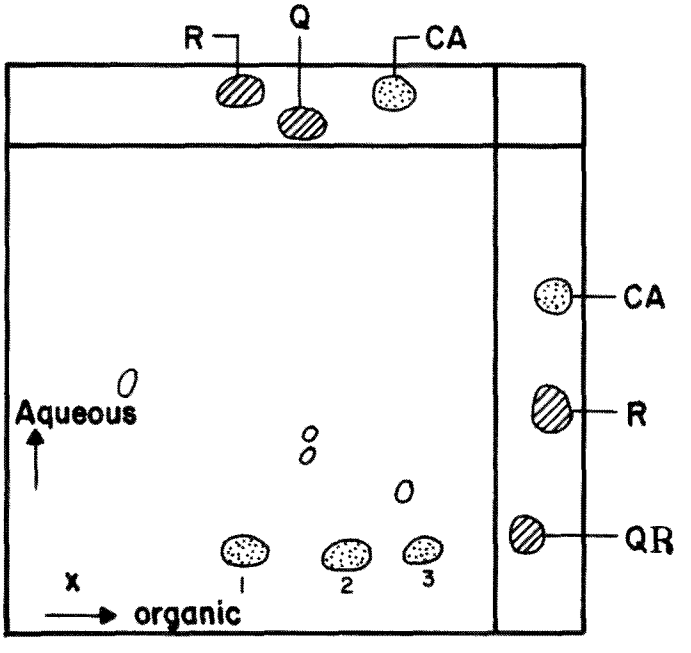
(a) Extract of 80 day-old root of Eston.

(b) Drawing of typical root pattern. Only major spots that stand out from the yellow smear produced in the organic solvent are indicated. Their blue fluorescence is shown by stippling.

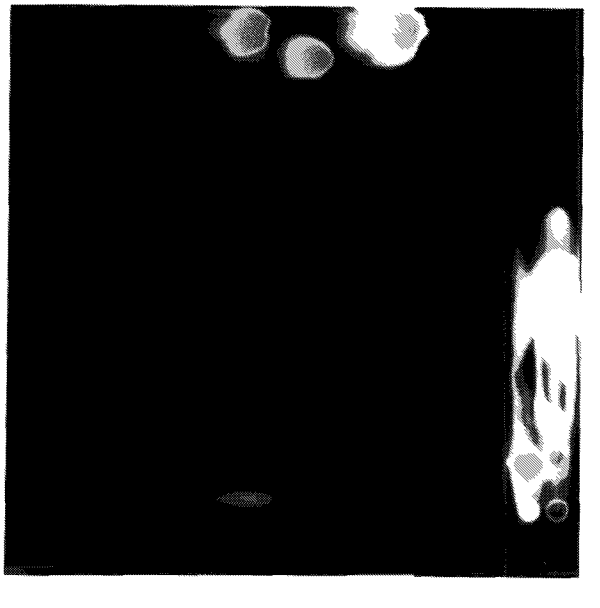
(c) Extract of 80 day-old root of P.I.345635.



a



b



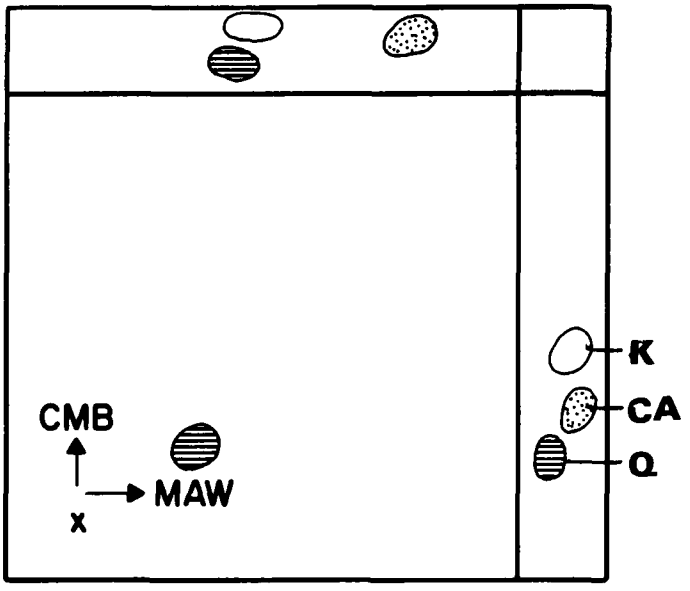
c

Figure 18. Photographs and drawing of 2-D chromatograms of petals of Eston and P.I.345635 showing aglycone distribution. Chromatographic conditions and standards as in Fig. 14.

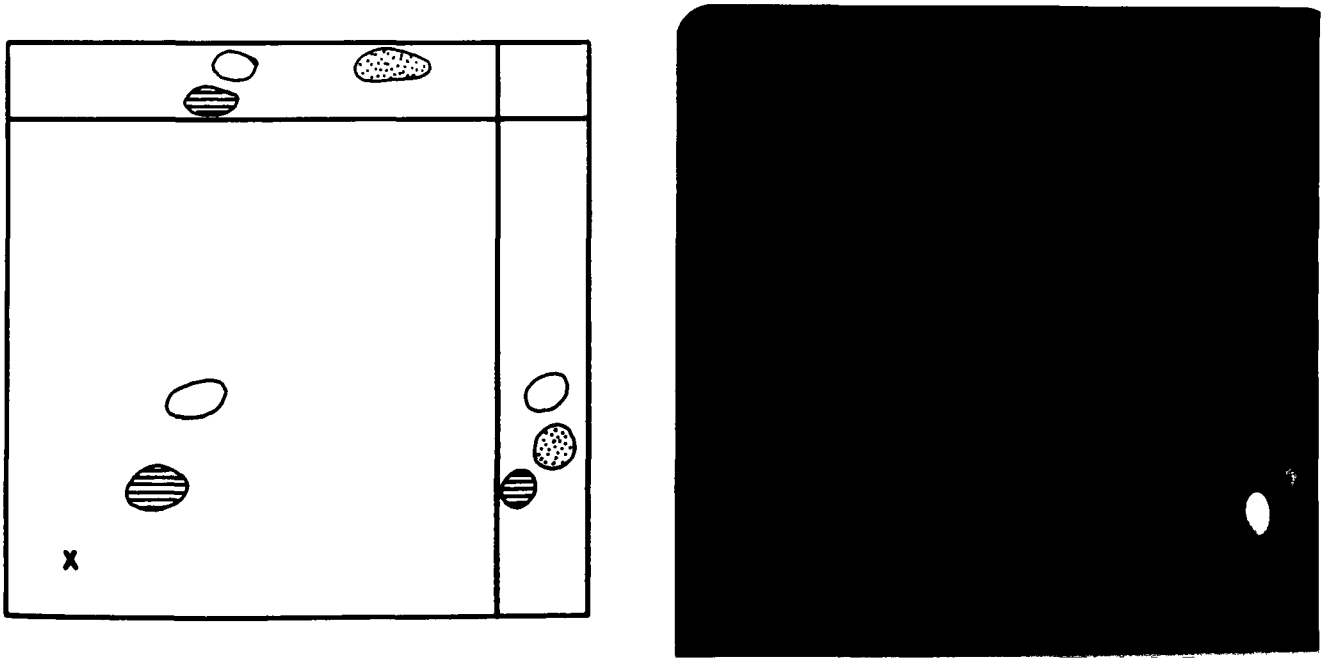
(a) Drawing of Eston petal.

(b) (i) Drawing and photograph of P.I.345635 petal.

(ii) Photograph of P.I.345635 petal.



a



b

whereas the yellow spot showed the same chromatographic behaviour as that of standard quercetin. Hydrolysed alcoholic extract of the pericarp of Eston similarly had only 1 yellow spot (Fig. 19a). The pericarp of P.I.345635 showed the presence of only a green spot with Rf value similar to that of standard kaempferol (Fig. 19b).

3.3.2.1 DISTRIBUTION OF FLAVONOID GLYCOSIDES IN THE REPRODUCTIVE PARTS (PERICARP AND PETALS) OF ESTON AND P.I.345635:

The petals of Eston predominantly contained quercetin glycosides (bright yellow spots) with only one spot of kaempferol (green spot) evident amongst the quercetin spots (Fig. 20a).

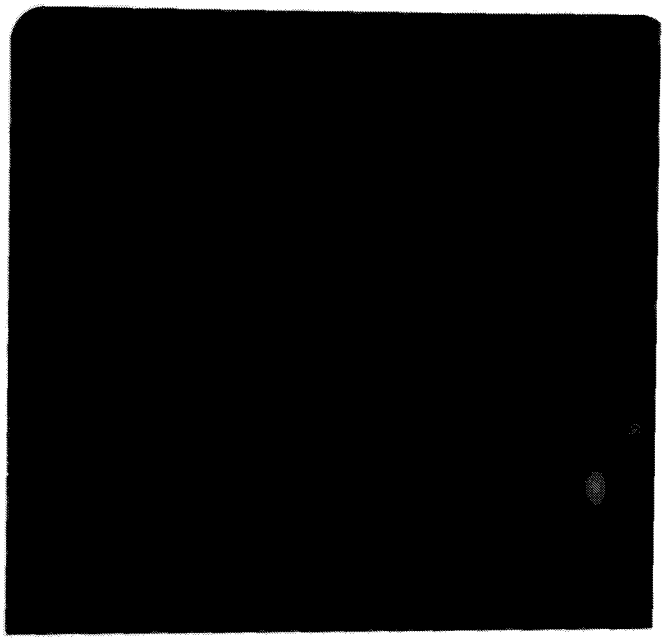
A distinct contrast in the glycosidic patterns of the petals of P.I.345635 was clearly discernable upon comparing their chromatographic fingerprints. In addition to the presence of quercetin glycosides in petals of P.I.345635 a number of extra spots similar to those found in leaves (#'s 10-24) ranging in colour from dull yellow to green were also detected (Fig. 20b). One spot (#30) was highly glycosylated and/or acylated.

The pericarp of Eston contained only the same bright yellow quercetin spots (nos. 7,8,15) with high mobility in organic solvent as were present in the petal (Fig. 21a) plus an additional one (#27) of high mobility in aqueous solvent. Pericarp extracts of P.I.345635 had a totally different flavonoid pattern from that of Eston (Fig. 21b). Apart from the presence of yellow and green spots, some blue spots were detected (Fig. 21b). The yellow and green spots (21,22,24,25,26) had mobility in the aqueous solvent, very similar to that of the spots present in the leaf. No yellow spots with high mobility in organic solvent were visible in this lentil line.

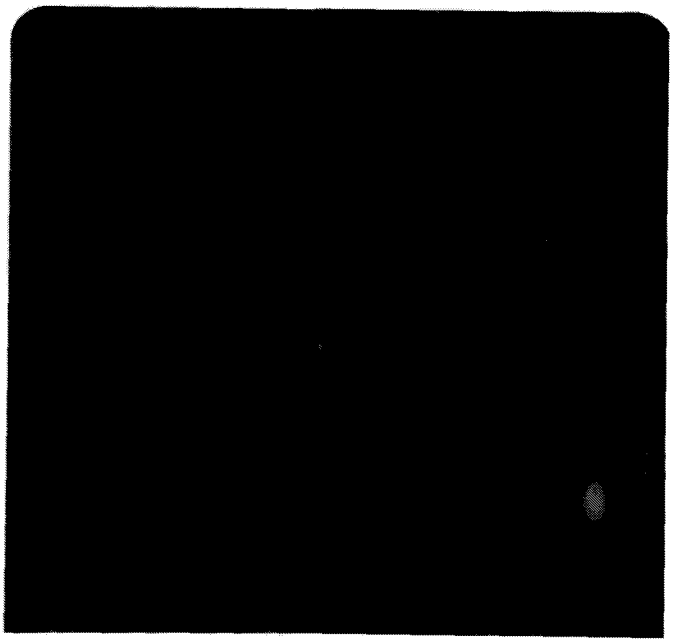
Figure 19. Photographs of the chromatographic patterns of the aglycones of the pericarp (ovary-wall) of Eston and P.I.345635 showing aglycone distribution. Chromatographic conditions as in Fig. 14.

(a) Photograph of Eston pericarp.

(b) Photograph of P.I.345635 pericarp.



a



b

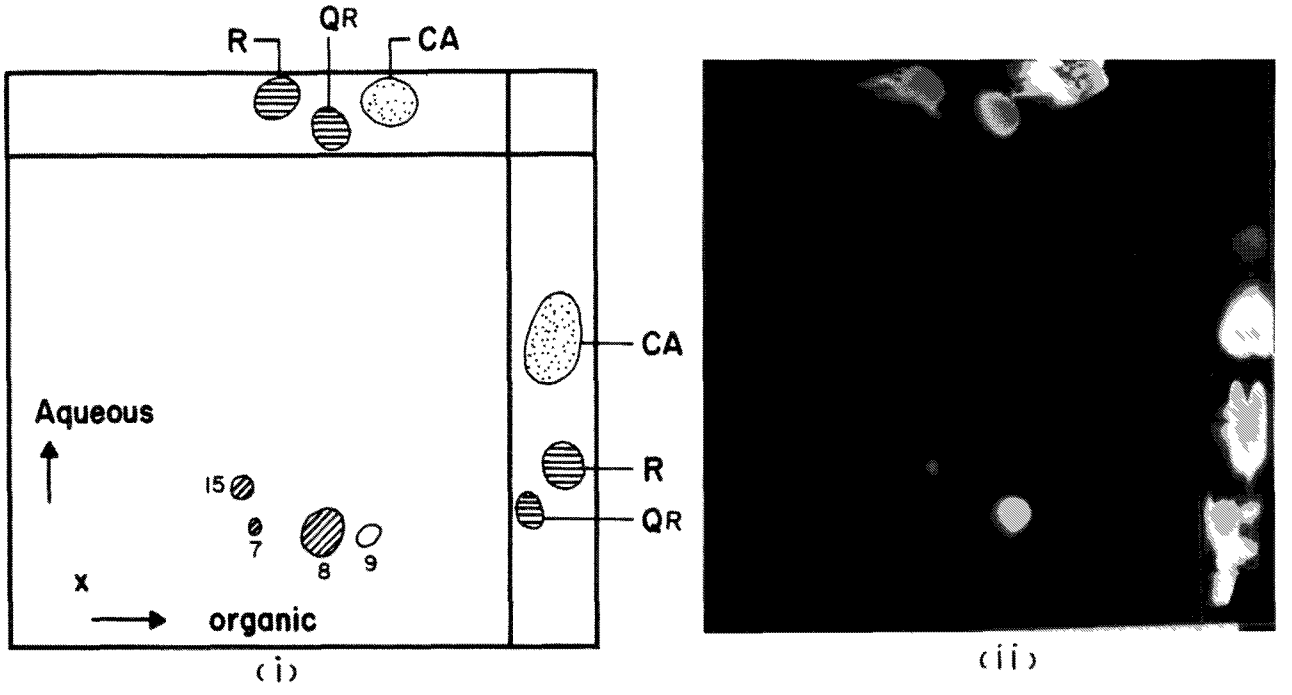
Figure 20. Photographs and drawings of typical 2-D chromatograms of flavonoid glycosides a of the petals of Eston and P.I.345635. Chromatographic conditions as in Fig. 15.

