Physiology of development, germination and ubiquitination in pea seeds.

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ABSTRACT

The development of entire seeds and axes of *Pisum sativum* L. cv. Spring was followed between 18 and 47 days after flowering, in terms of changes in fresh weight, dry weight, water content, protein level and chlorophyll content. The seed developed normally, but somewhat slower than suggested in other reports. Fresh immature seeds and axes placed in culture were able to germinate after a lag phase which varied depending upon the time of detachment of the seed from the legume pod and the axis from the seed. The seeds from all stages tested produced a viable seedling. Except for the least mature, the axes germinated fully, but grew only slightly. Root growth was particularly impaired.

Ubiquitin conjugates were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and labelling with rabbit anti-ubiquitin followed by ^{125} I goat antiRabbit-IgG. Ubiquitinated proteins were detected in seeds during development and germination, but not in dry mature seeds. Only free ubiquitin was observed in extract from dry seed. A correlation between higher concentration of ATP in the seed, and presence of ubiquitin conjugates was observed. These results demonstrate that pea seed can contain both unconjugated and conjugated ubiquitin.

INTRODUCTION

It is generally accepted that seeds have to attain a certain level of maturity before becoming capable of germination and subsequent seedling growth (Matthews, 1973; Dasgupta, Bewley and Yeung, 1982; Kermode, 1990). In some instances, maturation drying appears to be required for germination and subsequent growth (Long *et.al.*, 1981; Dasgupta and Bewley, 1982; Adams *et. al.*, 1983; Kermode and Bewley, 1985 a,b; Kermode *et. al.*, 1985). In many studies, however, the question of the capacity of the immature seed to produce a viable autotrophic seedling is not addressed. In addition, it implied in much of seed germination research, that isolated embryonic axes, once germinated, will produce a viable autotrophic seedling (Le Deunff and Rachidian, 1980). Contradicting this, some research suggest, the axis will not develop into autotrophic seedlings if deprived of storage reserves (Bain and Mercer, 1966c; Tabor and Barnett, 1987; Mapes and Zaerr, 1981). However, work on somatic embryos indicates that heterotrophically fed somatic embryos were able to germinate and produce a seedling (Durzan and Gupta, 1987; Boulay *et. al.*, 1988; Dunstan *et. al.*, 1988; Hakman and von Arnold, 1988; Roberts *et. al.*, 1990; Attree *et. al.*, 1992). Specifically in peas, it has been reported that whole plant regeneration can be achieved via somatic embryogenesis from immature zygotic embryos provided heterotrophic nutrients are supplied (Kysely *et. al.*, 1987). This led us to re-examine whether isolated pea embryonic axes could grow autotrophically. The first objective of this study was to investigate the

relationship between seed maturity and subsequent autotrophic growth in developing pea seeds.

The second objective of this study was to investigate the dynamics of ubiquitin conjugation in pea seed during development and germination. The presence of ubiquitin and ubiquitin conjugates in higher plants and its involvement in the proteolytic systems has been documented (e.g. Vierstra, 1987; Hatfield and Vierstra, 1989; Veierskov and Fergusson, 1991). Understanding of the dynamics of ubiquitin conjugation in seed development and germination is the first step in studying the potential developmental role of ubiquitin and the related proteolytic system.

LITERATURE REVIEW

1. Morphology and physiology of developing seed in relation to the establishment of the autotrophic seedling.

Peas (*Pisum sativum* L) were chosen for this study because the anatomical and physiological development of the seeds are relatively rapid and well described (Bain and Mercer,1966a). The seed developmental sequence starts with fertilization which occurs at full bloom (Cooper,1938). The subsequent development of a legume seed consist of four phases: 1. cell formation; 2. cell expansion; 3. synthesis of reserves; 4. maturation and dormancy (Bain and Mercer, 1966a; Briartry *et. al.*,1969). Although metabolism of seed development and germination has been studied extensively (e.g. Danielsson, 1952; McKee *et.al.*, 1955; Robertson *et.al.*, 1962; Bain and Mercer, 1966a,b; Millerd, 1975; Stafford and Davies, 1979; Hedley and Ambrose, 1980; Long *et.al.* 1981; Dasgupta *et.al.* 1982; Obendorf and Wettlaufer, 1984; Misra and Bewley, 1985a,b; Misra and Bewley, 1986; Misra *et.al.* 1993; Kermode, 1990), relatively few studies have focused on the relationship between the degree of seed development and ultimate capacity for autotrophic seedling growth. For example, soybean seeds which have moisture content below 60% (63 days after flowering) germinated and grew into seedlings. However, immature seeds (day 35, 68-79% moisture) were also able to germinate, but the germination did not lead to comparable seedling growth (Rosenberg and Rinne, 1986). Poor germination and weak seedling growth also occurred when immature pea seeds (84-55% moisture) were placed in moist folded filter paper (Le Deunff

and Rachidian, 1988). It has been documented that during development, embryo growth is controlled by the surrounding maternal tissues (Howell *et.al.*, 1959; Marinos, 1970; Matthews, 1973). When embryonic axes were excised from seeds and put either in water or in tissue culture condition, they germinated precociously or continued to develop embryonically for a period of time before they germinated (Quebedeaux *et.al.*, 1976; Crouch and Sussex, 1981; Long *et.al.*, 1981; Triplett and Quatrano, 1982; Cook *et.al.,* 1988; Kermode and Bewley, 1988). Whole immature seeds, when excised from the mother plant, also showed a similar pattern to axes (Millerd *et.al.*, 1975; Thompson, *et.al.*, 1977; Wang *et.al.*. 1987; Stafford and Davies, 1979; Fountain and Outred, 1990). Tomato or castor bean seeds, when placed in a fully hydrated environment, were able to germinate without drying (Berry and Bewley, 1991, 1992; Kermode and Bewley, 1989).

In regard to production of viable seedlings, immature embryonic axes and immature seeds detached from the parent plant and put in heterotrophic embryo culture were induced by drying to produce a vigorous seedling (Adams and Rinne, 1981; Kermode and Bewley, 1985; Rosenberg and Rinne, 1986; Gray *et.al.,* 1987; Hakman and von Arnold, 1988; Kermode and Bewley, 1988; Le Deunff and Rachidian, 1988; Roberts *et. al.*, 1990). In addition, drying in combination with specific embryo culture, i.e. sucrose and ABA, also stimulated somatic embryos to produce vigorous seedlings (Finer, 1987; Kysely *et.al.,* 1987; Anandarajah and McKersie, 1990a,b).

2. ATP and ubiquitin conjugate levels in pea seed.

Germination requires ATP, but mature dry seeds do not contain significant levels of ATP (Perl,1986). Rapid increase in ATP levels occurs in the early stages of seed germination (Ching and Ching, 1972; Moreland *et.al.,* 1974; Pradet *et al.* in 1968 cited by Bewley and Black, 1978; Perl, 1980; Linn and Madsen, 1981). In lettuce and sunflower a rapid gain of ATP was measured over the first four hours after imbibition and no further increases in ATP levels occurred until after visible germination commenced (Hourman and Pradet, 1981; Attuci *et al.*, 1991). In cucumber and mung bean seeds, ATP concentration increased rapidly during the first hour of imbibition (Morohashi and Sugimoto, 1988). Similarly, the ATP content of isolated wheat embryos increased gradually in the first hour of imbibition, then stabilized with a slight increase over the next 15 hours (Obendorf and Marcus, 1974). In imbibing radish seeds, a large increase in ATP occurred during first hour of imbibition, then it remained constant until radicle emergence at 16 hours (Moreland *et al.*, 1974). Brown and Wray (1968) reported that in intact cotyledons of pea (var-Meteor), ATP levels increased over the first two days after imbibition and then gradually declined during the following six days. It is apparent that ATP is the main energy source for biological activities during seed germination. Energy for axis growth is derived mainly from storage reserves (Ashton, 1976).

Seed germination is always accompanied by an increase in proteolytic activity in the cotyledons (Csoma and Polgar, 1984). Various proteolytic enzymes have been described in seeds (Beevers, 1968; Guardiola and Sutcliffe, 1971; Yomo and

Srinivasan, 1972; Hobday *et.al.*, 1973; Ryan, 1973; Yomo and Varner, 1973; Basha and Beevers, 1975; Chripeels and Boulter, 1975; Leung and Bewley, 1983; Mitsuhashi *et al.*, 1986; Yang and Malek, 1990), but nothing is known about the role of the ATP-requiring ubiquitin pathway in seed development and germination, or about the ATP-requiring proteosome activity. Ubiquitin was first isolated by Goldstein *et al.*, 1974 during their experiment on purification of peptide hormone from thymus. Ubiquitin was also isolated from a variety of eukaryotic species, and its primary sequence is nearly identical (Finley and Varshavsky, 1985). Ubiquitin was purified from yeast (Wilkinson *et al*, 1986) and oat (Vierstra *et al.*, 1986). Only three amino acid residues differ between plant and mammalian forms of ubiquitin.

Ubiquitin is a small (8.5 kDa), heat stable protein, containing 76 amino acids, and has been identified in the nucleus, in the cytoplasm, and on the plasma membrane. It is proposed to have two main functions: conjugation with short-lived proteins targeted for degradation and conjugation with histone H2A and H2B (Goldknopf and Busch, 1977). Sommer and Suefert (1992) reported that ubiquitin proteolysis is essential for cell viability. This may be due to the involvement of ubiquitin in the control of the half lives of important cellular regulators. Ubiquitin has also been identified as a heat shock protein (Bond and Schlesinger, 1985; Muller-Tanberger *et al.*, 1988).

The basic ubiquitin pathway is well understood (Finley and Varshavsky, 1985; Hershko and Ciechanover, 1986; Rechsteiner, 1987, Vierstra, 1987; Hershko, 1988), and the ATP-requiring proteolytic system involved was recently described (Mayer

et.al., 1989; Haas *et.al.*, 1990; Monia, *et al.*, 1990; Jentsch, *et al.*, 1990; Rechsteiner, 1991). Even though the ubiquitin proteolytic pathway originally was identified in reticulocyte, a similar pathway also occurs in plants (Vierstra, 1987; Hatfield and Vierstra, 1989). In plants, it has been demonstrated that ubiquitin is involved in phytochrome turnover (Shanklin *et.al.*, 1987; Jabben,*et. al*, 1989;), in metabolizing altered protein following high temperature stress (Shimogawara and Muto, 1989; Ferguson, *et al.*, 1990), and participates in photoinhibitory response (Schuster *et.al.*, 1988; Wettern *et al.*, 1990). Ubiquitin covalent conjugation to protein targets serves as a commitment step for degradation (Hershko *et al.*, 1983; Ciechanover, *et al.*, 1984) The ubiquitin protein conjugate is a multi-step process requiring ATP hydrolysis (Haas, *et al.*, 1980; Finley and Varshavsky, 1985; Vierstra, 1987). The ubiquitin conjugated target protein is then rapidly degraded by proteases specific for ubiquitin protein conjugates, again with ATP hydrolysis, and free ubiquitin is released (Hough, *et al*, 1986).

