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Storage protein degradation and proteolytic enzyme activities during germination of water-stressed impatiens seeds

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Iowa State University, 1990

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Storage protein degradation and proteolytic enzyme activities during germination of water stressed impatiens seeds

by

Mehrassa Khademi

A Dissertation Submitted to the
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GENERAL INTRODUCTION

The bedding plant industry is a major segment of the floriculture industry. Bedding plant production has been increasing at an annual rate of approximately 10% for the past 20 years. Future expansion of the bedding plant industry will depend in part on technical advancements made in single-cell-plant production, or plug production (Koranski, 1988). In this production system, seedlings are grown at a high density in individual compartments of specialized trays. Koranski (1987) has shown that the optimum method of germinating many flower seeds is to leave the seeds uncovered. As a result of this technique, a potential problem of germinating seeds in the plug system is water stress.

Water uptake by seeds is an essential initial step activates a number of metabolic processes necessary for germination. Under optimum conditions, water uptake by seeds is considered to be triphasic. Phase I, or imbibition, is characterized by rapid water uptake, largely as a consequence of the very low water potential of the mature dry seed. Metabolism commences during this phase, often within minutes of the introduction of water to the seed. Phase II is the lag phase of water uptake. During this phase, major metabolic events take place in preparation for radicle emergence from nondormant seed. Only germinating seeds enter phase III, which is concurrent with radicle elongation, and marks the completion of germination (Bewley and Black, 1985).

The role of water as a limiting factor in germination has been reviewed by Koller and Hadas (1982), and McIntyre (1987). They suggested that water is required for promotion of activity of hydrolytic enzymes.

Synthesis of cell wall material and cell expansion is inhibited under water stress conditions, (Bradford and Hsiao, 1982). Low levels of moisture, or mild water stress, will increase the length of the lag phase and lead to delayed or prevented germination (Hegarty, 1977). Water stress has been shown to stimulate the conversion of polyribosomes to monoribosomes in plant cells, and this conversion also has been related to a reduction in the synthesis of certain enzymes (reduction in *in vivo* protein synthesis capacity) (Armstrong and Jones, 1973; Bewley et al., 1983). Synthesis of hydrolytic enzymes, such as α-amylase in barley and pea, have been shown to be under osmotic control (Jones and Armstrong, 1971; Morohashi and Ueno, 1980). This observation was not confirmed in the case of bean cotyledons (Gepstein and Ilan, 1981). Seed sensitivity to water stress and the minimum water potential that inhibits protein synthesis and germination differs among species (Kaufmann, 1969; Palit, 1987).

During germination, the high molecular weight reserves in the storage organs of the seed are converted into transportable forms and are transported to metabolizing and growing tissues where they are utilized for energy producing and synthetic events (Koller and Hadas, 1982). Proteins are one of the major types of storage reserve in seeds (Bewley and Black, 1985). Major storage proteins of legumes and other dicotyledonous plants are salt-soluble globulins (Higgins, 1984). In mature seeds, storage proteins are found in small organelles (1 to 20 μm), called protein bodies, which are surrounded by a single membrane of tonoplast or endoplasmic reticulum origin (Tully and Beevers, 1976; Youle and Huang, 1976; Higgins, 1984). These organelles change to vacuoles during germination (Nishimura and Beevers, 1979).

Storage proteins are degraded to provide amino acids for synthesis of functional and structural proteins in the embryonic axis (Pusztai and Duncan, 1971; Murray, 1979; Davis and Chapman, 1980). Hydrolysis of storage proteins to their constituent amino acids requires a class of enzymes called proteinases. Proteolytic enzymes, which are involved in degradation of storage proteins in the germinating seeds, are classified as endopeptidases, including sulfhydryl, acidic, and serine proteases, and exopeptidases, including amino- and carboxypeptidases (Ryan and Walker-Simmons, 1981). These enzymes occupy a central role in the biochemical mechanisms of seed germination, but not much is known about the regulation of their synthesis and activity.

Different storage proteins within storage tissues often are hydrolyzed at different times and rates during and after germination (Youle and Huang, 1978; Manickam and Carlier, 1980; Davis and Slack, 1981; Minamikawa et al., 1983). Many proteolytic enzymes have been isolated from ungerminated and germinating seeds (Elleman, 1974; Kern and Chrispeels, 1978; Tully and Beevers, 1978). Aminopeptidases generally are present in dry seeds (Salmia and Mikola, 1975) and endopeptidases are synthesized *de novo* (Yomo and Srinivasan, 1973; Minamikawa et al., 1983). Ryan (1973) suggested that endopeptidases primarily are responsible for initiating storage protein breakdown. Ashton (1976) proposed a multiple-enzyme system hypothesis. According to Guardiola and Sutcliffe(1971), Basha and Beevers (1975), and Minamikawa et al. (1983), there is a lag period of several hours to several days before the main protein reserves of seeds are mobilized from the cotyledons, and that the embryonic axis may degrade and utilize self-contained reserve proteins. Murray (1979), however, reported that the

requirement for amino nitrogen in the growing axis is met by the immediate export of amino acids from the cotyledons rather than by massive breakdown of stored proteins *in situ*. In mung bean, vicilin peptidohydrolase (endopeptidase) and carboxypeptidase function together in the complete digestion (peak at day three) of vicilin in cotyledons (Minamikawa et al., 1983).

Aminopeptidase present in the cotyledons of dry seeds may participate in supplying amino acids during the early stages of germination (day one). In castor bean, aminopeptidases are involved in the early (days one to four) mobilization of endosperm storage proteins, whereas proteases, carboxypeptidase and BANAase (α-N-benzoyl-DL-arginine-naphthylamide) may take part in later (days five and six) protein turnover and senescence of the cotyledon (Tully and Beevers, 1978). Cotyledons of *Phaseolus vulgaris* were tested for their activity on BAPNA (α-N-benzoyl-DL-arginine-*p*-nitroanilide) and azocasein (endopeptidase) during a ten-day germination period. Both activities increased throughout germination (Nielsen and Liener, 1984). Using mercaptoethanol and the thiol-protease inhibitors, N-ethylmaleimide and E-64 (N-(N-[L-3-trans-carboxoxiran-2-carbonyl]-L-leucyl)-agamatine), it was shown that two enzymes were responsible for these activities. A thiol-protease with an acid pH optimum is responsible primarily for the disappearance of the major storage proteins during germination.

In protein bodies of castor bean endosperm, the matrix protein (water soluble albumin) degrades before the crystalloid proteins (salt soluble globulin) (Youle and Huang, 1976 and 1978).

This literature survey shows that there is no common degradation

pattern for storage proteins of seeds, and that the regulation of protein breakdown still is unknown. The degradation of storage proteins and the activity of corresponding proteolytic enzymes as factors controlling radicle protrusion has received little attention. Water stress seldom has been related to degradation of storage proteins even though they are a direct source of reduced nitrogenous compounds for the protein-synthesizing system. The objectives of this research were to study the pattern of storage protein degradation in terms of qualitative and quantitative changes in protein content during germination of impatiens seeds and its possible relation to radicle protrusion. To determine the activity of some of the proteolytic enzymes and their relation with the depletion of storage proteins and germination. And to determine the effect of water stress on the germination, the degradation of storage proteins, and the activity of proteolytic enzymes associated with protein degradation.

Explanation of Dissertation Format

This dissertation is arranged in the alternate format consisting of two papers that will be submitted to scientific journals. Mehrassa Khademi was the principal investigator on all research reported herein, and she is the first author on both papers. Drs. Koranski and Gladon served as co-major Professors for Mehrassa in her research and are listed as authors on both papers.

SECTION I. WATER STRESS AND STORAGE PROTEIN DEGRADATION DURING GERMINATION OF IMPATIENS SEEDS

WATER STRESS AND STORAGE PROTEIN DEGRADATION DURING GERMINATION OF IMPATIENS SEEDS

Mehrassa Khademi, David S. Koranski,

David J. Hannapel, and Richard J. Gladon

ABSTRACT

Water uptake of impatiens (*Impatiens wallerana* Hook. f. cv. Super Elfin Coral) seeds was measured as an increase in fresh weight every 24 hr during 144 hr of germination. Seeds absorbed most of the water required for germination within the first 3 hr of imbibition, and thus germinated at 60 to 67% moisture on a dry-weight basis. Germination started at 48 hr and was complete by 96 hr at 25C. Water stress of -0.1, -0.2, -0.4, and -0.6 MPa, induced by polyethylene glycol 8000 (PEG) reduced germination by 13, 49, 91, and 100%, respectively, at 96 hr. Under the same water-stress conditions, increases in fresh weight were inhibited by 53, 89, 107,and 106% respectively. Three distinct groups of storage proteins with estimated molecular weights of 35, 33, 31 kilodalton (kd) ; 26, 23, 21 kd; and two bands less than 14 kd were present in dry seed. Major depletion of storage proteins coincided with the time of maximum germination. Water potentials inhibited germination also inhibited degradation of storage proteins. The soluble-protein fraction increased and coincided with a decrease in the insoluble fraction during germination under optimum conditions. Threshold concentrations of soluble proteins were required for germination.

INTRODUCTION

A major portion of the bedding plants are produced in the plug. Plug production is a single cell-plant production system in which seeds are placed on the surface of germinating media by a mechanical seeder. Koranski (1987 and 1988) has shown that the optimum method of germinating impatiens is to leave the seed uncovered. As a result of this technique, one of the major problems of germinating seeds in the plug system is water-stress.

The uptake of water by seeds is an essential initiating step which activates a number of metabolic processes necessary for germination (Koller and Hadas, 1982). Water uptake by seeds is considered to be triphasic. Phase I, or imbibition, is a rapid water uptake, Phase II is the lag phase or water plateau, and Phase III, is a second rapid uptake due to germination (Bewley and Black, 1985). Low levels of moisture or mild water stress will increase the length of the lag phase and lead to delayed or prevented germination (Hegarty, 1977; McIntyre, 1987).