(a) (i) Drawing of Eston petal pattern

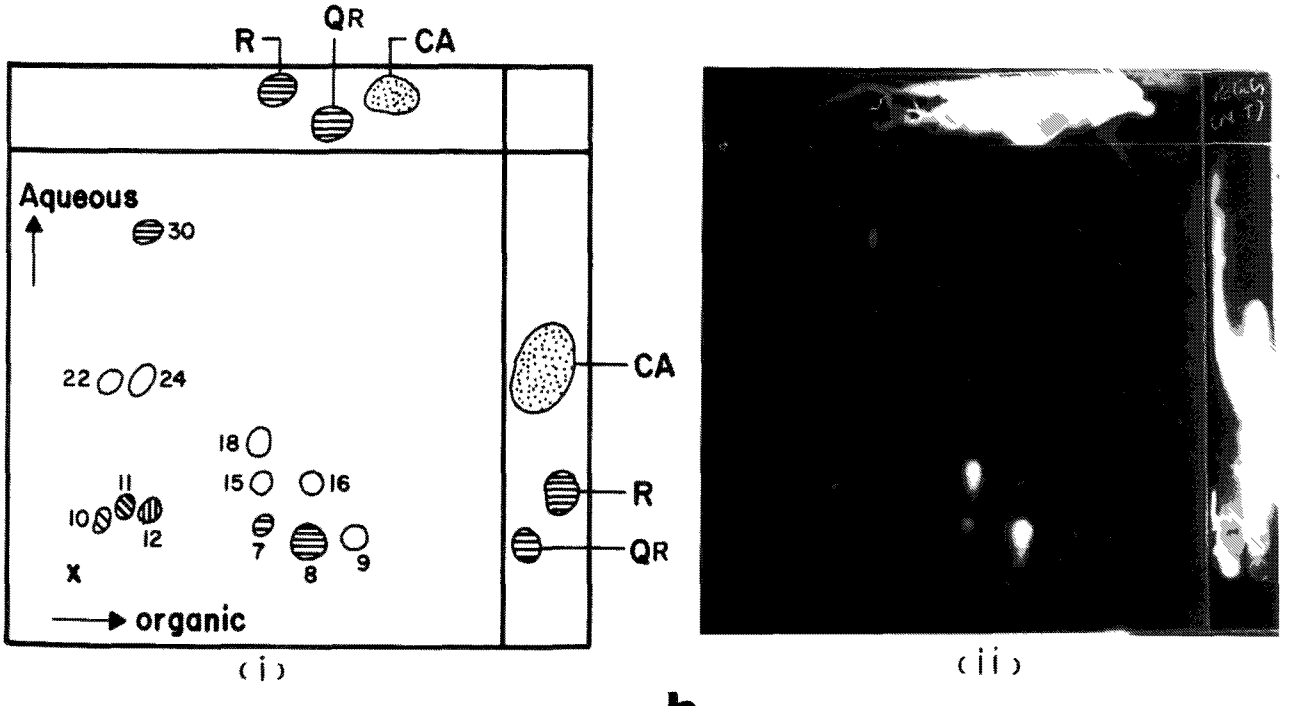
(ii) Photograph of extract of Eston petal

(b) (i) Drawing of P.I.345635 petal pattern

(ii) Photograph of chromatogram of extract from P.I.345635 petal


R
QR
CA
Aqueous
15
7
8
9
x
organic
CA
R
QR
(i)
(ii)

a


R
QR
CA
Aqueous
30
22
24
18
15
16
11
10
12
7
8
9
x
organic
CA
R
QR
(i)
(ii)

b

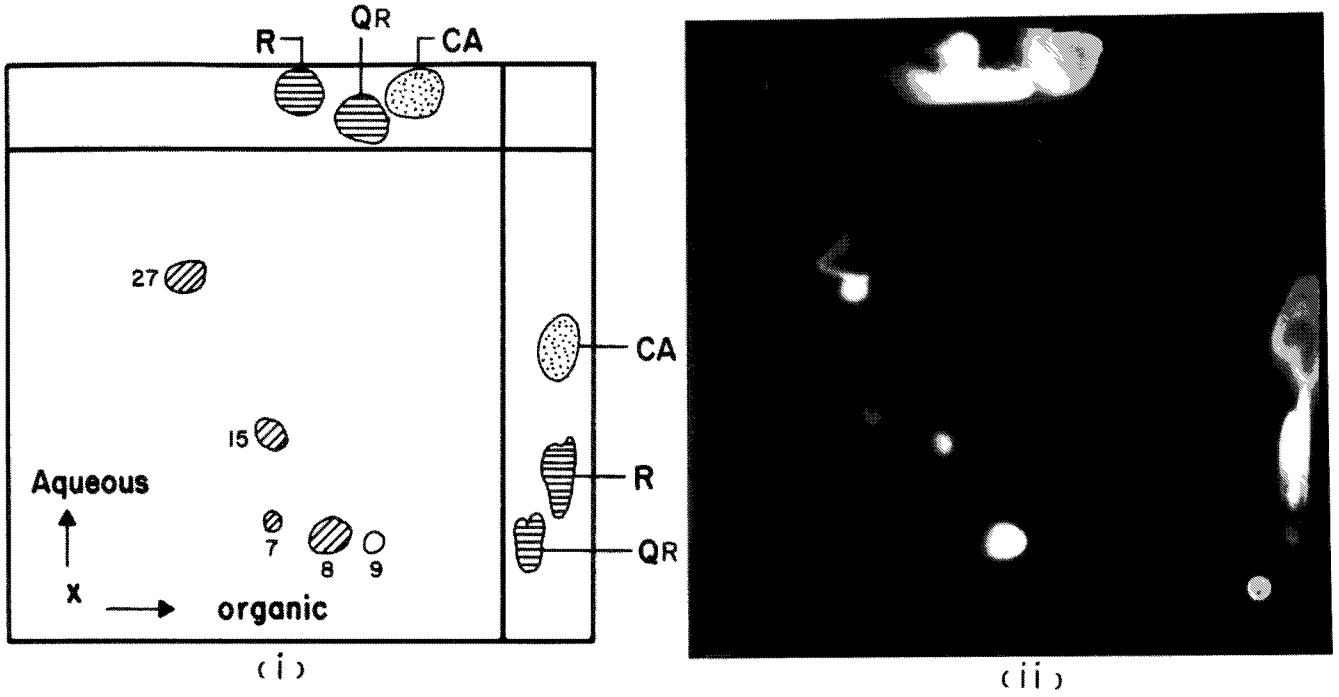
Figure 21. Photographs and drawings of typical 2-D chromatograms of flavonoid glycosides of pericarp of Eston and P.I.345635. Chromatographic conditions as for Fig. 15.

(a) (i) Drawing of Eston pericarp pattern

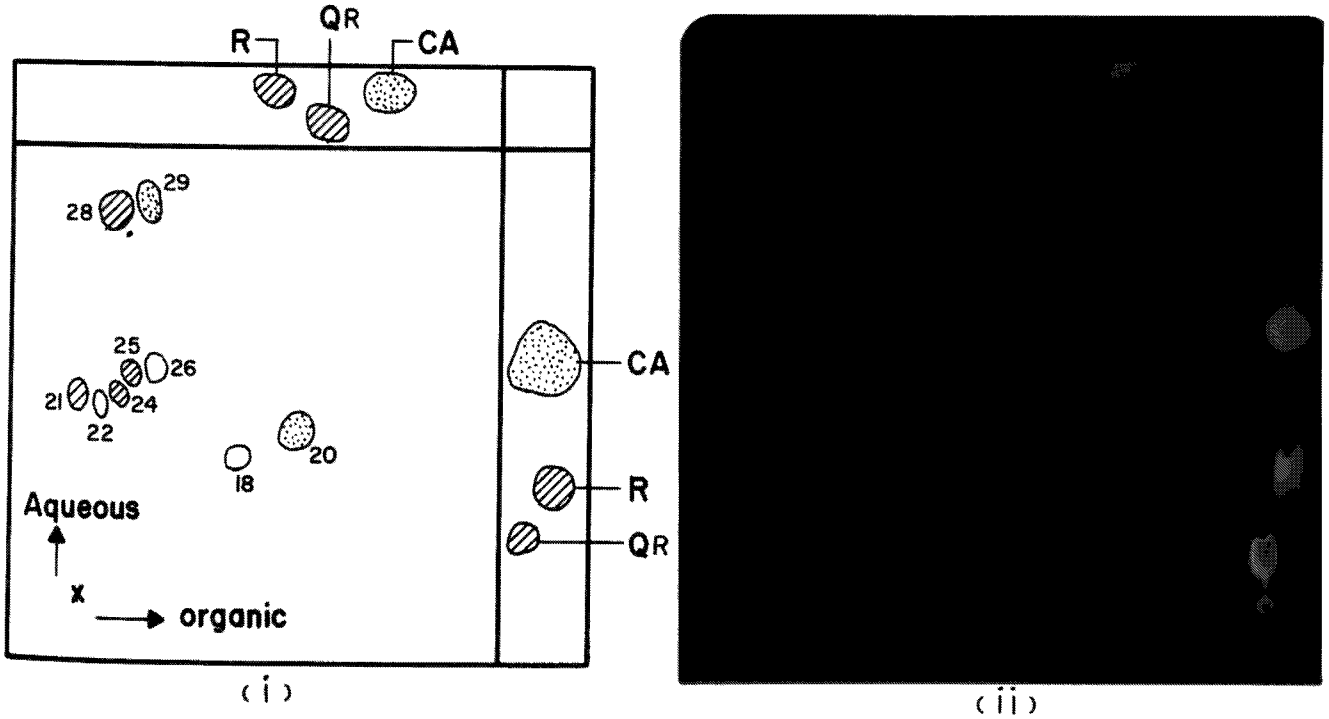
(ii) Photograph of chromatogram of pericarp of Eston. This particular chromatogram was accompanied by a chromatogram of a stem extract on the reverse side of the 2-sided polyamide sheets. As a result, additional spots of the stem extract show through (cf. Fig. 16a).

(b) (i) Drawing of P.I.345635 (pericarp) pattern.

(ii) Photograph of chromatogram of extract from pericarp of P.I.345635.


R
QR
CA
27
15
7
8
9
Aqueous
X
organic
CA
R
QR
(i)
(ii)

a


R
QR
CA
28
29
25
26
21
24
22
20
18
Aqueous
X
organic
CA
R
QR
(i)
(ii)

b

Table 10 summarizes the various glycoside spots in the reproductive parts of both lentil lines. No obvious differences with development were noted. Spot numbers correspond with numbers used for vegetative parts. K9 is common to the petals of both Eston and P.I.345635. No other green spot was visible on the chromatogram of Eston petal whereas several (K16,18,22 and 24) were present in the non-tannin line. Three yellow spots, Q7,8 and 15 were common to both Eston and P.I.345635 petal, but P.I.345635 had a few more (Q10,11,12 and 30). None of the spots of the Eston pericarp were held in common with the pericarp of P.I.345635. In Eston, only K9 and Q7,8 and 15 were found in both petal and pericarp. In addition the pericarp had Q27, absent from the petal. In P.I.345635, only K18, 22 were found in both pericarp and petal. Highly glycosylated K26, Q28 were found in the pericarp whereas the petal had K9, K16, K24, Q7, 8, 10, 11, 12, 15 and 30. Only the pericarp of P.I.345635 had blue spots.

The bright yellow spot (Q8) present in the unhydrolyzed extracts of pericarp and petals of Eston was isolated and purified, and subjected to further tests as shown in Table 11. This lentil pigment and standard quercetrin showed similar Rf values when run in several solvent systems. When the diagnostic reagents, sodium methoxide, sodium acetate, sodium acetate and boric acid were added to a methanolic solution of this pigment, spectra obtained were very similar to the ones cited in the literature (Table 11) (Mabry et al. 1970). The retention time on HPLC for both the pigment and standard quercetrin was similar (about 3.5 minutes; Table 11).

The lentil pigment (Q8) from pericarp and petal was hydrolyzed in order to separately identify the sugar and the aglycone. The aglycone was again run along with standard quercetin on polyamide and cellulose in different solvents and their Rf values came out to be quite similar (Table 11). The hydrolyzed pigment and standard quercetin were spotted together on polyamide and run in n-butyl acetate:methanol: formic acid:water without any indication

Table 11 : The results of tests for the identification of Quercetin 3 - rhamnoside (Quercetrin).

