Endo-β-mannanase activity and seed germination of thermosensitive and thermotolerant lettuce genotypes in response to seed priming

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Abstract

The effects of seed priming on germination and endo-β-mannanase activity at inhibitory and non-inhibitory temperatures in thermosensitive 'Dark Green Boston' (DGB) and in thermotolerant 'Everglades' (EVE) lettuce were investigated. A single-seed endo-β-mannanase assay was used to follow the enzyme activity during priming. Seeds were primed at 15°C in aerated solutions of polyethylene glycol (PEG) with constant light, then redried. Primed and non-primed seeds germinated 100% at 20°C. At 35°C, non-primed and primed EVE seeds germinated 100%, whereas non-primed seeds of DGB germinated only 4%. During priming, endo-β-mannanase activity increased between 24 and 48 h in EVE and between 24 and 72 h in DGB after the beginning of osmotic imbibition. Endo-β-mannanase activity persisted in primed seeds following seed drying, was detected before radicle protrusion and was present in the micropylar region in front of the radicle tip. Higher enzyme activity was observed in primed seeds and EVE compared with non-primed and DGB seeds. The results suggest that priming may overcome the inhibitory effect of high temperature in thermosensitive lettuce seeds due to increased endo-β-mannanase activity, possibly leading to a weakening of endosperm, thus overcoming thermodormancy.

Keywords: *Lactuca sativa*, endo-β-mannanase, endosperm weakening, osmotic conditioning, seed priming, thermo-dormancy, thermoinhibition

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Introduction

High temperature conditions during sowing in the greenhouse (transplant industry) or field can cause lettuce seed germination to be erratic or completely inhibited. Depending upon the genotype, lettuce seeds imbibed at temperatures above 25°C often fail to germinate (Gray, 1975). This can lead to two different phenomena. The first, known as thermoinhibition, occurs when lettuce seeds are imbibed at temperatures above the optimum for a specific cultivar; the seeds will germinate if the temperature decreases below this level. The second phenomenon, known as thermodormancy or secondary dormancy, occurs when lettuce seeds are imbibed and maintained under high temperature for 72 h or more, and the seeds will not germinate even if the temperature decreases to a favourable level for germination (Khan, 1980/81). In this case, a seed treatment or removal of the outer seed coverings is necessary for germination to occur (Ikuma and Thimann, 1963; Keys *et al.*, 1975; Guedes and Cantliffe, 1980).

Since the lettuce endosperm cell walls are composed largely of galactomannans (Halmer *et al.*, 1975), endo-β-mannanase may be the enzyme involved in the cell wall degradation leading to endosperm weakening and subsequent radicle protrusion. Endo-β-mannanase, which hydrolyses mannan-polymers, is produced and secreted by the lettuce endosperm (Halmer and Bewley, 1979). Endo-β-mannanase is also involved in the degradation of storage galactomannans in legume seeds (Reid *et al.*, 1977). In lettuce, early studies detected mannan hydrolysis only as a post-germinative event (Halmer *et al.*, 1975; Bewley and Halmer, 1980/81; Dulson and Bewley, 1989; Halmer, 1989). Recently, Dutta *et al.* (1997) reported that a cell-wall-bound endo-β-mannanase was expressed in lettuce seed endosperm prior to radicle protrusion and noted that enzyme levels were regulated by the same conditions that govern seed germination.

Seed priming circumvents thermodormancy of lettuce seeds and allows germination at higher temperatures (Guedes and Cantliffe, 1980; Khan, 1980/81; Cantliffe *et al.*, 1981; Wurr and Fellows, 1984; Valdes *et al.*, 1985). The mechanism by which seed priming overcomes thermodormancy in lettuce is not known. Guedes *et al.* (1981) observed a progressive loosening of the endosperm during priming, possibly indicative of endosperm weakening, thus allowing lettuce seed germination to proceed at high temperature. Cantliffe *et al.* (1984) reported that seed priming appeared to lead to the irreversible initiation of cell elongation, thus overcoming thermodormancy. Weges *et al.* (1991) measured the osmotic potentials of primed lettuce seeds and reported that the temperature requirement for 50% germination was not associated with osmotic adjustment of cells. Thus, the changes that occurred in the cells were due to cell wall extensibility; these changes were suggested as the priming effect in lettuce seeds (Karssen *et al.*, 1989).