Water deficits have a profound effect on plant metabolism, including cell wall synthesis and cell expansion (Hsiao, 1973; Bewley, 1981; Bradford and Hsiao, 1982). It is proposed that this effect is enacted through a rapid reduction of protein synthesis by affecting the processes of translation, rather than the production of a specific set of proteins (Armstrong and Jones, 1973; Bewley et al., 1983). It also has been suggested that limited growth of osmotically stressed plants is the result of a reduced capacity for protein synthesis, and not related to turgor loss

(Mason and Matsuda, 1985).

Seed storage protein is defined as any protein accumulated in significant quantities in the developing seed and rapidly hydrolyzed upon germination. Major storage proteins of legumes and others dicotyledonous plants are believed to be globulins, which are insoluble proteins (Higgins, 1984). During seed germination, storage proteins in the cotyledon are degraded to produce amino acids and amides, most of which are translocated to the growing parts of the developing seedling where they are used for the synthesis of new functional and structural proteins (Pusztai and Duncan, 1971; Murray, 1979; Davis and Chapman, 1980; Mitsuhashi et al., 1984). Degradation of storage proteins has received little study as a factor affecting radicle protrusion. Little is known about the effect of water stress on the degradation of seed storage proteins. This investigation was conducted to study the relationship of qualitative and quantitative changes in storage proteins to the germination of impatiens seed under both optimum and water stress conditions.

MATERIALS AND METHODS

Water Uptake

Samples of 50 mg (about 100 seeds) of impatiens seeds (George J. Ball Co., West. Chicago, IL) were placed in acrylic germination boxes (12 x 12 cm) with two layers of blue blotter paper (Anchor Paper Co., St. Paul, MN). Blotters were pre-soaked in deionized (D.I.) water and drained to remove excess water. Another 10 ml of D.I. water then was added to each box. Boxes were sealed with parafilm and kept in a growth chamber at 25 ± 0.5C with continuous irradiance at 4 $\mu mol \cdot s^{-1} \cdot m^{-2}$ photosynthetic photon flux. At 1-hr time intervals (for the first 8 hr) and every 24 hr (for the remaining 136 hr) seeds were collected, surface-dried, and the fresh weight of samples was recorded (Hegarty, 1977).

PEG Treatment

Polyethylene glycol (PEG 8000 Fisher Scientific) solutions of osmotic potentials -0.1, -0.2, -0.4, and -0.6 MPa (50, 75, 100 and 125 g/500 ml respectively) were prepared. Water potentials were determined at room temperature by using a Wescor 5100 C Vapor Pressure Osmometer. Germination papers were soaked in the appropriate solution and then used for germination as in the water uptake measurements. For fresh weight measurements, PEG-soaked seeds were washed with distilled water for 2 min, surface-dried, and weighed. One hundred seeds were placed in each germination box as above and incubated under the same conditions. Germinated seeds were counted at 24 hr intervals for a total of 144 hr.

Germination was defined as radicle protrusion through the seed coat. D.I. water was used as the control (0.0 MPa).

Experimental Design and Analysis

A split-plot arranged in a completely randomized design was used for germination percentages, with PEG at five levels (including control) as the whole plot factor, and time at six levels as the split-plot factor. A factorially arranged, completely randomized design was used for fresh weight measurements, with PEG and time at five and six levels, respectively. Three replications in time were used for each experiment. Data were analyzed using Statistical Analysis System (SAS) GLM procedure (SAS Institute, Raleigh, N.C.).

Electrophoresis and Protein Measurement

Seeds germinated as described above, were harvested at 24 hr intervals as in the water uptake experiment, and at 96 hr for PEG-treated seeds. Seeds were washed and then dried at 37C for 48 hr. Roots and hypocotyls were removed and 50 mg samples of dry cotyledons were used for electrophoresis and protein measurement.

Samples were ground in 400 μl of extraction buffer over ice (Laemmli, 1970). Extracts were centrifuged for 20 min at 11000X g. Six μl of the supernatant was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (0.75 mm discontinuous slab gel) by the method of Laemmli (1970).

Fifty mg samples of dry cotyledons were ground in 400 μl of 5 mM Tris

(pH 8.00) to extract soluble protein (Tully and Beevers, 1976). Extracts were centrifuged for 20 min at 11000X *g*. After collecting the supernatant, the pellet was freeze dried overnight and dissolved in a 50:50 solution of 5% SDS:0.5 N NaOH (Thorn, 1978). After standing for 1 hr at room temperature the protein suspension was centrifuged as above and the supernatant was measured for insoluble protein. The soluble and insoluble protein content of the cotyledons was measured according to Peterson's modification of the micro Lowry method (Peterson, 1977). Data were analyzed by using the ANOVA procedure of SAS (SAS Institute, Raleigh, N.C.).

RESULTS

Water Uptake

The increase in fresh weight of seeds during germination followed a typical triphasic water-uptake curve (Fig. 1). A rapid increase in fresh weight was followed by a lag period up to 48 hr. Germination started at 48 hr and was followed by a second rapid increase in fresh weight. By 96 hr 94% of the seeds had germinated. Fresh-weight measurements every hr for eight consecutive hr provided more detailed information about the kinetics of water uptake during the early hours of imbibition (Fig. 2). Water uptake reached a plateau after 8 hr, but seeds had absorbed most of the water needed for germination in the first 2 to 3 hr (Fig. 2).

PEG Treatment

The majority of seeds germinated between 48 to 96 hr from imbibition at 0.0 MPa for the D.I. water (control)(Fig. 3). Water potentials of -0.1 and -0.2 MPa delayed germination for 24 hr (compared with control) but germination was less inhibited as time of germination proceeded. Germination percentage under -0.1 and -0.2 MPa was significantly less than the control and greater than lower water potentials. At -0.4 MPa, germination started at 96 hr, and reached only 31% by 144 hr. A water potential of -0.6 MPa totally inhibited germination. At 96 hr under -0.1, -0.2, -0.4, and -0.6 MPa, germination was inhibited by 13, 49, 91, and 100% respectively.

The effect of PEG on seedling growth, as measured by an increase in

fresh weight (Fig. 4), was parallel to its effect on germination. Significant increases in fresh weight of seeds at 0.0 MPa (control) began at 48 hr. With PEG treatment, the increase in fresh weight at -0.1 and -0.2 MPa occurred at 72 hr and with -0.4 MPa occurred at 96 hr, which corresponds to the time of germination under these treatments. but -0.4 MPa was not significantly different from -0.6 MPa, under which there was no second increase in fresh weight (seeds do not enter phase III) and no germination. At 96 hr under -0.1, -0.2, -0.4, and -0.6 MPa, increases in fresh weight were inhibited by 53, 89, 107, and 106%, respectively.

SDS-PAGE and Protein Content

The SDS-PAGE profile of protein extracts from dry impatiens seeds separated into three distinct groups (Lane 0, Fig.5). The molecular weights of the proteins in these groups were estimated to be 35, 33, 31 kd; 26, 23, 21 kd; and two bands less than 14 kd for groups 1, 2, and 3, respectively. The general patterns of degradation of storage proteins during 144 hr of germination are shown in Lanes 2-6 (Fig. 5). Degradation started at 72 hr (Lane 3, 31 kd) and proceeded rapidly through 96 hr, corresponding to the greatest percentage of germination (see Fig. 3, control). By 144 hr most of the insoluble storage proteins disappeared, and new bands started to appear at 96 hr (Lanes 4, 5, and 6; see arrow). It was not clear if all of the products of the degradation had been moved to the embryonic axis or were broken down to small polypeptides not resolved on this gel.

At water potentials of -0.1 and -0.2 MPa, conditions under which some

germination occurred, degradation of storage proteins was detected (Fig. 6, Lanes 2 and 3). However, at -0.4 and -0.6 MPa, conditions under which no germination occurred (Lanes 4 and 5), the integrity of the major storage proteins was similar to that in dry seed (Fig. 6, lane 0). Fully germinated seeds (Fig. 6, Lane 1) showed a considerable degree of storage protein degradation after 96 hr. The protein bands in Lanes 4 and 5, compared to Lane 0, indicated that very little degradation of the major storage proteins occurred in seeds when germination was prevented by water-stress.

The protein content of seeds germinated in water (Table 1) and PEG (Table 2) reflected the changes in protein profiles shown in Figs. 5 and 6. As germination proceeded, soluble protein content increased and insoluble storage protein decreased. The imposition of water stress by lowering the water potential caused decreased germination which, in turn, was reflected by an increased conservation of protein content. Although there were no significant differences in insoluble protein content during the 96 hr of germination under water stress, soluble protein decreased with a decreasing water potential (Table 2). According to Table 2, the lowest levels of soluble protein content in cotyledons correlated to the lowest levels of germination (-0.4 and -0.6 MPa, Fig. 3).

DISCUSSION

The three components of the water potential of a seed are osmotic potential, matric potential, and pressure potential (Bewley and Black, 1985). In mature dry seeds, the water potential can exceed -100 MPa due to a high matric potential (Hegarty, 1978). Phase I, or imbibition, is largely a consequence of matric forces (starch and protein reserve). During phase II, the matric potential no longer plays a significant role in water uptake, and there is transition to greater dependence of water uptake on osmotic or solute potential (Bradford, 1986; McIntyre, 1987). Germination of impatiens seeds under the conditions of this study started at 48 hr after imbibition. Seeds absorbed most of the water needed for germination by 3 hr. Therefore, 45 hours of lag phase (water plateau) was required for developing the growth potential necessary for germination. This growth potential is provided by osmotic substances such as amino acids probably derived from storage protein (Takeba, 1980a; Takeba, 1980b). Impatiens seeds must gain water up to 60-67% of their dry weight to germinate. This increase in fresh weight correlates with an increase in soluble protein content which coincides with the first sign of radicle protrusion.