Chromatography on	Solvents used	Putative Quercetrin Rf x 100	Standard Quercetrin Rf x 100
Polyamide	water:pyridine: cyclohexanone	14	12
	n-butyl acetate: methanol: formic acid: water	53	54
Cellulose	BAW	71	72
	water	18	18
	15% acetic acid	46	48
Spectral data	Diagnostic reagents	Absorbance peaks nm.	
	MeOH	260, 350	256 350
	NaOMe	270, 330, 400	270 326 393
	NaOAC	270, 355	272 372
	NaOAC + H_3BO_3	260, 370	260 367
HPLC	Retention time in minutes	3.5	3.4
Chromatography on	Solvents used	Putative Quercetin Rf x 100	Standard Quercetin Rf x 100
Polyamide	water:pyridine: cyclohexanone	16	15
	n-butyl acetate: methanol: formic acid: water	31	36
Cellulose	BAW	8	6
	water	0	0
	15% acetic acid	6	5
Cellulose	ethyl acetate: pyridine: water	unknown sugar .66	Rhamnose .69

of separating into two different spots.

The sugar fraction when co-chromatographed on cellulose thin layer chromatogram with glucose, arabinose and rhamnose and run in ethyl acetate:pyridine:water showed the same Rf value as rhamnose. When standard rhamnose and the unknown sugar were spotted together, they ran to the same spot as shown in Fig. 22 and Fig. 23.

All these tests clearly indicated that the bright yellow spot present in petals and ovary wall of Eston was quercetrin (quercetin-3 rhamnoside).

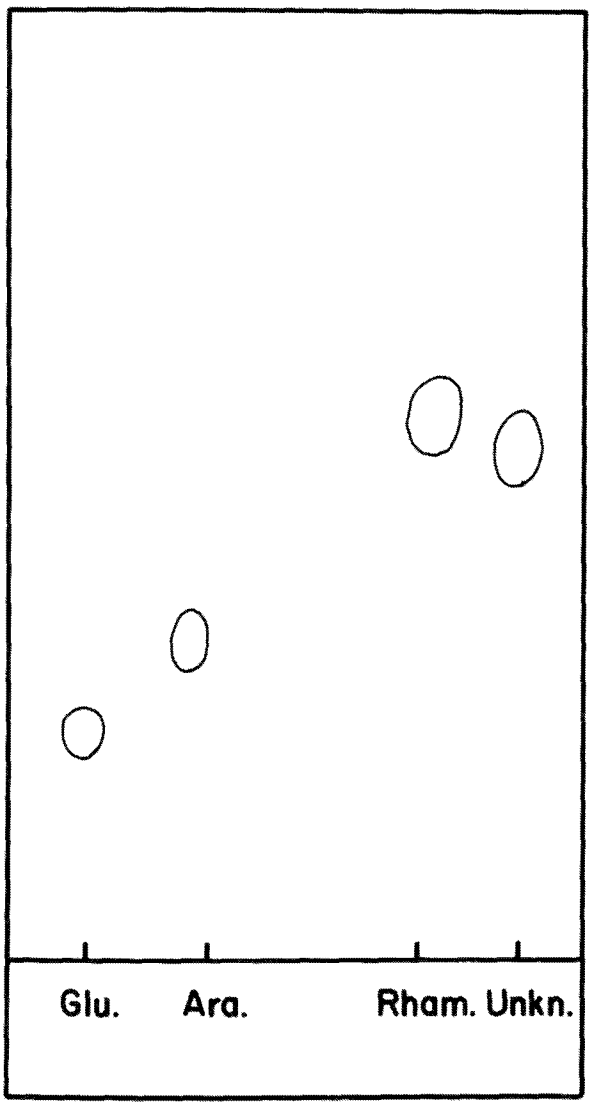
3.3.3 AGLYCONES IN THE TESTA AND EMBRYO OF ESTON AND P.I.345635:

Two green spots were present in the hydrolyzed extract of Eston testa (Fig. 24a). Only one light green spot showed up in P.I.345635 testa (Fig. 24b) with similar Rf value as that of standard kaempferol. D'Arcy and Jay (1978) reported that the lentil testa contains two flavones, tricetin and luteolin, but the aglycones of both Eston and P.I.345635 testa had Rf values very different from those reported for tricin and luteolin (Table 12).

Hydrolysed extract of Eston and P.I.345635 embryo showed the presence of two green spots (Fig. 25). The Rf value and colour of one of the spots (#1) was similar to kaempferol. A kaempferol and 5-deoxykaempferol have been identified by D'Arcy and Jay (1978) in lentil embryo (cotyledons). It is possible that the spot #2 in the embryo of these two lentil lines is 5-deoxykaempferol.

3.3.3.1 FLAVONOID GLYCOSIDES IN THE TESTA AND EMBRYO OF ESTON AND P.I.345635:

Figure 22. Drawing and photograph of cellulose thin layer chromatogram showing the unknown sugar and standard sugars, glucose, arabinose and rhamnose. The chromatogram was run in ethyl acetate:pyridine:water (10:3.2:2), dried and sprayed with aniline hydrogen phthalate. Glucose and rhamnose are brown, arabinose is pink.


Glu.
Ara.
Rham.
Unkn.

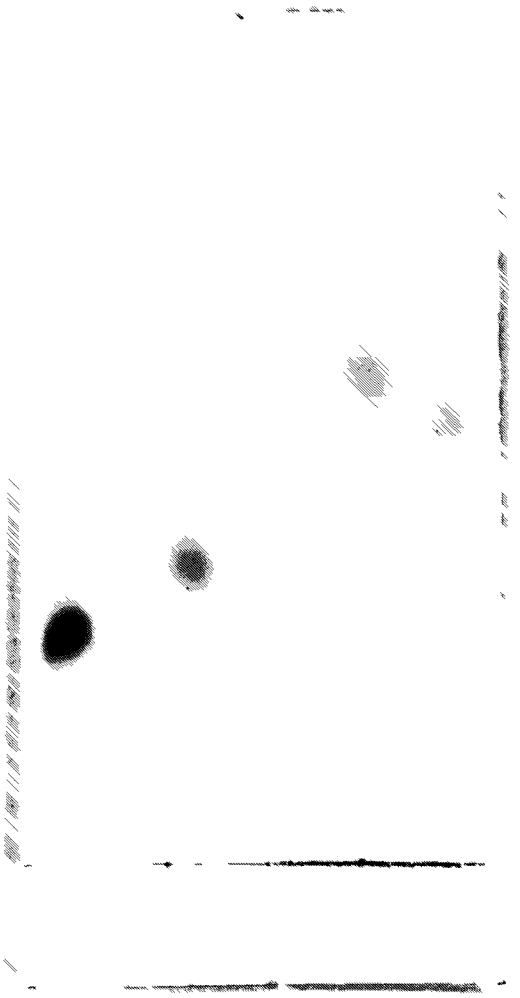


Figure 23. Drawing and photograph of cellulose thin layer chromatogram showing standard and unknown sugars (dark spot) run separately and together. Chromatographic conditions as in Fig. 22.

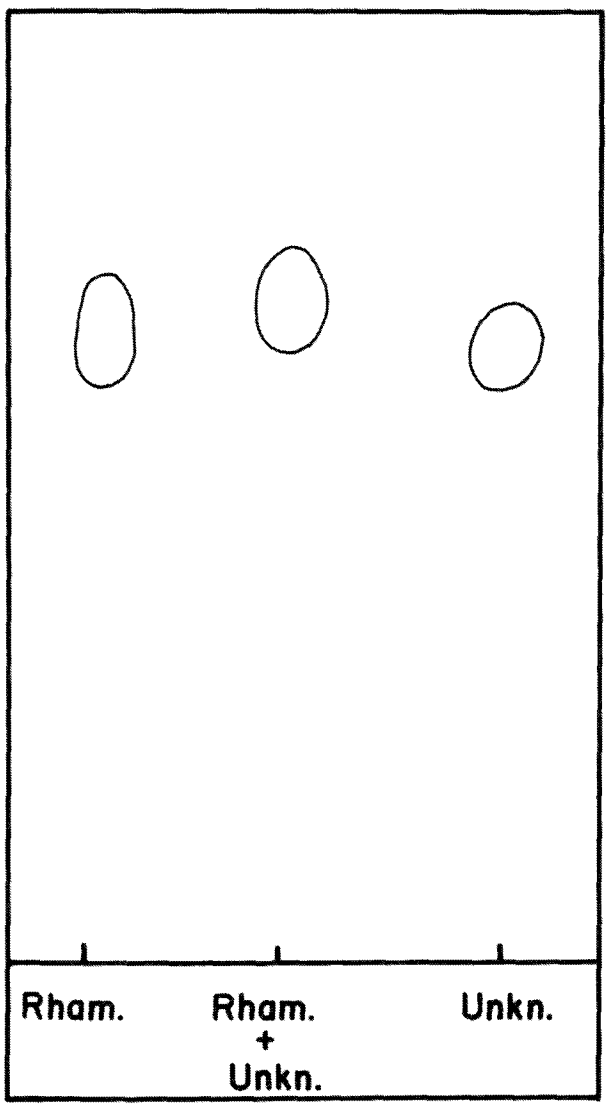

Rham.
Rham.
+
Unkn.
Unkn.

Table 12 : Rf values of aglycone, present in Eston testa, as compared with Rf values given in the literature (Classen 1981).

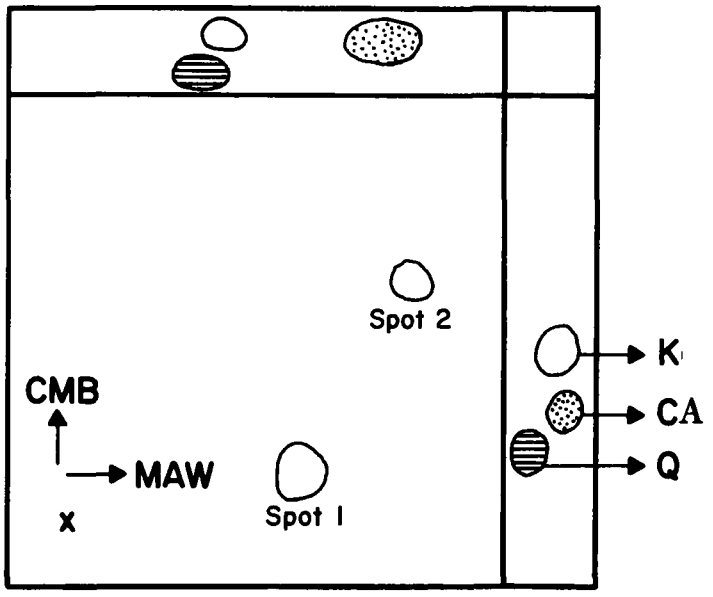
	Rf values in CMB	MAW
Luteolin (classen 1981)	.19	.22
Tricin "	.73	.25
*Quercetin "	.09	.16
*Kaempferol "	.20	.18
*Quercetin (present study)	.18	.31
*Kaempferol "	.43	.34
Spot 1 (Fig. 25a)	.12	.54
Spot 2 (Fig. 25a)	.56	.80
Spot 1 (Fig. 24b)	.43	.30

* The Rf values of standard quercetin and kaempferol (in both CMB and MAW) used in the present study are approximately double the value from the same standards used by Classen (1981). But the Rf values of spot 1 and spot 2 do not show the same ratio (double the value) with standard luteolin and tricin indicating thereby that these could be some other compounds.

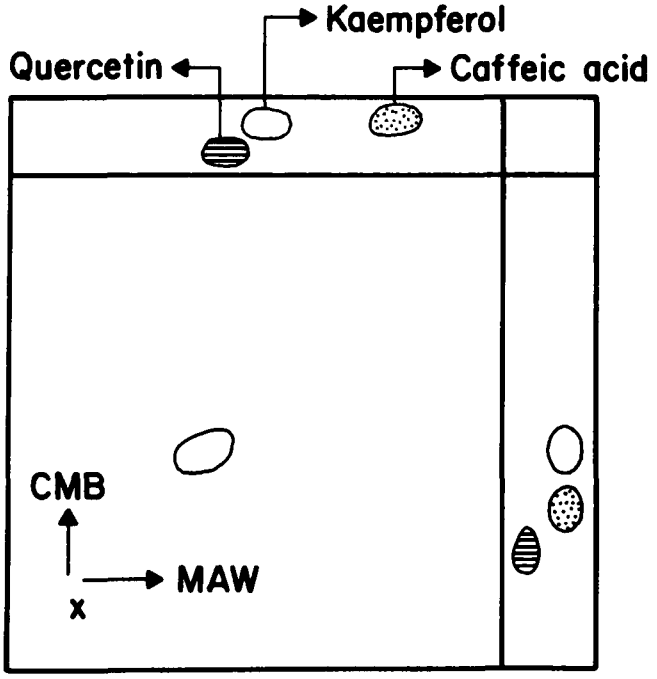
Figure 24. Drawings of the chromatographic patterns of the aglycones of the testa of Eston and P.I.345635. Chromatographic conditions as in Fig. 14.