Using a water relations analysis of the initiation of radicle growth, Bradford and Somasco (1994) suggested that the beneficial effects of priming in lettuce occurred primarily on the growth potential of the embryo, rather than surrounding envelope tissues. In tomato, Haigh (1988) proposed that priming resulted in more rapid imbibition, increased the extensibility of radicle cell walls and weakened the endosperm. Small *et al.* (1993) suggested that increased respiration and ATP production during priming might be the primary mechanism in alleviating thermoinhibition in lettuce seeds; however, seed priming did not markedly alter the pattern of respiration and ATP production (Cantliffe, 1976; Cantliffe *et al.*, 1984). Sung (1996) observed weakening of the lettuce endosperm layer by seed priming. Using a puncture test, Sung *et al.* (1998b) also verified that priming results in a reduction of the initial force necessary to penetrate the endosperm. Thus, the mechanism of priming in lettuce seeds that allows embryonic growth and/or causes endosperm weakening is still unclear. In tomato seeds, increased endo-β-mannanase (Karssen *et al.*, 1989) and galacto-mannan-hydrolysing activity (Nonogaki *et al.*, 1992) were noted during seed priming, and the enzymes were suggested to be responsible for the endosperm weakening leading to improved germination.

Assuming that the weakening of the endosperm must occur for lettuce seed to germinate at high temperature, priming might overcome thermo-inhibition and/or thermodormancy by weakening the endosperm via increased endo-β-mannanase activity. To test this idea, a sensitive single-seed assay for endo-β-mannanase activity (Still *et al.*, 1997) was adapted to monitor enzyme activity in seeds during priming and seed germination at optimal and high germination temperatures after priming in both thermosensitive and thermotolerant lettuce genotypes.

Materials and methods

Plant material

Lettuce (*Lactuca sativa* L.) seeds (achenes) from thermosensitive 'Dark Green Boston' (DGB) and thermotolerant 'Everglades' (EVE) genotypes were used in this study. Thermotolerance was defined as the ability of seeds to germinate above 90% at 35°C in the light (Guzman *et al.*, 1992; Sung, 1996). DBG and EVE were chosen because of the genetic relation between these two genotypes (Guzman *et al.*, 1992). All seeds were produced in the same season and region of San Joaquin Valley, California, in 1994. Seeds were stored at 10°C, 40% RH until used.

Seed priming

Optimum conditions for priming these two lettuce cultivars were determined by Sung *et al.* (1998a). Seeds were primed in 200 mm test tubes for three (DGB) or two (EVE) days at 15°C with constant light ($\sim$26 µmol m^{-2} s^{-1}) in an aerated solution of polyethylene glycol (PEG), at an osmotic potential of −1.2 MPa (DGB) or −1.3 MPa (EVE), (30 ml of solution g^{-1} of seed). An aquarium pump provided aeration. The air was pre-hydrated by passing it through water to minimize evaporation of the soak solution. At the end of the prescribed time period, the seeds were placed in a Buchner funnel, then rinsed three times with 100 ml of distilled water, and redried to their original moisture content in an incubator at 15°C and 45% RH for 2 days.

Seed moisture content

Seed moisture content was determined by oven drying 100-mg samples for 1 h at 130°C, placing the seeds in a desiccator at room temperature for 20 min and weighing (AOSA, 1993). Samples were replicated twice.

Seed germination

Four replications of 25 seeds were placed on two layers of 5.0 cm diameter germination paper (Anchor Paper, Hudson, WI, USA) moistened with 3 ml of distilled water. Distilled water was added as needed to keep the filter paper moist. Blotters were covered with 5.5 cm glass Petri dish lids and incubated at 20 or 35°C under constant light ($\sim$26 µmol m^{-2} s^{-1}) on a one-dimensional thermogradient bar (Type DB 5000,

Van Dok & De Boer, B.V., The Netherlands). Seeds were examined hourly for evidence of visible germination. Germination was defined as visible radicle protrusion through the pericarp and was noted as time to first radicle protrusion and of 50% radicle protrusion of those seeds that germinated.

Enzyme activity

A gel-diffusion assay (Still *et al.*, 1997) was used to monitor endo-β-mannanase (EC 3.2.1.78) activity during seed priming and germination. Gel plates were prepared by dissolving 0.05% (w/v) galactomannan (locust bean gum, Sigma Chemical Co., St. Louis, MO, USA) in incubation buffer (0.1 M citric acid, 0.2 M sodium phosphate, pH 5.0), stirring and heating for 30 min. Afterward the solution was clarified by centrifugation at 11,000 g for 15 min at 4°C. Phytagar (Gibco Lab., Grand Island, NY, USA) at 0.7% (w/v) was added to the clarified solution, stirred and heated to boiling. Thirty milliliters of the solution were dispensed into 150 × 25 mm disposable Petri dishes (Falcon, Franklin Lakes, NJ, USA). After solidification, 32 wells per plate were made using a 2-mm disposable plastic pipette and removing the excised gel by aspiration.