Factors such as low water potential cause a delay in water uptake, increase the length of the water uptake plateau, and subsequently delay or prevent germination. Phase I of water uptake is not dramatically affected by PEG treatment because of the very low water potential of the dry seed. Delay in germination under PEG concentrations representing water potentials

of -0.1, -0.2, and -0.4 MPa demonstrate this induced inhibition (Fig. 4). The extent of this inhibition is shown by the fact that water potential of -0.1 MPa causes a 24 hr delay in initiation of germination and a 40% reduction in germination.

A water potential of -0.6 MPa totally inhibits germination in impatiens, whereas other species required lower water potentials for complete inhibition of germination -- carrot, -1.0, calabreese, -1.5 MPa (Hegarty, 1977), jute, -0.8 MPa (Palit, 1987), and tomato, -1.0 MPa (Haigh, 1987). This increased sensitivity of impatiens seeds could be due to the soft seed coat, relatively small size of the seed, low moisture content of the seed (only 5%), and the origin of this plant (high humid regions of East Africa).

Although at 144 hr 31% of seeds germinate under -0.4 MPa, the very small increase (nonsignificant compared with -0.6 MPa) in fresh weight (Fig. 4) shows that radicle growth after germination is inhibited. Root elongation and cotyledon expansion are also inhibited at a water potential as high as -0.1 MPa. Therefore, higher water potentials are required after germination has occurred, than before germination to allow radicle elongation to proceed. This is in contrast with what has been reported for calabreese and cress by Hegarty and Ross (1978). They measured differential sensitivity of radicle growth to moisture stress, and reported that, after germination, seedlings develop water potentials lower than those permitting germination (approximately 0.8 MPa lower).

As germination proceeds, the pattern of these protein bands changes considerably. This shows that the increase in soluble protein is probably

derived from storage protein degradation. Degradation is needed to supply amino acids for metabolism in the germinating seeds (Pusztai and Duncan, 1971). Although at water potentials of -0.1 and -0.2 MPa the extent of degradation is less than the control, the water potential is still high enough to permit germination. In contrast, at water potentials of -0.4 and -0.6 MPa, where no germination takes place, very little degradation occurs. Therefore, under PEG induced water stress conditions, a gradual decrease in water potential causes a gradual decrease in germination, and this is correlated with the inhibition of storage protein degradation. It is likely that germination and the breakdown of storage proteins are both affected independently by low water potentials. But, from these results we cannot rule out the possibility that degradation of storage proteins is required for germination processes. From this data, it would seem that the breakdown of insoluble storage proteins accompanies radicle protrusion in impatiens.

Some of the metabolic processes which are inhibited under water stress conditions are the cell wall extension, protein synthesis, and enzyme activity (Hsiao, 1973). Yomo and Srinivasan (1973) and Minamikawa et al. (1983), reported cycloheximide, α-amanitin, and cordycepin inhibited protease formation and the degradation of the globulin (storage protein) in bean and cowpea. Their results indicated that *de novo* synthesis of some of the proteolytic enzymes was occurring during germination. Therefore, integrity of storage proteins of impatiens seed under stress conditions in this experiment may be explained as inhibition of *de novo* synthesis of some of the proteolytic enzymes responsible for degradation of storage proteins.

Degradation of storage proteins (soluble or insoluble) in the embryo axis and cotyledons after the start of imbibition is complex. New synthesis of proteolytic enzymes in the axis is not a prerequisite for degradation of albumin (soluble proteins) during the initial stages of germination (Minamikawa et al., 1983). However it is required for degradation of globulin (insoluble proteins) in the embryo axis of cowpea. The presence of soluble proteins in dry seed may be representative of the same situation in impatiens. Although we have shown the disappearance of insoluble proteins during germination, changes in insoluble proteins under water-stress are not significantly different. It is possible that part of the increase in soluble proteins comes from the soluble storage proteins. Another possible explanation for this could be that a small amount of storage protein degradation is required for germination, but cannot be detected by these techniques.

There is a significant difference in the soluble protein content of impatiens seeds treated with different concentrations of PEG. Soluble and insoluble protein content under -0.4 and -0.6 MPa are similar to 48 hr of incubation in water (Table 1) prior to the first sign of radicle protrusion. The same is true for -0.2 MPa and 72 hr. This shows that a threshold amount of accumulated soluble proteins may be necessary for radicle emergence. This accumulated soluble protein may serve as building blocks for synthesis of new proteins necessary for germination, or act as osmotic substances to produce enough growth potential.

In practice, imposition of any water-stress during the three phases of germination is not recommended. But after germination, seedlings may

actually benefit from a small amount of stress up to -0.1 MPa in order to be hardened for shipping and handling. Water potentials lower than -0.1 MPa will inhibit the efficient growth and production of impatiens seedlings, and are not recommended. The correlation between the time of germination, degradation of storage proteins and increase in soluble proteins, and the inhibition of these processes under PEG-induced water-stress implies that breakdown of storage protein (soluble or insoluble) is required for germination. It is possible that the role of water stress in the prevention of degradative processes along with germination is exerted through the inhibition of synthesis or activity of preexisting proteolytic enzymes.

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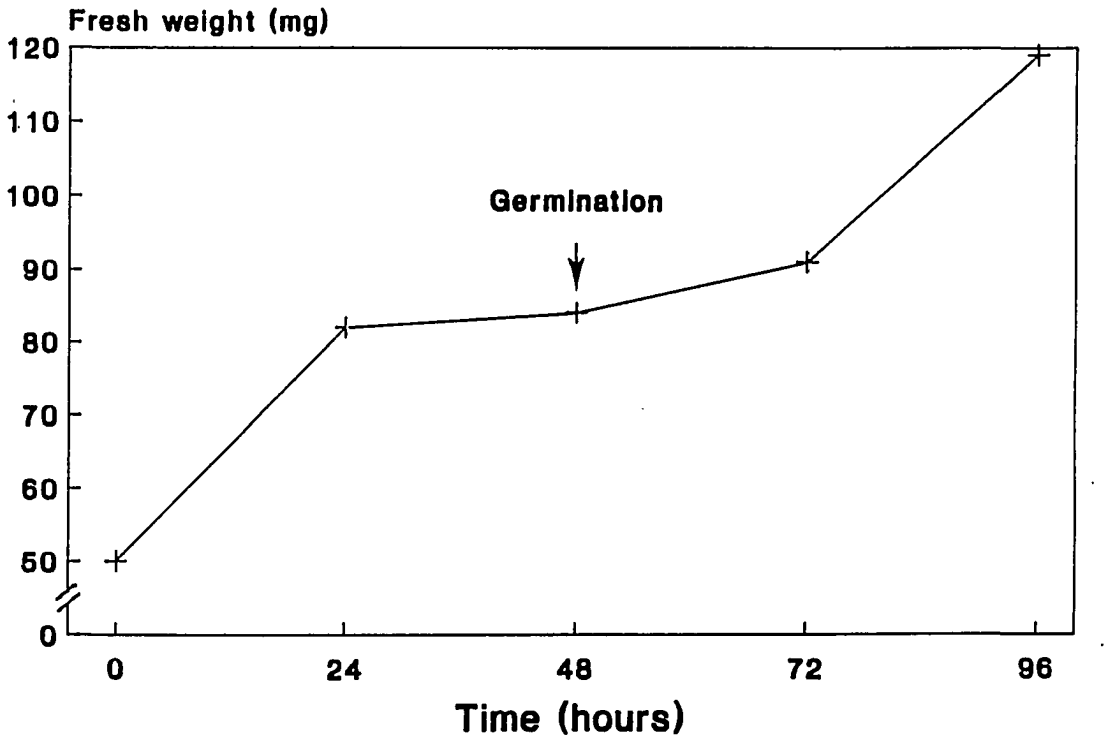

Figure 1. Water uptake curve for impatiens seeds imbibed in deionized water. Fifty mg samples of seeds were imbibed for a designated time at 25C, Kimwipe dried and measured for fresh weight. Each point is the mean of 3 replications

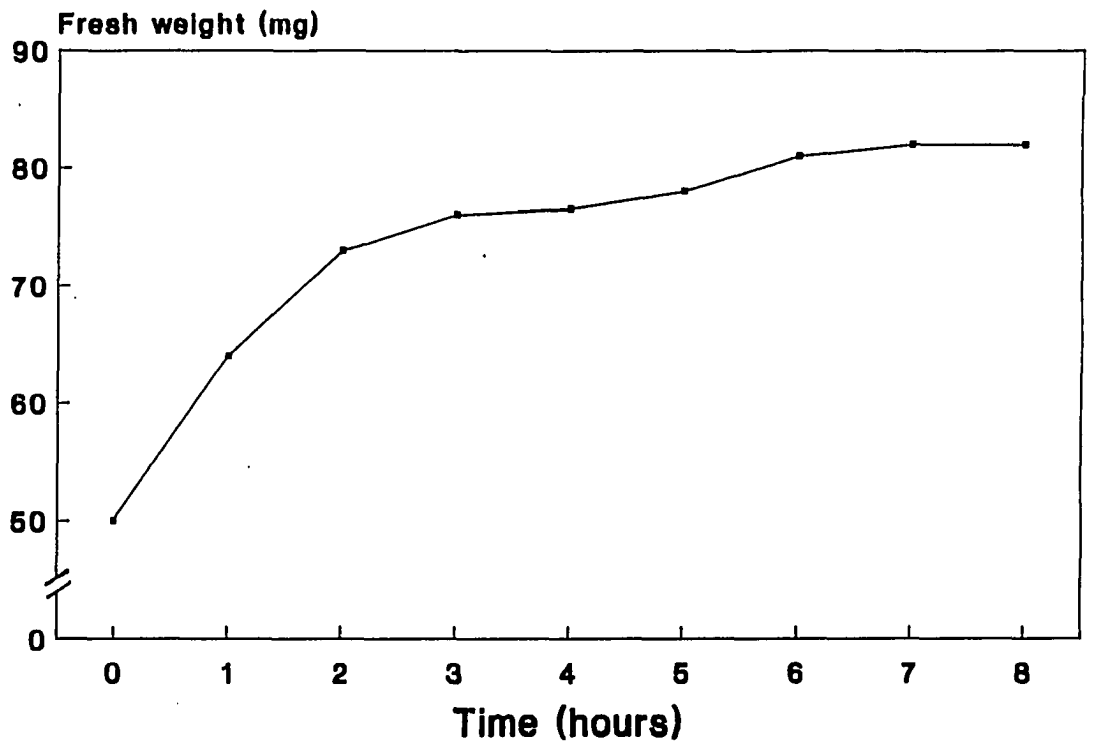

Figure 2. Water uptake curve for impatiens seeds imbibed in deionized water. Fifty mg Samples of seeds were imbibed for a designated time at 25C, Kimwipe dried and fresh weights recorded for 8 consecutive hours. Each point is the mean of 3 replications