Nothing is known about the possible involvement of ubiquitin in desiccation tolerance and germination of seeds. Furthermore, the existence of ubiquitin conjugate proteolytic enzyme has not been demonstrated in seeds. Seed ubiquitin may have a role in the degradation of proteins which are no longer needed following the transition from development to germination. Ubiquitin and ubiquitin conjugates has not been demonstrated in pea seeds; as well, shifts in relative amounts of free and conjugated ubiquitin during development and germination has also not been recorded.

MATERIALS AND METHODS

1. Plant Materials and Growth Conditions.

Peas (*Pisum sativum* L) c.v. Spring, Asgrow Seeds, Bradford, Ontario treated with Captan, were sown in shallow plastic trays of vermiculite and grown in a growth cabinet with 16 hours light from fluorescent tubes at about 200 uE $s^{-1}m^{-2}$ at 20°C and dark for 8 hours at 15°C. The seedlings were watered twice a week with a quarter strength Hoagland's solution (1.25 mM KNO_3, 1.25 mM $Ca(NO_3)_2$, 0.25 mM KH_2PO_4, 0.5 mM $MgSO_4.7H_2O$, 0.05 mM Fe EDTA, 0.125 mg/L $MnCl_2.4H_2O$, 0.125 mg/L H_3BO_4, 0.0125 mg/L $ZnSO_4.7H_2O$, 0.005 mg/L $CuSO_4.5H_2O$, 0.0125 mg/L $H_2MoO_4.H_2O$). Initial flowering occurred approximately 30 days after sowing. Flowers were tagged on the day of appearance and the seed age counted as days after flowering (DAF). The developing seed pods were collected at 18-47 DAF in 10 different stages: stage 1: 18-20 DAF, stage 2: 21-23 DAF, stage 3: 24-26 DAF, stage 4: 27-29 DAF, stage 5: 30-32 DAF, stage 6: 33-35 DAF, stage 7: 36-38 DAF, stage 8: 39-41 DAF, stage 9: 42-44 DAF, and stage 10: 45-47 DAF. Based on morphological and physiological changes during seed development of *P. sativum* L., the 10 developmental stages arbitrarily overlap the last two stages (III and IV) recognized by Bain and Mercer (1966a). The characteristics of the phases are described in Table 1.

Table 1. Description of developing pea seed from fertilization to maturity (based on Bain and Mercer, 1966).

Developmental stages	Days After Flowering	Description
I	0-10	cell formation active; embryo undifferentiated up to day 7; mitochondria and plastid was differentiated but immature, embryo was differentiated by the 10th day.
II	10-18	cell expansion active; the number of ribosomes increases; water content increases greatly; trace of storage protein appears.
III	18-28	protein synthesis starts increasing in early phase III; then the rate greatly increases until about day 28; dry weight gain throughout this phase, as well as fresh weight; chlorophyll reduction starts at the end of phase III. Phase III was equivalent to stages 1,2,3 and 4 in this study.

IV	28-54	protein deposition continues but very slowly; water content decreases; by day 35 the fresh weight is maximum, then decreases; dry weight increases but slows down as the seeds mature and become dormant. Phase IV was subdivided into stages 5,6,7,8,9, and 10 in this study.

2. Culture of seeds and embryonic axes.

Intact pods were surface sterilized with 1% sodium hypochlorite for 2 minutes and rinsed several times with sterile distilled water, and under aseptic conditions the seeds were removed from the pods. Seeds and excised embryonic axes at the 10 developmental stages were placed aseptically in an autoclaved 9 cm Petri dish containing 50 grams of acid washed sand (white quartz 50-70 mesh, Aldrich Chemical Co. Inc. USA), wetted with 20 ml of quarter strength Hoagland's solution. Each petri dish contained five seeds or five axes as an experimental unit. Nine replicates were used in this experiment. The seeds or excised axes were germinated and grown in a Conviron growth chamber (CMP 3023, Controlled Environment Ltd., Winnipeg, Man.) at the same photoperiod and temperature as during growth. Light intensity was between 70-100 uE m^{-2} s^{-1}.

3. Measurement of germination.

Due to the use of isolated embryonic axes, the doubling of the axis length was chosen as a criterion of completion of germination.

4. Weight changes during seed and excised axis development.

Calculation of whole seed and axis fresh weight, dry weight and water content were based on weight taken before and after oven drying at 90^0C for 48 hours. The changes in water content were calculated as a percent of the fresh weight using:

(fresh weight - dry weight)/ fresh weight x 100

5. Chlorophyll extraction.

All extractions were carried out under dim light. Five replicate samples, each of one whole seed (or 7-10 excised axes) harvested from seeds at different stages, were used. Seeds were ground in a chilled mortar and pestle and axes were ground in a chilled glass homogenizer in 6 ml of 80% acetone (2 ml for axes). The homogenate was centrifuged for 10 minutes at 10,000 x g in a refrigerated centrifuge (Sorvall RC2B, DuPont, Wilmington, Delaware) in an SS34 rotor. Absorbance was recorded at 663 nm and 645 nm with Beckmann DU-50 Spectrophotometer (Beckmann Instruments Inc. Fullerton CA) and Chlorophyll content calculated according to Arnon (1949).

6. Protein extraction and assays.

Eight to ten excised axes (or one whole seed) harvested at 10 different stages were ground in a small chilled mortar and pestle in 3 ml buffer containing 20 mM Tris NaOH pH 7.8, 0.5 M NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was decanted, the mortar rinsed with an additional 1 ml of above buffer, and then was extracted for two hours at 4^0C. The homogenate was centrifuged at 20,000 x g for 20 minutes in a refrigerated centrifuge (Sorvall RC2B DuPont Wilmington, Delaware) in an SS-34 rotor. The pellet was reextracted three times in a glass homogenizer with the same amount of above buffer. For axes, a glass homogenizer was used, and 4 x 1 ml of buffer. The concentration of protein was determined using the Bio Rad Coomassie brilliant blue dye binding method (Bradford, 1976), with bovine serum albumin (BSA) as a standard. Spectrophotometric measurements were made on a Beckmann Model DU-50 spectrophotometer (Beckmann Instrument Inc., Fullerton, CA) at 595 nm.

7. ATP determination.

The levels of ATP were determined according to Lemaster and Hackenbrock (1978). Five seeds were extracted with 5 ml of cold 5% trichloroacetic acid (TCA) using a chilled mortar and pestle. The extract was centrifuged for 10 minutes at

15,000 x g in a Sorvall SS 34 rotor. The supernatant was collected and kept on ice. The pellet was reextracted and centrifuged with an additional 5 ml of 5% TCA. For seed development, 5 ml of 5% TCA were used in total. A 50 ul aliquot from combined supernatant was rapidly mixed with 0.5 ml of luciferin-luciferase (Sigma Chemical Co., St. Louis,MO.) by forceful injection from a syringe into the cuvette of a M600 photometer (Oriel Optics Corp.,Co.) set at an appropriate sensitivity level. Light produced in the reaction was recorded with a chart recorder (Linear Instruments Corp.) and compared with ATP standards.

8. Extraction of protein from pea seeds for electrophoresis , Western transfer, and immunoblotting.

8.1. Germinating seeds.

Protein was extracted from 0, 12, 24, 48, and 96 hours germinated seeds. Five seeds were homogenized in a chilled mortar and pestle in 4 ml of 50 mM Tris-HCl pH 8. One hundred ul of each supernatant were treated with an equal volume of sodium dodecyl sulfate (SDS) treatment buffer (0.125M Tris-HCl pH 8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol) and boiled for 90 seconds. Samples were stored at 4^0C until needed for electrophoresis.

8.2. Developing seeds.

Four different developmental stages were used (stage 0: day 15 to 17, stage 1: day 18 to 20, stage 6: day 33 to 35, stage 10: day 45 to 47). Samples of five previously frozen seeds were ground in liquid nitrogen and buffer as in 8.1. For dry mature seed 1 gram of seed powder was used in 4 ml of buffer. After 90 second boiling treatment with SDS treatment buffer, samples were stored until required.

8.3. SDS Polyacrylamide Gel Electrophoresis and Western transfer.

Approximately 30 ug of proteins were loaded onto each lane. The gels were prepared according to standard methods (Hoeffer catalog, based on Laemmli, 1970). Bio Rad Mini Gel apparatus was used to run electrophoresis at 100 V for about 90 minutes. Following electrophoresis, proteins were electrotransferred to a nitrocellulose membrane (Schleicher & Schuell) using Bio Rad Western Transfer Apparatus at 40 V in Western transfer buffer (20%(v/v) methanol, 25 mM Tris, 192 mM Glycine pH 8) for approximately 20 hours. The nitrocellulose was autoclaved for 5 minutes in the same buffer. The non-specific binding to membranes was "blocked" overnight with 3% dried skim milk powder in Tris Buffered Saline (TBS; 20 mM Tris HCl, 50 mM NaCl, pH 7.5), and subsequently washed in TBS for 2x5 min. The membranes were then incubated with rabbit anti-ubiquitin antibody (Sigma) in 0.2% Tween 20-TBS for 90 minutes and then washed 2 x with TBS as above to remove unbound antibody. Bound primary antibody was detected with ^{125} I-goat anti-Rabbit IgG in 0.2% Tween 20-TBS for 60 minutes. Unbound ^{125} I-goat anti-Rabbit IgG was

then washed out as above. Radioimmunolabelled proteins were detected by autoradiography using Kodak X-Omat RP film enhanced with an intensifying screen (Du Pont Cronex Lightening Plus) at -60^{0}C for about 48 hours.

RESULTS

1. Seed and axis development.

Fresh weight of the seeds increased up to 29 days (stage 4) and then started to decrease (Fig.1). As the seed entered the maturation drying stage, fresh weight decreased greatly. For a mature dry seed, at stage 10 (day 45 to 47), the fresh weight continued to decrease. Axes followed the same pattern (Fig.2). Fresh weight of both seeds and axes reached a maximum between stages 4 to 6 (29 to 35 days). The dry weight of whole seeds increased only slightly from stage 3 until the final harvest. The axes' dry weight increased continuously until stage 6 (day 35) and then remained stable. At maturity, the dry weight of seeds and axes reached 84% and 89% of fresh weight, respectively. Water content decreased throughout the maturation period. The rate of decline of the seed water content was rapid between stage 2 (about 80%) and stage 3, then again at about the beginning of stage 6 (Fig.1). Water loss slowed temporarily after the seed approached maximum dry weight (stage 3) (Fig.3). Minimum water content reached about 16% at stage 10. The axes dried down more gradually from about 70% to 10% with significant water loss starting after full dry weight was attained at stage 5-6, 30 to 35 days (Fig.2).