(a) Eston testa

(b) P.I.345635 testa


Spot 2
K
CA
Q
CMB
MAW
X
Spot I

a

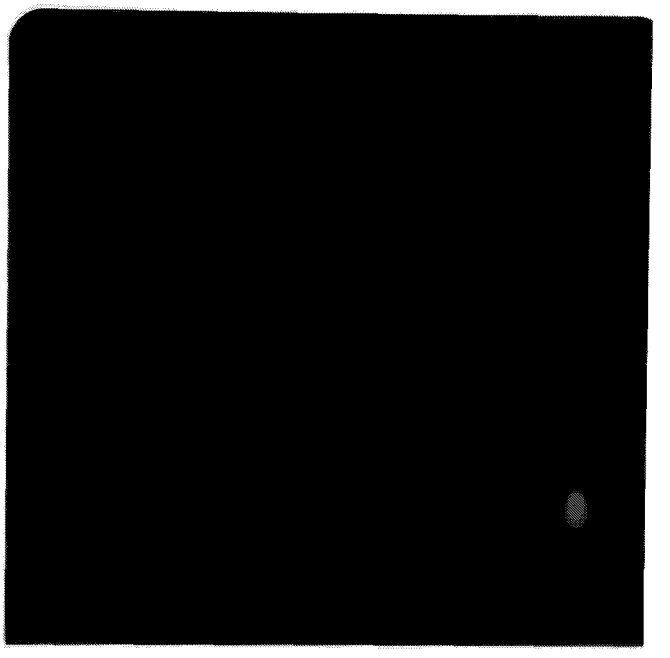

Kaempferol
Quercetin
Caffeic acid
CMB
MAW
X

b

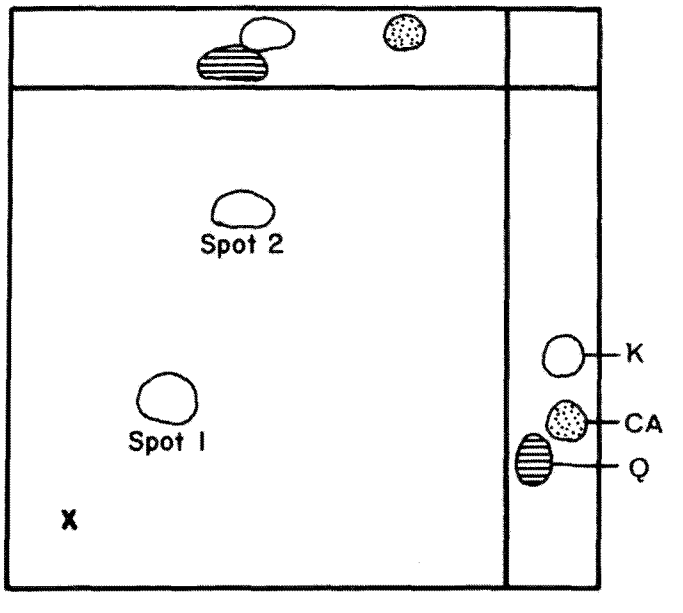
Figure 25. Photograph and drawing of the chromatographic patterns of the aglycones of the embryo (cotyledons) of Eston and P.I.345635. Chromatographic conditions as in Fig. 14. The distribution pattern was similar in both these lentil lines.

(a) Photograph of extract of Eston embryo.

(b) Drawing of the 2-D chromatogram of both Eston and P.I.345635.



a



b

Only green spots, but several of them, could be detected in the extract of Eston testa (Fig. 26a). Only one spot, green, was present in Eston embryo (Fig. 26b). No spots were visible at the concentrations used on chromatograms made from crude extracts of P.I.345635 testa and embryo. This indicates very low levels of flavonoids in embryo and testa of this lentil line.

3.3.4 FLAVONOIDS IN EXTRACTS OF TESTA SPRAYED WITH TURNBULL'S REAGENT:

When 2-D chromatograms of vegetative or floral parts from both lentil lines were sprayed with Turnbull's blue reagent, compounds that reacted with flavone reagent stained a blue colour characteristic of phenolic compounds. Both flavone reagent and Turnbull's reagent seemed equally sensitive to detect these compounds in leaves, stem, root and petals. In extracts of Eston testa, however, some extra spots were detected with Turnbull's reagent. These were present even in the youngest testa examined (first stage, 11 days after anthesis) (Fig. 27a) and are probably proanthocyanidin oligomers and polymers. Most of the compounds remained at the origin streaking somewhat in the aqueous solvent at all the developmental stages. Discrete spots moving only in the organic solvent are apparent in the youngest seeds (11 days after anthesis) (Fig. 27a). Mobility of these compounds in the aqueous solvent increased with age (18 days and 30 days after anthesis) (Fig. 27b,c). No such spots were visible in the non-tannin line.

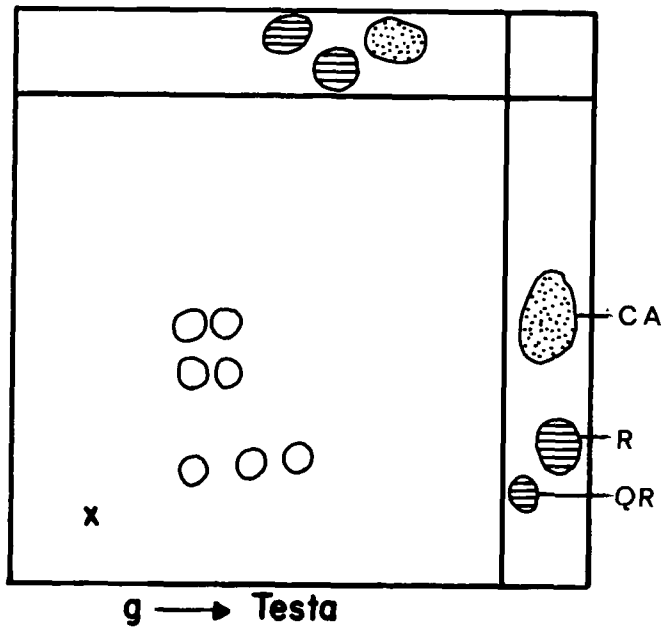
3.4 HISTOCHEMICAL LOCALIZATION OF PHENOLIC COMPOUNDS IN OVULES/SEEDS:

The reagents used for staining seed sections react with phenols to give a brightly coloured

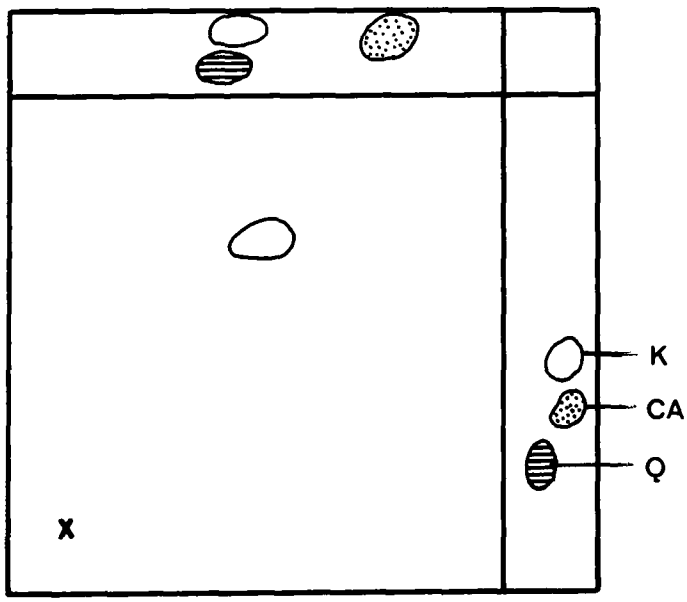
Figure 26. Drawings of typical 2-D chromatograms of flavonoid glycosides of Eston testa and embryo. No glycosides could be detected on the chromatograms of P.I.345635 testa and embryo. Chromatographic conditions as in Fig. 15.

(a) Eston testa

(b) Eston embryo (cotyledons)



a



b

Figure 27. Photographs of 2-D chromatograms of ovules/seeds of Eston prepared at various stages of growth and sprayed with 1% $FeCl_3$-K_3FeCN_6 (Turnbull's reagent)

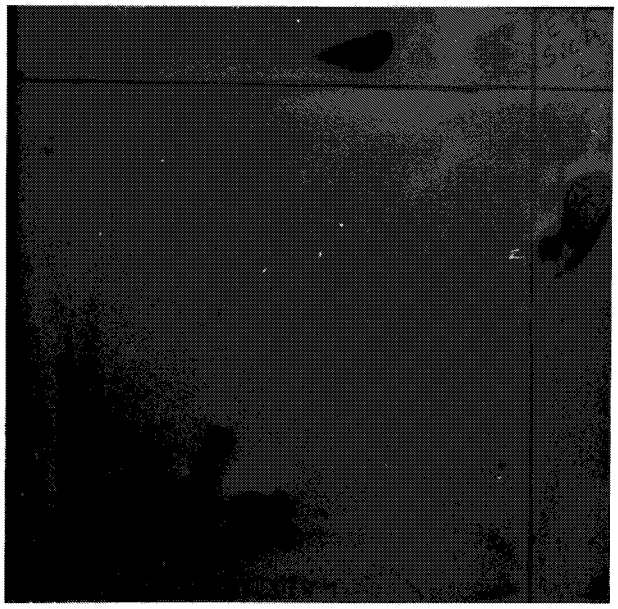
a) stage 1 - 11 days after anthesis

b) stage 2 - 18 days after anthesis

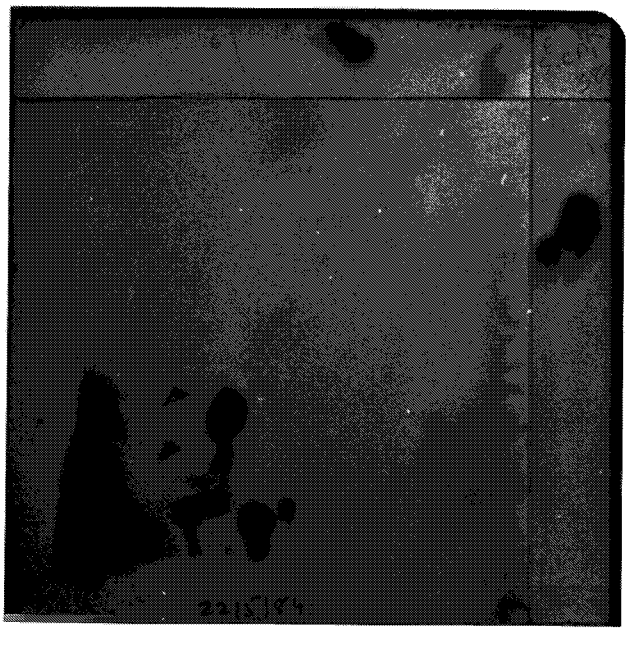
c) stage 3 - 30 days after anthesis

Other chromatographic conditions as in Fig. 15.

a



b



c

nitroso product at concentrations as low as 4 mg/l chlorogenic acid (Reeve 1951). The reaction of nitrous acid with phenols produces either a quinone oxime or a nitrosophenol (Reeve 1959). Apart from Eston testa no plant part of either lentil line showed any formation of yellow or red colour and therefore no indication of presence of phenols.

Section from 11 day old ovule (stage 1) of cultivar Eston stained red in the subepidermal region. The red colour was in the form of patches in the endosperm (region below the sub-epidermal layer) (Fig. 28a). The epidermal layer of the testa, composed of sclereids with unevenly thickened walls, was also stained red but the colour was not as dense (Fig. 28) as in the subepidermal region. In sections from 18 day old ovule (2nd stage), the red colour was mostly confined to the subepidermal layer. The region immediately beneath the subepidermal layer, the endosperm, also showed a slight presence of red colour in the form of patches (Fig. 28b). The red colour in sections of 30 day old ovules (3rd stage) was confined to the sub-epidermal layer only (Fig. 28c) and the rest of the section did not show any traces of red stain. This region consists of columnar cells with parenchymatious tissue underneath (Esau 1977). Sections of 68 day old (mature) seeds were not obtained, since the testa was too dry and too hard for microtome sectioning.

3.4.1 AUTOFLUORESCENCE:

A fluorescent blue colour was predominant in the epidermal layer of 30 day old ovules (3rd stage) of cultivar Eston (Fig. 29). No fluorescence was visible in the endosperm or the regions immediately around the embryo. Sections from 18 day old embryos (2nd stage) showed only a few patches of blue fluorescent colour visible in the epidermal layer. Sections of 11 day old ovule (1st stage) were devoid of any blue fluorescent colour.

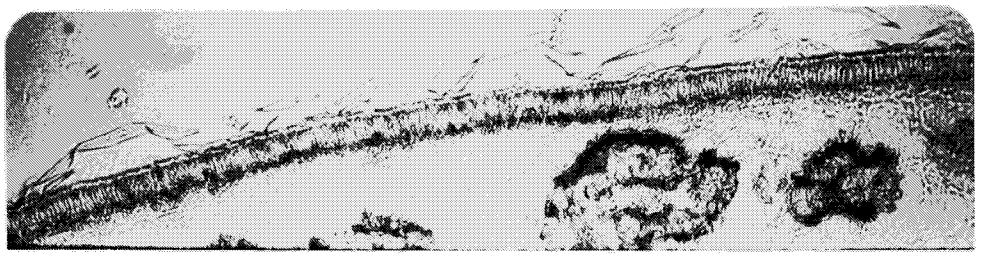
Figure 28. Photographs showing fresh ovule sections from Eston stained with nitrous acid reagent.

a) ovule - 11 days after anthesis (stage 1) -colour in testa and in patches in the embryo

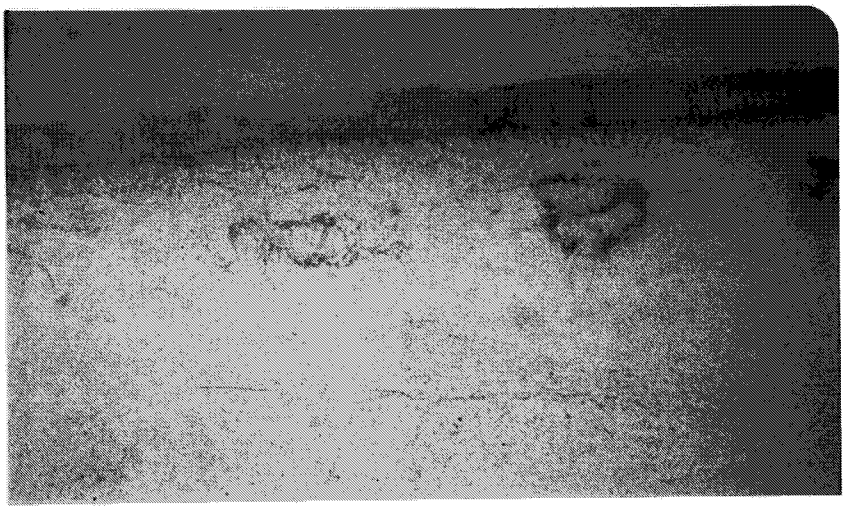
b) ovule - 18 days after anthesis (stage 2) -colour as in a)

c) ovule - 30 days after anthesis (stage 3) -colour only in the palisade layer of the testa, none in parenchyma layer below, nor in cotyledon.

