

**AN INVESTIGATION ON THE ROLE OF LEA  
PROTEINS IN DESICCATION TOLERANCE OF  
SEEDS**

**Aida Glazer**

**Ecophysiology Project**

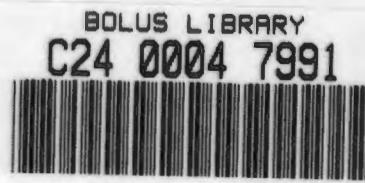
**Supervisor: Dr J.M. Farrant**

**University of Cape Town**

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## ABSTRACT

A set of heat stable proteins that accumulates late in embryogenesis (LEA proteins) has been hypothesised to have a role in protecting mature orthodox seeds against desiccation damage. A test of whether LEA proteins are indeed involved in desiccation tolerance of orthodox seeds, would be that they are absent from recalcitrant seeds. This study aimed to test this suggestion, using 1-Dimensional SDS-Polyacrilamide gel electrophoresis to assay for the presence of LEA proteins during various stages of development in the seeds of the following species: *Podocarpus henkelii* and *Aesculus hippocastanea* (recalcitrant species); an orthodox (control), *Pisum sativum* var *Knight*. It was found that in the orthodox control, at least four new heat stable proteins, characteristic of LEAs, accumulate during the final stages of seed maturation. Furthermore, in the recalcitrant seeds, no new heat stable proteins characteristic of LEAs accumulate at maturity; although heat stable proteins are present in axes and/or cotyledons at a mature stage, they are still present from an early stage. Thus it is concluded that the results of this study are in accordance with the findings of other researchers. However, it is suggested that the use of gradient gels, 2-D gel electrophoresis and even Fluorography would have been more appropriate for this study.

## INTRODUCTION

In recent years, a great deal of interest has been generated regarding the phenomenon that some seeds can be stored while others cannot. Seeds can essentially be divided into two groups- orthodox or recalcitrant seeds (Roberts 1973), and it is the orthodox seeds which have the ability to be stored. Ultimately, this storage ability is a result of their ability to withstand desiccation. Such desiccation tolerance is believed to be developmentally controlled (Kermode 1990) and it has been established that embryos become more tolerant as they mature, and less tolerant shortly after they germinate (Bewely 1979).

When they are shed from the parent plant, orthodox seeds have a low moisture content, and are metabolically inactive (Roberts 1973). At this stage, they are able to tolerate low levels of desiccation, and in the dry state, of sub-zero temperatures. Thus, successful storage for long periods of time can be achieved by storing the dry seeds under low temperature and low relative humidity conditions. In doing so, the dry metabolically inactive state is maintained while the onset of the less tolerant germinating phase is prevented.

Recalcitrant seeds, however, have a high moisture content and are metabolically active when shed (Farrant, Pammenter and Berjak 1988, 1989; Farrant, Berjak and Pammenter 1992a; Vertucci and Farrant 1993). At this stage they are intolerant of even the slightest amount of drying and, in most species, of chilling. Because of this, storage of recalcitrant seeds under conditions optimal for orthodox seeds, is lethal. Furthermore, recalcitrant seeds initiate germination on, or shortly after shedding from the parent (Farrant *et al.* 1986, 1988, 1989). Thus, storage under natural conditions (where high seed moisture content at ambient

temperatures are maintained), allows the progression of the germination process. This process cannot be halted, and ultimately, additional water and/or nutrients are required to continue the germination process. As these are not supplied in store, damage accumulates and viability is lost. This is usually exacerbated by microbial contamination. Seed viability ranges from hours to a few months depending on the species (Farrant *et al.* 1993).

The mechanism whereby orthodox seeds are able to tolerate drying to low water contents is still poorly understood (reviewed by Kermode 1990). Because it is known that these seeds become tolerant of drying at a very precise stage during their embryological development, many researchers have concentrated on events prior to and after the onset of desiccation tolerance in order to ascertain what changes might be ascribed to the ability to tolerate drying. Several hypotheses have been advanced (Clegg 1986; Koster and Leopald 1988; Rosenburg and Rinne 1988; Chen and Burris 1990; Kermode 1990; Blackman, Wettlaufer; Koster 1991; Obendorf and Leopald 1991; Farrant *et al.* 1992b; Farrant *et al.* 1993).

One such hypothesis has found that in many orthodox seeds, a set of proteins called Late Embryo Abundant (LEA) proteins are produced during the late stages of embryological development (Rosenburg and Rinne 1988; Farrant *et al.* 1993). The expression is more or less coincident with the onset of desiccation tolerance, and expression ceases when desiccation tolerance is lost during germination of the seeds. Furthermore, these proteins appear to have no metabolic or enzymic role, they are hydrophilic and very robust (they remain soluble during heating to 80°C) (reviewed by Kermode 1990; Blackman *et al.* 1991). Thus it has been suggested that these proteins might play a role in the ability of orthodox seeds to tolerate desiccation.

Recalcitrant seeds are not tolerant of desiccation. Thus the mechanism of desiccation tolerance must be absent or non-functional in such seeds (Farrant *et al.* 1993). A test of whether LEA proteins are indeed involved in desiccation tolerance of orthodox seeds would be that they are absent from recalcitrant seeds. Farrant, *et al.* (1992a) have shown that the highly recalcitrant seeds of *Avicennia marina* do not have LEA proteins. If LEA proteins are truly involved in desiccation tolerance, then they will be universally absent from desiccation sensitive tissues, and universally present in desiccation tolerant tissues.