Protein content of whole seeds increased most rapidly between stages 2 and 3 (20 to 26 days) with axes acquiring protein more gradually between stage 3 and 7 (24 to 38 days)(Fig.3). Mature seed (stage 10) contained about 4% of dry weight as protein in its axis (Fig.3) compared to about 1.5% of dry weight (Fig.1 and 2). As shown in Fig.4, seeds harvested at stage 1 (day 18 to 20), contained 38 ug

chlorophyll per seed. This decreased to 19 ug Cp in mature seeds. Embryonic axes' Cp content declined from 0.75 ug to 0.2 ug (Fig.4). The period of most rapid Cp loss was more abrupt in the embryos (stage 2 to 3) but more gradual in whole seeds (stages 3 to 6).

2. Embryonic axis and seed germination.

The germination of embryonic axes of pea seeds was defined as doubling in size. The three figures described below give different perspectives of the same experiment. Typical germination curves are shown for three different developmental stages of axes (Fig.5). Pea axes older than 35 days (stage 6) germinate nearly 100% within 6 days. Those younger than stage 6 (day 33) showed little growth for several days before they started to germinate. Over the four week experimental period, axes from nearly mature seeds (stages 6-10) attained 50% germination within 3-4 days, while the intermediate stages (stage 3-5) took about 4-5 days (Fig.6). Axes of stage 2 (21 to 23 days) seed required about 7 days to reach 50% germination, while stage 1 axes did not germinate to 50% after 40 days (Fig.6). Total germination (Fig.7) of the youngest axes studied reached only 27%. The least mature seeds, however, germinated 80% in the same period. (Fig.7).

Not surprisingly, when entire seeds were germinated (radicles doubled in length) the germination kinetics were more rapid than for isolated axes (Fig.8 vs. Fig.5). Even the least mature seeds initiated germination on day 10 and were 80% germinated on day 18 (Fig.8). A linear relationship between T_{50} and developmental

stage was observed, with the least mature seeds taking 13 days to reach T_{50} (Fig.9).

3. Autotrophic seedling growth.

Germinated seeds from stage 1 to 10 were all capable of producing a viable autotrophic seedling. Roots and shoots showed good growth (Table 2). After approximately 30 days the seedlings started flowering and produced pods which were comparable in size to those grown from in vivo matured seeds. Germinated axes from stage 1 to 10 were not able to produce autotrophic seedlings. Seedlings from axes from the immature stages (stage 1 and 2) showed very little growth for a few days and then died (Table 2, Fig.10). More mature axes grew better (Fig. 10), but still were not able to produce viable autotrophic seedlings. Seedlings kept more than four weeks remained green but did not elongate further, even when transplanted into vermiculite and supplied with quarter strength Hoagland's. Morphological development of the seedlings growth from seeds and axes collected on day 30 (stage 5) after 3 and 5 days in culture is shown in Fig 11 , and Fig 12.

4. Ubiquitin conjugation in developing and germinating seeds and correlation with ATP.

There were several ubiquitinated proteins present reacting with anti-ubiquitin antibody: 12 kDa, 17 kDa, 28 kDa, 48 kDa and a smear >60 kDa (Fig.13). Bands on the bottom of the gels were free ubiquitin (8.5 kDa). In the early developmental stages, immature pea seeds contained high levels of both free ubiquitin and conjugated ubiquitin (lane A). The concentration of free ubiquitin decreased slightly as the seed matured, whereas the conjugates appeared to be at their highest at stage 1 (Fig.13, lane B) and then decline with maturation drying (lanes C,D). In the mature dry seed, there appeared to be few large molecular weight ubiquitin conjugates with a free ubiquitin signal stronger than in the other lanes (lane G). Conjugation of ubiquitin increased rapidly within 12 hours from the start of imbibition, (lane H), with little change subsequently (lanes I,J,K). Free ubiquitin appeared to decrease slightly (compared to lane G). In the early hours of germination the levels of ATP increased about 16-fold and at 24 hours, about 30-fold (Fig.14). The level of ATP was high in the immature seeds and then declined to about 20 nmoles per seed as the seed matured (Fig.15). This was higher than in the commercially obtained dry seeds, 5 nmoles per seed (Fig. 14), which were stored for at least one year.

Fig.1. Changes in fresh weight (○), dry weight (●), and water content (□) of pea seed during development. Each point was based on forty five seeds (nine replicates of five) ± SE. Means without vertical lines indicate that the standard error is smaller than the symbol.

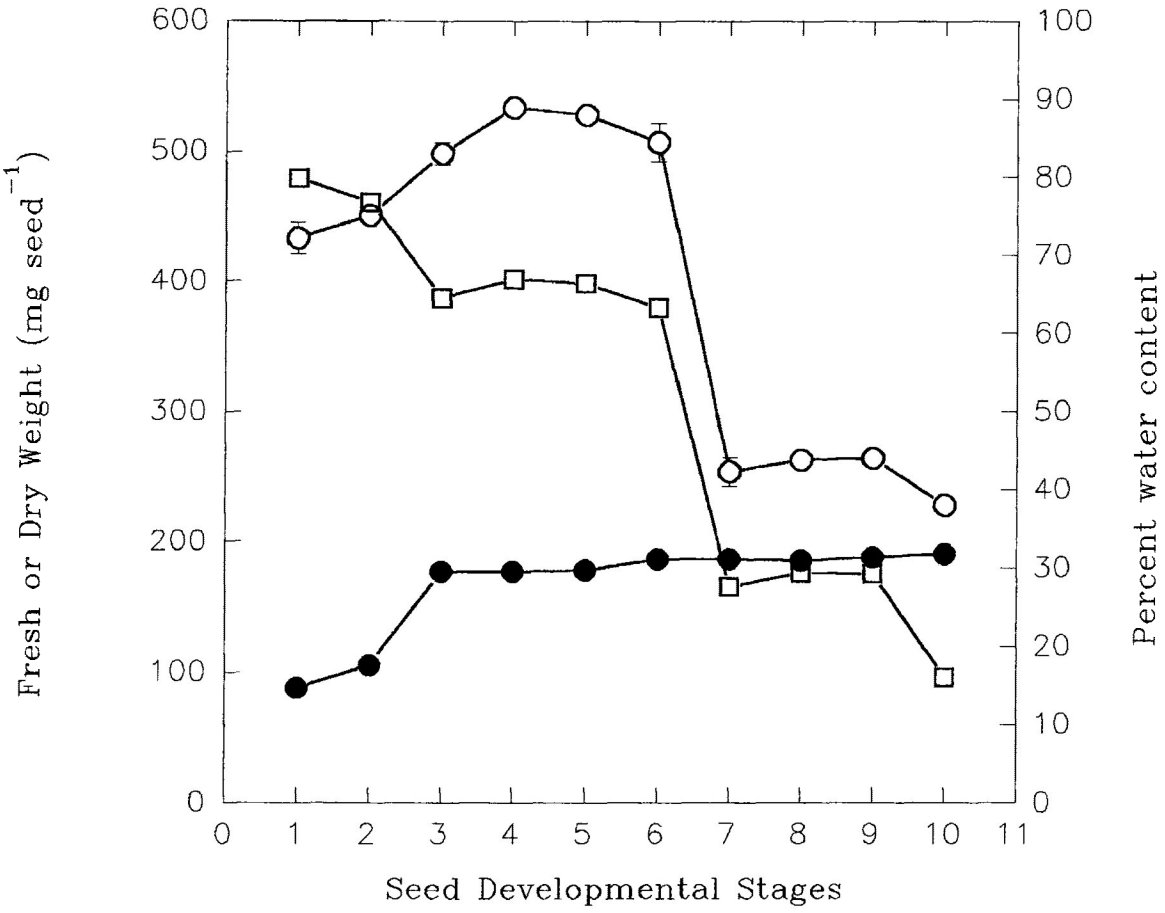

Fresh or Dry Weight (mg seed^{-1})
Percent water content
Seed Developmental Stages
600
500
400
300
200
100
0
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11

Fig.2. Changes in fresh weight (○), dry weight (●), and water content (□) of axes during pea seed development. Each point was based on forty five seeds (nine replicates of five) ± SE. Means without vertical lines indicate that the standard error is smaller than the symbol.

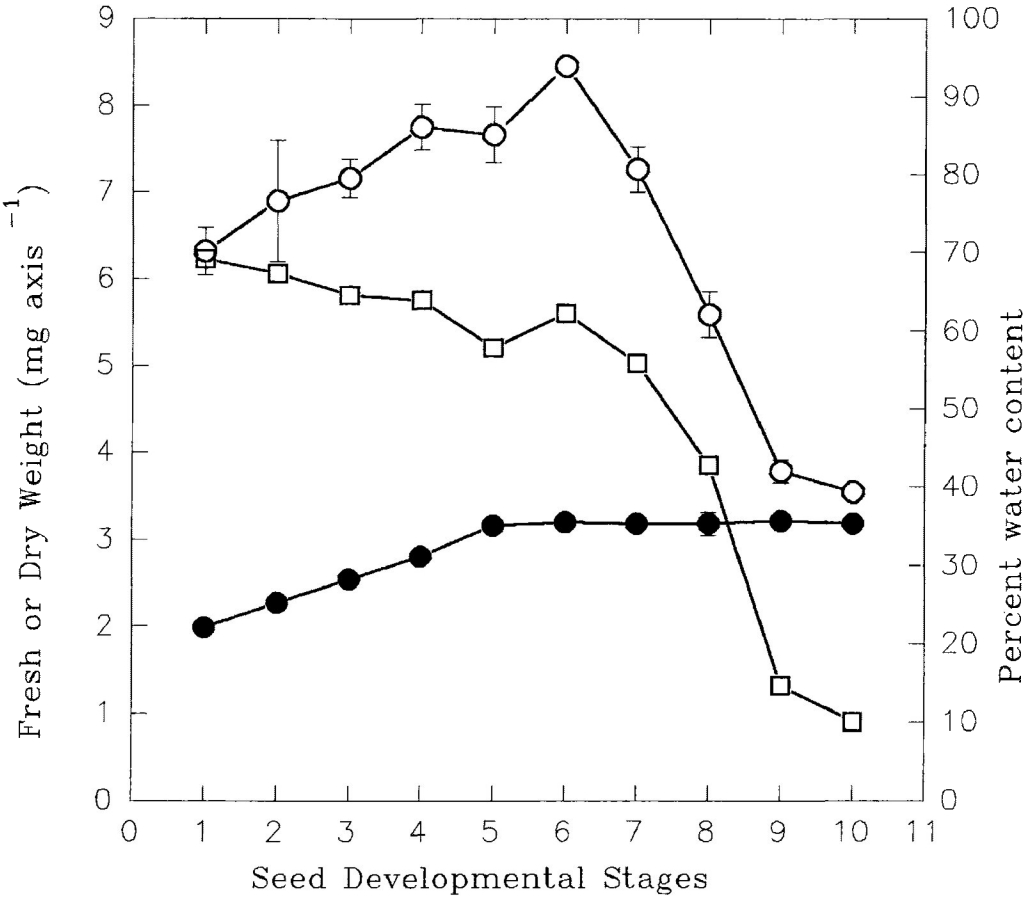

Fresh or Dry Weight (mg axis $^{-1}$)
Percent water content
Seed Developmental Stages
0
1
2
3
4
5
6
7
8
9
10
11
0
10
20
30
40
50
60
70
80
90
100

Fig.3. Protein content in seed (●) and axis (○) during development. Each point is a mean of five replicates ± SE. Means without vertical lines indicate that the standard error is smaller than the symbol.

