Using a Puncture Test to Identify the Role of Seed Coverings on Thermotolerant Lettuce Seed Germination

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ABSTRACT. Temperature is an important environmental factor that affects lettuce (*Lactuca sativa* L.) germination. The present research was conducted to determine the role of seed coverings on lettuce seed germination at high temperature. Five lettuce genotypes were primed in order to bypass thermoinhibitional effects on germination. During germination of primed and nonprimed seeds, imbibition followed a normal triphasic pattern. Primed seeds had higher final water content, a decreased imbibitional phase II, and germinated at 36 °C compared to nonprimed seeds of thermosensitive genotypes, which did not germinate at 36 °C. Puncture tests were conducted to determine the force required to penetrate the whole seed or endosperm of the five genotypes at 24 and 33 °C. 'Dark Green Boston', a thermosensitive genotype, had the highest mean resistance (0.207 N) and PI 251245, a thermotolerant genotype, had the lowest (0.139 N). Resistance to penetration of the endosperm of the five genotypes was different at both temperatures. However, three thermotolerant genotypes had lower endosperm resistance than two thermosensitive types. At 36 °C, the penetration force for primed and nonprimed seeds was compared after the first hour of imbibition and 1 hour before radicle protrusion. The force required to penetrate the seed was affected by genotype, seed priming, and duration of imbibition. Puncture force decreased as imbibition time at 36 °C increased in primed and nonprimed seed of each thermotolerant genotype but not in the thermosensitive genotypes. Priming reduced the initial force necessary to penetrate the seed and endosperm in all genotypes. Thus, for radicle protrusion to occur, there must first be a decrease in the resistance of the endosperm layer as evidenced by priming or thermotolerant genotype. Then, the pericarp and integument are sufficiently weakened so that tissue resistance is lower than the turgor pressure of the expanding embryo, allowing germination to be completed.

Seed coverings that surround the embryo of lettuce include the pericarp, integument, and endosperm (Borthwick and Robbins, 1928). For germination to occur, the embryonic axis must penetrate these layers. In certain lettuce genotypes, when the temperature is above 30 °C, seed germination is erratic or completely inhibited, a condition termed thermoinhibition (Gray, 1975; Khan, 1980–81). At high temperature, germination of lettuce seed is problematic because the seed coverings may act as a physical barrier restricting germination, (Ikuma and Thimann, 1963; Speer, 1974). Cutting or removing the endosperm, integument, and pericarp from lettuce seed alleviates thermoinhibition (Ikuma and Thimann, 1963). Weakening of the tissues opposite the radicle tip can be sufficient to remove this constraint, permitting the radicle to emerge. Seed priming is another technique that can improve lettuce germination especially at high temperatures (Guedes and Cantliffe, 1980).

To analyze the role of the seed coverings in seed germination of pepper (*Capsicum annuum* L.), tomato (*Lycopersicon esculentum* L.), muskmelon (*Cucumis melo* L.), and lettuce, puncture tests were conducted to quantify the force required to penetrate the covering tissues (Groot and Karssen, 1987; Tao and Khan, 1979; Watkins and Cantliffe, 1983; Welbaum et al., 1995). At radicle emergence in tomato seed, the force required to puncture endosperm tissue was 0.2 N (Groot and Karssen, 1987), and in pepper seed it was 0.3 N (Watkins and Cantliffe, 1983). In a similar test, the net force required to puncture the perisperm envelope of muskmelon at radicle emergence was 0.1 N (Welbaum et al., 1995).

To identify the role of the seed coverings in controlling seed germination at high temperature, Tao and Khan (1979), Drew and Brocklehurst (1984, 1990), and Wurr et al. (1987) used a puncture test to measure the force required to penetrate the different lettuce seed tissues. In 'Grand Rapids' and 'Cobham Green', the major barrier to embryo growth was reported to be the endosperm layers, which contributed 60% and 40%, respectively, of the total resistance needed to puncture the intact seed (Drew and Brocklehurst, 1984; Tao and Khan, 1979). However, Wurr et al. (1987) suggested that the strength of the pericarp had a more important role in determining germination than did that of the endosperm.

If endosperm resistance is a control mechanism for germination of lettuce at high temperature (35 °C), then bypassing thermoinhibition should lead to a reduction of endosperm resistance, thus, permitting radicle protrusion. To test this hypothesis, we measured puncture force and compared it to normal genotypes, those that germinate at 36 °C, and primed seeds, which germinate at 36 °C. Potentially, priming thermotolerant genotypes might accelerate the onset of endosperm force reduction.