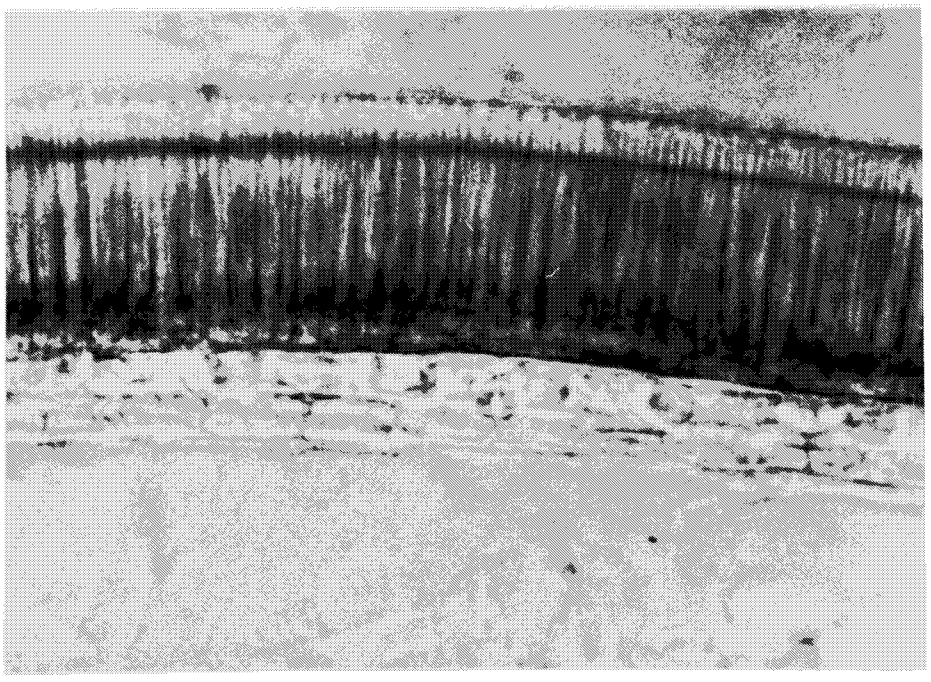
Catechols and other dihydroxyphenol give a red colour in this reaction, but in photographs a & b, the red colour has largely been replaced by a yellow to yellow-brown colour.



a ×63

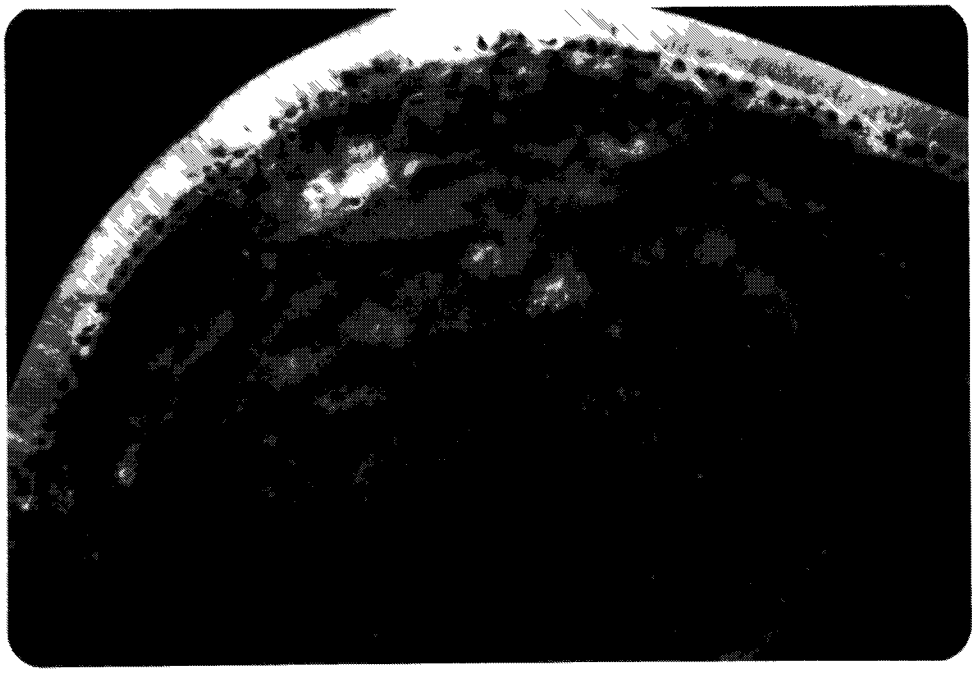


b ×252



c ×630

Figure 29. Photograph showing the seed section (30 days after anthesis) as observed under the fluorescent microscope. The blue fluorescent colour is confined to the palisade layer of the testa alone. Reddish color indicates presence of chlorophyll in the embryo and parenchyma layer of the testa. The round structure in the centre is a cross-section of the shoot-root axis (magnification 250X)



3.5 MEASUREMENT OF FUNGAL GROWTH IN THE PRESENCE OF SEED EXUDATES:

Exudates of 30 seeds of the two tannin-containing lentil lines, Eston and TB406M had 4.2 mg/g dry wt. and 4.06 mg/g dry wt. of total phenols respectively. No phenols were detected in exudates from mature seeds of the non-tannin line of lentils, P.I.345635.

Fig. 30 and Fig. 31 show the growth patterns of the fungi *Fusarium oxysporum* isolate 82A and *Fusarium oxysporum* var. *callistephi* in the presence of these seed exudates. The growth of isolate 82A in control (without seed exudate) and in the presence of seed exudate shows a continuous increase with passage of time until it reaches a plateau after about 50 hrs (Fig. 30). No significant difference in growth is evident amoung the four conditions except for a temporary setback in presence of the exudates of the tannin lines and a similar enhancement in control and the non-tannin exudate at 22 hrs. However at 5% level of significance the t-test indicated that all data at 22 hrs. in Fig. 30 belonged to the same distribution (Appendix III). Similarly the differences in Fig. 31 at 30 hrs. are not significant at either (Appendix III).

Growth of var.*callistephi* was much less and exhibited a pronounced lag phase under all conditions (Fig. 31). Final wt of mycelium was significantly less in control, than in the presence of seed exudates. Those from tannin lines tended to permit less growth than that from the non-tannin line.

Figure 30. The growth pattern of *Fusarium oxysporum* isolate 82A in the presence of PDA extract plus water (control) or seed exudates of Eston, non-tannin variety (P.I.345635) and line TB406M (#23). Experimental conditions as described in Section 2.6.2. Vertical bars represent S.D.

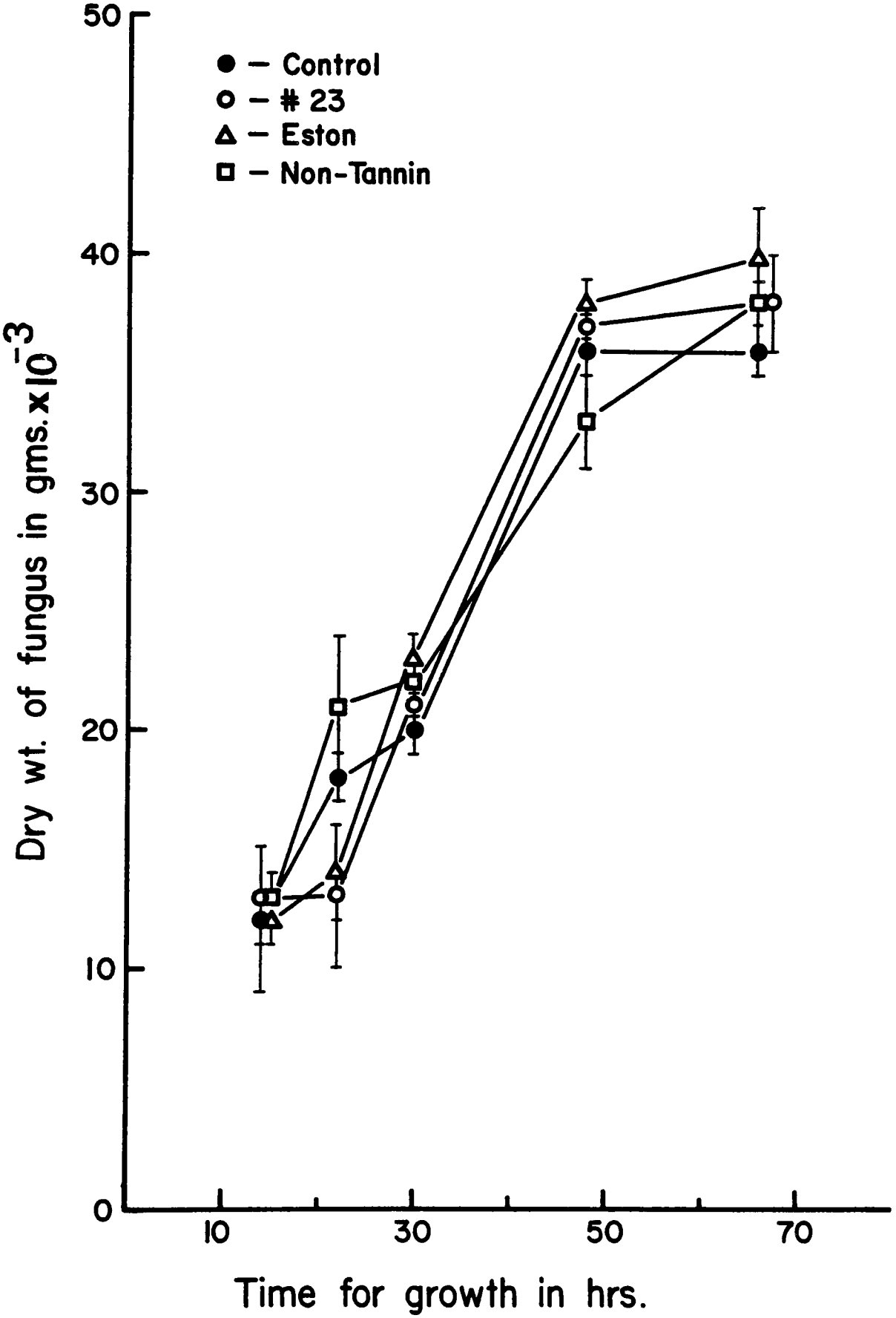
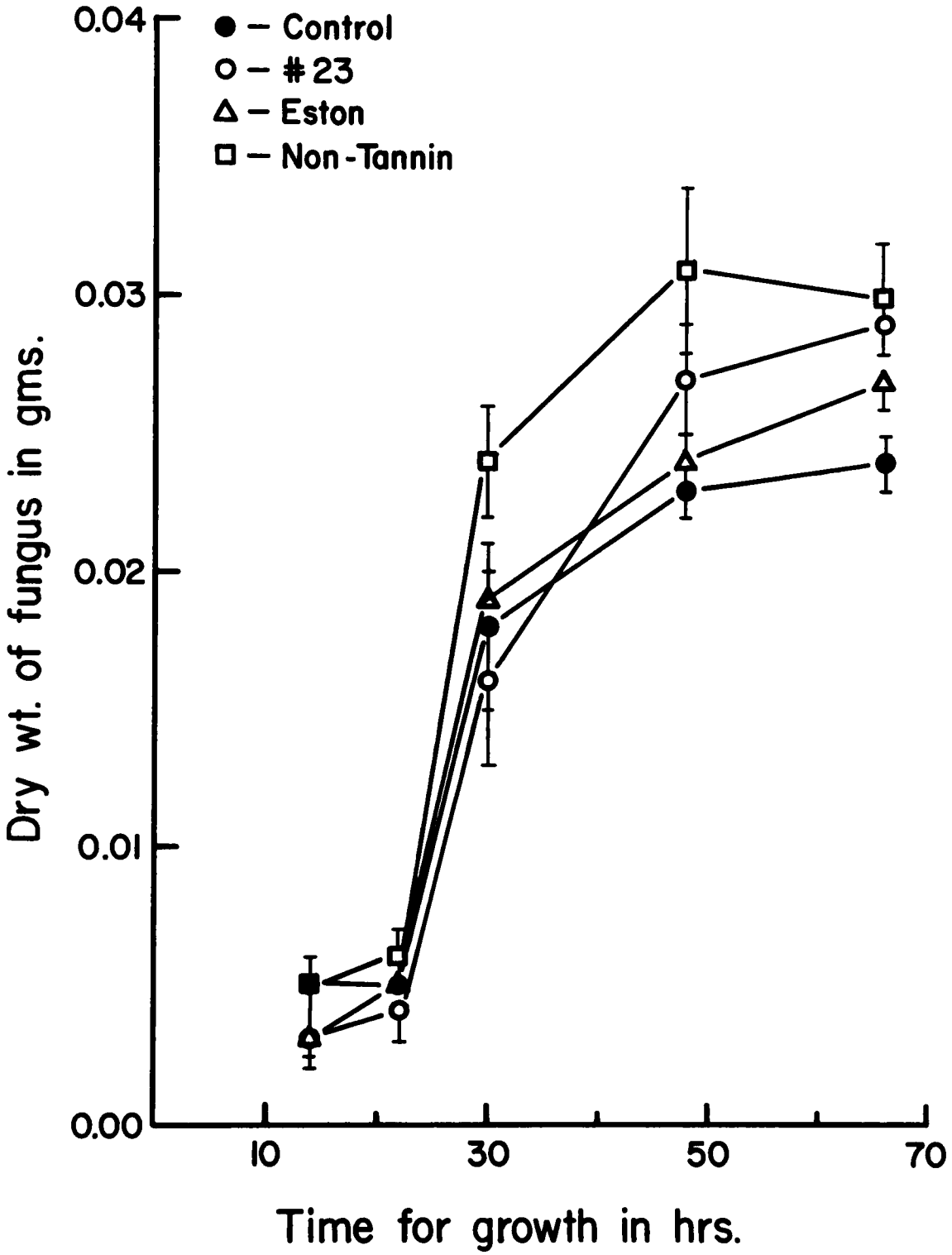

● — Control
○ — # 23
△ — Eston
□ — Non-Tannin
Dry wt. of fungus in gms. x 10^-3
0
10
20
30
40
50
10
30
50
70
Time for growth in hrs.