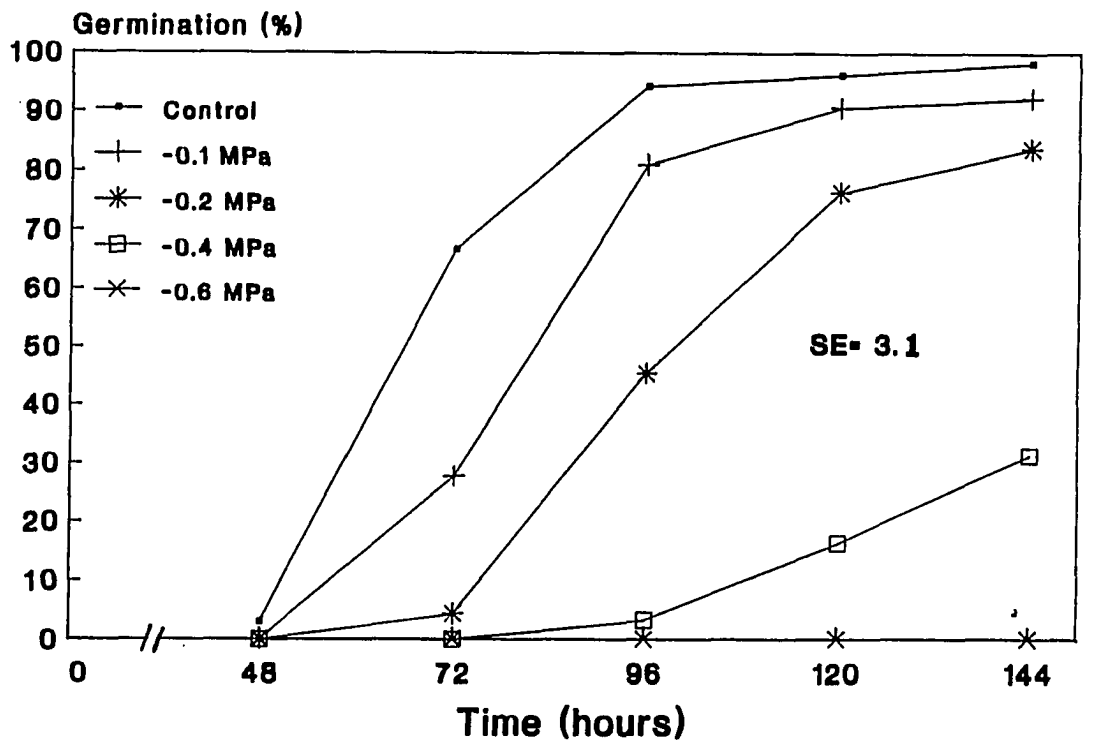

Figure 3. Time course of germination (radicle emergence) of impatiens seeds in PEG solutions of -0.1, -0.2, -0.4, and -0.6 MPa water potentials at 25C for 144 hr. Each point is the mean of 3 replications. Deionized water was control (0.0 MPa)

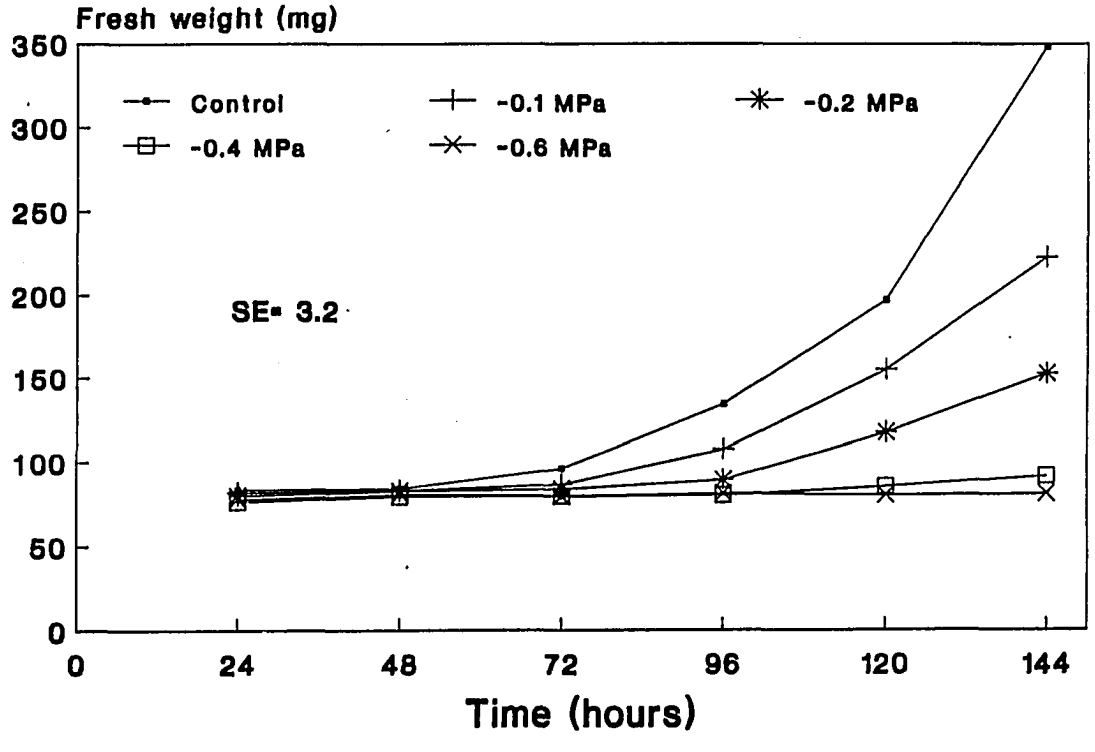

Figure 4. Fresh weight of 50 mg samples of impatiens seeds germinated in PEG solutions of -0.1, -0.2, -0.4, and -0.6 MPa water potentials at 25C for 144 hr. Each point is the mean of 3 replications. Deionized water was control (0.0 MPa)

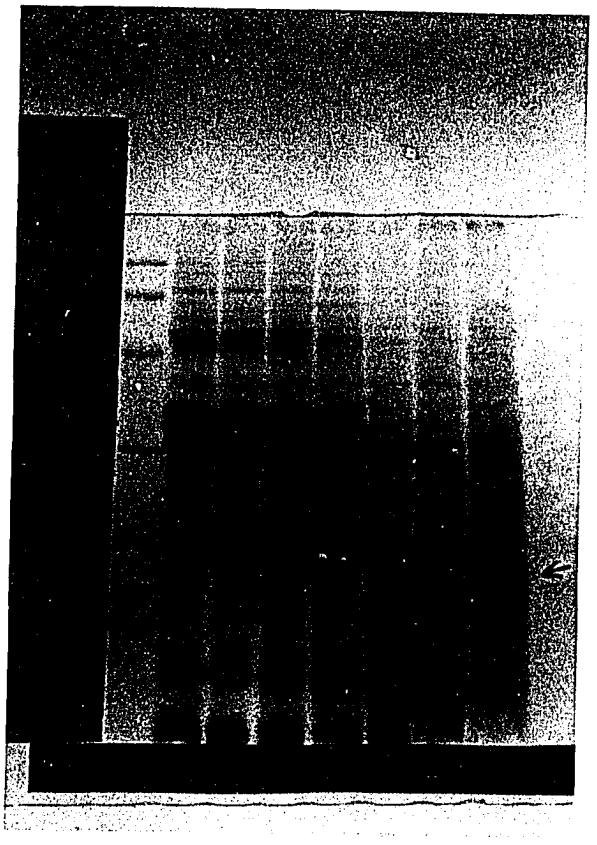

Figure 5. SDS-PAGE profile of proteins extracted from germinating seeds. Abbreviations are: S, molecular weight markers; Lane 0, mature impatiens seeds prior to germination; lanes 1-6, after 24-144 hours of germination respectively. Molecular weights in kd are indicated on the y-axis

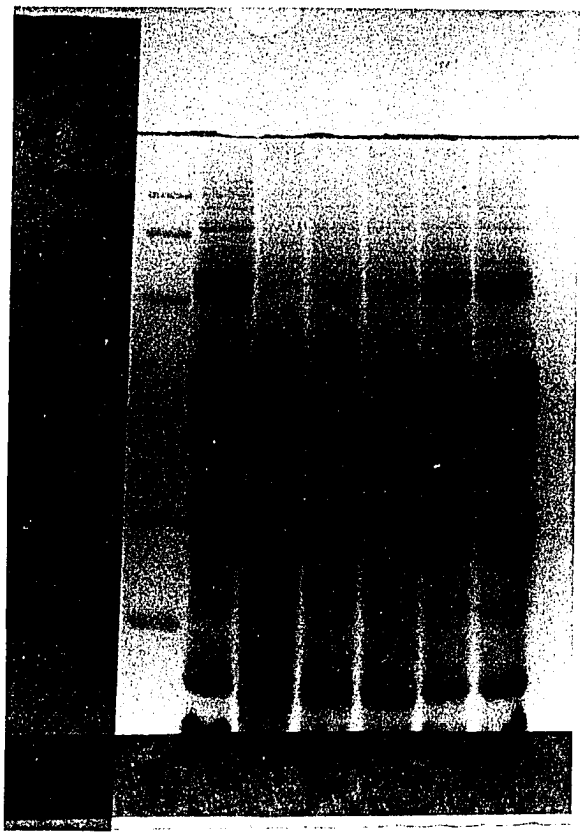

Figure 6. SDS-PAGE profile of proteins extracted from seeds germinating under PEG induced water stress conditions. Abbreviations are: S,molecular weight markers; lane 0, dry seed; lanes 1-5, seeds germinated for 96 hr under water potentials of 0, -0.1, -0.2, -0.4, and -0.6 MPa. Molecular weights in kd are indicated on the y-axis

Table 1. Changes in soluble and insoluble protein content of cotyledons during 144 hours of germination of impatiens seeds

Incubation time (hr)	Cotyledon protein content as percent dry weight[z]	
	Soluble	Insoluble
0	9.3 a[y]	13.4 ab
24	9.4 a	13.8 a
48	10.4 ab	13.1 ab
72	11.7 bc	12.7 b
96	12.6 c	12.7 b
120	16.4 d	9.4 c
144	19.2 e	9.0 c

[z]Protein content of 50 mg dried cotyledons.