Twenty-eight whole individual endosperms (Figs 1 and 2) or 14 radicle tips and 14 lateral endosperms (Figs 3–5) from lettuce seeds primed and/or imbibed at different temperatures for different periods of time were used for enzyme activity measurement. After drying, lettuce seeds were placed on blotter paper moistened with distilled water and incubated for 3 h at 4°C. This procedure was necessary in order to moisten the pericarp and endosperm before excision. Time for radicle protrusion was previously determined at both temperatures for each genotype. Three replications were utilized for each treatment. Endosperms were excised by pressing the cotyledon end using the tip of a conical glass rod. Micropylar and lateral regions were separated with a surgical blade. Each endosperm, radicle tip, or lateral part was placed into an individual microtitre plate (Nalge Nunc, Naperville, IL, USA) well containing 20 µl of incubation buffer and incubated in the dark for 2 h at 20°C.

After the 2 h incubation, 10 µl of buffer from each well were transferred to the gel-diffusion plates. Petri dishes were covered with a lid, wrapped in Parafilm (American National Can., Greenwich, CT, USA) and incubated for 24 h at 20°C. Gels were stained by adding 10 ml of Congo Red (Sigma Chemical Co.) in water (0.4%, w/v) to each plate. Plates were shaken for 20 min at 60 rpm during staining. The Congo Red solution was decanted and the gel was washed gently (1 min) in distilled water; then 10 ml of 0.1 M citric acid + 0.2 M sodium phosphate (citrate-phosphate),

pH 7.0, were added. After 2–3 min on an orbital shaker at 60 rpm, the buffer was decanted. Plates were scanned within 5–10 min using a Hewlett Packard Scan Jet 3c/T. The diameters of cleared areas were measured using MacRhizo™ (Regent Instruments Inc., Quebec, Canada) software. Enzyme activity was calculated from standard curves using regression analysis. Purified endo-β-mannanase (Megazyme, Ireland) was used as a standard.

Experimental design and statistical analysis

Germination tests and enzyme activity were conducted using a completely randomized design, using three replications for each treatment. Each experiment was repeated at least two times. Analysis of variance (ANOVA) of data was performed by means of Statistical Analysis System Software (SAS, 1987). Treatment means were separated by the Duncan Multiple Range test.

Results and discussion

The seed moisture content (SMC) of quiescent seeds was 5.9% for DGB and 5.6% for EVE. Lettuce seed water uptake during seed priming followed the classical triphasic pattern (Bewley and Black, 1994). Rapid water uptake was observed in the first 6 h of soaking, but the water uptake period was extended by seed priming (Fig. 1A, B). Endo-β-mannanase activity during seed priming increased between 24 and 72 h for DGB (Fig. 1A) and 24 and 48 h for EVE (Fig. 1B). Nonogaki *et al.* (1992) also observed an increase of galactomannan hydrolysing enzyme during seed priming in tomato. At the end of the soak period (72 and 48 h for DGB and EVE, respectively), the SMC was 46.8% for DGB and 38.9% for EVE. No radicle growth was observed.

Following seed dry back, endo-β-mannanase activity, using whole endosperm, persisted at levels equal to the activity of the hydrated seed at the end of priming (Fig. 1A, B). In tomato, Nonogaki *et al.* (1992) reported that about 30% of the activity observed after the soak period was lost during drying of tomato.