This project was undertaken as a preliminary study to test this suggestion; the rationale being that the LEA proteins will not occur in the recalcitrant seed types, but become evident during the late stages of development of the orthodox varieties. Although there are a large number of recalcitrant species to be tested, two were used in this project. Those chosen, however, were diverse in characteristics (gymnosperm versus angiosperm) and natural habitat (subtropical versus temperate). These results can then be compared with the finding for the tropical, recalcitrant species, *Avicennia marina*. The presence of LEA proteins were assayed for, during various stages of development of *Podocarpus henkelii* and *Aesculus hippocastanea*. As a control, similar studies were performed on an orthodox seed, *Pisum sativum* var *Knight*. *not italicis*

7cm in length. Axial samples are termed P2, and cotyledonary material, P3. This allowed for cotyledon proteins to be differentiated from axes proteins in the immature phase. Mature axes (P4) and cotyledons (P5), were excised from mature seeds, which had commenced maturation drying. After material had been collected at the relevant stages, it was lyophilised and stored at  $-20^{\circ}\text{C}$ , until extraction.

Seeds of *Podocarpus henkelii* were collected from Pietermaritzburg Botanical Gardens, and couriered to Cape Town. On arrival, embryos were excised from the female gametophyte, and axes and cotyledons were separated. These were then lyophilised and stored at  $-20^{\circ}\text{C}$  until extraction. Developmental stages (immature and mature), were identified by, and based on the developmental study by Dodd, van Staden and Smith (1989). According to that study, flowering<sup>gymnosperm!</sup> occurs in January, while seeds ripen in late May. Immature material for this study was collected in March, and mature material was collected in May.

A recent study performed on *Aesculus hippocastanea*, has shown that 16 developmental stages can be recognised in this species (Farrant, *pers comm*; Figure 1).

**Materials:**

The following solutions were used in the extraction processes, and in the preparation, loading and running of gels:

**1. Extraction Buffer (pH 7.5)**

0.1M Tris

0.1M EDTA

2mM Phenylmethylsulfonyl fluoride (PMSF)

Stored at 4°C

**2. Sample Buffer (pH 6.8)**

10% Glycerol

5% 2-mercaptoethanol

0.06M Separation gel buffer

2% SDS

Stored at 4°C

**3. 10% SDS**

Stored at room temperature



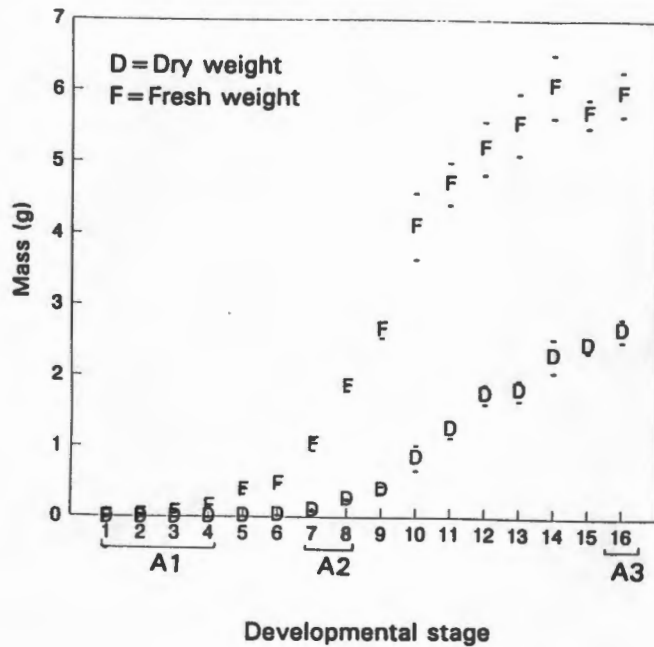


Figure 1: Mass changes associated with developmental changes in the cotyledon of *Aesculus hippocastanea*

From Farrant (unpublished data)

Seeds, from each of these stages, were collected in Colorado, USA. Where possible, embryos and cotyledons were separated, and the material was then lyophilised and stored at  $-80^{\circ}\text{C}$ . For the purposes of this study, some material was air freighted from Colorado to Cape Town, where extractions were performed immediately. The following developmental stages were chosen for experimentation (Figure 1): stages 1-4 (pooled tissues [A1]); stages 7&8 (A2); and stage 16 (A3). These were selected on the basis that A1 represented tissues that had just completed histodifferentiation; A2 represented tissues which had just commenced reserve accumulation and A3 was mature tissue.