Materials and Methods

Five lettuce genotypes (Asgrow Seed Company, Woodland, California) varying in their degree of thermotolerance were used: 'Dark Green Boston' and 'Valmaine' (thermosensitive), and 'Floricos 83', 'Everglades', and PI 251245 (thermotolerant). All seeds were produced in a single field in the Salinas area of California in 1992. The seeds were stored in a room controlled at 10 °C and 50% relative humidity (RH).

PRIMING TREATMENT. Polyethylene glycol (PEG) was used as osmoticum. The osmotic potentials of the PEG solutions were –1.0, –1.1, –1.2, and –1.3 Mpa (Michel and Kaufmann, 1973). Seeds were weighed and placed in 200-mL test tubes with 30 mL of PEG

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Table 1. Germination of primed and nonprimed seeds of lettuce genotypes at 36 °C.

Genotype	Germination (%)		GT ₅₀ (h) ^y	
	Primed	Nonprimed	Primed	Nonprimed
Dark Green Boston	86	0**	16	0**
Valmaine	85	0**	12	0**
Floricos 83	100	23**	12	38**
Everglades	100	95 ^{NS}	12	26**
PI 251245	100	93**	12	17**

^yGT₅₀ = time to 50% of final seed germination.

^{NS, **} Nonsignificant or significant comparison of primed and nonprimed at *P* = 0.05 or 0.01, respectively, by *F* test.

solution/g of seed. Test tubes were covered with Parafilm and placed in an incubator at 15 °C with constant light ($\approx 10 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Aeration of the solution during priming was provided by means of a glass tube connected by a rubber hose to an aquarium pump. After priming, the seeds were placed in a Buchner funnel, and rinsed with 100 mL of distilled water. Surface moisture was removed by applying vacuum for 1 min. Seeds were rinsed twice. Seeds were distributed in 9-cm petri dishes with filter paper and placed into an incubator at 10 °C and 45% RH until the mass of the seeds returned to the preprimed mass. Seeds were stored at 10 °C and 45% RH until needed for further testing.

GERMINATION TESTS. Germination tests were conducted at 36 °C in 12 h of light ($\approx 20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and 12 h of dark. Each replication consisted of 30 seeds and germination was conducted in 5.5-cm petri dish with two layers of 4.5-cm-diameter Whatman no. 3 filter paper moistened with 2 mL of distilled water. Distilled water was added as needed to keep the filter paper moist during the course of the experiments. Germination was defined as visible radicle protrusion through the pericarp, and total percent germination was calculated.

MOISTURE CONTENT. Primed and nonprimed seeds were imbibed in an incubator at 36 °C for lengths of time as listed in the results under 12 h light and 12 h dark. Primed seeds were weighed hourly during imbibition until the radicle emerged. Because of poor or no germination in nonprimed seeds of 'Dark Green Boston', 'Valmaine', and 'Floricos 83' at 36 °C, water content of these seeds was measured at the same time as those of primed seeds. To measure dry mass, the seeds were oven dried for 1 h at 130 °C, then cooled in a desiccator for 20 min, following the rules of the International Seed Testing Association (1985). Seed moisture content as a percentage of mass was calculated by the formula: (fresh mass – dry mass)/fresh mass $\times$ 100.

PUNCTURE TESTS. Primed and nonprimed seeds of the five

genotypes were imbibed by the same method used for the germination test. Eighty seeds of each genotype were imbibed for 6 h at 24 and 33 °C. An additional 80 seeds of each genotype at each duration were imbibed at 36 °C as follows: 'Valmaine', 'Floricos 83', and 'Everglades', for 1, 3, and 5 h; PI 251245 for 1, 2, and 3 h; and 'Dark Green Boston' for 1, 3, 5, and 9 h. For each genotype the final puncture test was done ≈ 1 h before the radicle protruded the pericarp. For the puncture test, imbibed seeds were cut 1 mm above the radicle tip. For each genotype at each time the resistance for the pericarp, integument, endosperm and embryo (seed) were tested or with pericarp and integument removed (endosperm). To avoid damage to the endosperm, the embryo was left intact.

The puncture test was conducted using an Instron Universal Testing Machine (model 1132; Instron Corporation, Canton, Mass.). The load cell was set to 200 g full scale load. Crosshead and chart speed were 5 and 10 $\text{cm}\cdot\text{min}^{-1}$, respectively. The seed tissue was placed (radicle tip down) in a countersink on an aluminum block (4 $\times$ 4 cm) directly above a hole drilled through the block. A circular flat-faced no. 92 drill bit (0.20 mm diameter) was attached to the load cell. With this system the drill bit penetrated the cotyledons, embryonic axis, endosperm, integument, and pericarp, thus constituting one measurement. This process was monitored using an illuminated 2 $\times$ magnifier.