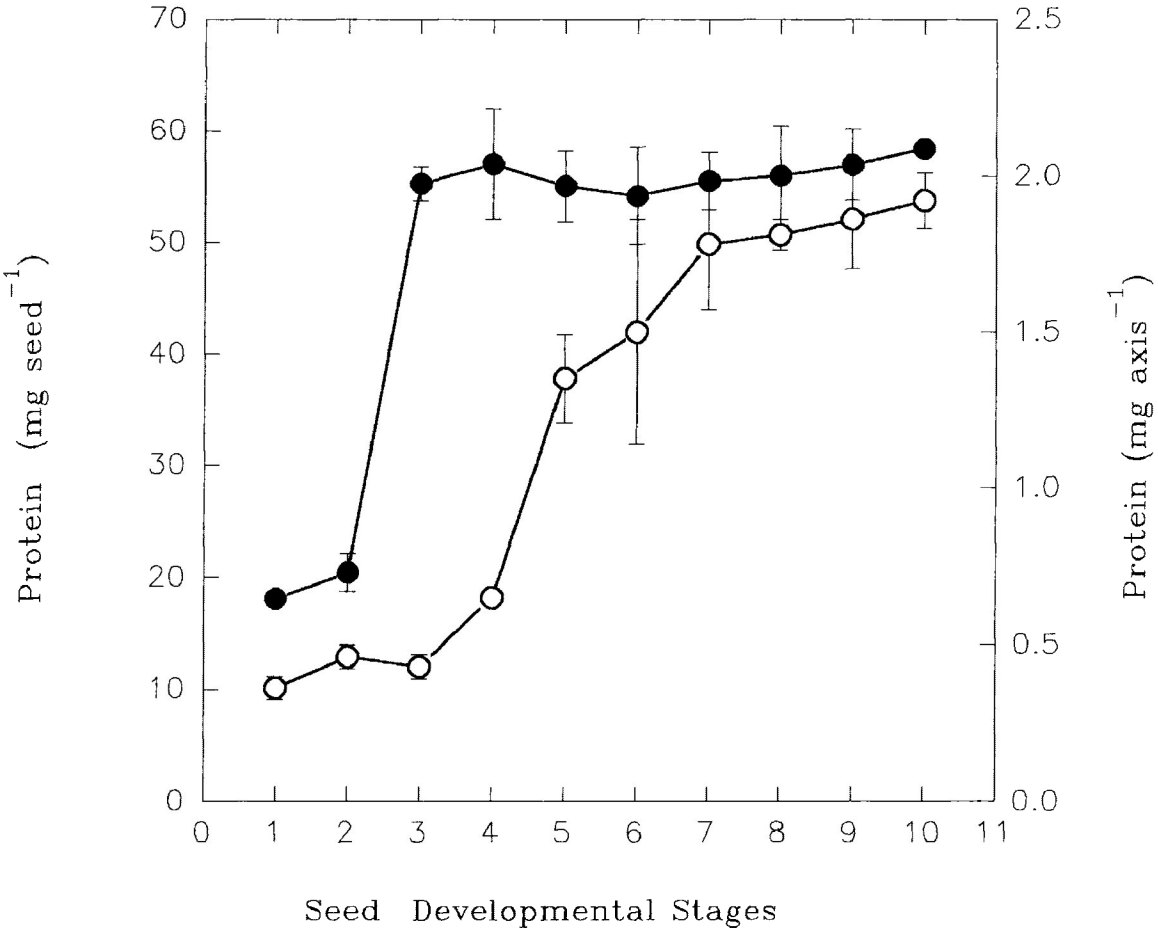

70
60
50
40
30
20
10
0
2.5
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11
Protein (mg seed^{-1})
Protein (mg axis^{-1})
Seed Developmental Stages

Fig.4. Changes in chlorophyll content of pea seed (●) and axis (○) during seed development. Each point is a mean of three replicates ± SE. Means without vertical lines indicate that the standard error is smaller than the symbol.

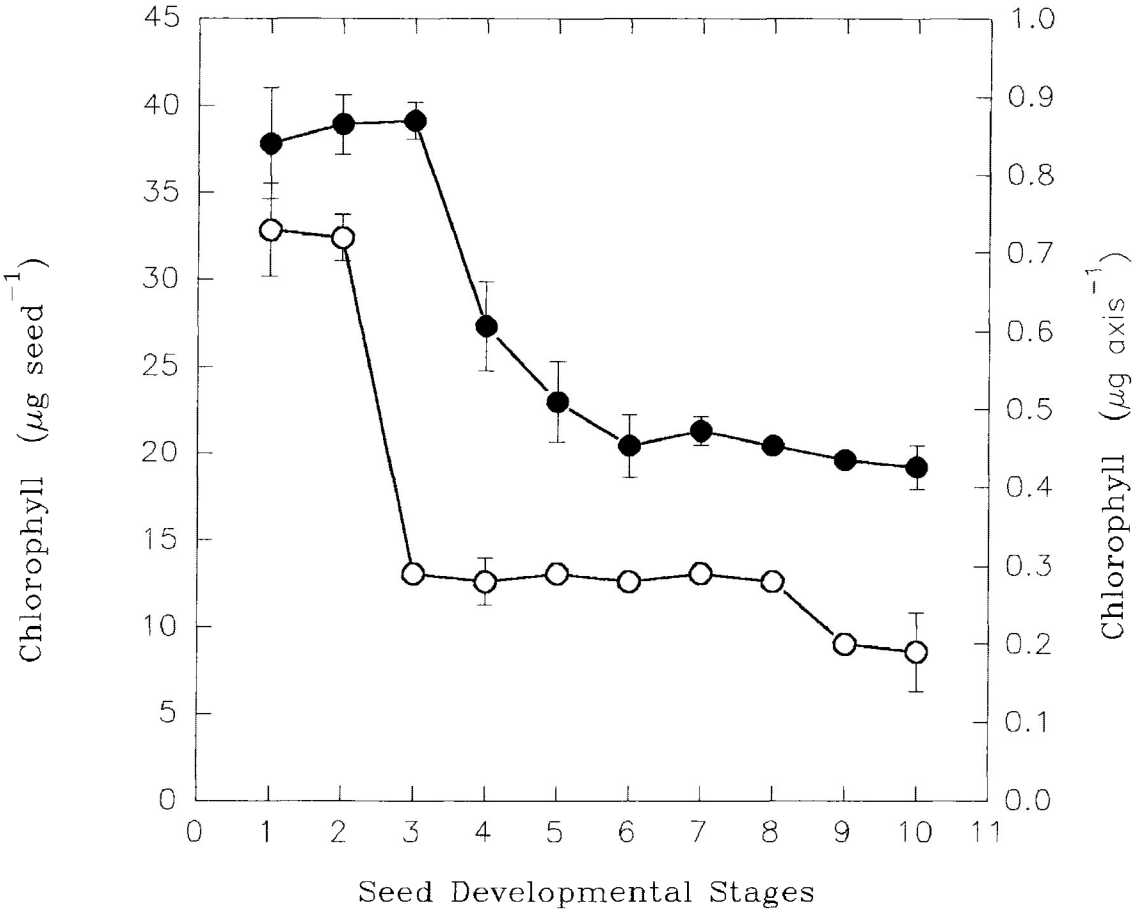

Chlorophyll (μg seed^{-1})
Chlorophyll (μg axis^{-1})
Seed Developmental Stages
0
5
10
15
20
25
30
35
40
45
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0 1 2 3 4 5 6 7 8 9 10 11

Fig.5. Percent germination of immature and mature pea axes, ▲ 45-47DAF (stage10), ■ 33-35 DAF (stage 6), ● 18-20 DAF (stage 1). Each point is a mean of nine replicates of five ± SE. Means without vertical lines indicate that the standard error is smaller than the symbol.

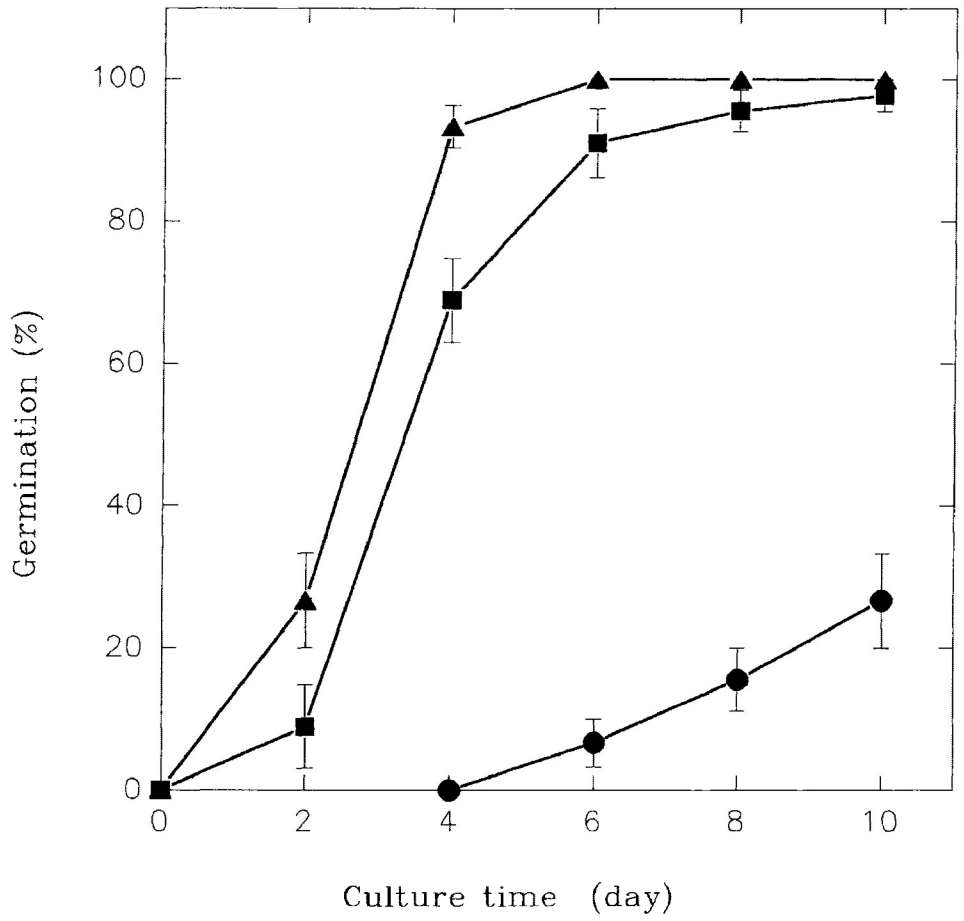

100
80
60
40
20
0
Germination (%)
0
2
4
6
8
10
Culture time (day)

Fig.6. Time to 50% germination (T_{50}) of pea axes at the different developmental stages. Bars show standard error. Each point is a mean of forty five axes (nine replicates of five).