In the present study, enzyme activity at the completion of lettuce seed priming reached 65–70% of the total activity assayed from seeds immediately after visible radicle protrusion. No enzyme activity was detected in non-primed 'dry' seeds. It is also interesting to note that endo-β-mannanase activity was 60 times higher in EVE, a thermotolerant genotype, than in DGB, a thermosensitive genotype, after 48 h of priming (Fig. 1A, B). Endo-β-mannanase synthesis and action during priming might assist in endosperm weakening, circumventing the need for additional wall weakening or enzyme synthesis.
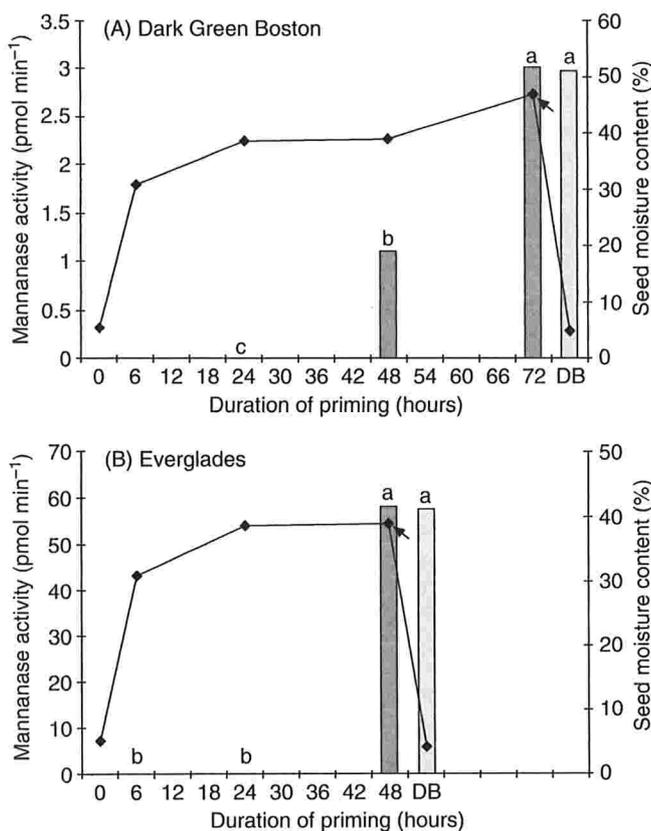
Figure 1. Endo-β-mannanase activity (bars) and seed moisture content (line) during priming in the light at 15°C of: (A) 'Dark Green Boston' lettuce seeds in PEG at –1.2 MPa; (B) 'Everglades' lettuce seeds in PEG at –1.3 MPa. Arrow indicates the end of soaking. DB = Dried back to original storage moisture content. Means followed by the same letter were not significantly different by Duncan's Multiple Range test at $P \leq 0.05$. Vertical bars (shown when larger than the symbol) indicate standard error.

At 20°C, DGB and EVE seeds germinated 100% (Table 1). Non-primed seeds of EVE germinated 50% after 10 h and DGB after 17 h, whereas primed seeds germinated 50% after 3 h (EVE) and 5 h (DGB). At 35°C, EVE (primed and non-primed) and primed DGB seeds germinated 100%, while non-primed seeds of DGB germinated 4%. Fifty per cent of the primed seeds of EVE and DGB germinated after 3 and 4 h, respectively, whereas half of the germinable population of non-primed seeds germinated after 10 h (EVE) and 18 h (DGB) at 35°C. Time to initial radicle protrusion and time to 50% radicle protrusion of the seeds which germinated were similar within each treatment. These results corroborate the previous studies of lettuce seed priming (Guedes and Cantliffe, 1980; Khan, 1980/81; Cantliffe *et al.*, 1981; Wurr and Fellows, 1984; Valdes *et al.*, 1985).

Endo-β-mannanase activity was observed during priming and after reimbibition, but before radicle protrusion, when seeds of DGB were germinated at either temperature. More endo-β-mannanase activity was found in primed compared to non-primed DGB seeds at either temperature (Fig. 2A, B). Enzyme activity in the micropylar area was high (about 50% of the total activity), considering the smaller mass of the micropylar tissue (less than 20% of the total tissue) compared to the lateral part (Fig. 3A, B). Prior to radicle protrusion, endo-β-mannanase activity in the micropylar region was considerably higher in primed compared with non-primed DGB seeds at either temperature. Micropylar endo-β-mannanase activity was observed after 2 h of imbibition in DGB primed seeds, whereas in non-primed seeds, enzyme activity was observed only after 16 h at 20°C (Fig. 3A) and 20 h at 35°C (Fig. 3B). Endo-β-mannanase was also observed in the lateral endosperm region before radicle protrusion, even at an early stage of germination (Fig. 3).

In tomato, endo-β-mannanase is first produced in the micropylar area and, after radicle protrusion, in the entire endosperm tissue (Nonogaki and Morohashi, 1996; Toorop *et al.*, 1996; Voigt and Bewley, 1996; Nonogaki *et al.*, 2000). Nonogaki and Morohashi (1996) reported some differences in the products of galactomannan hydrolysis in tomato endosperm between the pre-germinative and post-germinative enzymes, indicating that the action pattern of the two types of enzymes was different. Nonogaki *et al.* (2000) found two electrophoretic isoforms of endo-β-mannanase that were sequentially expressed in tomato endosperm; initially at the endosperm micropylar cap, then in the remaining lateral endosperm. During priming of tomato seeds, galactomannan-hydrolysing activity was detected only in the micropylar region (Nonogaki *et al.*, 1992).

After radicle protrusion, mannanase activity increased in the lateral endosperm (Fig. 3A), possibly for endosperm carbohydrate mobilization. In general, enzyme activity was greater in seeds incubated at 20 than 35°C, corroborating results from Dutta *et al.* (1997), who observed a lower endo-β-mannanase activity in 'Pacific' lettuce seeds germinated at 32°C compared with 25°C. These observations suggest that high temperature might inhibit synthesis of endo-β-mannanase.