[y]Mean separation in column by LSD, 5% level.

Table 2. Changes in soluble and insoluble protein content of impatiens seeds germinated under PEG induced water stress conditions for 96 hours

Water potential (MPa)	Cotyledon protein content as percent dry weight[z]	
	Soluble	Insoluble
0.0	13.0 a[y]	12.5 a
-0.1	12.1 b	12.7 a
-0.2	11.5 bc	13.0 a
-0.4	10.7 c	13.0 a
-0.6	10.6 c	13.2 a

[z]Protein content of 50 mg dried cotyledons.

[y]Mean separation in column by LSD, 5% level.

SECTION II. STORAGE PROTEIN DEGRADATION AND AMINOPEPTIDASE ACTIVITY DURING GERMINATION OF STRESSED AND NONSTRESSED IMPATIENS SEEDS

STORAGE PROTEIN DEGRADATION AND AMINOPEPTIDASE ACTIVITY DURING GERMINATION OF STRESSED AND NONSTRESSED IMPATIENS SEEDS

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ABSTRACT

Seeds of impatiens (*Impatiens wallerana* Hook. f. cv. Super Elfin Coral) were germinated for 0, 48,and 96 hr at 25C. After 48 hr of imbibition, two proteins, 35 and 26 kilodalton (kd), were degraded in the radicles. The remainder of the storage proteins were degraded by 96 hr. Under optimum conditions, the sharp increase in activity of leucine aminopeptidase (LAPase) during 144 hr of germination coincided with the major depletion of storage proteins from radicles and cotyledons. Isoelectric focussing (IEF) showed that only one form of LAPase was present in impatiens seeds, and it appears to be a sulfhydryl protease with peak activity at pH 8. An increase in specific activity indicated synthesis of this enzyme during germination. LAPase may be one of the proteases involved in major degradation of impatiens seed storage proteins. Decreasing water potentials induced by polyethylene glycol 8000 (PEG) caused a decrease in activity of the enzyme. The loss of enzyme activity under water stress was related to both inhibition of enzyme synthesis and inactivation of preexisting enzymes. In LAPase assays *p*-hydroxymercuribenzoate (*p*HMB) was the most potent inhibitor but it did not inhibit germination while N-ethylmaleimide (NEM) inhibited the LAPase activity by 70%. Total inhibition of germination by iodoacetamide (IAc-NH_2), (NEM), and phenylmethylsulfonyl fluoride (PMSF) indicated involvement of other sulfhydryl proteases or enzymes in germination processes.

INTRODUCTION

Seeds of dicots contain a large amount of storage protein sequestered in protein bodies in the radicles and cotyledons. During germination, these proteins are hydrolyzed to free amino acids, which support protein synthesis in the germinating axis (Koller and Hadas, 1982). These hydrolytic events are mediated by a series of proteinases, with varying substrate specificity and activity during germination. These proteases are classified as endopeptidases, including sulfhydryl, serine, and acid proteinases; and exopeptidases, including carboxy- and aminopeptidsases (Ryan and Walker-Simmons, 1981). Proteinases of plants from different genera or species show different patterns and levels of activity during seed germination (Ryan, 1973). Temporal variation in activity of endo-, carboxy-, and aminopeptidases during germination of bush bean (Feller, 1979), mung bean (Mitsuhashi et al., 1984), and ostrich fern (Cohen and DeMaggio, 1986) is representative of different types of regulation in seed.

In a previous paper we reported the pattern of storage-protein degradation during germination in stressed and nonstressed impatiens seeds (see Section I). The results showed that, under water-stress, storage proteins were not degraded and that this stability coincided with the inhibition of germination. The objective of this study was to partially characterize some of the proteolytic enzymes active during germination, and to correlate their patterns of activity with the breakdown of storage proteins and radicle protrusion in both stressed and nonstressed impatiens seeds.

MATERIALS AND METHODS

Preparation of Plant Material

Experiment 1

Samples of 150 mg (about 300 seeds) of impatiens seeds (George J. Ball Co. W. Chicago, IL) were placed in acrylic germination boxes (12 x 12 cm) with two layers of blue blotter paper (Anchor Paper Co., St. Paul, MN). Blotters were pre-soaked in deionized (D.I.) water and drained to remove excess water. Another 10 ml of D.I. water was then added to each box. Boxes were sealed with parafilm and kept in a growth chamber at 25 ± 0.5C and received continuous irradiance at 4 $\mu mol \cdot s^{-1} \cdot m^{-2}$ photosynthetic photon flux. At 48 hr time intervals (for a total of 144 hr) seeds were collected, freeze-dried and kept for further tests. Freeze-dried, ungerminated seeds were the control.

Experiment 2

Polyethylene glycol (PEG 8000, Fisher Scientific) solutions of different osmotic potentials -0.1, -0.2, -0.4, and -0.6 MPa (50, 75, 100 and 125 g/500 ml, respectively) were prepared. Water potentials were determined at room temperature using a Wescor 5100 C Vapor Pressure Osmometer. Germination papers were soaked in the appropriate solution and used for germination as described above. Samples of 150 mg seeds germinated in PEG were collected at 96 hr, washed with distilled water for 2 minutes, surface dried, freeze-dried, and kept for extraction. D.I. water was used as the control (0.0 MPa).

Both experiments were arranged in a completely randomized design.

Three replications in time were used for each experiment. Data were analyzed by using the ANOVA procedure of SAS (SAS Institute, Raleigh, N.C.).

Experiment 3

Inhibitors of proteolytic enzymes, such as *p*HMB, NEM, IAc-NH_2, and PMSF; and leupeptin and N-(N-[L-3-trans-carboxoxiran-2-carbonyl]-L-leucyl)-agamatine (E-64) were used in the enzyme assay at final concentrations of 2 mM and 10 μM, respectively. These chemical solutions were also used as germination media at the same concentrations. Samples of 50 mg seeds were placed for 144 hr on blotters soaked with these chemical solutions, collected, freeze-dried, and kept for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis

Radicles and cotyledons from samples of 0, 48, and 96 hr germinated seeds (experiment 1) were excised manually with a scalpel blade. Enough seeds were dissected to collect 40 mg samples (approximately 80 cotyledons and 600 radicles) of seed material. Samples were ground in 400 μl of SDS extraction buffer at 4C, centrifuged for 20 minutes at 11000x g, and 6 μl of the supernatant was subjected to SDS-PAGE (0.75 mm discontinuous slab gel) (Laemmli, 1970). Samples of seeds treated with inhibitors were prepared and used for SDS-PAGE as described above.

Enzyme Activity

Freeze-dried samples (150 mg) of 0, 48, 96, and 144 hr D.I. water germinated seeds (Experiment 1), and 96 hr PEG germinated seeds (Experiment 2), were homogenized in a cold mortar and pestle with 1.2 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM 2-mercaptoethanol and centrifuged at 25000xg for 20 min (Mitsuhashi et al., 1984). The supernatant solution was used as the enzyme solution for the leucine-aminopeptidase assay and for the detection of LAPase activity on polyacrylamide gel.

LAPase was assayed using 1 ml of 0.4 mM L-leucine-*p*-nitroanilide (in 50 mM Tris-HCl buffer, pH 8) and 7 μl of enzyme solution (Mitsuhashi et al., 1984). LAPase activity was determined by measuring the rate of production of *p*-nitroaniline at 405 nm over a 5 minute period at 30C (Pfleiderer, 1970 and Gwynne et al., 1976). One unit of enzyme activity was defined as the amount of enzyme catalyzed the formation of 1 μmole of product in 1 min under the assay condition. Protein was determined by the modified Lowry method (Peterson, 1977).

Detection of LAPase Activity on the Gel

Isoelecric focussing was performed in a vertical polyacrylamide mini-gel system (Ni et al., 1987). Enzyme solutions (see enzyme activity) from dry, 96 hr, and PEG germinated (-0.6 MPa) seeds were loaded based on activity (25, 15, and 25 μl, respectively). Analysis was performed in the pH range of 3 to 10 at 4C for 4 hr. The gel was assayed for LAPase activity by the method of Vallejos (1983). Migration distance of methyl red was used to establish the R_f value for the enzyme.

RESULTS

Changes in Storage Proteins During Germination

The SDS-PAGE profile of protein extracts from dry impatiens seed (Fig. 1, lane W) showed three distinct groups of major storage proteins. Estimated molecular weights of the bands within these groups were 35, 33, 31 kd; 26,23,21 kd; and two bands smaller than 14 kd. Dissected cotyledons and radicles contained the same groups of protein (Lanes C and R, 0). Changes in protein bands were detected in the radicle at 48 hr from the start of imbibition. Two bands, 35 and 26 kd, degraded in the radicle at this time (Lanes C and R, 48). Major degradation of storage proteins took place between 48 and 96 hr (Lanes C and R, 96) and this corresponded with the maximum germination count (see Section I).