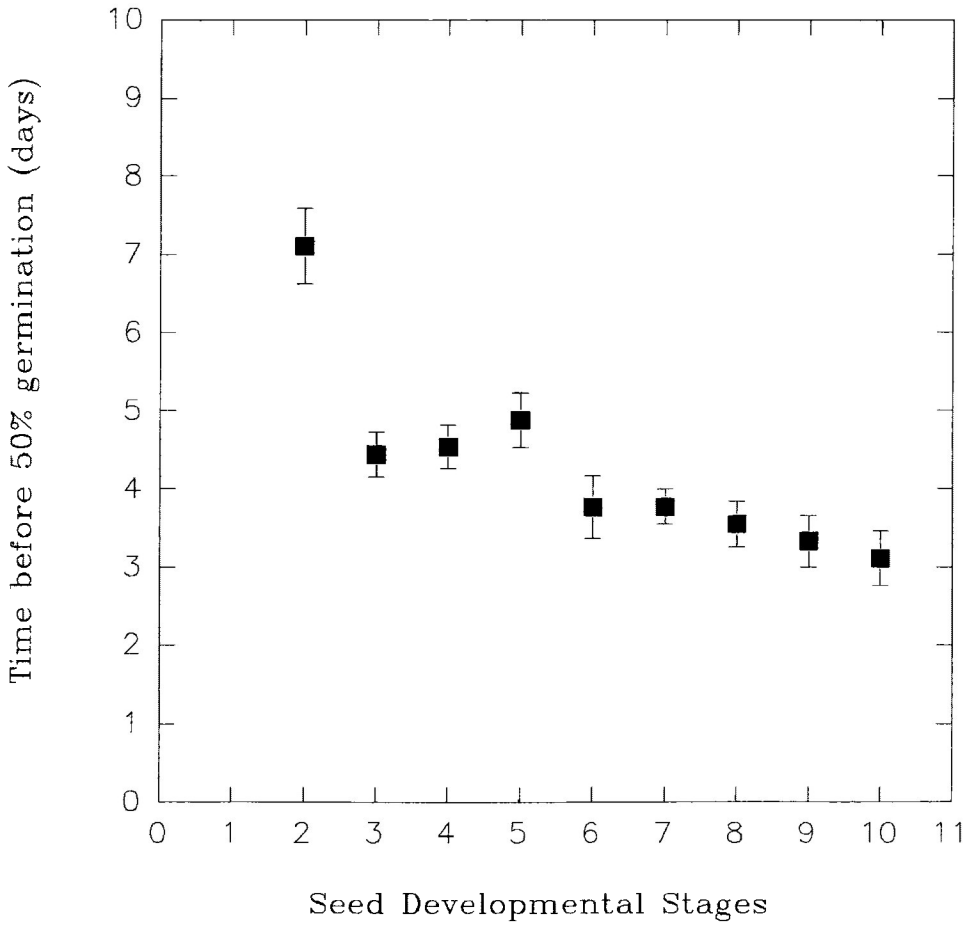

Time before 50% germination (days)
10
9
8
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7 8 9 10 11
Seed Developmental Stages

Fig.7. Total percent germination after four weeks of seed (■) and axis (□) of pea seeds at different developmental stages. Each bar is a mean of forty five seeds or axes (nine replicates of five) with standard error.

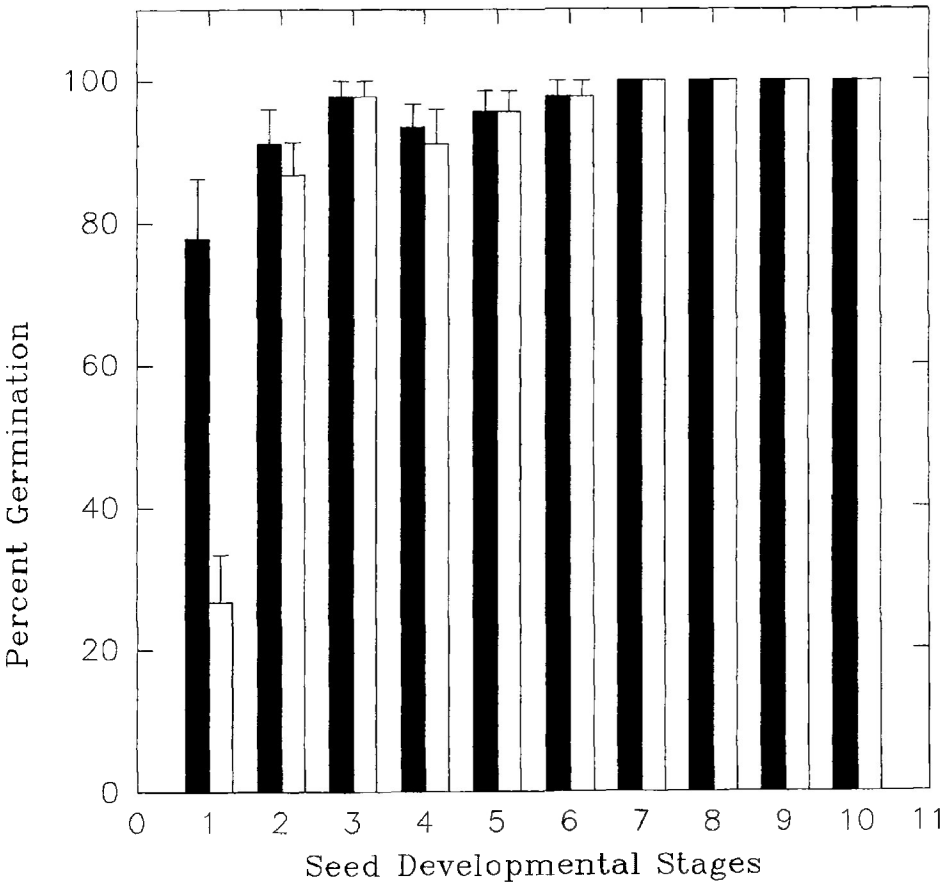

Percent Germination
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11
Seed Developmental Stages

Fig.8. Percent germination of pea seed ▲ 45-47 DAF(stage 10), ■ 33-35 DAF(stage 6), ● 18-20 DAF (stage 1). Each point is a mean of nine replicates of five ± SE. Means without vertical lines indicate that the standard error is smaller than the symbol.

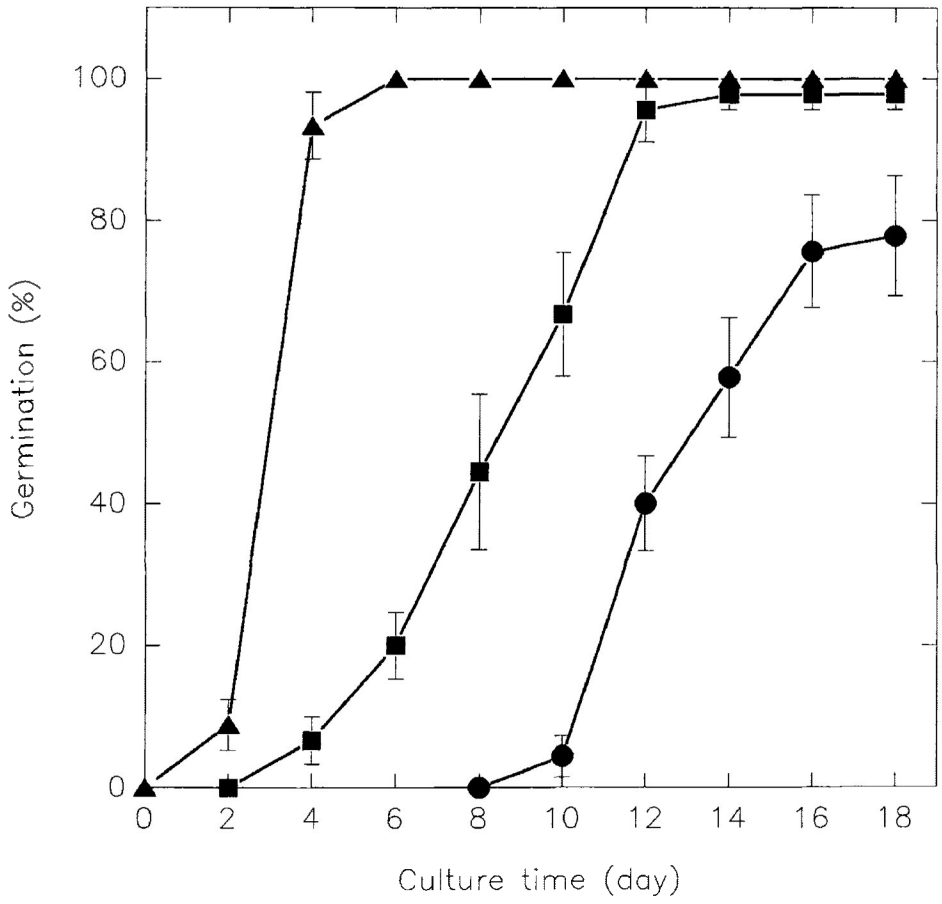

Germination (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
Culture time (day)

Fig.9. Time to 50% germination (T_{50}) of pea seed at different seed developmental stages. Bars show standard error. Each point is a mean of forty five seeds (nine replicates of five). Means without vertical lines indicate that the standard error is smaller than the symbol.