During germination at 35°C, but before radicle protrusion, primed EVE (thermotolerant) seeds had approximately 50 times more endo-β-mannanase (Fig. 4B) than primed DGB (thermosensitive) seeds (Fig. 3B). This difference in enzyme activity was consistent with the differences observed in endo-β-mannanase activity between these genotypes during priming (Fig. 1A, B). In a previous study by Nascimento *et al.* (2000), seeds of thermotolerant lettuce genotypes had greater

Table 1. Germination of 'Dark Green Boston' (DGB) and 'Everglades' (EVE) lettuce primed and non-primed seeds in light at two temperatures

|	Treatments		
	Primed	Non-primed	Significance
DGB			
20°C germination temperature			
Total germination (%)	100	100	NS[b]
Time to first germination (h)	4	17	**
Germination rate (h)[a]	5	17	**
35°C germination temperature			
Total germination (%)	100	4	**
Time to first germination (h)	3	18	**
Germination rate (h)[a]	4	18	**
EVE			
20°C germination temperature			
Total germination (%)	100	100	NS
Time to first germination (h)	3	10	**
Germination rate (h)[a]	3	10	**
35°C germination temperature			
Total germination (%)	100	100	NS
Time to first germination (h)	3	8	**
Germination rate (h)[a]	3	10	**

[a] Germination rate is defined as the time when 50% of the germinating seeds exhibit radicle protrusion.
[b] NS, non-significant and **, $P \leq 0.01$, by F-test.

mannanase activity before radicle protrusion at high temperature than did thermosensitive genotypes. Sung et al. (1998b) verified, via a puncture test, that the same thermotolerant genotypes used in the present work had lower endosperm resistance than the thermosensitive genotypes during germination at 36°C. Moreover, thermotolerant genotypes, including EVE, were shown to have reduced physical resistance of the endosperm by weakening of the cell wall and depletion of stored reserves prior to radicle protrusion (Sung, 1996; Sung et al., 1998b). With the same seed lots used in the present study, Sung et al. (1998b) also showed that less force was required to penetrate endosperm of primed compared with non-primed seeds. In response to seed priming, Sung (1996) observed structural changes in the micropylar (or tip) endosperm region of thermosensitive cultivars at high temperature before radicle protrusion. Endosperm cells of lettuce opposing the radicle were highly vacuolated and had storage materials mobilized before radicle protrusion (Psaras et al., 1981; Sung, 1996). Sung (1996) also observed that the cells at the lateral and cotyledonary end of the lettuce endosperm appeared unchanged prior to radicle protrusion at 36°C. Therefore, since the same seeds and conditions were used here as in Sung's work, a relationship between seed germination at high temperature, a lower resistance to endosperm rupture, and an increase in endo-β-mannanase before radicle protrusion were confirmed in the present study.

In EVE, although primed and non-primed seeds both germinated 100%, primed seeds produced much higher levels of mannanase activity immediately before radicle protrusion than did non-primed seeds at 20°C or 35°C (Fig. 4A, B). At 20°C, endo-β-mannanase activity was observed after 2 h of imbibition in endosperm from primed EVE seeds and after 12 h in non-primed seeds. The amount of enzyme produced among individual non-primed seeds was quite variable (coefficient of variation (CV) = 331%). However, germinating primed seeds exhibited a greater uniformity (CV = 25%) of mannanase activity than did germinating non-primed seeds, in agreement with the results obtained for tomato seeds (Karssen et al., 1989). The greater uniformity and activity of mannanase may have contributed to uniform radicle emergence in primed seeds, regardless of temperature. As observed at 20°C, endo-β-mannanase activity at 35°C was higher in primed compared with non-primed seeds (Fig. 4B). High enzyme activity in the micropylar area was also observed prior to radicle protrusion in primed seeds (Fig. 5A, B).