Changes in LAPase Activity During Germination

The activity of LAPase increased during 144 hr of germination (Fig. 2a). Dry seeds showed a low level of LAPase activity which remained unchanged for the first 48 hr. However, there was a significant increase in activity by 96 hr (germination started at 48 hr and 95% of seeds were germinated by 96 hr (see Section I)). The activity increased up to 144 hr, but at a much lower rate than that found for the period between 48-96 hr.

Under PEG simulated water stress (Fig. 3a), LAPase activity at the 96 hr of germination decreased gradually as the water potential decreased. Decreased activity at -0.1 and -0.2 MPa was significantly different from control and other treatments. Decreases in activity paralleled the decreases in germination percent (germination was totally inhibited at -0.4

and -0.6 MPa (see Section I)).

Enzyme activity was determined as a function of pH by buffering the reaction mixture with different buffer systems (Gomori, 1955). The results indicated the presence of a single form of the enzyme, and the optimum activity was at pH 8 (Fig. 4).

Zymogram Pattern of LAPase During Germination

LAPase was assayed for activity after IEF (Fig 5). R_f value was 0.91 which indicated that the enzyme was a protein with a low isoelectric point. Bands started to appear five minute after incubation of the gel in the substrate and continued to develop up to 45 min. Only active band in lane DS (Fig. 5a) indicated the existence of one form of LAPase in impatiens seeds. The enzyme was present in dry seed and its activity increased during germination. Although enzyme solutions were loaded based on equal activities, the band from 96 hr germinated seeds was more intense than the band from dry seeds. LAPase was detected in extracts from seeds germinated for 96 hr under nonstress and PEG simulated water stress conditions (Fig. 5b).

Inhibitors

LAPase was sensitive to -SH inhibitors, especially *p*HMB, which caused 100% inhibition of activity in enzyme assay (Table 1). NEM treated extracts showed about 70% inhibition after 7 hr. PMSF, IAc-NH_2, leupeptin, and (E-64) had a less inhibitory effect on the enzyme during assay. The enzyme appeared to contain an essential thiol group.

*p*HMB, when used as germination media, prevented further growth of the

radicle after germination. NEM, PMSF, and IAc-NH_2 completely prevented germination. Leupeptin and E-64 did not inhibit germination. SDS-PAGE of protein extracts from the seeds treated with *p*HMB, NEM, PMSF, IAc-NH_2 showed that chemicals which inhibited germination also inhibited storage protein degradation (Fig. 6).

DISCUSSION

Impatiens seed germination was accompanied by the degradation of storage proteins in both the radicle and cotyledon. Two proteins (35 and 26 kd) disappeared from the radicle before radicle protrusion (Fig. 1, lane R-48). A similar observation was made on a small protein (13 kd) in the embryonic axis of pea (Murray, 1979) and of mung bean (Manickam and Carlier, 1980). These two proteins have been classified as albumin soluble storage protein, while those proteins found in impatiens have been classified as insoluble storage proteins. Proteins found in the cotyledon also were represented in the radicle. Despite significant homology between the storage proteins of distinct groups of plants (Templeman et al., 1987), during germination there is a great variation in proteolytic activity in seeds from different genera (Ryan, 1973). Solubility and size of the protein does not determine the degradation priority, which depends more on the type of regulation present in a species.

In an attempt to correlate proteolytic enzyme activity with storage protein degradation during germination, we were unable to measure activity for endopeptidase (azocaseolytic activity) and carboxypeptidase (activity toward N-carbobenzoxy-L-phenylalanine-L-alanine) in extracts of germinating impatiens seeds. These two enzymes probably had an activity lower than the limits of detection. Increased aminopeptidase activity did correlate with the time of major depletion of storage proteins.

On the basis of the inhibitor study (Table 1), LAPase in impatiens seed is an -SH peptidase, because its activity was totally inhibited by *p*HMB, an inhibitor of -SH active site (Glick, 1972). Seventy percent

inhibition by NEM also supports this conclusion (Glick, 1972). Two aminopeptidases of pea (Elleman, 1974) and those of castor bean (Tully and Beevers, 1978) and mung bean (Chrispeels and Boulter, 1975) are -SH enzymes. LAPase exhibited an optimum activity at pH 8, which agrees with the pH range for aminopeptidases (Mikola, 1983). Based on our results from IEF, there is only one LAPase in impatiens seeds. Four LAPase have been detected in mung bean (Mitsuhashi et al., 1984) and three in barley (Sopanen and Mikola, 1975).

LAPase was present in dry impatiens seeds and increased in activity at 48 hr from the start of imbibition. This increased activity coincided with the start of germination. The sharp increase in enzyme activity coincided with the time of rapid storage protein degradation indicating that LAPase is one of the proteases involved in the germination process. The observed decline in LAPase activity after day one in pea (Beevers, 1968) and bush bean (Feller, 1979) suggested that the enzyme may not play an important role in storage protein metabolism. However, according to Tully and Beevers (1978) and Mitsuhashi et al. (1984), LAPase present in dry seed participates in the early mobilization of storage protein. The two other proteases (endo- and carboxy-), which have their peak after aminopeptidase activity declines, are responsible for later protein turnover and senescence of the cotyledons. In scots pine, total aminopeptidase activity (including LAPase) is sufficient to account for the rate of storage protein mobilization during germination (Salmia and Mikola, 1975; Salmia and Mikola, 1976). Yet in ostrich fern, all three proteases have their peak activity at the same time before germination (Cohen and DeMaggio, 1986).

Our results showed that under PEG induced water-stress there is a

significant decrease in LAPase activity. Protein synthesis (Dhindsa and Cleland, 1975) and degradation (Cook et al., 1979) have been shown to be susceptible to water stress, and it seems that declines in enzyme activity are more instantaneous than declines in protein synthesis (Hanson and Hitz, 1982). However, different enzymes are affected to different extents, and certain enzymes seem to be more susceptible to water stress than others (Bardzik et al., 1971; Cook et al., 1979).

Most investigations on the effect of water stress on metabolic activity are performed on actively growing plant tissues (mostly young or recently mature leaf tissue). In these experiments the activity of an enzyme, i.e., nitrate reductase (Bardzik et al., 1971) or ribulose bisphosphate carboxylase (Vu et al., 1987), is measured as a direct or an indirect response to induced water stress (Vassey and Sharkey, 1989). Seed tissues are very different from leaf tissue in that they maintain minimal metabolic activity in the dry state and that stress during imbibition prevents protein synthesis and enzyme activation from starting. Therefore, the observed decreased activity of LAPase under -0.1 MPa water potential was due probably to the direct effect of water stress on lowering the activity of preexisting enzyme. However, the decreased specific activity (Fig. 3b) of LAPase shows that inhibition of synthesis of the enzyme also is involved. This is supported by the observation that LAPase specific activity increased during the 144 hr of germination (Fig. 2b). We suggest that, at lower water potentials (<-0.2 MPa), inactivation and inhibition of synthesis of LAPase are responsible for the reduction in enzyme activity. At lower water potentials the activity of LAPase reached a steady state level. This response was observed in maize seedlings (Bardzik et al.,

1971). Decreased LAPase activity under water-stress correlates with decreased and inhibited germination (Khademi and Koranski, 1988).

Less inhibition of LAPase activity by leupeptin and E-64 is probably because of the unstable nature of these inhibitors. The fact that IAc-NH_2, NEM, and PMSF (Serine protease inhibitor) totally inhibit germination when used as germination media shows that there are other proteases and probably other -SH enzymes which are directly or indirectly involved in germination. *p*HMB did not inhibit germination, but after germination further growth of the radicle was inhibited. One possible explanation for this observation is that *p*HMB can not enter the seed. Kubota et al. (1973) suggested that *p*HMB only can react with free -SH groups and that there must be conformational changes in the enzyme before *p*HMB can exert its inhibitory effect.

The fact that increases in the activity of LAPase coincided with the time of major depletion of storage proteins and germination indicates that this enzyme is involved in the germination processes of impatiens seed. Decreased activity of LAPase under water stress also coincided with decreased germination percentages and inhibited storage protein degradation. It appears that the maintenance of storage proteins under water stress is due to both inactivation of existing, and inhibition of synthesis of proteolytic enzymes. Other proteases besides LAPase probably are involved in the germination process. More research is required to find out if degradation of storage proteins in the radicle is sufficient for germination. Further work on the activation mechanism of proteolytic enzymes and their role in germination is needed.

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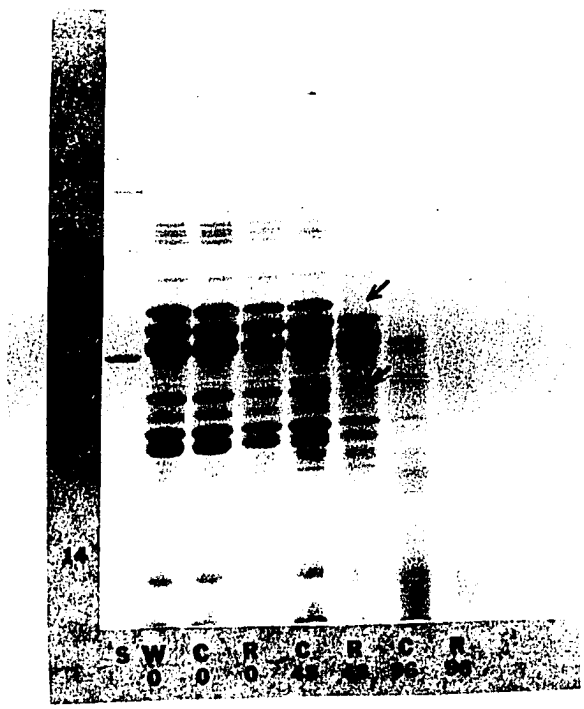

Figure 1. SDS-PAGE profile of proteins extracted from germinating impatiens seeds. Abbreviations are: S, molecular weight markers; Lanes W, C, and R (0), whole seeds, cotyledons, and radicle of mature dry impatiens seeds, respectively; and Lanes C and R (48 and 96), cotyledons and radicles after germination for 48 and 96 hr, respectively. Arrows denote protein bands which degraded at 48 hr in the radicles. Molecular weights in kd are indicated on the y-axis