Figure 31. The growth pattern of *Fusarium oxysporum* var. *callistephi* in the presence of seed exudates of Eston, P.I.345635 and line TB406M (#23). Conditions as in Fig. 30.


● – Control
○ – #23
△ – Eston
□ – Non-Tannin
0.04
0.03
0.02
0.01
0.00
Dry wt. of fungus in gms.
10
30
50
70
Time for growth in hrs.

CHAPTER 4

DISCUSSION

No difference was apparent in the flavonoid pattern of the vegetative parts of cultivar Eston and P.I.345635. The leaf showed a complex mixture of kaempferol and quercetin glycosides with the number of kaempferol glycosides being more than quercetin glycosides. Apart from assessing the colour and relative level of glycosylation by mobility on chromatogram, no attempt was made to identify them. The leaves of other closely related legumes of the tribe Viceae have also been reported to contain kaempferol and quercetin glycosides although their glycosylation patterns are quite different from lentils. For example, the major leaf flavonoids in pea (*Pisum sativum*) have been reported to contain both quercetin and kaempferol 3-triglucosides as such and also acylated with p-coumaric acid (Statham et al. 1972). The leaves of broad bean (*Vicia faba* L.) contain kaempferol and quercetin 3-O-B-D-glucosyl-7-O-rhamnosides, kaempferol 3,7-O-bis-B-D-glucoside and rutin (quercetin 3-rutinoside) (Knackstedt et al. 1981). None of these compounds has the same chromatographic mobility as the lentil pigments.

These results clearly show that a great diversity in flavonoid patterns can exist among species of the same tribe. Flavonoids are considered to be of taxonomic value since they tend to have chemical complexity and structural variability; and are environmentally and physiologically stable (Buttery and Buzzell 1973); their widespread distribution and variation between populations makes them well suited to chemotaxonomic studies (Harborne 1975). To date only leaves have been used to provide the biochemical data useful in taxonomic structuring of the genus. In the present study, it has been shown that glycosidic patterns of

leaf can vary with development. The diversity in flavonoids at different stages of plant growth is not usually taken into consideration by taxonomists, since the leaves are examined at only one particular stage of growth. A few other studies have also pointed out the differences in flavonoid patterns of a leaf at an early and mature stage of growth (eg. Classen 1981).

In the lentil stem kaempferol glycosides also predominate over quercetin glycosides. Not much reference in the literature is available on the flavonoid pattern of stems of other legumes. Usually only leaves are used for assessing flavonoid patterns. The flavonol pattern of lentil stem is very similar to lentil leaf but difference in what appears to be quercetin glycosides is noticeable. In *Pisum sativum* also, the flavonoid pattern of stem is similar to leaf (Nozzolillo, personal communication). *Cicer arietinum*, not a closely related species, has been reported to contain isoflavones, biochanin A and formononetin, rather than flavonols (Ingham 1976).

The flavonoid pattern of lentil root was quite different from that of either leaf or stem. The roots of both lentil lines showed the presence of flavones as reported by D'Arcy-Lameta (1986). The compounds identified in the lentil root exudates were three desoxy-flavones. This is of interest since all other legumes examined have isoflavonoids in the roots. Not only *Pisum sativum* L. (Burden et al. 1974), a closely related species but also *Cicer arietinum* (Barz 1969) (previously in tribe Viciae, now in its own tribe) has been reported to contain isoflavonoids in roots. Other legumes examined are *Vigna radiata* (Barz 1969), *Trifolium pratense* (Chang et al. 1969) and *Phaseolus vulgaris* (Burden et al. 1974).

A dramatic change in the flavonoid pattern was observed in the reproductive parts of lentils. The difference in the pattern was discernible not only in the vegetative and reproductive parts of the same lentil line but the flavonoid 'fingerprint' of the reproductive organs of the two lentil lines, Eston and P.I.345635, was also quite different. Quercetrin (quercetin 3-rhamnoside) was the major flavonol present in the pericarp, pistils and petals of cultivar Eston whereas it was totally absent from the pericarp of P.I.345635 and present in

relatively small amounts in petals.

The present study is the first report on the flavonoid pattern of the different reproductive organs of lentil. The phenolic compounds of only seed coats (testa) of lentil have previously been reported (D'Arcy & Jay 1978; Vaillancourt 1984).

The present study shows the diversity of flavonoid patterns that exists among organs of the same lentil plant. Roots differ from the shoot. Vegetative organs differ from reproductive organs. Thus, a complete description of the flavonoid patterns would provide information on the variation within a genus and aid in the identification of introduced plants.

The quantitative analysis of total phenols in vegetative parts showed that P.I.345635 contained appreciably smaller amounts of total phenols than the corresponding plant parts of Eston at all stages of development. Since the flavonoid fingerprints of the vegetative parts of both lentil lines were similar and no other form of polyphenols (tannins) were detected in the vegetative parts of Eston, it can be suggested that the quantities of flavonoid glycosides in vegetative parts are produced in greater amounts in Eston than in P.I.345635. In particular, the total phenol accumulation in the non-tannin (P.I.345635) leaves reached a plateau between 50 to 80 day-old seedlings, whereas no such plateau was evident in the total phenols of leaves of Eston until the plants were 80 day-old (total phenols in vegetative parts were not monitored after 80 days). A plateau as observed in the non-tannin lentil line in the present study, has also been observed elsewhere in a study on the developmental changes in flavonoids in barley (Blume & McClure 1979) and some tissues of Victoria plum tree (Hillis & Swain 1959). Blume and McClure (1979) showed that the enzyme activity involved in the polyphenolic biosynthesis in leaves reached a maximum between 15 to 20 days and then declined. The primary leaves of barley enlarge, mature and senesce in about 35 days from planting and hence is a model system to observe changes in metabolic activity during development. During the senescence of leaves, membrane integrity decreases and the flavonoid

may be converted into simple protein-flavonoid complexes or oxidation products formed by the action of high levels of various oxidases; resulting in complexes which are not extracted from the plant material (Blume & McClure 1979). The growth pattern of the two lentil lines, Eston and P.I.345635 was observed to be similar since the flowering stage and the maturity of fruits was attained approximately at the same time. Thus the possibility of senescence being triggered earlier to result in lower levels of phenols, in the non-tannin lentil line can be precluded.

Analysis of proanthocyanidins in the two lentil lines revealed that P.I.345635 was totally devoid of proanthocyanidins. Vaillancourt (1984) working on the inheritance of tannin in lentils concluded that a single recessive gene is responsible for the complete absence of tannins in P.I.345635. Proanthocyanidins were also not detectable in the vegetative parts of cultivar Eston. Only the reproductive parts of this lentil line showed the presence of proanthocyanidins. The proanthocyanidin content in the Eston testa was found to be the highest of all the reproductive tissues. The continued production of proanthocyanidins in the Eston testa from the first stage of development to maturity is evident from the continuous increase in recovery of anthocyanidins with acid solution in both methanol extracts and the residues. Gupta & Haslam (1979) state that any plant produces a range of procyanidins of different molecular weights. Only those flavan-3-ols with molecular weights up to about 3000 are soluble, and it is not known whether those of higher molecular weight are simply insoluble or are in fact attached to skeletal components, e.g. cell wall, of the plant. Alternatively, the monomers might react with the various proanthocyanidins already present to produce higher oligomeric forms which would have decreased solubility (Lea 1978). The Eston testa contained both lower and higher molecular weight proanthocyanidins as evident by the results with Turnbull's spray. The amount of low molecular wt. tannins increased with maturity of the seed until 68 days after anthesis. The higher molecular wt. tannins also increased with maturity as shown by the deeper intensity of the red colour of the residue of the testa, suggesting progressive insolubilization of the polymeric proanthocyanidins. Haslam (1977) contends that as

chlorophyll develops in the seed coat there is an apparently rapid synthesis of polyphenols and both dimers and catechins are present. He observed that in red sorghum as the grain ripens (to a red-brown appearance) the monomeric and dimeric flavan-3-ol species appear to decline rapidly in concentration to leave the polymeric procyanidin as the principal and in many cases the sole procyanidin in the seed coat. In lentil seeds proanthocyanidins increased in amount until at least 68 days after anthesis. The bright green color of the seed at this time shows that chlorophyll content also increased.

Proanthocyanidin analysis by heating in acid solution is subject to errors of at least two kinds (Gupta & Haslam 1979). First the yield of anthocyanidins is probably quite low since only the "lower" flavan-3-ol structural unit is potentially available to yield cyanidin from a dimer, trimer, tetramer, oligomer or polymer (Fig.3); also the transformation is not quantitative since a large amount of brown 'phlobaphene-like' substances is formed along with the anthocyanidin as shown in figs. II.1 (Appendix II).

Quantitative variation in total phenol content of the two lentil lines and the qualitative differences in the flavonoids of vegetative and reproductive parts could be a result of either different genomes or of the same genome being regulated differentially. For instance, although the genes in Eston and P.I.345635 governing flavonoid synthesis seem to be similar for leaf, stem and root pigments, the quantities synthesized are not. The different flavonoid patterns in the reproductive organs from those of the vegetative organs shows that the same genome can be expressed differently in various organs. The expression of genes controlling the enzyme activities that catalyze the different reactions of the biosynthetic pathway of flavonoids could be modified at the transcriptional or translational level (Steyns & Brederode 1986). Generally pigments in flowers are more complex. Genes controlling hydroxylation, methylation and glycosylation have been reported mainly in reproductive tissue only (Harborne 1965). Any possibility of differences brought about by environmental variation, was minimized by growing

both lines in the controlled atmosphere of a growth chamber. When grown in slightly different environmental conditions (greenhouse vs. growth chamber) cultivar Eston did not show any significant change in the flavonoid pattern at any developmental stage of growth, thus indicating that environmental differences had little effect on genotype expression. The question, how genes are regulated differently in various organs of lentil plant (or for that matter any other plant) still remains to be answered. The variation in flavonoid pattern in different plant organs presumably relates to their different functions. For example, flavonols in leaf could play a role in photosynthesis, in root the flavones might be used in defence mechanism and in flowers the flavonols could aid in pollination.

Flavonoids are derived via the shikimic acid pathway through the intermediacy of phenylalanine (Fig.32) (Harborne 1986). The role of acetate-malonate units in biosynthesis is important, since these are needed together with phenylpropanoid moieties, for the production of flavonoids. Recently Stafford and Lester (1981; 1982) have proposed a more detailed metabolic pathway leading to the monomers and oligomeric proanthocyanidins (Fig.33). Dihydroquercetin (DHQ), a flavanonol, is a key intermediate. DHQ is suggested to be synthesized by two possible routes, one of them involving naringenin, a trihydroxyflavanone, as the first C15 intermediate, the other involving the tetrahydroxyflavanone eriodictyol. Both dihydroxykaempferol (DHK) and DHQ are postulated precursors of quercetin. The synthesis of the various flavonoids in different plant organs of lentil can be explained by this scheme. For example, in leaf and stem, the presence of more kaempferol glycosides than quercetin suggests that quercetin possibly comes from kaempferol via DHK rather than from DHQ. In reproductive organs in Eston on the other hand, the accumulation of quercetin 3-rhamnoside and production of large amounts of proanthocyanidins suggests that DHQ is the major intermediate. Proanthocyanidin synthesis proceeds from intermediate production of the flavan-3-ols, catechin and epicatechin. There must be an immense production of DHQ and catechins to allow for production of such quantities of quercetrin in the petal and pericarp and

Figure 32. Pathway of biosynthesis of flavonoids and related plant phenolics (after Harborne 1986)