**4. Stacking Gel Buffer (SGB) (pH 6.8)****0.5M Tris****Stored at 4°C****5. Resolving Gel Buffer (RGB) (pH 8.8)****1.5M Tris****Stored at 4°C****6. Monomer Solution****30% Acrylamide****2.7% N,N Bis Acrylamide****Stored at 4°C****7. 10% Ammonium persulphate****Made up fresh before use****8. Running Gel Buffer****0.025M Tris****0.192M Glycine****1% SDS****Stored at 4°C**

## 9. Stain Solution

0.025% Coomassie Blue

7% Acetic Acid

40% Methanol

Stored at room temperature

## 10. Destain Solution

50% Methanol

10% Acetic Acid

Stored at room temperature

## Protein Extraction:

With the exception of *Pisum sativum*, where material was considerably limited, approximately 100mg of tissue was extracted in 1ml extraction buffer. For *Pisum sativum*, <sup>9.5 mg</sup> 0.00933g of P1; <sup>10.3 mg</sup> 0.01030g of P2; 0.06g of P3; 0.02636g of P4; and 0.15786g of P5 was used. The powder was suspended in 150 $\mu$ l (for P1 and P2), 600 $\mu$ l (for P3), 300 $\mu$ l (for P4), and 1.5 ml (for P5) extraction buffer. Lyophilised material was ground using a pestle in a pre-chilled mortar. After the addition of the extraction buffer, the suspension was kept on ice for 10 minutes. Samples were centrifuged at 4°C at 16000 rpm in a Beckman Model J2-21 for 20 min. Because of the small sample size of *P. sativum*, extractions were centrifuged in a Heraeus Sepatech Biofuge 15. Samples were spun at 11000 rpm for 30 minutes. Supernatants were stored at -20°C before heating and preparation for electrophoresis as described below. The maximum length of storage was 1 week.

### **Preparation of heat stable proteins**

Total protein supernatants were divided into two. Half remained unheated (control; ie. total protein). The second half was heated to 80°C in a water bath for 10 minutes. Heat coagulated proteins were removed by centrifugation at 10000rpm, for 15 minutes. The protein content of the control and heat stable samples was determined using the Bradford (1976) assay. Samples containing approximately 80µg protein were removed from both control and heat stable solutions, and an equal volume of sample buffer was added. All samples were heated to 95°C for 2 minutes in order for SDS to associate with the proteins. Samples were cooled to room temperature, 5µl Bromothymyl Blue added (which served as a tracking dye), and proteins were separated by electrophoresis as describe below. In order to determine molecular weights of the proteins separated, molecular weight samples (molecular weight range: 12,3-78,0Kd), BDH Laboratory supplies, were included for each gel run. Molecular weight standards were made up according to instructions, and heated to 95°C for 2 minute prior to loading.

### **Preparation of gels:**

It was found that the best gels resulted when the following constituents for the resolving and stacking gels were used (Table 1):

Table 1: Constituents for the resolving and stacking gels

	Resolving gel	Stacking gel
1. Monomer	10ml	1.33ml
2. SGB		2.5ml
3. RGB	7.5ml	
4. 10% SDS	3.0ml	0.1ml
5. Water	12.05ml	6.1ml
APS	66 $\mu$ l	50 $\mu$ l
TEMED	10 $\mu$ l	5 $\mu$ l

APS was made up fresh for each run. The solution consisting of 1-5, was degassed for 20 minutes before APS and TEMED were added. Gels were cast in the SE 245 Mighty Small Dual Gel Caster.

#### Loading gels:

Protein was extracted, as described above, and the following procedure was employed for loading the gels. Appropriate volumes of sample, containing approximately 80 $\mu$ g of protein were loaded in each well. Different species were separated on different gels. Except for the *Pisum sativum*, samples were loaded such that axial material was situated together, and separate from cotyledon material. Thus, the *Podocarpus henkelii* gel was loaded as follows: lane 1, immature axis (control); lane 2, immature axis (heat stable); lane 3, mature axis

(control); lane 4, mature axis (heat stable); lane 5, immature cotyledon (control); lane 6, immature cotyledon (heat stable); lane 7, mature cotyledon (control); lane 8, mature cotyledon (heat stable); lane 9, molecular weight marker. Due to the fact that 3 developmental stages were chosen in *Aesculus hippocastanea*, two gels were loaded; one with the axial material, and the other with the cotyledonary material. The axial material was loaded as follows: lane 1, immature (A1) axis (control); lane 2, immature (A1) axis (heat stable); lane 3, immature (A2) axis (control); lane 4, immature axis (A2) (heat stable); lane 5, mature (A3) axis (control); lane 6, mature (A3) axis (heat stable); lane 7, molecular weight marker. The cotyledonary material was loaded as follows: lane 1, immature (A1) cotyledon (control); lane 2, immature (A1) cotyledon (heat stable); lane 3, immature (A2) cotyledon (control); lane 4, immature cotyledon (A2) (heat stable); lane 5, mature (A3) cotyledon (control); lane 6, mature (A3) cotyledon (heat stable); lane 7, molecular weight marker. Because 3 stages of development were also chosen in *Pisum sativum*, two gels were loaded for this species as well. However, the axial material was not separated from the cotyledonary material. The first gel was loaded as follows: lane 1, whole embryo (P1) (control); lane 2, whole embryo (P1) (heat stable); lane 3, immature axis (P2) (control); lane 4, immature axis (heat stable) (P2); lane 5, immature cotyledon (P3) (control); lane 6, immature cotyledon (P3) (heat stable); lane 7, mature axis (P4) (control); lane 8 mature axis (P4) (heat stable); lane 9, molecular weight marker. The second gel was loaded as follows: lane 1, mature cotyledon (P5) (control); lane 2, immature cotyledon (P5) (heat stable); lane 3, molecular weight marker.

**Running gels:**

Running gel buffer was added to the wells and to the upper and lower buffer tanks. Electrodes were connected to the Hoefer PS 500X Power Supply, and gels were run (approximately 45 minutes) at a constant current of 30A per gel. The voltage was monitored to ensure that it was comparable between gels. Gels were then stained for 2 hours in the stain solution, removed and destained over two days during which the destain solution was changed on a regular basis.