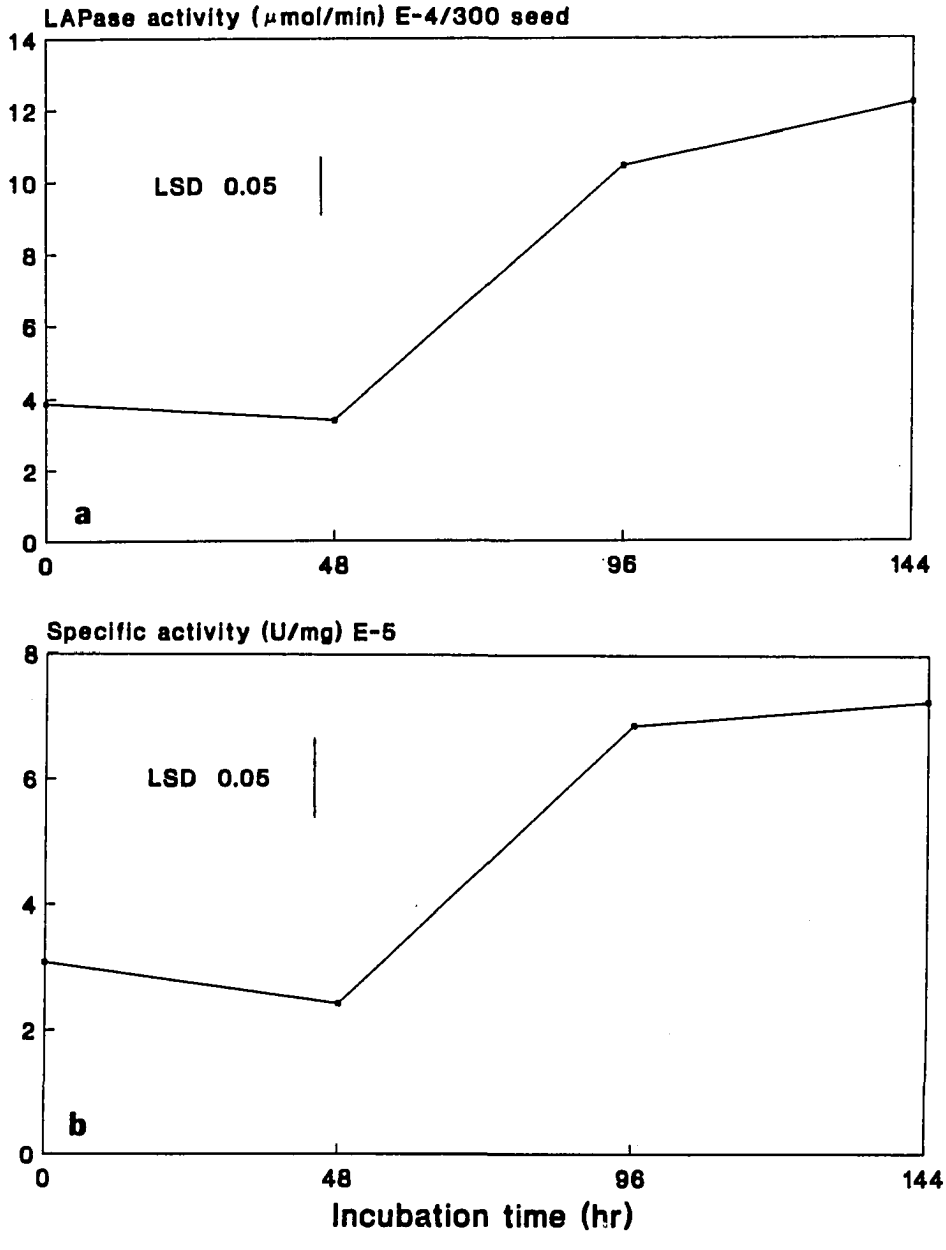

Figure 2. LAPase activity. Changes in LAPase activity (a) and specific activity (b) in samples of impatiens seeds germinated for 0, 48, 96, and 144 hr at 25C. Activity was measured as μmol of *p*-nitroaniline released per minute

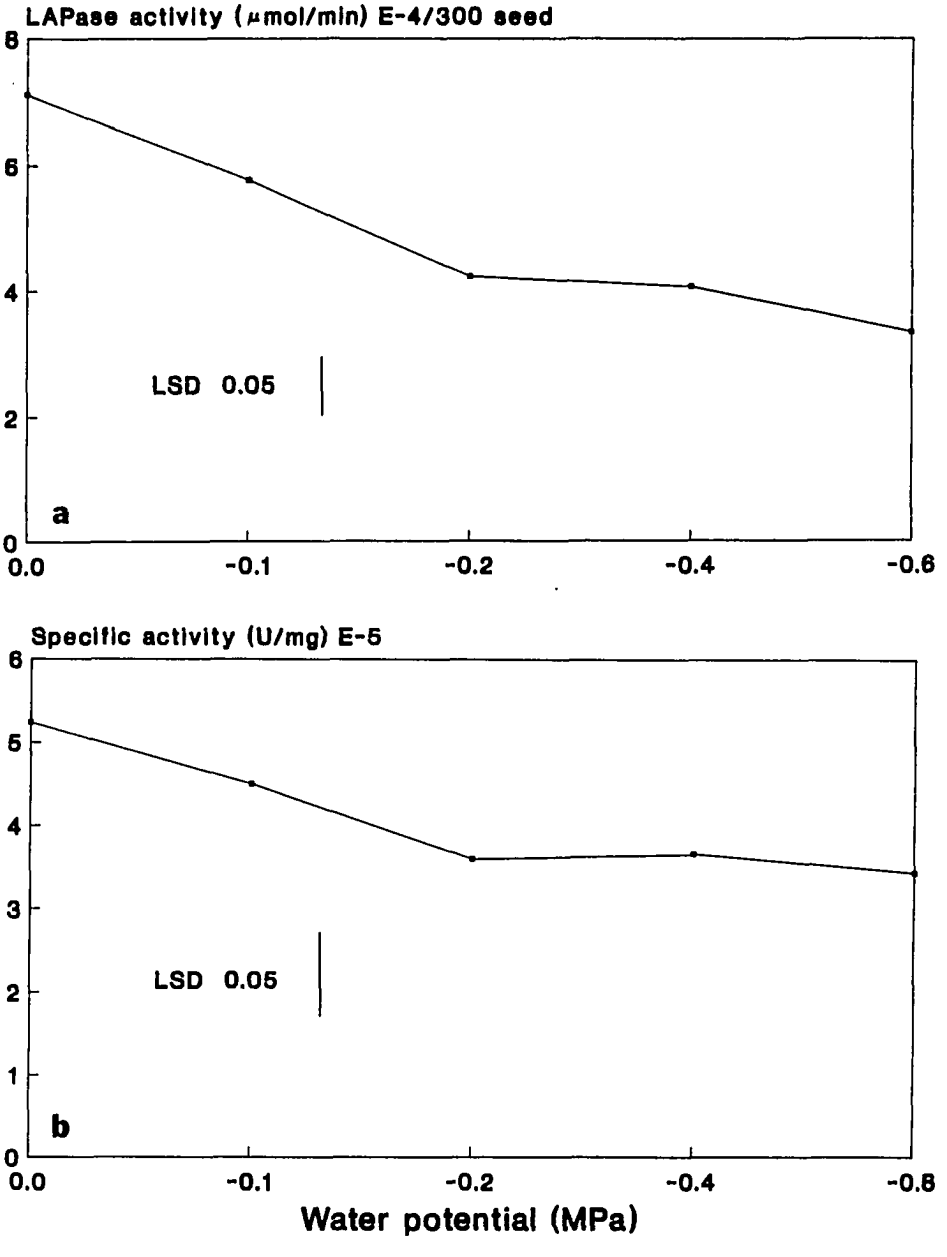

Figure 3. Effect of water stress on LAPase activity. Changes in LAPase activity (a) and specific activity (b) during germination under PEG-simulated water-stress. Impatiens seeds were germinated for 96 hr at 25C at 0.0,-0.1, -0.2, -0.4, and -0.6 MPa water potentials. Activity is measured as μmol of *p*-nitroaniline released per minute

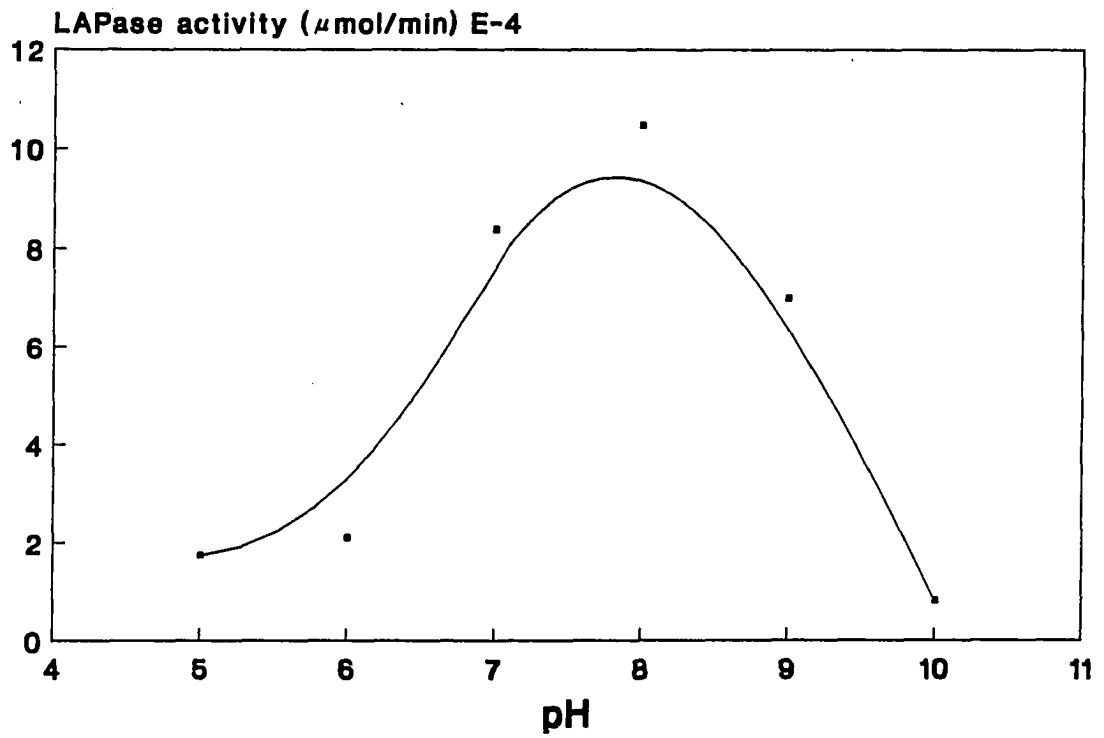

Figure 4. Effect of pH on the activity of LAPase in extracts of impatiens seeds, germinated for 96 hr. The following buffers (50 mM) were used for the indicated pH ranges: citrate-phosphate, pH 5 to 6; Tris-HCl, pH 7 to 8; boric acid-borax, pH 9; and borax-NaOH, pH 10