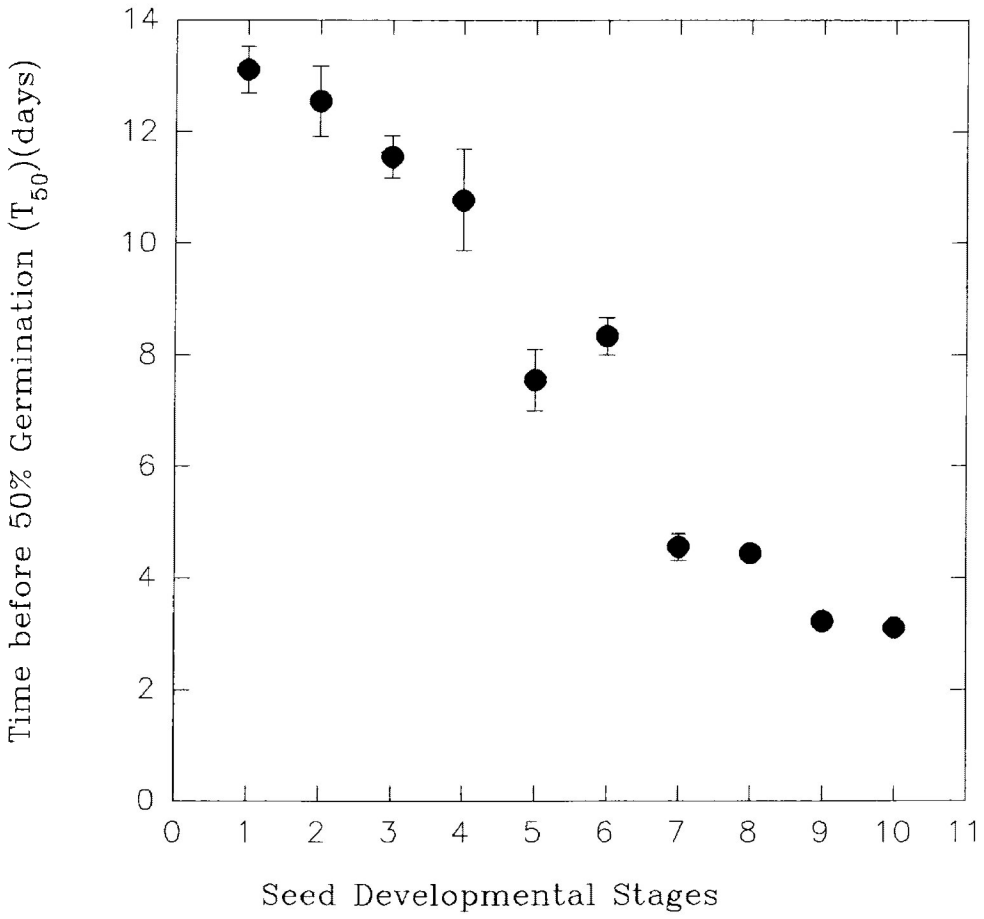

Time before 50% Germination (T_{50})(days)
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11
Seed Developmental Stages

Fig.10. Growth in culture of axes of *Pisum sativum* following excision at various stages. Each point is a mean of forty five axes (nine replicates of five) ± SE ▲ 45-47 DAF, ■ 33-35 DAF, ● 18-20 DAF. Means without vertical lines indicate that the standard error is smaller than the symbol.

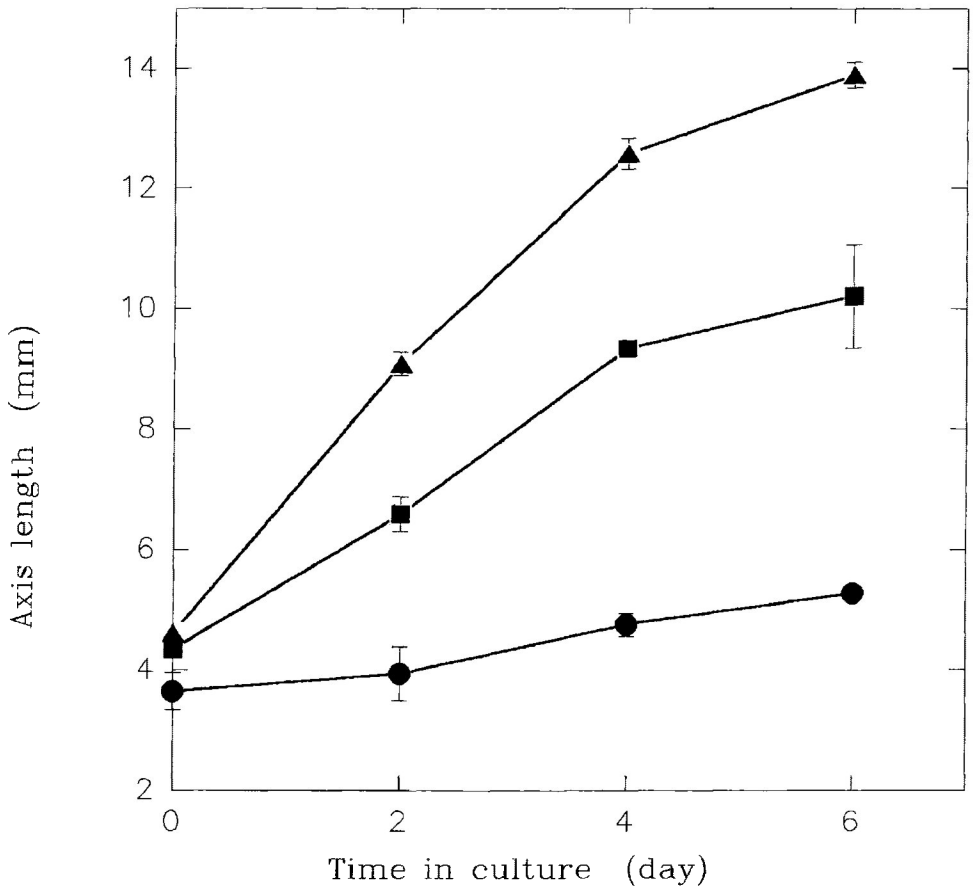

Axis length (mm)
14
12
10
8
6
4
2
0
2
4
6
Time in culture (day)

Table 2. The growth of seedlings from immature and mature seeds and axes

Stages	seedlings from axes	seedlings from seeds
1 (18-20)		++
2 (21-23)		++
3 (24-26)	+	++
4 (27-29)	+	++
5 (30-32)	+	++
6 (33-35)	+	++
7 (36-38)	+	++
8 (39-41)	+	++
9 (42-44)	+	++
10(45-47)	+	++

Shoot and roots grew poorly

\+ shoots grew well, roots grew poorly

++ shoots and roots grew well, normal seedling

Fig.11. Seedling developed from a 30 day, stage 5, A: whole seeds, B: excised embryonic axis of ***Pisum sativum*** , 3 days after culture.

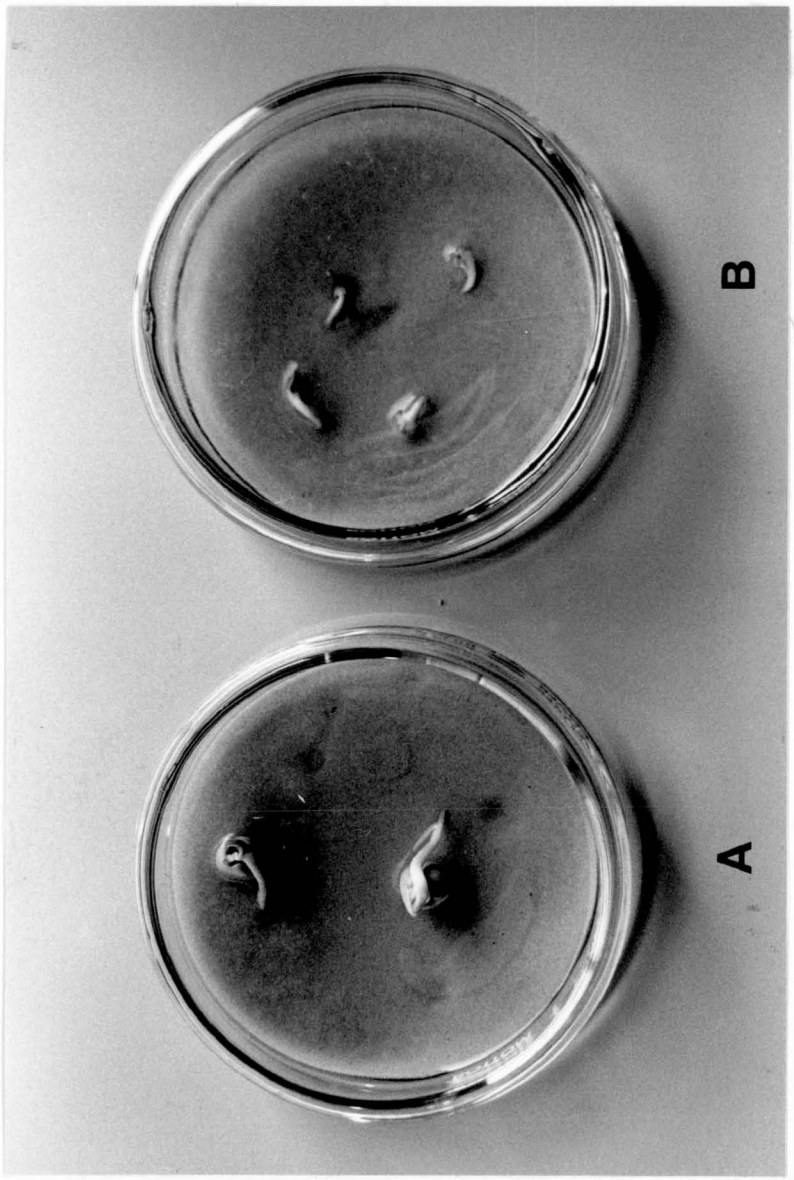

A
B

Fig.12. Seedling developed from a 30 day, stage 5. A: whole seeds; B: excised embryonic axes of *Pisum sativum*, 5 days after culture.