## RESULTS

### *Pisum sativum* var *Knight*

Figure 2 shows the control and heat stable proteins of the various stages of *P. Sativum* var *Knight*. While protein bands in the whole embryo were distinct in the control (lane 1), bands in the heat stable proteins (lane 2) were faint. However, four new proteins (1, 2, 3 and 4) could clearly be seen in both the axes (lanes 8) and cotyledons (lane 11) in the mature stage. Another protein (5) also appeared in the mature stages, but was only new to the cotyledons (lane 11). Bands in the P4 control were very dark. This is attributed to the fact that most proteins have accumulated at this mature phase, and the lanes were overloaded as a consequence of the high protein levels. Despite this, the relevant protein bands, 1, 2, 3 and 4 could still be seen.

### *Podocarpus henkelii*

Figure 3 shows the control and heat stable proteins of the various stages of *Podocarpus henkelii*. Three major heat stable proteins (6, 7 and 8) of low molecular weight, were found to be present. They occurred in the cotyledons and axes of both the immature and mature stages and were present in the control and heat stable samples. High molecular weight proteins (1, 2, 3, 4, and 5) were also present at all stages of development in the axes and cotyledons of both control and heat stable samples. No new proteins could be seen in these bands at any stage.



*Aesculus hippocastanea*

Figures 4 and 5 show the control and heat stable proteins of the various stages of the axes and cotyledons, respectively for *Aesculus hippocastanea*. As in the *Pisum sativum*, protein bands in the post-histodifferentiation stage were clear in the controls (lane 1; Figures 4 and 5), but faint in the heat stable lanes (lane 2; Figures 4 and 5). The heat stable proteins 8 and 10, occur at all stages in the axes and cotyledons. However, heat stable proteins 2, 3, 4, 5, 6, and 7, occur in the later immature stage (lane 4; Figure 4) and mature stage of the axes only. They could all faintly be seen in the control lane of the post-histodifferentiation phase (lane 1; Figure 4). Furthermore, all were absent from the post-histodifferentiation phase of the cotyledons (lane 2; Figure 5), and only proteins 3 and 4 were found in the next immature stage (lane 4; Figure 5). However, they are all faintly present in the immature controls (lanes 1 and 3; Figure 5).

In the cotyledons, protein 5 seems to be newly formed in the mature stage (lane 6; Figure 5) it was absent in both immature controls and heat stable samples. Another new heat stable protein (1) appears in the immature stage (after post-histodifferentiation) in both the axes and cotyledons, (lane 4; Figures 4 and 5). It persists to the mature phase (lane 6; Figures 4 and 5). Protein 9 (lane 2; Figures 4 and 5) occurs in the immature post-histodifferentiation phase in both axes and cotyledons. It has a heat stable capacity at this stage, but does not reappear in heat stable lanes of later stages (lanes 4-6; Figures 4 and 5).