The force required to penetrate the seed tissues was recorded as the maximum of the load-deflection curve. The force required to penetrate the seed tissues was recorded in Newtons. To be sure the drill passed through the tip of the seed, the procedure for each treatment was viewed and only data of five to seven similar punctures were used.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS. The tests were conducted using a randomized complete block design with each treatment replicated three times. A Statistical Analysis System (SAS Institute, Cary, N.C.) software program was used to analyze the data. The effects of seed priming and imbibition hours were separated by the least significant difference (LSD) test.

Results

SEED PRIMING TREATMENT. Although germination at 36 °C was completely inhibited in the two thermosensitive genotypes, 'Dark Green Boston' and 'Valmaine', priming almost completely circumvented this inhibition (Table 1). When primed, all thermotolerant genotypes germinated 100% at 36 °C, while some seeds germinated without priming.

WATER CONTENT. Seed water content increased with time and had the same triphasic pattern for primed seeds and nonprimed seeds (data not shown). The final water content of primed and

Table 2. Mean force required to penetrate lettuce seed tissues after 6 h of imbibition at 24 and 33 °C.

Genotype	Seed		$\bar{x}$	Endosperm + Embryo		$\bar{x}$
	24 °C	33 °C		24 °C	33 °C	
				Force (N)		
Dark Green Boston	0.208	0.206	0.207	0.080	0.104	0.092
Valmaine	0.178	0.202	0.190	0.097	0.118	0.108
Floricos 83	0.214	0.148	0.181	0.114	0.093	0.103
Everglades	0.180	0.144	0.162	0.077	0.088	0.083
PI 251245	0.128	0.150	0.139	0.099	0.092	0.096
$\bar{x}$	0.182	0.170		0.093	0.099	
Genotype (G) (LSD _(0.05) = 0.043)			*			*
Temperature (T)			NS			NS
G $\times$ T (LSD _(0.05) = 0.02)			NS			*

^{NS, *} Nonsignificant or significant at *P* = 0.05 by *F* test.

Fig. 1. Force required to penetrate seed of primed and nonprimed seeds imbibed at 36 °C with 2 various h of imbibition. DGB = Dark Green Boston; Val = Valmaine; Flo = Floricos 83; Eg = Everglades; PI = PI251245. Vertical bars indicate standard error.

nonprimed seeds of 'Dark Green Boston' was 45%. Primed seeds germinated after 11 h imbibition, while nonprimed seeds did not germinate. Water content of primed 'Valmaine' seeds was 5% more than that of nonprimed seeds after 1 h of imbibition. Primed seeds germinated after soaking in water for 7 h. Water content of primed seeds of 'Floricos 83', 'Everglades', and PI 251245 after 1 h of imbibition was 5% more than that of nonprimed seeds. Water content of the primed seeds continued to increase gradually for the next 4 to 5 h, at which point the radicle emerged. Water content of nonprimed seeds of 'Floricos 83', 'Everglades', and PI 251245 lagged behind that of primed seed, then increased 7% to 10% at 3, 6, and 4 h of imbibition, respectively. After this, water content in each nonprimed genotype remained unchanged. Nonprimed seeds of 'Floricos 83', 'Everglades', and PI 251245 germinated at 36 °C, but germination of 'Floricos 83' was less than 25%. Radicles of nonprimed 'Everglades' and PI 251245 seed protruded through the seed coverings after 10 h imbibition and primed seeds germinated in 12 h instead of 26 and 17, respectively, for nonprimed seeds.

PUNCTURE TEST. When seeds of all genotypes were imbibed at 24 or 33 °C for 6 h, there was no significant interaction between genotype and imbibition temperature for the force required to penetrate intact seed or the endosperm plus embryo (Table 2). However, the force required to penetrate the seed alone was different among the five genotypes regardless of imbibition temperature. For instance, 'Dark Green Boston' had the greatest average resistance (0.207 N) and PI 251245 the least (0.139 N).

There was a significant interaction in the force required to penetrate the endosperm in the five genotypes over time (Table 2). For example, at 24 °C, the force required for 'Valmaine' and 'Floricos 83' was 0.097 and 0.114 N, respectively. At 33 °C, the force required for 'Valmaine' increased to 0.118 N, but in 'Floricos 83' decreased to 0.093 N. In 'Dark Green Boston' and 'Everglades', the forces were

similar and both seed and endosperm embryo forces increased at 33 °C as compared to those at 24 °C, but the increased force in 'Dark Green Boston' was more than that in 'Everglades'. In the PI line, this force significantly decreased at 33 °C.