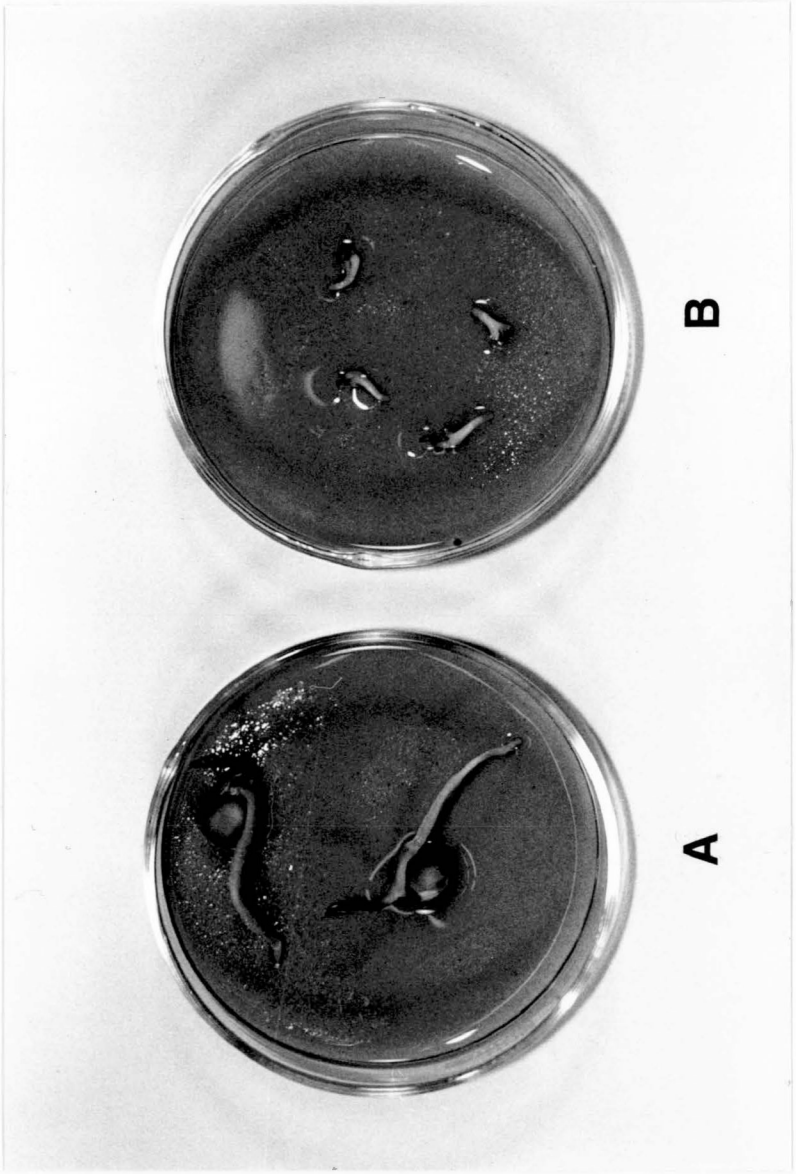

B
A

Fig.13.Radioimmunoblot detection of ubiquitin-protein conjugates in pea seed extracts.Total protein extract from developing seeds (lanes A-D), and germinating seeds (lanes G-K) were electrophoresed and blotted on nitrocellulose by Western-transfer method, and the reaction between protein and ubiquitin-antibody were detected by radioimmunoblot. Lane A: day 15 to 17; lane B: day 18 to 20 (stage 1); lane C: day 33 to 35 (stage 6); lane D: day 45 to 47 (stage 10); lane E and F: ubiquitin standard; lane G: 0 hr imbibition; lane H: 12 hr imbibition; lane I: 24 hr imbibition; lane J: 48 hr imbibition; lane K: 96 hr imbibition. Bands on the bottom of the gels are free ubiquitin (8.5 kDa). The high mol. wt. smear band (>60 kDa) and several other bands, 12 kDa, 17 kDa, 28 kDa, 48 kDa detected with anti-ubiquitin antibody were ubiquitin-protein conjugates. Numbers on the right of gel are mol. wt. of marker proteins.

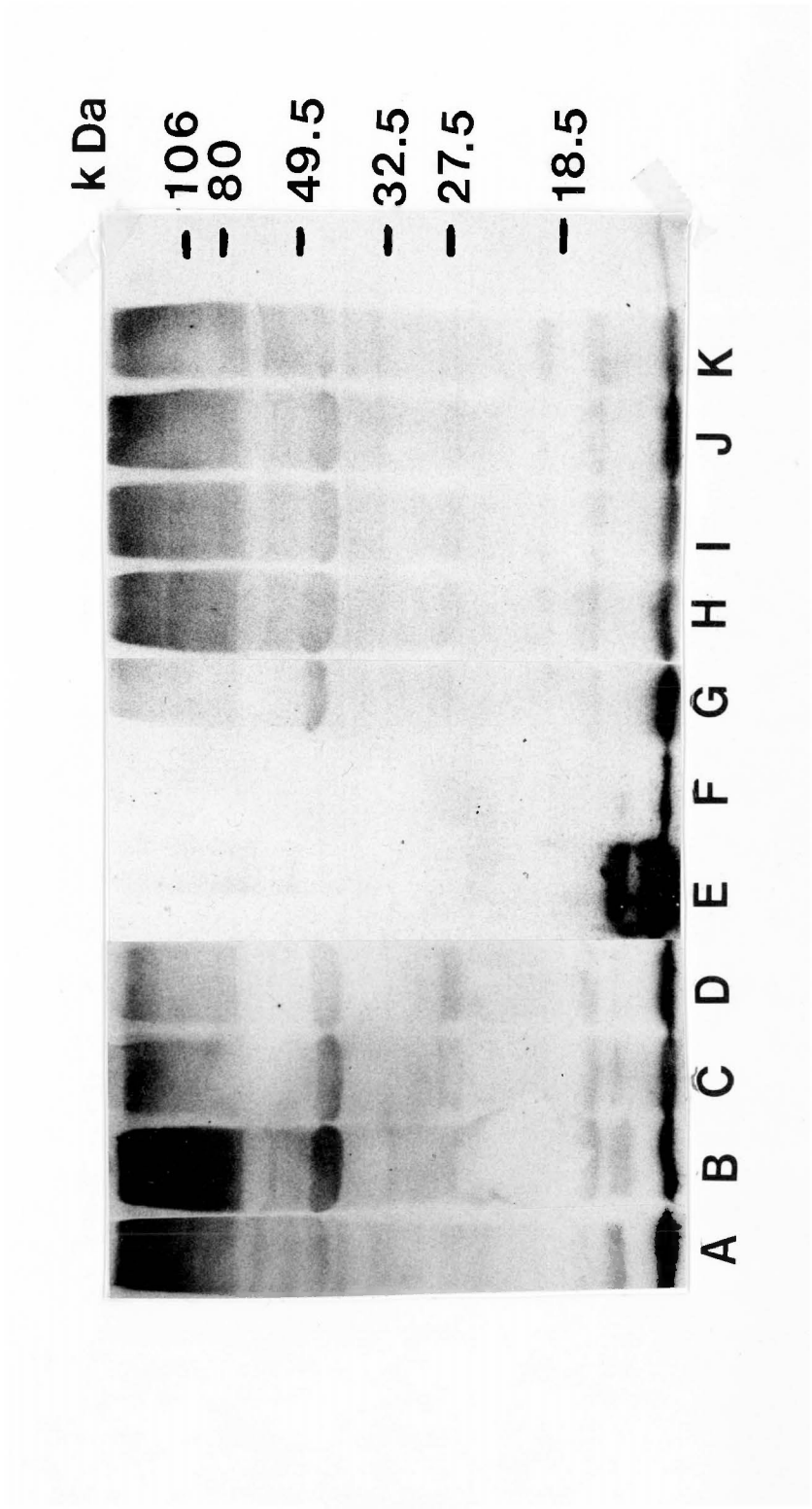

k Da
106
80
49.5
32.5
27.5
18.5
A B C D E F G H I J K

Fig.14. ATP levels in pea seed during germination. Each point is a mean of three replicates ± SE. Means without vertical lines indicate that the standard error is smaller than the symbol.

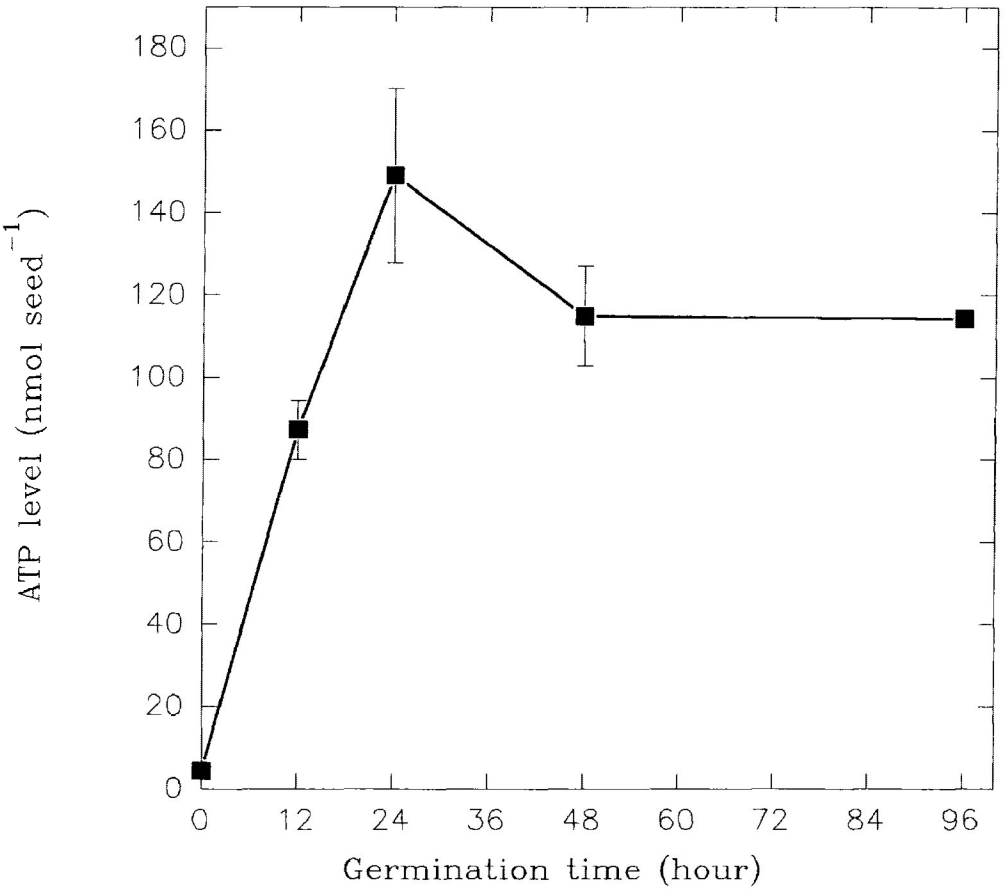
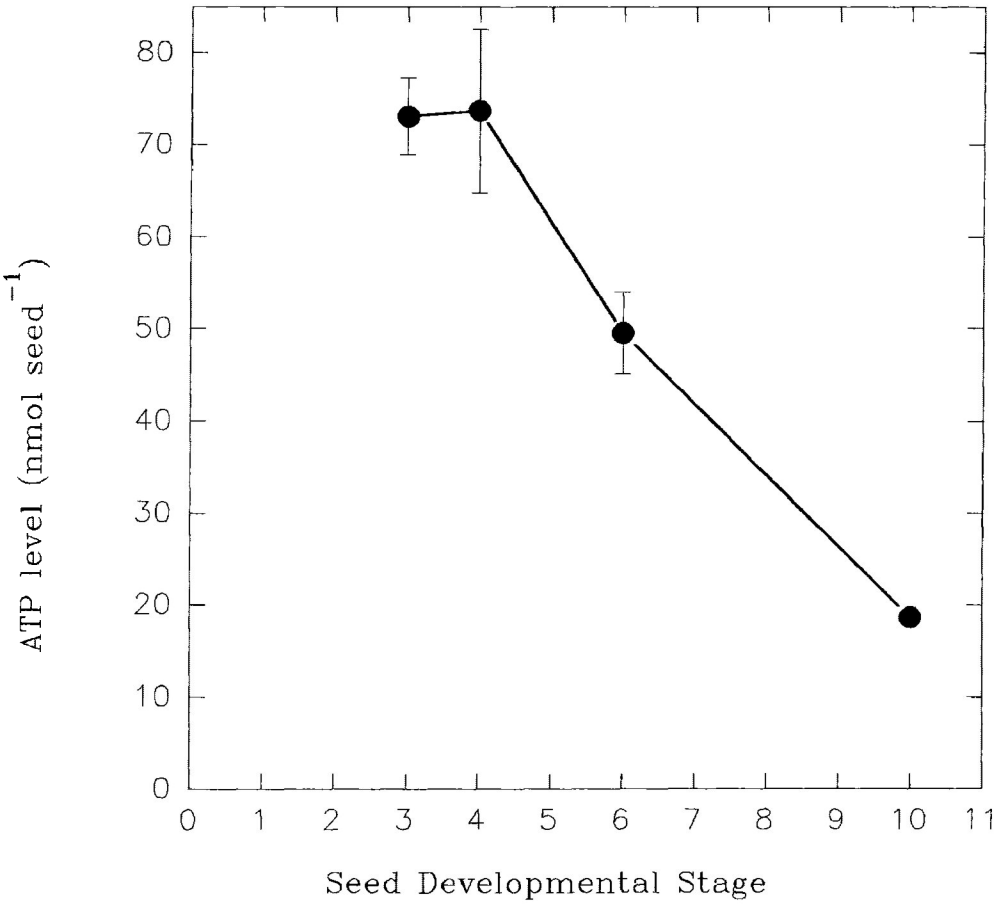