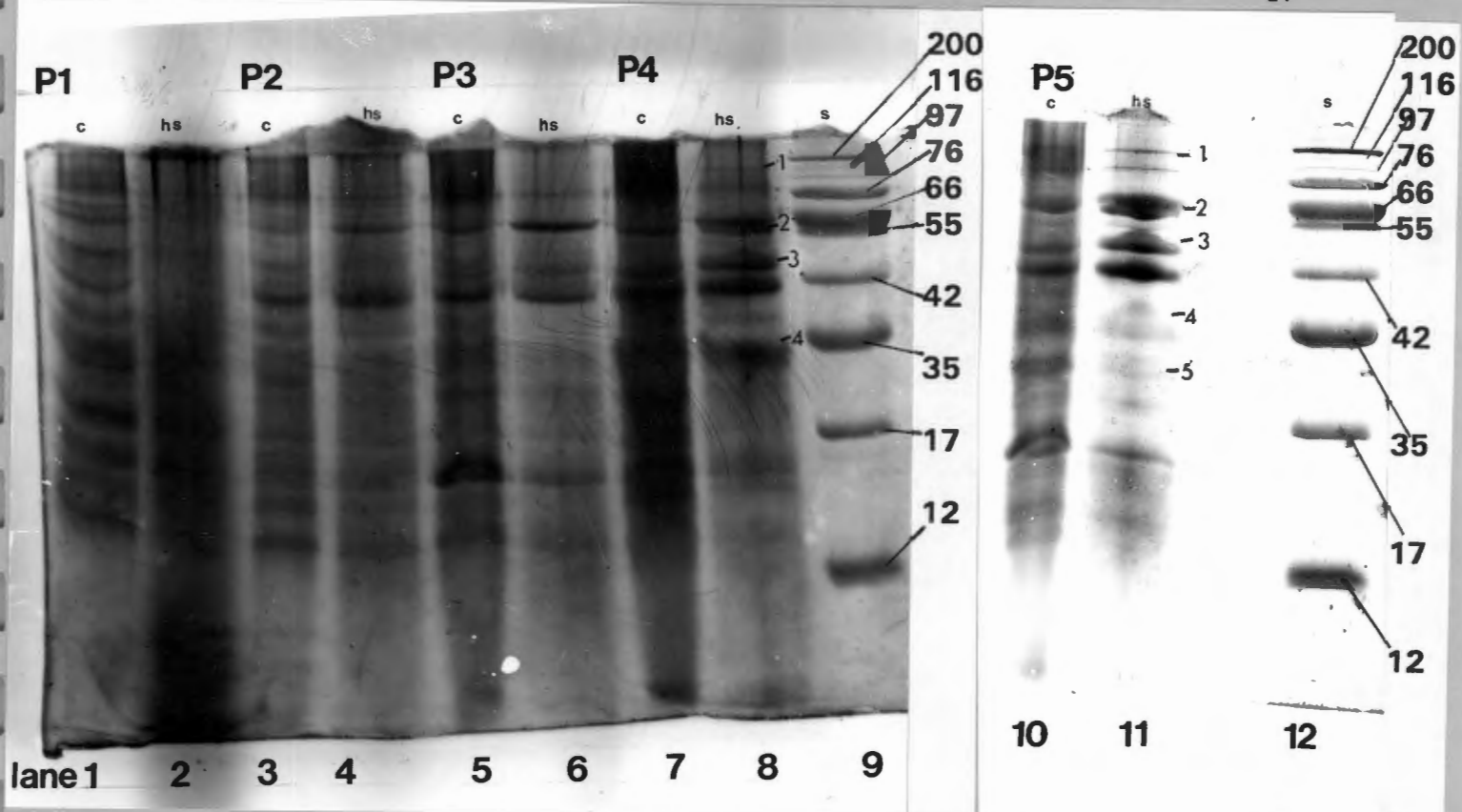


Figure 2: One-dimensional separation of control (c) and heat stable proteins (hs) at different developmental stages of *P. Sativum* var *Knight*. Molecular mass standards (s) are indicated to the right of lanes 9 and 12. Numbers assigned to new heat stable proteins are indicated to the right of lane 8.

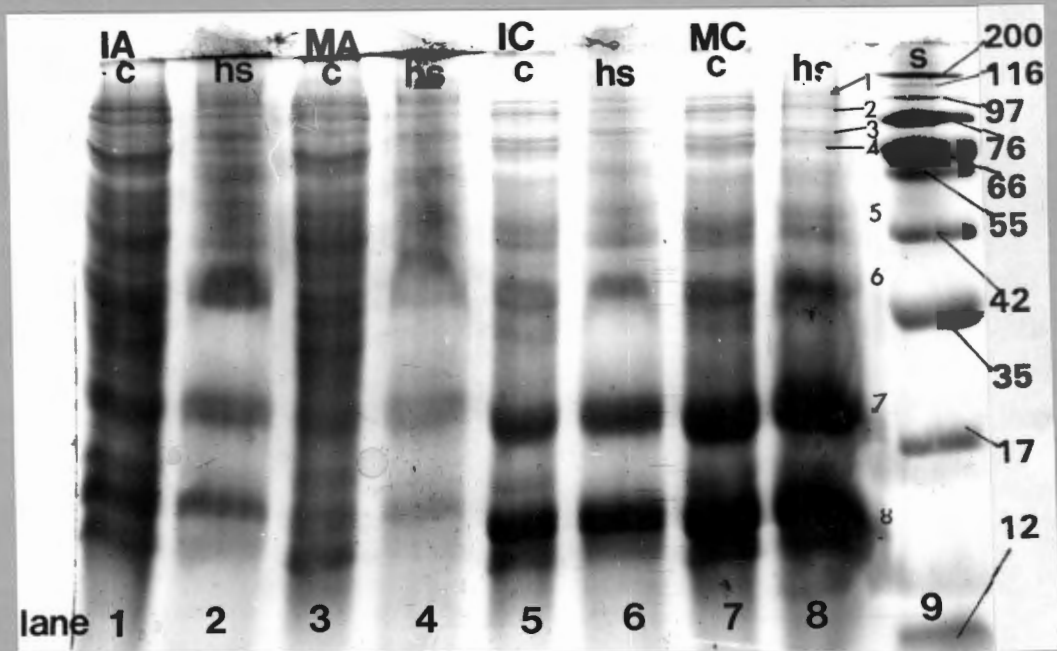


Figure 3: One-dimensional separation of control (c) and heat stable proteins (hs) at different developmental stages of *Podocarpus henkelii* (IA=immature axes; IC=immature control; MA=mature axes; MC=mature control). Molecular mass standards (s) are indicated to the right of lane 9. Numbers assigned to heat stable proteins are indicated to the right of lane 8.