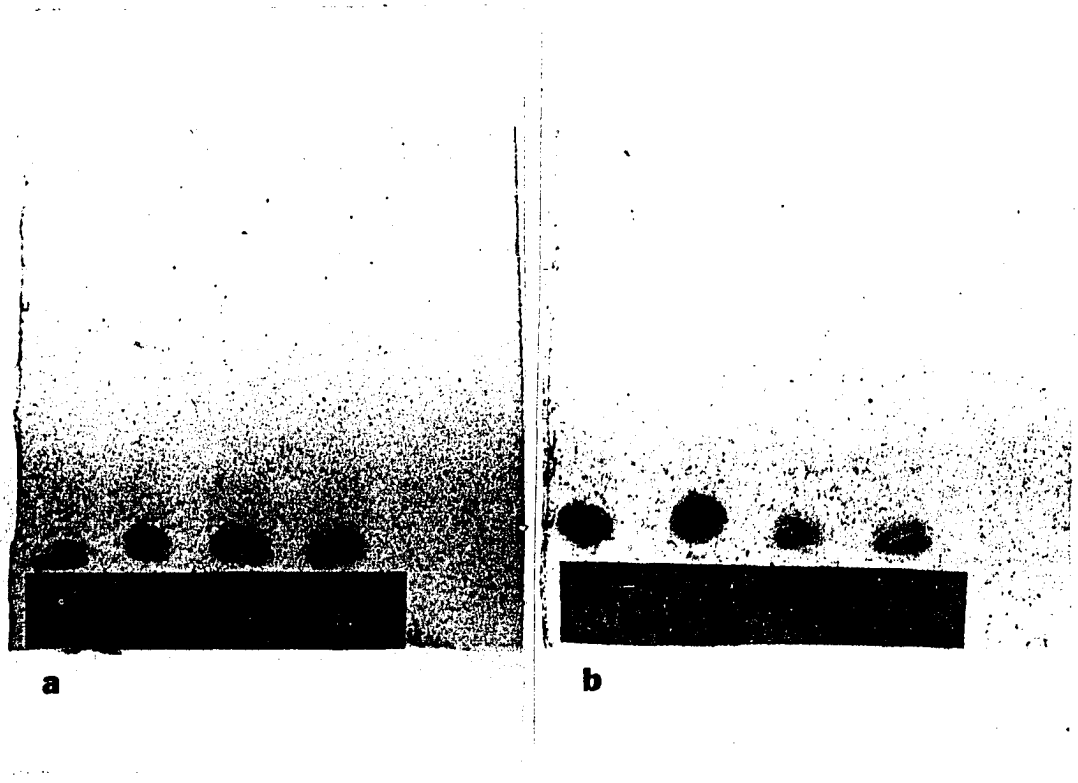

Figure 5. Zymogram patterns of LAPase in impatiens seeds. Samples of seeds germinated in deionized water for 0 and 96 hr at 25C (a) and samples of seeds germinated for 96 hr at -0.6 MPa (PEG simulated water-stress) (b). Abbreviations are: DS, dry seed; DI, deionized water; and PEG, polyethylene glycol

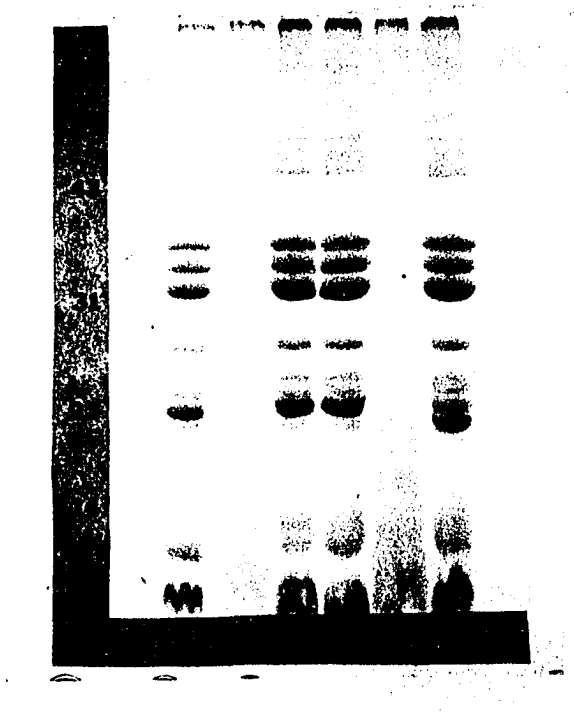

Figure 6. SDS-PAGE profile of proteins extracted from seeds treated with proteolytic enzyme inhibitors for 144 hr of germination. Abbreviations are: S, molecular weight markers; DS, dry seed; DI, deionized water; NEM, N-ethylmaleimide; PM, phenylmethylsulfonyl fluoride; MB, *p*-hydroxymercuibenzoate; IAc, idodoacetamide. Molecular weights in kd are indicated on the y-axis

Table 1. Effect of protease inhibitors on LAPase activity. Extracts from seeds germinated for 96 hr were treated with inhibitors for 0 and 7 hr at 0-4C. Inhibitors were used at final concentration of 2 mM except for Leupeptin and E-64 which were used at a final concentration of 10 μM. Data are means of three replications

Inhibitors	Activity (% of control)	
	0 hr	7 hr
Control	100	100
IAc-NH_2	93	82
NEM	77	34
PMSF	82	72
*p*HMB	23	0
Ethanol	95	82
Leupeptin	90	84
E-64	89	83

SUMMARY AND DISCUSSION

Water uptake of impatiens (*Impatiens wallerana* Hook. f. cv. Super Elfin Coral) seed was measured as an increase in fresh weight every 24 hr during 144 hr of germination. Seeds absorbed most of the water required for germination within 3 hr of imbibition, and germinated at 60-67% moisture on a dry weight basis. Germination started at 48 hr and was complete by 96 hr at 25C. Three distinct groups of storage proteins with estimated molecular weight of 35, 33, 31 kilodalton (kd); 26, 23, 21 kd; and two bands less than 14 kd were present in dry seeds. At 48 hr from the start of imbibition, two proteins, 35 and 26 kd, degraded in the radicles. The remainder of the storage proteins degraded by 96 hr. Major depletion of storage proteins coincided with the time of maximum germination. The soluble protein fraction increased and coincided with a decrease in the insoluble fraction during germination under optimum conditions. Under these conditions the activity of leucine aminopeptidase (LAPase) during 144 hr of germination correlated with the major depletion of storage proteins from radicles and cotyledons. Isoelectric focussing showed that only one form of LAPase was present in impatiens seeds. This was determined to be a sulfhydryl protease with peak activity at pH 8. An increase in specific activity indicated synthesis of this enzyme during germination. LAPase was one of the proteases involved in degradation of impatiens seed storage proteins.

Water-stress of -0.1, -0.2, -0.4, and -0.6 MPa, induced by polyethylene glycol 8000, reduced germination by 13, 49, 91, and 100%, respectively at 96 hr. Under the same water-stress conditions, increases

in fresh weight were inhibited by 53, 89, 107, and 106% respectively. Water potentials which inhibited germination also inhibited degradation of storage proteins, and the protein profile remained unchanged. At these levels insoluble protein content remained unchanged, and a small increase in soluble proteins was not enough to proceed germination. Threshold concentrations of soluble proteins were required for germination. Water-stress caused a decrease in activity of LAPase. The loss of enzyme activity under water-stress conditions was due to both inhibition of enzyme synthesis and inactivation of preexisting enzymes. *p*-hydroxymercuribenzoate was the most potent inhibitor of LAPase in enzyme assays, but did not inhibit germination. N-ethylmaleimide (NEM) inhibited the LAPase activity by 70%. Total inhibition of germination by iodoacetamide, NEM, and phenylmethylsulfonyl fluoride indicated involvement of other sulfhydryl proteases or enzymes in germination processes.

In practice, imposition of any water-stress during the three phases of germination is not recommended. But after germination, seedlings may actually benefit from a small amount of stress up to -0.1 MPa in order to be hardened for shipping and handling. Water potentials lower than -0.1 MPa will inhibit efficient growth and production of impatiens seedlings, and are not recommended. Correlation between the time of germination, degradation of storage proteins, increase in soluble protein, and increase in LAPase activity, and the inhibition of these processes under PEG-induced water-stress implies that activity of proteolytic enzymes and breakdown of storage proteins (soluble or insoluble) are required for germination. Further research is required to find out if degradation of storage proteins of radicles is sufficient for germination. Further work

also is required to understand the activation mechanism of proteolytic enzymes and their role in germination through providing amino acids for protein synthesis and increasing the solute potential as a driving force for water uptake.

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