When primed and nonprimed seeds of the five genotypes were imbibed at 36 °C for various time periods, there was no significant interaction between priming and imbibition time for penetration force. In 'Dark Green Boston', the force required to penetrate nonprimed whole seeds was significantly greater than for primed seeds, even after 9 h of imbibition (Fig. 1). In 'Valmaine', priming did not affect the force necessary to penetrate the seed (Fig. 1). The penetration force of the whole seed was affected by imbibition time and sharply declined after 2 h of imbibition (data not shown). The force required to penetrate a nonprimed 'Floricos 83' seed (0.169 N) was greater than that for primed seed (0.149 N). For the 'Floricos 83' endosperm, there was no significant difference in the penetration force between primed and nonprimed seed (Fig. 2). In 'Everglades', priming had a significant effect on decreasing the force necessary to penetrate both the intact seed and the endosperm (Figs. 1 and 2). The puncture force for the intact seed and endosperm of PI 251245 was the same for nonprimed and primed seeds (Figs. 1 and 2).

The force required to penetrate the endosperm and embryo in nonprimed seed of 'Dark Green Boston' actually increased after 9 h of imbibition (Fig. 1). This puncture force was less in primed seeds. These seeds did not germinate, however, while primed seeds did. The force required to penetrate the intact seed of 'Valmaine' decreased with imbibition time, especially in primed seed. The force necessary to penetrate the seed was less in primed than nonprimed seeds of 'Valmaine' (Fig. 1). Again, the nonprimed seeds did not germinate. In the thermotolerant genotypes 'Floricos 83', 'Everglades', and PI 251245, penetration forces for

Fig. 2. Force required to penetrate endosperm and embryo of primed and nonprimed seeds imbibed at 36 °C with 2 various h of imbibition. DGB = Dark Green Boston; Val = Valmaine; Flo = Floricos 83; Eg = Everglades; PI = PI251245. Vertical bars indicate standard error.

the seed and the endosperm decreased during imbibition approaching radicle emergence for both primed and nonprimed seeds (Figs. 1 and 2).

Discussion

To determine if endosperm restraint was related to genotypic thermotolerance, the five lettuce genotypes used in the present research had different abilities to germinate at high temperatures. Generally, penetration forces either increased or remained unchanged over time in the thermosensitive genotypes. At imbibition temperatures of 24 and 33 °C, less force was required to penetrate the intact seed or endosperm of seeds of thermotolerant genotypes than to penetrate these same tissues in thermosensitive genotypes. These differences among thermotolerant genotypes suggested that the resistance of the seed coverings could be reduced at high germination temperature.

The critical role of the endosperm or the pericarp on resistance to lettuce seed germination at high temperature still is unclear from previous reports (Drew and Brocklehurst, 1984, 1990; Tao and Khan, 1979; Wurr et al., 1987). Perhaps the position used to penetrate the seeds, which varied among studies, lead to inconclusive results. In our work, we attempted to measure the precise force required to penetrate the seed coverings. To measure the force required to penetrate the seed coverings, we felt that the puncture area must be located at the site from which the radicle would naturally protrude through the seed coverings. Thus, the measurement of seed-layer strength may be more correlated to restriction of embryo growth. Drew and Brocklehurst (1984) and Wurr et al. (1987) used a 0.7 mm probe and penetrated the center of whole-seed specimens dorsoventrally. In our puncture tests, we used a 0.2-mm probe and penetrated through the micropyle to imitate the

route of the radicle, since it has been reported that only the micropylar endosperm cells undergo significant structural alterations before radicle emergence in germinating lettuce seeds (Dutta et al., 1997; Georgiou et al., 1983; Psaras et al., 1981).

During phase II of the imbibition process, water uptake by a seed slows or stops, and the seed undergoes many processes essential for germination. In this phase, water content of lettuce seed was ≈40%, compared to the 3% to 4% present in dry seed. Phase II is primarily a period of chemical activation and its length can be affected by temperature, water deficit, or irradiation (Bradford, 1990). Primed lettuce seeds appeared to have a greater water content and a shorter lag phase than nonprimed seeds. Perhaps the tissues surrounding the embryo offered less resistance after priming, which enhanced their ability to take up more water and thus expand more.