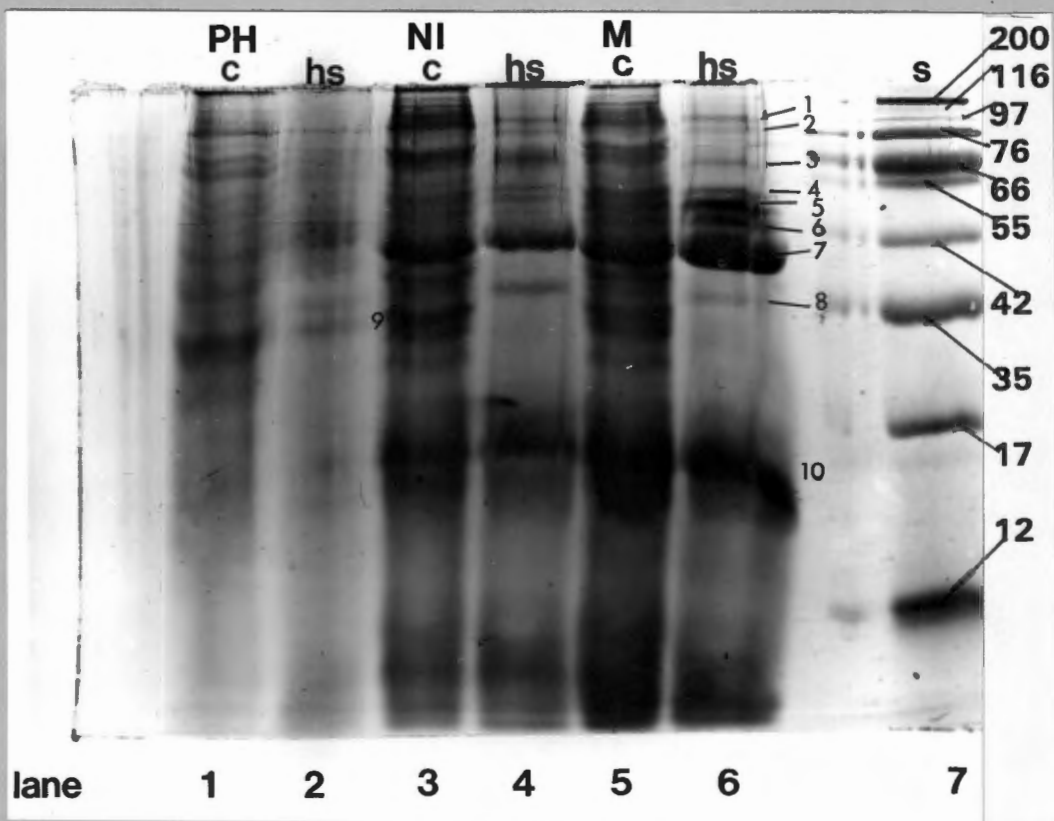


Figure 4: One-dimensional separation of control (c) and heat stable proteins (hs) at the various developmental stages of the axes of *Aesculus hippocastanea* (PH=post-histodifferentiation; NI=next immature stage; M=mature stage). Molecular mass standards (s) are indicated to the right of lane 7. Numbers assigned to heat stable proteins are indicated to the right of lane 2 and lane 6.

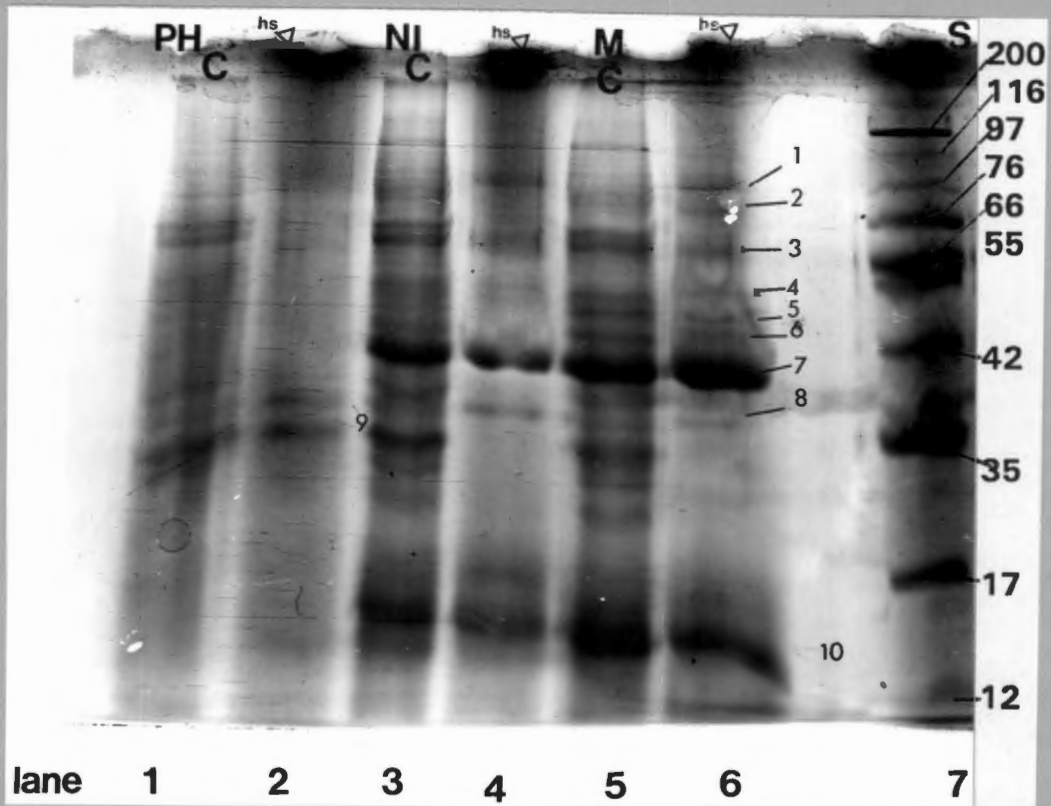


Figure 5: One-dimensional separation of control (c) and heat stable proteins (hs) at the various developmental stages of the cotyledons of *Aesculus hippocastanea* (PH=post-histodifferentiation; NI=next immature stage; M=mature stage). Molecular mass standards (s) are indicated to the right of lane 7. Numbers assigned to heat stable proteins are indicated to the right of lane 2 and 6.