In the present study, levels of 1 pmol min⁻¹ of endo-β-mannanase appeared adequate to weaken the lettuce endosperm. Lettuce endosperm polysaccharide composition is different in the micropylar and lateral regions, where the former has a higher proportion of arabinose (Dutta et al., 1994). Consequently, other enzymes (Ikuma and Thimann, 1963; Halmer et al.,
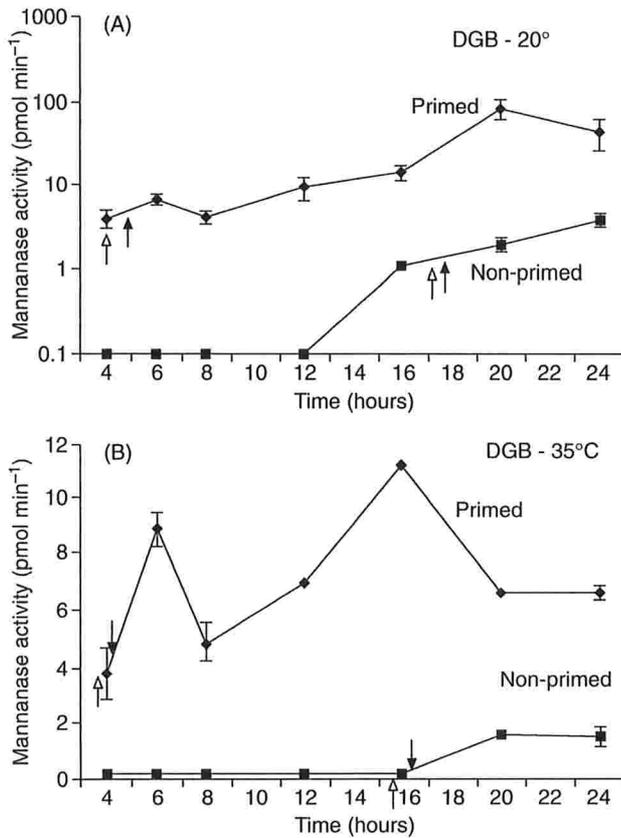
Figure 2. Endo-β-mannanase activity during germination of whole primed and non-primed 'Dark Green Boston' lettuce seeds in light at (A) 20°C or (B) 35°C . Arrows indicate time of radicle protrusion, initial (⇪) or when 50% (⬆) of the germinating seeds exhibited radicle protrusion. Germination was 100% in both treatments at 20°C. However at 35°C, primed seeds germinated 100%, while non-primed seeds germinated only 4%. Vertical bars (shown when larger than the symbol) indicate standard error.

1975; Pavlista and Valdovinos, 1975; Bewley *et al.*, 1983; Dutta *et al.*, 1997) might be involved in endosperm weakening. The appearance of endo-β-mannanase in lettuce was reported to be exclusively a post-germinative event (Halmer *et al.*, 1975; Bewley and Halmer, 1980/81; Dulson and Bewley, 1989; Halmer, 1989; Nonogaki and Morohashi, 1999). The present study shows that it is not. Perhaps the methods utilized in previous studies (Bewley, 1997) were not sensitive enough to detect low amounts of mannanase. Endo-β-mannanase activity in the present and other studies was assayed using locust bean galactomannan as substrate, which could underestimate mannanase activity (Dutta *et al.*, 1994). In the present study, using a single seed assay method, it was possible to measure enzyme levels at 1 pmol min^{-1} or higher; the isolated tissues were incubated for 2 h prior to conducting gel-diffusion assays of the bathing medium, while in other
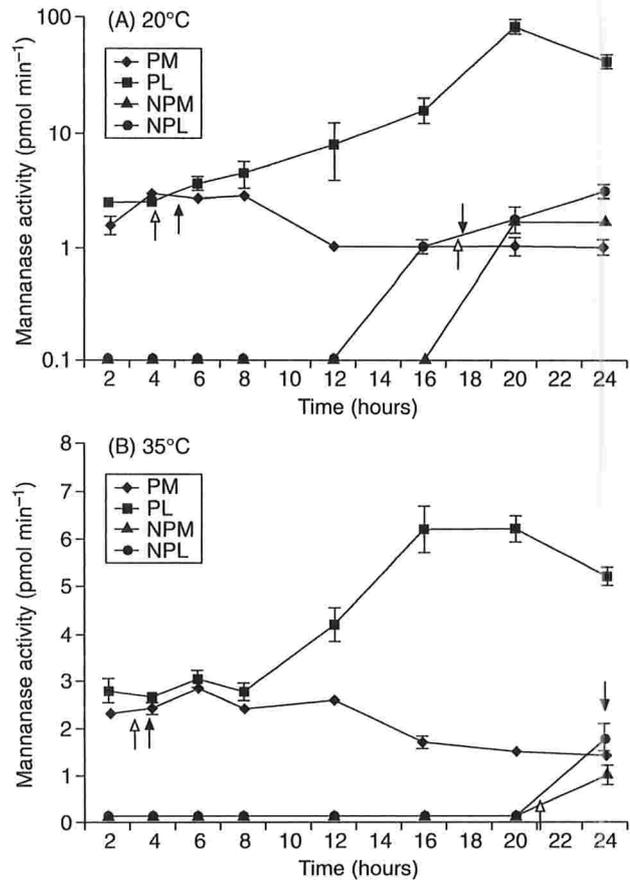
Figure 3. Endo-β-mannanase activity during germination of primed (PM = micropylar region; PL = lateral region) and non-primed (NPM = micropylar region; NPL = lateral region) 'Dark Green Boston' lettuce seeds in the light at (A) 20°C or (B) 35°C. Arrows indicate time of radicle protrusion, initial (⇪) or when 50% (⬆) of the germinating seeds exhibited radicle protrusion. At 20°C, both seed treatments germinated at 100%, while at 35°C primed seeds germinated 100% and non-primed seeds germinated 4%. Vertical bars (shown when larger than the symbol) indicate standard error.
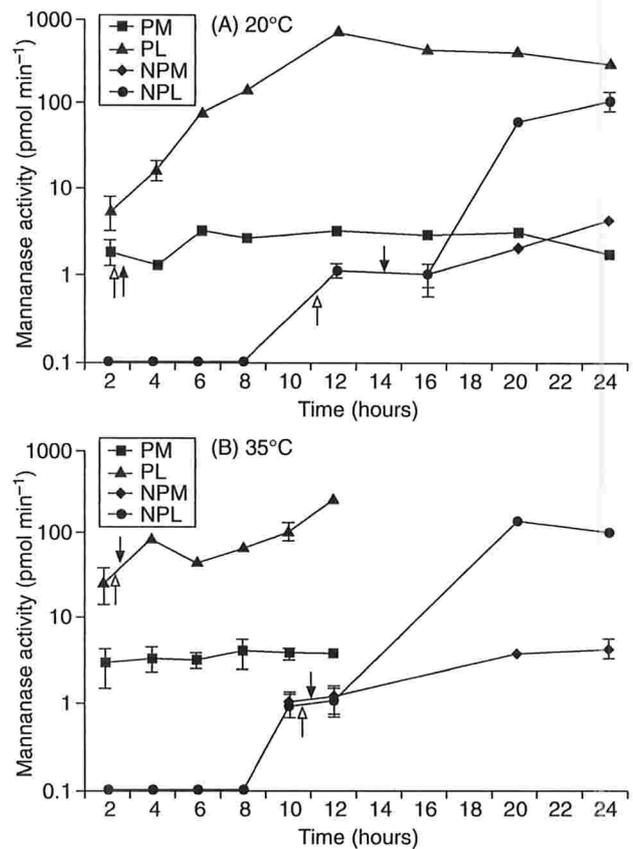
studies tissues were homogenized directly without pre-incubation.