Bradford (1990) indicated that the length of phase II imbibition in seed was determined by the time required to reduce the resistance of the seed coverings, although at the time of radicle emergence the barrier formed by the seed coverings constitutes only a minimal resistance to radicle growth. Additionally, the turgor pressure of the embryo may be sufficient for the radicle to overcome the remaining resistance of the seed coverings. Seed coverings also help establish a water potential gradient to initiate phase III of imbibition. Georgiou et al. (1983) observed cracks and breaks between the pericarp, integument, and endosperm just prior to visible radicle growth of lettuce seeds, leading to a reduction in the yield threshold of penetration of the seed coverings (minimum embryo turgor required for penetration of seed coverings). Since intact seeds are at zero water potential during phase II of imbibition, no further water uptake can occur at that time. At the transition between phase II and phase III, when the seed coverings rupture due to the combined effects of embryo turgor and tissue weakening, tension on the embryo is released and the radicle can

protrude, signifying that germination has been successfully completed (Welbaum and Bradford, 1990). It is at this point, at phase III when the radicle protrudes through the seed coverings, that the water content of seeds increases again due to continued cell division and cell elongation. In the present research, nonprimed seeds did not germinate uniformly and this rapid increase in water uptake at phase III was not detected.

The puncture forces for intact seed and endosperm of primed and nonprimed seeds of the three thermotolerant lettuce genotypes decreased during imbibition at 36 °C. Priming appeared to lower the initial force required to penetrate the intact seed and the endosperm, especially in 'Everglades'. In primed seed, measurement of puncture force was stopped just before radicle protrusion. At this time, nonprimed seeds of 'Floricos 83' and 'Valmaine' did not have as great a reduction in puncture force as primed seeds. Possibly, the amount of reduction was not sufficient to allow penetration of the intact seed, which may explain why these seeds did not germinate well at 36 °C. Primed 'Dark Green Boston' seeds required less force to penetrate the endosperm than nonprimed seeds, while the puncture force of nonprimed seeds remained the same or increased slightly after 9 h of imbibition. It appeared that priming lead to a lowering of the initial force necessary to penetrate the seed coat and that this reduction was enough to overcome the resistance of seed coverings when germination took place at 36 °C.

These results suggested that for lettuce seeds to germinate at high temperature, there first must be a decrease in the resistance of the endosperm layers, and second, the pericarp and integument must be weakened enough so that the resistance is less than the turgor pressure of the embryo. Bradford (1990) suggested that the yield threshold (minimum turgor of growth) for germination is determined by the yield threshold of tissues surrounding the embryo (minimum embryo turgor required for penetration of the seed coverings) and the radicle itself (minimum turgor for growth of the embryonic axis). For example, Tao and Khan (1979) reported that the force required to penetrate the endosperm of 'Grand Rapids' lettuce seed treated with gibberellic acid was 0.15 N lower than that of nontreated seeds. In pepper seed, application of gibberellic acid resulted in earlier germination at low temperature and a decrease in endosperm strength earlier than in untreated seeds (Watkins and Cantliffe, 1983). Gibberellic acid increased galactomannanase activity during germination before radicle emergence in pepper. These findings indicated that before radicle emergence, a breakdown and loss of cellular integrity of the endosperm and a subsequent reduction in endosperm thickness occurred in the micropylar area adjacent to the radicle (Watkins et al., 1985). Germination studies with gibberellin-deficient mutant tomato seeds proved that endosperm weakening was absolutely dependent on gibberellin since it was shown to induce enzymatic hydrolysis of the relatively thick cell walls of the endosperm. These enzymes included endo- β -mannanase, α -galactosidase, and mannanase (Groot et al., 1988). Increased mannanase activity preceding radicle protrusion of the endosperm has recently been reported in lettuce (Dutta et al., 1997). It has been suggested for lettuce seed that the emerging radicle can develop enough force to overcome the estimated mechanical resistance of the endosperm without first degrading the endosperm cell wall (Nabors and Lang, 1971). In our study, the turgor of the embryonic axis was not measured; however, it did not appear that the embryo forces constituted a considerable barrier (Bradford, 1990). Rather, our study suggests that the endosperm represented the source of resistance when radicle growth began. This force may have been reduced as a result of priming via preliminary weakening that reduced the penetration resistance of the seed coverings of

thermotolerant genotypes. Thermotolerant genotypes and primed seeds required less penetration force and may either have had a weakened integument and pericarp or a weakened endosperm. The results of our study indicate that the timing of radicle emergence was controlled primarily by the rate at which weakening of the seed coverings occurred.

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