## DISCUSSION AND CONCLUSIONS

Several authors have noted the accumulation of certain proteins and their mRNA's during the late stages of seed development (eg. Kermode 1990). These have been called different things eg. LEAs (Dure, Crouch, Harada, Ho, Mundy, Quatrano, Thomas and Sung 1989), dehydrins (Bradford and Chandler 1992) and maturation proteins (Blackman *et al.* 1991). In addition it is believed that they may all have the same role in protection against desiccation. To date, Blackman *et al.* (1991) are the only authors who have identified actual Lea proteins (as opposed to the mRNA for LEAs) by heat stable methods. That study will be used to compare results from the present study.

The poor protein separation in the immature post-histodifferentiation phase in *Pisum sativum* and *Aesculus hippocastanea* could be attributed to the fact that protein levels are low at this stage. On completion of histodifferentiation, the only proteins present are enzymic or structural, and it is unlikely that tissues have accumulated storage proteins. Furthermore, it is likely that Bradfords' assay (1976) is insensitive at low protein concentrations and thus, error arises in protein determination in this range. This could lead to underloading. Underloading is especially likely to occur in heat stable samples, where proteins of already low concentrations, are spun out. Furthermore, interfering substances in the extraction buffer may give artificially high readings in the Bradford assay, resulting in underloading. This would be particularly critical in samples, such as these, with low protein contents. | such as

Consequently, comparing proteins from such an early stage is questionable, and little confidence will be placed in the discussion of results from this stage. It is felt that

comparison between proteins in later immature and mature stages is acceptable because the later immature stage is well before the onset of drying (the moisture content was still high) (Higgins-Opetz, *pers comm*).

In the present study, the increase in heat stable proteins in the mature stage of orthodox *Pisum sativum* seeds was consistent with the findings of Blackman *et al.* (1991). Furthermore, all of them had similar molecular weights to some LEAs identified in the study by Blackman *et al.* et al (1991) (Table 2).

Table 2: Comparison of the molecular weights of maturation proteins of *Pisum sativum* (present study) with LEA proteins in *Glycine max* [taken from Blackman *et al.* (1989)]; a+c=found in both axes and cotyledons; c=found only in cotyledon.

	Molecular weights (kD)	
	<i>Pisum sativum</i> var <i>Knight</i>	<i>Glycine max</i>
Protein 1 (a+c)	116.0	120.6
Protein 2 (a+c)	55.0	50.78
Protein 3 (a+c)	49.7	50.78
Protein 4 (a+c)	30.0	30.0
Protein 5 (c)	22.5	26.25

However, while proteins 1, 2, 3 and 4 were new to both the axes and cotyledons, protein 5, was new only to the mature stages of the cotyledons. To date no distinction has been made on whether LEAs are restricted to axes and/or cotyledons, or whether this would vary

between species. In the study by Blackman *et al.* (1991) only proteins from excised axes were examined.

It could be argued that LEAs are most likely to occur in both axes and cotyledons. Presence in both would presumably provide maximum protection against desiccation in all tissues. Based on this, only proteins 1, 2, 3 and 4 would be LEAs. However, it could also be argued that LEA accumulation in the axes alone, is <sup>more</sup> most important, because the axis is the structure from which the new plant develops. LEA accumulation in the cotyledons would presumably be wasteful since cotyledons serve primarily as food reserves.

Yes, but they do retain/regain structure + function on rehydration.

It is suggested that the former argument is more feasible although this would require further testing for the presence of LEAs in the cotyledons in *Glycine max*. Bearing this in mind, as well as the fact that these proteins have similar molecular weights to the LEAs in *Glycine max*, it is likely that proteins 1-4 are all LEAs. Protein 5, however, may be a storage protein; the cotyledons being the primary site for storage protein accumulation. In addition, while there is some sense in restricting LEAs solely to the axes, there appears no obvious reason why LEAs would be restricted solely to the cotyledons.

If it is a storage protein, surely there would be a lot more of it

Farrant *et al.* (1992a) are the first to have published data on the nature and pattern of protein synthesis during development and early germination in desiccation sensitive seeds. They concluded that in *Avicennia marina*, although heat stable proteins are present in axes and/or cotyledons at a mature stage, they are <sup>all</sup> still present from an early stage. Thus, they cannot be characterised as LEAs. In that study, these findings were also backed up with 2D protein separation as well as fluorography on labelled 2-D gels. Similar findings can tentatively be



said to occur from this study for the recalcitrant species, *Podocarpus henkelii* and *Aesculus hippocastanea*.

For instance, in *Podocarpus henkelii*, low molecular weight proteins 6, 7 and 8 were found in immature and mature heat stable samples. Due to the amount present, they are suggested to be storage proteins. Dodd, van Staden and Smith (1989) have reported the occurrence of such proteins in this species. Furthermore, no new proteins could be seen in the high molecular bands in the axes and cotyledons of *Podocarpus henkelii* at any stage of development (proteins 1, 2, 3, 4 and 5 occurred at all stages in both the axes and cotyledons).

In *Aesculus hippocastanea*, proteins 8 and 10 were the only proteins that occurred in all stages of both axes and cotyledons. The high concentrations of protein 10 suggests that it might have been a storage protein. Although proteins 2, 3, 4, 6 and 7 were absent as heat stable proteins in the immature cotyledons, they were present at all stages in the axes and in the immature control of the cotyledons. This strongly suggests that these proteins may all be present in the immature cotyledons, but are not seen as a consequence of underloading. Further evidence for underloading may be that there is a faint hint of protein 3 in the heat stable lane of the immature control. This suggests that they are not new to the mature cotyledons, and are thus unlikely to be LEAs.