Halmer *et al.* (1976) found a low amount of endo-β-mannanase activity in both germinable and dormant Grand Rapids lettuce seeds before radicle emergence. Potentially, only a low quantity of the enzyme is needed for endosperm weakening and termination of dormancy. This might be contributed from a low amount of highly bound cell wall endo-β-mannanase that is produced prior to the completion of germination (Bewley, 1997). Work by Sung (1996) and Bewley (1997) suggest that only a localized lesion of the cell walls is required for radicle penetration through the endosperm; then perhaps only minor hydrolytic enzyme activity is required.
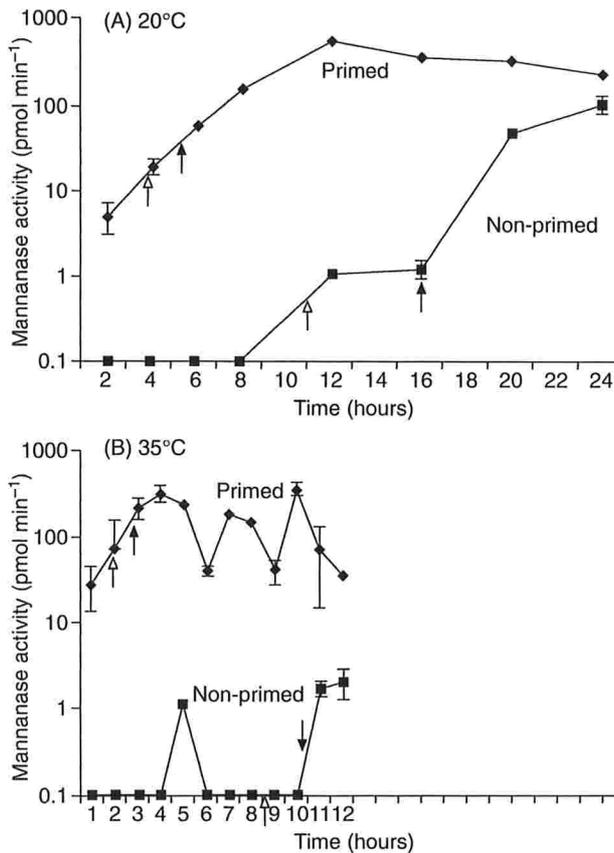
Figure 4. Endo-β-mannanase activity during germination of whole 'Everglades' primed and non-primed lettuce seeds in light at (A) 20°C or (B) 35°C. Arrows indicate time of radicle protrusion, initial (⇧) or when 50% (⬆) of the germinating seeds exhibited radicle protrusion. Both seed treatments germinated at 100%. Vertical bars (shown when larger than the symbol) indicate standard error.