ATP level (nmol seed^{-1})
180
160
140
120
100
80
60
40
20
0
0
12
24
36
48
60
72
84
96
Germination time (hour)

Fig.15. ATP levels in pea seed during seed development. Each point is a mean of three replicates $\pm$ SE. Means without vertical lines indicate that the standard error is smaller than the symbol.


ATP level (nmol seed^{-1})
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
Seed Developmental Stage

DISCUSSION

1. Seed and axis development.

The overall pea seed development described here is similar to that reported previously for peas (Millerd and Spencer, 1974) and also for other species i.e. soybean (Rosenberg and Rinne, 1986), french bean (Walbot ***et.al.,*** 1972), and castor bean (Greenwood and Bewley, 1981). In terms of fresh weight, dry weight, and water content (Figs. 1 and 2), seed and axis development was slower than reported in previous studies. The maximum fresh weight of seed was reached at stage 4 (27-29DAF) somewhat later than the 22 days in the work of Matthews (1973), and Rogerson and Matthews, (1977). The differences could be due to varietal or cultural variation. The dry weight increased during the same period of the increase of fresh weight and remained almost the same until the end of the study. It is known that seeds reach physiological maturity when the dry weight is maximum, which is followed by a period of water loss and developmental maturation. This dehydration started at stage 6 (33-35 DAF) (Figs. 1 and 2).

Developmental studies have indicated that the rate of protein deposition varies with the developmental age of the seeds (Fig.3). Between stages 2 and 3 (21-26 DAF) the seed had accumulated most of the protein present at maturity. Thomson ***et.al.*** (1979) reported that in peas, this amount of protein had accumulated by day 20. Again, the difference could be due to the cultural and varietal differences.

From the present study, the period of rapid synthesis and deposition of protein reserves started about day 23 (the onset of stage 2) to day 26 (stage 3). A similar result was reported by Beevers and Poulson (1972). The accumulation of storage protein legumin and vicilin was detected in seeds as early as four days after fertilization (Gatehouse ***et.al.***,1981). Smith (1973) observed that in developing cotyledons of ***Pisum arvense*** protein accumulation continued until the seeds were ripe. In this study the deposition of storage protein still occurred until the final harvest (day 47), but in very small amounts. Protein content of mature pea seed usually lies within the range of 15-35% (Pate, 1975). The protein content of our seed was about 30%. The pattern of protein accumulation in developing excised axis followed the same pattern as the seeds, with a delay of a few days (Fig.3). Rapid synthesis and accumulation of axis protein began at about day 30 and it continued until maturation. Axis from mature seed (day 45 to 47) contained about 60% protein on a dry weight basis.

The seed chlorophyll content declined during development from 38 ug to 20ug (Fig.4). This result is similar to that reported by Millerd and Spencer (1974) in which they found that chlorophyll in a 21-day old single cotyledon (seed minus testa and axis) was about 20 ug. Our result is also in agreement with that reported by Flinn and Pate (1968) for both pea and cowpea.

2. Germination and growth of seeds and isolated axes.

Under our experimental conditions (autotrophic media), both embryonic axes and entire seeds were able to germinate (i.e. doubled length of axis and exhibited root and shoot growth). Youngest seeds tested (stage 1, day 18 to 20) germinated with difficulty and the percent germination was only about 80%. In contrast, fresh pea seeds harvested at 25 DAF were unable to germinate (Matthews, 1973). Le Deunff and Rachidian (1988) found that in cv Finale and cv Solara pea seeds, 56% germination occurred with seeds harvested at 67 day after planting ($\pm$ 37 DAF). The differences in germination competence of immature seeds could be due to cultural or varietal differences. In this study, seeds older than stage 1 reached 100% germination within four weeks (Fig.7). It is apparent that partially developed seed have the capability to germinate even though they did not reach a maximum fresh or dry weight, and the water content was still high (Fig.1).

There was a clear inverse relationship between seed degree of maturity and time required to achieve germination (Fig.9). The less mature seed presumably require additional time to go through further development. Similar results were reported in pea (Millerd ***et.al.***, 1975) and french bean (Long ***et.al***, 1981). In addition, it was reported by many researchers that immature seed that detach from the mother plant in an early stage of development cannot germinate, but that, the ability to do so is acquired at ages closer to maturity (Thompson ***et.al.***, 1977; Stafford and Davies, 1979; Cook ***et.al.***, 1988)).

Axes excised from different developmental stages also have the ability to germinate. However, the youngest axes excised from seeds at stage 1 germinated at a very low level compared to the entire seeds of the same age (Fig.7). Stage 1 axes likely did not acquire sufficient quantity of storage reserves to support their germination. In this study, the lag phase of stage 1 axes to achieve complete germination was shorter (7 days) than for entire seeds (13 days)(data not shown). However, no differences were detected in mature seeds. The lag phase of both, seed and axis, was reported to be the same in french beans (van Onckelen ***et.al.***, 1980; Long ***et.al.,*** 1981), rape (Crouch and Sussex, 1981), and cotton (Dure, 1975).

3. Autotrophic seedling growth.

At all developmental stages used in this study, germination of seeds and axes was possible. All whole seeds which germinated were able to produce viable autotrophic seedlings. However, germinating immature seed (28 DAF) did not produce a vigorous seedlings which was also reported for peas by Le Deunff and Rachidian (1988). In contrast, immature soybeans do not germinate (Rosenberg and Rinne, 1986). Even though axes excised from immature and mature seeds in this study germinated in autotrophic culture, they were unable to produce normal seedlings after transplanting into pots. This is in accordance with the results of Bain and Mercer (1966c) on ***P. sativum*** cv. Victory Freezer. They cultured the axes of pea seedling, which were excised at different germination stages, either in sand wetted with distilled water or in heterotrophic culture solution. Bain and Mercer

(1966c) demonstrated that the axes likely do need support material from the cotyledons to continue growth after germination. However, they found that water alone was insufficient to support the development of assimilatory metabolism. It remains to be studied whether the failure of the small green seedlings to grow autotrophically is due to insufficient respiratory reserves or a more subtle nutritional or growth regulator imbalance.

Mature embryos of pines were able to produce viable seedlings when they were cultured in media containing sucrose (Haddock, 1954; Berlyn and Miksche, 1966), but not in a basic medium (Tabor and Bennett, 1987). In autotrophic culture media, embryonic axes of pea would not be able to produce a viable seedling (Bain and Mercer, 1966c). There is considerable evidence that the germination and post germination of seeds are characterized by the transfer of reserve material from the cotyledons to the growing axes (Barker and Douglass, 1960; Okamoto, 1962; Beevers, 1968; Beevers and Splittstoesser, 1968; Guardiola and Sutcliffe, 1972). Axes excised from the mature seed produced good growth for about one week, then no further growth occurred. It is apparent that axes do depend on the cotyledons in some way after germination is finished, even though the morphological development would indicate structural self sufficiency. It is well known that somatic and zygotic embryos have the ability to grow and regenerate into viable seedlings.

Success here usually has been the result of manipulating the osmotic pressure, pH and the composition of the organic nutrient media (Maheswaran and Williams, 1986; Finer, 1987; Kysely ***et.al.***,1987; Cyr ***et.al.***, 1991; Attree ***et.al.***, 1992). Recently, researchers working with somatic embryos have successfully produced seedlings (Senaratna ***et.al.,*** 1989, 1990; Anandarajah and McKersie, 1990a,b; Roberts ***et.al.,***1990; Anandarajah ***et.al.***,1991). However, factors required to produce high quality and vigorous seedling still remain to be established (Redenbaugh, 1990). It is usually suggested that the germination media used for somatic embryo germination do not replace entirely the surrounding maternal tissues.

4. Ubiquitin conjugates in relation to the presence of ATP.

The formation of ubiquitin conjugates, as well as their hydrolysis requires ATP as an energy source (e.g. Hershko ***et.al.***, 1984; Finley, 1985; Vierstra, 1987;). In dry mature seed there is only a very small amount of ATP, which is barely detectable (Figs.14 and 15). Since ubiquitin conjugation is an ATP-requiring process, no conjugation occurred in dry seed. During germination, when the production of ATP increased, the concentration of ubiquitin conjugates was found to increase. The reverse pattern occurred during seed development, declining ATP correlating with the disappearance of ubiquitin conjugates. The change in concentration of ubiquitination is affected by the presence of ATP level in the substrate. It was documented that ubiquitin exists in seeds, etiolated shoots and green leaves of oats (Vierstra ***et.al.,***1985; Vierstra, 1987) Our study supports this

observation. Since ubiquitin conjugated protein was found in pea, this provides indirect evidence that peas have enzyme activities needed for ubiquitin to bind to and to degrade conjugated proteins. This is not unexpected since wheat germ has proven to be a good source of ubiquitin conjugation enzymes (Hatfield and Vierstra, 1989; Sullivan and Vierstra, 1989). Even though the relevance of ubiquitin in the development and germination of plants is not yet established, these results support the expectation that ubiquitin might have a role in seed protein turnover.

SUMMARY

An interesting finding of this study was the failure of excised axis to produce viable autotrophic seedling after germination was completed. It should be rewarding to examine growing excised embryonic axis after germination for substances such as hormones and nutrients and to test these substances on zygotic or somatic embryos. Other finding was the conjugation of ubiquitin to protein targets during developing and germinating seed. This result suggested that pea seed contains components needed for ubiquitin linked conjugation of protein targets. It would be of obvious interest to study the specific proteins which are degraded via ubiquitin proteolysis pathway during development and germination in order to understand the possible physiological relevance of ubiquitin conjugates in development.

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