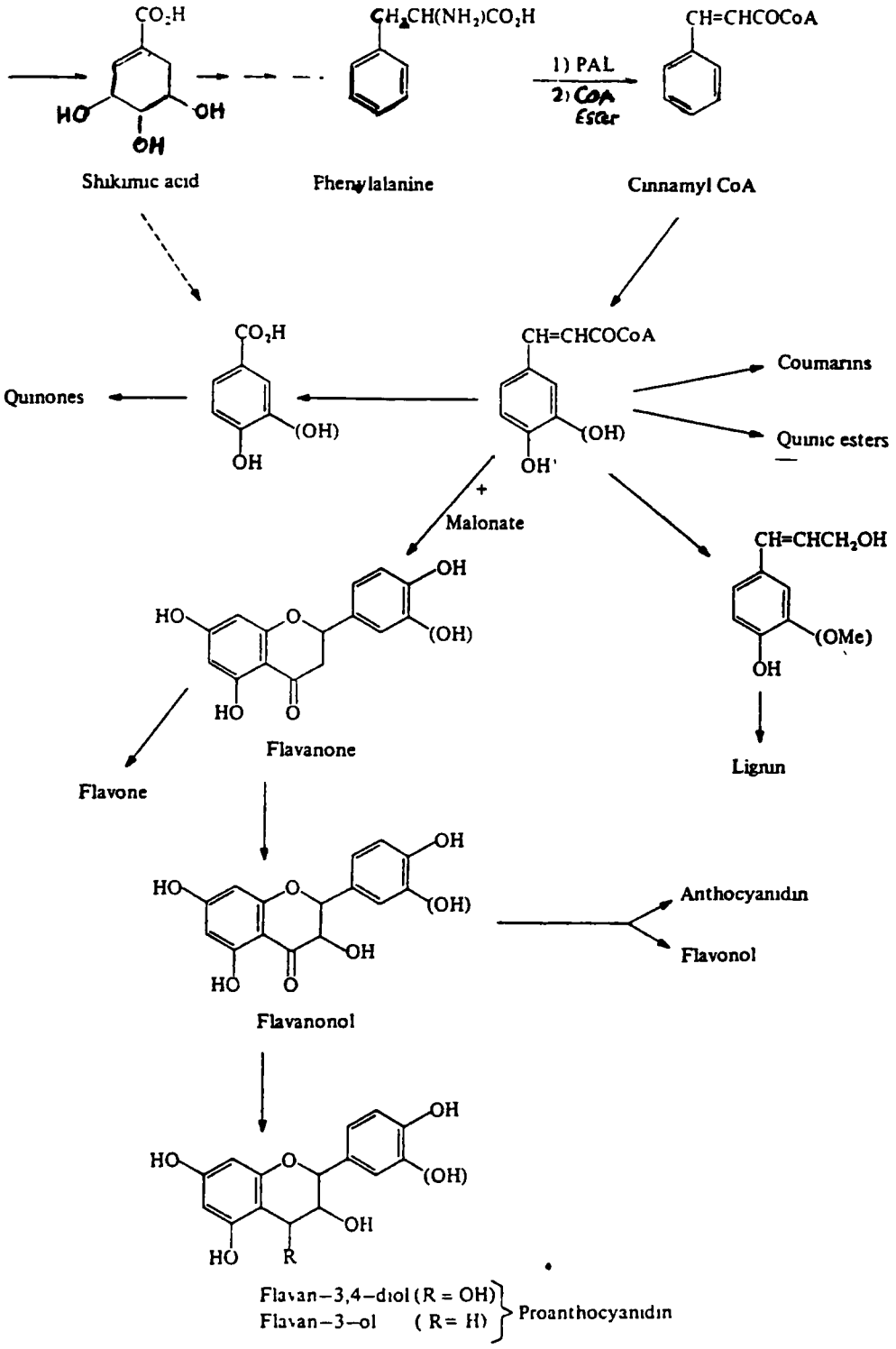

CO2H
HO
OH
OH
CH2CH(NH2)CO2H
1) PAL
2) CoA Ester
CH=CHCOCoA
Shikimic acid
Phenylalanine
Cinnamyl CoA
Quinones
(OH)
OH
Coumarins
Quinic esters
Malonate
HO
O
Flavanone
Flavone
CH=CHCH2OH
(OMe)
Lignin
Anthocyanidin
Flavonol
Flavanonol
R
Flavan–3,4–diol (R = OH)
Flavan–3–ol (R = H)
Proanthocyanidin

Figure 33. Diagram of possible metabolic pathways leading to procyanidins starting with C6-C3 or phenylpropane precursors (after Stafford & Lester 1981).

Abbreviations used:

CAF - caffeic acid Cinn. - cinnamic acid

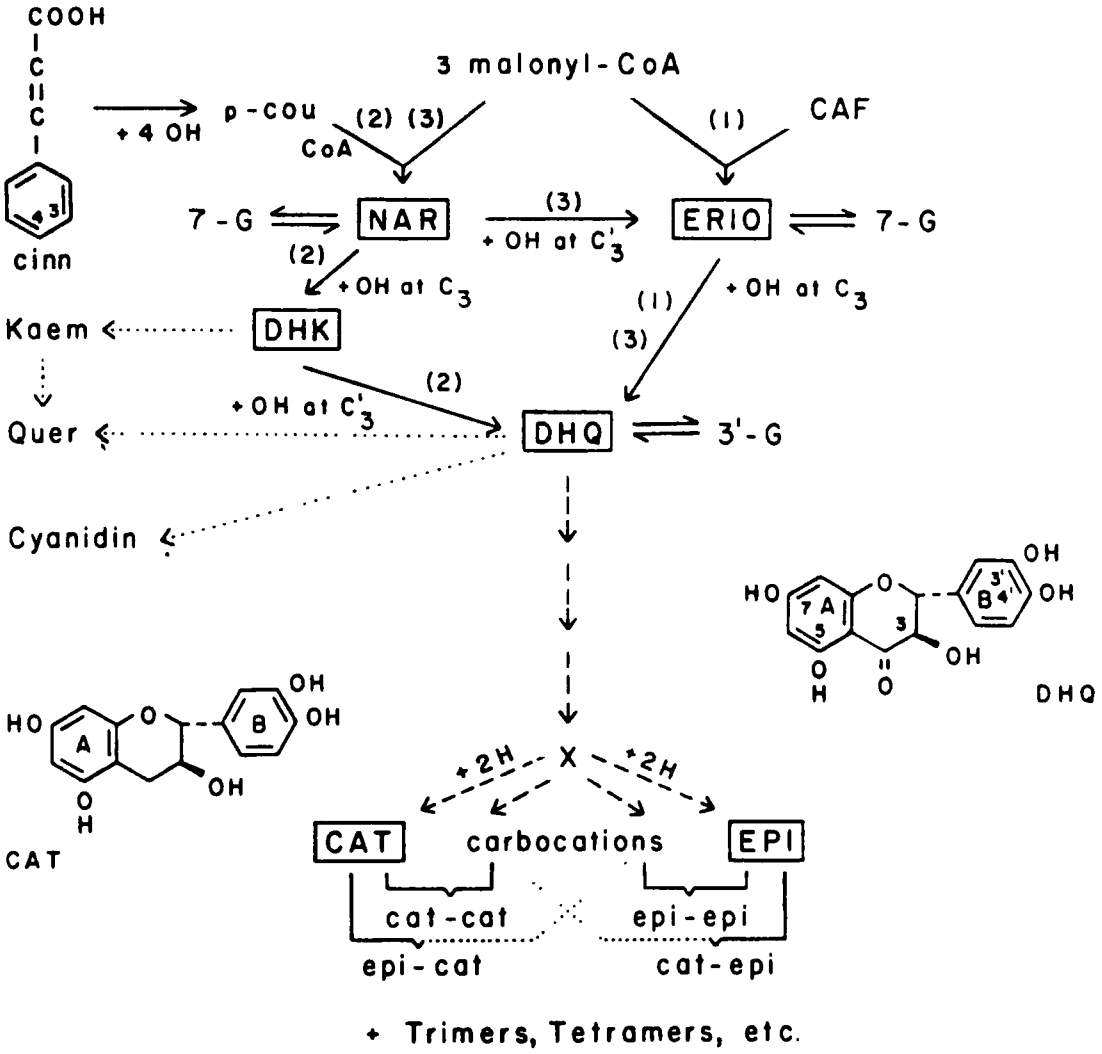
NAR - naringenin Kaem. - kaempferol

DHK - dihydrokaempferol Quer. quercetin

DHQ - dihydroquercetin P-cou - p-coumaric acid

ERIO - eriodictyol 7-G - 7-glucosides

CAT - catechin EPI - epicatechin


COOH
cinn
+ 4 OH
p-cou
CoA
(2) (3)
3 malonyl-CoA
(1)
CAF
7-G
NAR
(3)
+ OH at C'3
ERIO
7-G
(2)
+ OH at C3
(1)
(3)
+ OH at C3
Kaem
DHK
(2)
+ OH at C'3
Quer
DHQ
3'-G
Cyanidin
OH
HO
OH
OH
DHQ
HO
OH
OH
OH
CAT
+2H
X
+2H
CAT
carbocations
EPI
cat-cat
epi-epi
epi-cat
cat-epi
+ Trimers, Tetramers, etc.

of proanthocyanidins in the seed coat. Yet no evidence of either substance was found on the chromatographs. This indicates that probably all of DHQ formed is channeled into the formation of the final products on multi-enzyme systems. The absence of proanthocyanidins in P.I.345635 suggests that any DHQ formed is converted to quercetin only.

The histochemical studies also indicated that the vegetative parts of Eston and both vegetative and reproductive parts of P.I.345635 were devoid of proanthocyanidins. The histochemical tests were also negative for presence of other phenolic substances, yet chromatographic results clearly indicate their presence. The absence of a positive yellow color with the nitrous acid reagent, might result from 1) leaching of these soluble compounds from the thin hand section or 2) a lack of sensitivity to the low amounts that would be present in a thin section or from both these factors.

The presence of a blue fluorescent colour only in the nearly mature Eston testa indicates a relative abundance of substances such as ferulic acid, (Harborne 1975) etc. in the later stages of seed development. Since quantitative assay showed the presence of phenolics also in the initial stages of seed development, these must have been non-fluorescent molecules such as p-coumaric acid. No fluorescent phenols were present in the testa of non-tannin P.I.345635 at any stage of development.

Proanthocyanidins are often restricted to the seed coat tissues in plants, especially legumes (Bate-Smith & Lerner 1954). Proanthocyanidins are deposited in the hourglass and parenchymatous layers of the faba bean (*Vicia faba*) seed coat. In faba bean, the seed coat contains about 90% of the seed tannins (Crofts 1979). Likewise in bean (*Phaseolus vulgaris*) most of the condensed tannins are localized in the seed coat while the cotyledons contain either a small amount or no detectable tannin (Ma & Bliss 1978). In peanut (*Arachis hypogea*) only a small amount of condensed tannin is found in tissues beneath the seed coat (Sander & Mixon 1978). In lentil also histochemical analysis clearly showed that tannins were restricted to the

palisade layer of the seed coat.

In spite of the quantitative uncertainty, the average proanthocyanidin content of the mature Eston testa was calculated in the present study to average 24 mg g^{-1} of dry testa. Upon comparing these results with other studies (Table II.1 in Appendix II) only bean among the pulses reported to contain tannins has more tannin, up to 50 mg g^{-1} bean seed coat (Ma & Bliss 1978). Preliminary field trials on non-tannin P.I.345635 lentil by Vaillancourt (1984) showed that a high incidence of seed rot occurred and emergence rarely exceeded 5%. Phenolic compounds such as flavonoids and proanthocyanidins have been implicated in disease resistance (Van Sumere et al. 1975), thus suggesting that the failure of the non-tannin line in the field may be related to the absence of proanthocyanidins. In addition, the amount of total phenol in the non-tannin line of lentils when compared with the tannin line was much lower in all plant organs. The experiments carried out on the two strains of *Fusarium oxysporum* were an attempt to provide an insight into the reasons for this unhealthy behaviour of the non-tannin line in field. It is interesting to note that growth of the virulent strain 82 of *F. oxysporum* proceeded without a lag phase regardless of the presence of seed exudates whereas growth of the avirulent strain was equally delayed under all conditions tested. Thus no fungistatic effect of seed exudates was observed and if anything, non-tannin seeds of P.I.345635 appeared to stimulate growth. Perhaps the experimental simulation of certain factors *in vitro* was not adequate, for instance, far too many spores could have been introduced in the innoculum that were not effectively inhibited by the concentration of the phenols in the medium. Increasing the concentration of phenols in the medium might have proved conclusive in their possible role as a defence mechanism in lentils. Also, the possibility of the production of some phenolic compounds, induced by the plants *in vivo* on fungal attack cannot be overlooked.

Kraft (1974) showed that both resistant and susceptible lines of pea seeds produced like amounts of phenols and reducing sugars in exudates. However, only exudates from resistant

line inhibited sporulation of *F. solani*. Kraft (1977) showed that delphinidin in the resistance line of pea produced the fungistatic effect which could be overcome when an adequate supply of sugar was present. The similar growth patterns of *F. oxysporum* in both tannin and non-tannin varieties of lentil in the present study could be attributed to nutrients provided by seed exudates (Schroth et al. 1964). The exudation of organic compounds from seeds has been shown to supply the energy source needed for fungi to grow (Kraft 1974; Kraft & Erwin 1967). The liability of P.I.345635 lentil seeds to pre-emergence mortality in soil could be associated with the readiness with which these seeds exude electrolytes and soluble carbohydrates into steep water.

CHAPTER 5

CONCLUSION

In conclusion, a comparison of the developmental aspects of flavonoids in cultivar Eston and P.I.345635 illustrated that no qualitative differences existed in the flavonoid patterns of their vegetative parts. A dramatic change in the flavonoid pattern, different for each line, appeared in the reproductive parts. Quercetin-3-rhamnoside was the major flavonol glycoside present in the petal and pericarp of Eston whereas a different array of glycosides was observed in petal and pericarp of P.I.345635.

The tannin line contained higher amount of total phenols than did the non-tannin line P.I.345635 even in vegetative parts lacking tannins. This suggests a possible relationship of the presence of the gene controlling tannin synthesis and total flavonoid synthesis perhaps through the enzymes(s) controlling production of DHQ.

An antifungal activity of seed exudates of two tannin-containing lentil lines, Eston and TB406M, toward *Fusarium oxysporum* was not established in the present investigation. Perhaps higher concentration than those used are necessary to demonstrate a role of phenolic compounds as a defence mechanism.

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Appendix 1. Table I.1 compares the total phenol contents of variety Eston grown in the two different environments of greenhouse and growth chamber. The overlap of S.D. of the means of 3 replicates clearly indicates that minor environmental changes do not induce significant variation in the overall total phenol content of any organ at any stage of growth.

Table I.2 compares the total phenol contents of the non-tannin variety grown at two different times in the growth chamber. Again no significant differences were observed in the total phenol content between the two conditions of growth.

Table I.I. : Total phenols in various plant parts of Eston grown in two different environments of greenhouse and growth-chamber.