Figure 5. Endo-β-mannanase activity during germination of primed (PM = micropylar region; PL = lateral region) and non-primed (NPM = micropylar region; NPL = lateral region) 'Everglades' lettuce seeds in light at (A) 20°C or (B) 35°C. Arrows indicate time of radicle protrusion, initial (⇧) or when 50% (⬆) the germinating seeds exhibited radicle protrusion. Both seed treatments germinated at 100%. Vertical bars (shown when larger than the symbol) indicate standard error.

In tomato, abscisic acid (ABA) inhibits radicle protrusion (Toorop *et al.*, 1998). In the case of tomato endosperm, weakening occurs in two phases, where phase 1 correlates with a decrease in puncture force and an increase in endo-β-mannanase activity. The second phase has a slower decrease in puncture force, but a continued increase in mannanase activity. Application of ABA does not inhibit activity (Toorop, 1998) or expression (Nonogaki *et al.*, 2000) of the pre-germinative endo-β-mannanase isoform. Early reports suggested an ABA-mediated inhibition of endo-β-mannanase activity (Nomaguchi *et al.*, 1995; Voigt and Bewley, 1996). The discrepancy between these and other studies might be explained by the possibility that earlier experiments used more deeply dormant seeds (Toorop, 1998). In studies with ABA structural analogues, germination was affected similarly by all the analogues depending on their structure, but they had no effect on endo-β-

mannanase activity in the micropylar endosperm (Toorop, 1998).

Similar circumstances may prevail in lettuce. Regardless of the potential for inhibitors that may be present in dormancy situations, the inhibitor may only act at the level of the embryonic axis *per se* and not at the endosperm level. Several years ago, Dulson *et al.* (1988) postulated: (1) that a diffusible inhibitor, possibly ABA, could be leached or diluted from Grand Rapids lettuce endosperms; and (2) that ABA regulates mannanase production. Based on the above-mentioned work with tomato, it is doubtful that ABA regulates endo-β-mannanase synthesis or activity. Nonogaki and Morohashi (1999) identified three isoforms of endo-β-mannanase from Green Wrap lettuce. Further, these isoforms were identified by post-germinative anti-tomato-endo-β-mannanase antibodies. The molecular

masses of the polypeptides were similar. Tissue prints revealed that initial activity of the enzyme was in the micropylar endosperm region and then spread to the rest of the endosperm.

Using a similar gel-diffusion assay to that used in the present study, Nonogaki and Morohashi (1999) could not detect endo-β-mannanase activity in dark-germinated Green Wrap lettuce at 28°C. They used single endosperm assays of 11-h-imbibed ungerminated seeds and compared those results to germinated seeds with radicles of 1, 2 or 3 mm in length. At 28°C in the dark, their Green Wrap lettuce seeds had a slow germination pattern, beginning at 11–12 h and ending at 22 h. Potentially, the seeds tested by the gel diffusion assay had enzyme levels that were too low for the detection procedure, due to slow, erratic germination.

In other species, such as *Picea glauca*, three isoforms of endo-β-mannanase increase in the micropylar megagametophyte, then decline after the testa splits, typically a day before the radicle protrudes (Downie *et al.*, 1997). Activity of the enzyme increases in the cotyledons and axis as the embryo elongates. In other seeds, such as *Datura ferox*, treatments that maintain dormancy prevent increases in endo-β-mannanase activity (Bewley, 1997). For this species there is a good correlation between endosperm cell wall dissolution and radicle protrusion. Similarly, it has been suggested that lettuce seed germination was dependent on an increase in growth potential of the embryo (Nabors and Lang, 1971a, b), resulting from solute accumulation (Takeba, 1980) and from an increase in cell wall extensibility leading to germination (Carpita *et al.*, 1979). The findings of the present study help support the enzymatic endosperm-weakening hypothesis as a part of the ability of lettuce radicles to penetrate the endosperm layer during germination. The force of the expanding embryo most likely contributes to lettuce seed germination, as does endosperm weakening. In our experiments, endo-β-mannanase activity increased markedly in primed seeds before radicle protrusion, and this may be a mechanism for endosperm weakening for lettuce seed germination, especially at high temperatures. In yet another corollary to tomato, Toorop *et al.* (1998) concluded that although lowering endosperm restraint during priming decreased the time to germination of primed seeds, it was not a prerequisite for rapid germination.

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