Furthermore, high concentrations of protein 7 in the later stages suggests that it may be a storage protein. As a consequence, this protein would not be expected to be evident at an early stage anyway, since reserve accumulation occurs after histodifferentiation (Dure 1989).

Protein 5 is a newly formed protein, as it is not found in the immature control or heat stable samples of the cotyledons. Since it has the same molecular weight (49.7 kD) as protein 3 in *Pisum sativum* (Table 2), it may be suggested to be a LEA. An assessment of mass changes associated with the various developmental stages in *Aesculus hippocastanea*, reveals that the appearance of this protein coincides with the onset of seed drying <sup>by how much.</sup> during stages 7-14 of its development (Figure 1). Thus, it is possible that this protein is a LEA and is involved in enhancing tolerance to drying during seed development.

However, from Table 3 (Farrant 1993; unpublished data), the lethal moisture content below which damage occurs at this stage (days 8-10), is the same as that of early developmental stages (days 0-5). Thus the presence of this protein does not seem to enhance tolerance to desiccation.

Table 3: Lethal moisture content in *Aesculus hippocastanea* below which damage occurs. Days 0-5 are early developmental stages. Days 8-10 represent the growth spurt at the beginning of maturation.

Stage (days)	0-5	8-10
Moisture content below which damage occurs (g.g <sup>-1</sup> )	0.8	0.8

This could imply one of three things:

1) this protein is not a LEA, but rather a storage protein. It could also be a protein involved in cold hardiness for survival of temperate conditions. It has recently been shown that

*Not a new idea*

another class of "stress proteins" exists (Vertucci and Farrant 1993). They may be expressed when a stress such as heat is applied, and Vertucci and Farrant (1993) have alluded to the fact that they may also be expressed during cold hardiness. Since they are heat stable proteins, they may thus appear similar to LEAs. Furthermore, following the argument presented earlier, LEAs are more likely to be restricted to either the axis, or to the axes and the cotyledons, as in *Pisum sativum*. There seems no advantage in restricting LEAs to the cotyledons only. Also, since this protein is found in both axes and cotyledons at all stages in the mature axis, absence in the immature cotyledons is suggested to be a result of underloading.

2) this protein is a LEA. However, contrary to previous suggestions, Leas in general may not play a role in desiccation tolerance as this protein did not seem to protect against desiccation.

3) this protein is a LEA, but it had no role in desiccation tolerance because more than one LEA is required to protect against desiccation. Furthermore, LEAs may only be effective in desiccation tolerance when they occur in conjunction with other factors which are believed to play a role in desiccation eg. certain carbohydrates (Chen and Burris 1990). This is a more feasible explanation than the second scenario, because it is known that the expression of LEA proteins or their transcripts alone is not sufficient for tolerance (Blackman *et al.* 1991; Johnson-Flanagan, Huiwen, Geng, Brown, Nykiforuk and Singh 1992; Ried and Walker-Simmons 1993). However, in reviewing all arguments, it is suggested that the first argument is the most valid based on evidence in the literature.

Protein 1 may be considered to be a LEA as it is absent from the immature heat stable stages in the cotyledons. However, this protein is not new as it can be seen in the later immature

control and in the immature control and heat stable lanes in the axis. Thus, it is probably not a LEA, and may be absent from the immature heat stable lane as a consequence of underloading. An indication of underloading is that other high molecular weight proteins in this lane were not particularly clear either.

The loss of the heat stable protein 9 after stage A1, could be a consequence of this protein losing its heat stable capacity during seed development. An alternative suggestion is that it is not synthesised at later stages, and thus appears less in relation to other proteins at such a young developmental stage. As it occurred in the control and immature stages, it was suggested to have no relation to LEAs.

## CONCLUSION

The results of this study tentatively suggest that LEA proteins may indeed be absent from recalcitrant seeds. Although in *Podocarpus henkelii* and <sup>*Aesculus* ?</sup> *Pisum sativum* it appeared that "new" heat stable proteins developed with the onset of maturation, it could be argued with confidence that they were not LEAs. Either they were found to be absent from the immature phase because of underloading, or, they were uncharacteristic of LEAs in that they were effectively not new proteins. Thus the results of this study are in accordance with the findings of Farrant, Berjak and Pammenter (1992a).

Furthermore, the hypothesis that LEA proteins accumulate with the onset of maturation in orthodox seeds (Rosenburg and Rinne 1988; Farrant *et al.* 1993) was supported in this study. At least four new heat stable proteins, characteristic of LEAs, accumulated during the final stages of seed maturation in the orthodox control, *Pisum sativum*.

However, it is suggested that further validation of the findings in this study could have been achieved through the use of gradient gels, as well as 2D protein separation and fluorography on labelled 2-D gels similar to that used by Farrant *et al.* (1992a). This would have improved protein separation. Also, confirmation of tentative results may have been achieved if the nature of proteins in germinating material had been examined. Since LEAs are believed to disappear with the onset of germination, the new proteins in *Pisum sativum* would be predicted to have been absent at this stage. Thus, examination of germinating material would have provided more conclusive results as to the nature of these proteins. Similarly, uncertainties over the nature of "new" proteins in *Podocarpus henkelii* and *Aesculus*

*hippocastanea* could have been overcome had germinating material been considered.

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