Plant organ and stage of growth		March'84 Total phenols (G.H.) mg /g dry wt.±SD	Oct.'84 Total phenols (G.C.) mg /g dry wt.±SD	Average
Leaf	1st (20-day seedlings)	1.80 ±.31	2.20 ±.40	2.00 ±.39
	2nd (45 ")	2.32 ±.43	3.0 ±.20	2.66 ±.47
	3rd (65 ")	2.81 ±.27	4.31 ±.30	3.56 ±.86
	4th (80 ")	4.73 ±.51	5.54 ±.50	5.13 ±.63
Stem	1st (20 day)	0.11 ±.01	0.17 ±.01	0.13 ±.03
	2nd (45 ")	1.60 ±.28	1.87 ±.30	1.73 ±.30
	3rd (65 ")	2.43 ±.26	2.29 ±.03	2.36 ±.18
	4th (80 ")	2.41 ±.35	2.20 ±.40	2.30 ±.36
Root	1st (20 day)	0.12 ±.01	0.02 ±.00	0.07 ±.06
	2nd (45 ")	1.80 ±.36	2.30 ±.32	2.05 ±.41
	3rd (65 ")	2.90 ±.42	3.26 ±.25	3.08 ±.41
	4th (80 ")	2.80 ±.25	3.22 ±.31	3.01 ±.34
Peri-carp	1st (11-day after anthesis)	18.70 ±0.99	20.84 ±2.69	19.77 ±2.16
	2nd (18 ")	17.59 ± .66	12.51 ± .80	15.05 ±2.86
	3rd (30 ")	13.09 ±1.31	11.79 ± .33	12.44 ±1.12
	4th (68 ")	8.26 ±1.62	8.82 ±2.40	8.54 ±1.86
Testa	1st (11 day)	15.89 ±1.03	20.60 ±1.18	18.24 ±2.76
	2nd (18 ")	37.47 ±1.24	28.25 ±3.71	32.86 ±5.64
	3rd (30 ")	49.40 ±4.10	61.94 ±4.87	55.67 ±7.96
	4th (68 ")	72.83 ±2.8	77.06 ±1.81	74.94 ±3.07
Embryo	2nd (18 ")	0.22 ± .02	N.D.	.22 ±.02
	3rd (30 ")	0.48 ± .12	0.48 ±.14	.48 ±.12
	4th (68 ")	N.D.	N.D.	N.D.

G H - Green House

G C - Growth Chamber

Table I.2. : Total phenols in various plant parts of P.I. 345635 grown at two different times in the growth chamber.

Plant organ and stage of growth		Sept.'83 Total phenols (G.C.) mg /g dry wt. ±SD	May'84 Total phenols (G.C.) mg /g dry wt. ±SD	Average
Leaf	1st (20-day old seedlings)	0.58 ±.2	0.78 ±.3	0.68 ±.25
	2nd (45 ")	1.80 ±.3	1.40 ±.08	1.60 ±.29
	3rd (65 ")	2.10 ±.25	2.30 ±.1	2.20 ±.2
	4th (80 ")	2.06 ±.3	2.0 .2	2.03 ±.23
Stem	1st (20 ")	N.D.	0.11 ±.02	0.11 ±.02
	2nd (45 ")	0.75 ±.10	0.48 ±.10	0.61 ±.17
	3rd (65 ")	1.05 ±.20	1.32 ±.15	1.18 ±.22
	4th (80 ")	1.1 ±.10	1.29 ±.10	1.20 ±.14
Root	1st (20 ")	0.25 ±.05	0.20 ±.05	0.23 ±.05
	2nd (45 ")	1.12 ±.11	1.08 ±.20	1.10 ±.15
	3rd (65 ")	1.72 ±.15	1.66 ±.20	1.69 ±.16
	4th (80 ")	1.65 ±.05	1.60 ±.10	1.63 ±.08
Peri-carp	1st (11-day after anthesis)	4.30 ±1.26	4.36 ±.68	4.33 ±.91
	2nd (18 ")	3.19 ±.37	4.09 ±.82	3.64 ±.75
	3rd (30 ")	3.18 ±.60	3.34 ±1.08	3.26 ±.79
	4th (68 ")	3.16 ±.24	3.02 ±.80	3.09 ±.53
Testa	1st (11 ")	N.D.	N.D.	-
	2nd (18 ")	"	"	-
	3rd (30 ")	"	"	-
	4th (68 ")	"	"	-
Embryo	2nd (18 ")	N.D.	N.D.	-
	3rd (30 ")	"	"	-
	4th (68 ")	"	"	-

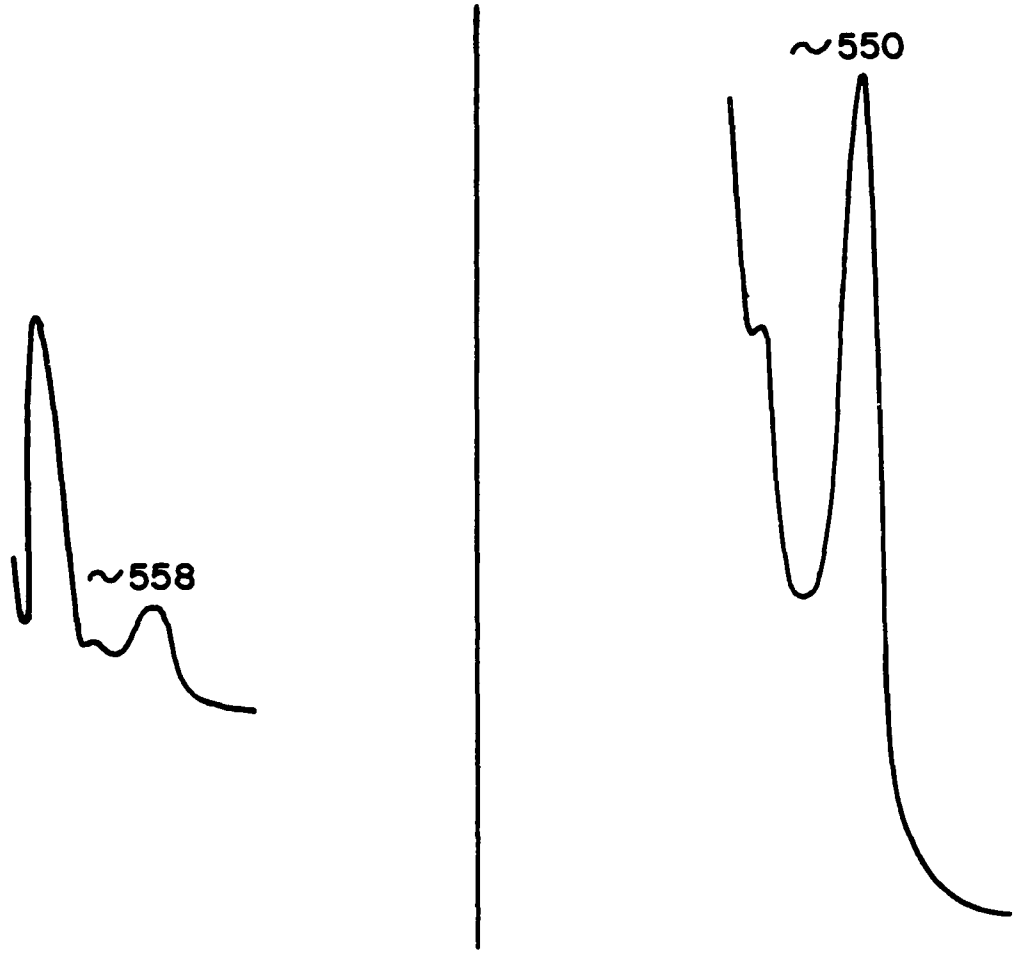
G C – Growth Chamber ND – Not Detectable

Appendix II.

(a) Fig. II.1 shows some of the spectra obtained on the Eston testa and ovary wall extracts after boiling them with the acid solution. The smaller shoulder/peak between 430-450 nm shows the presence of phlobaphene like polymers whereas the peak for anthocyanidins is about 550 nm (Swain & Hillis 1959).

(b) Table II.1 shows the average tannin content of a few species of legumes including the data collected in the present study.

(c) The presence of cyanidin and delphinidin in the isoamyl alcohol extracts of testa was established on the basis of the Rf values which were similar to standard cyanidin and delphinidin.


Extract
Residue
~558
~550

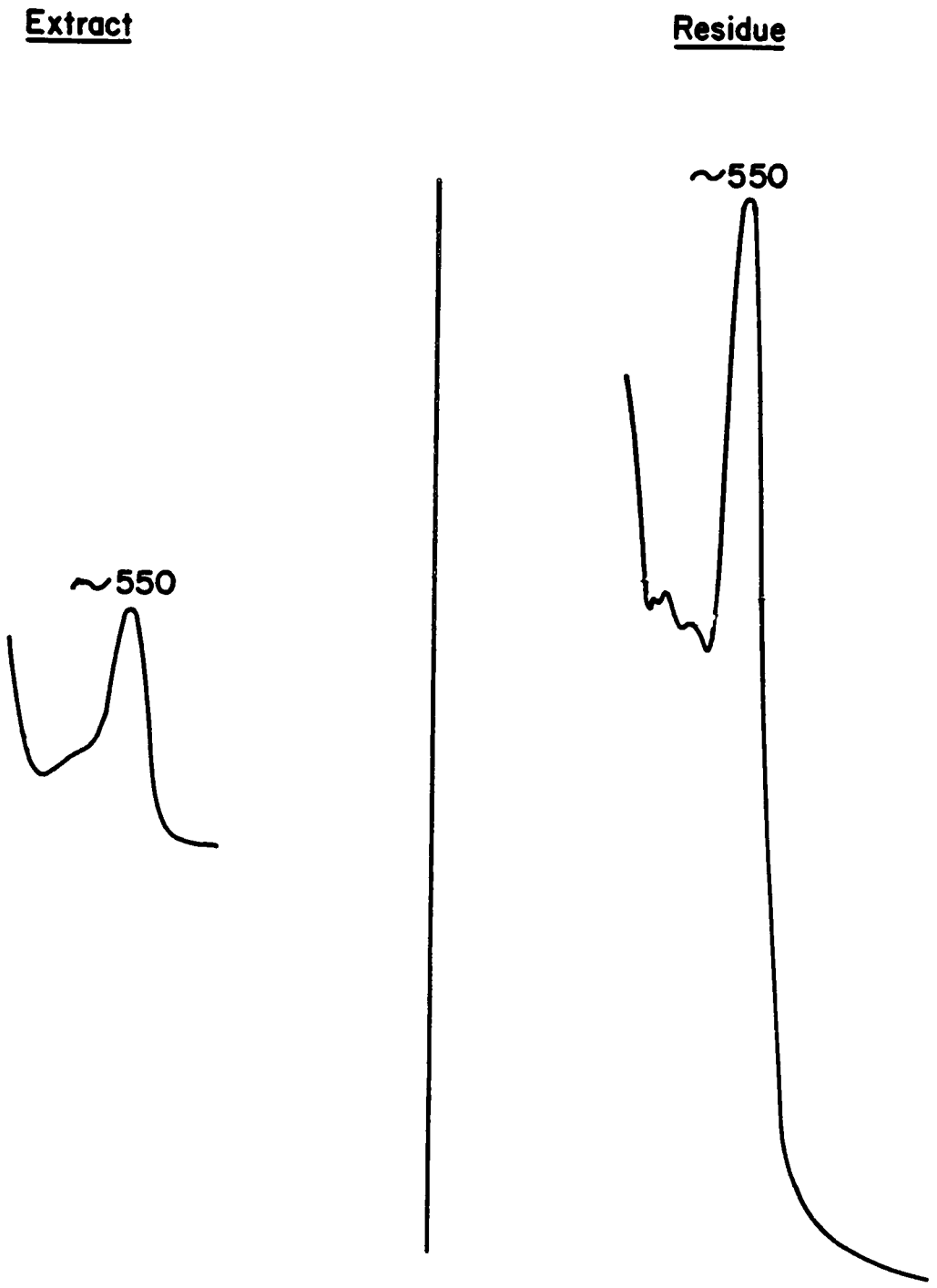

Extract
Residue
~550
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Table II.1. : Legume species containing tannin.

Species	Tannin content mg g^{-1}	Reference
Peanut (*Arachis hypogea*)	0.6 - 8.8 of seed coat	Sanders & Mixon 1978
Pigeon pea (*Cajanus cajan*)	0 - 0.8 seed dry weight	Price et al. 1980 b
Lentil (*Lens culinaris*)	0 - 23 of seed coat	Vaillancourt 1984
"	0 - 24 of seed coat	present study
Bean (*Phascolus vulgaris*)	0 - 50 of seed coat	Ma & Bliss 1978
Faba bean (*Vicia faba*)	0 - 5 of seed coat	Crofts 1979
Cow pea (*Vigna unguiculata*)	0 - 6 seed dry weight	Price et al 1980 b

Appendix III. Table III.1 and III.2 respectively show the results of t-test at 0.05 level of significance conducted on the data obtained on the growth of *F. oxysporum* isolate 082A after 22 hrs and *F. oxysporum* var. *callistephi* after 30 hrs. in the presence of seed exudates of P.I.345635, Eston, TB405M, and control which did not contain any seed exudates.

Table III.1 : Growth of Fusarium oxysporum isolate 082A after 22 hrs. at 0.05 level of significance.

Sample couples	t calculated from data	$t_{0.05}$ *	$t-t_{0.05}$
P.I.345635 and control	0.84	2.77	-ve
P.I.345635 and Eston	2.21	2.77	-ve
P.I.345635 and TB406M	2.26	2.77	-ve

* The value obtained form standard t distribution table.

Table III.2 : Growth of F. oxysporum var. callistephi after 30 hrs. at 0.05 level of significance.

Sample couples	t calculated from data	$t_{0.05}$*	$t-t_{0.05}$
P.I.345635 and control	2.35	2.77	-ve
P.I.345635 and Eston	1.96	2.77	-ve
P.I.345635 and TB406M	3.14	2.77	+ve

* The value obtained from standard t distribution table.