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Effect of Salicylhydroxamic Acid on Endosperm Strength and Embryo Growth of *Lactuca sativa* L. cv Waldmann's Green Seeds[1]

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ABSTRACT

Salicylhydroxamic acid (SHAM) stimulated germination of photosensitive lettuce (*Lactuca sativa* L. cv Waldmann's Green) seeds in darkness. To determine whether SHAM acts on the embryo or the endosperm, we investigated separately effects of SHAM on growth potential of isolated embryos as well as on endosperm strength. Embryo growth potential was quantified by incubating decoated embryos in various concentrations of osmoticum and measuring subsequent radicle elongation. Growth potential of embryos isolated from seeds pretreated with 4 millimolar SHAM was equal to that of untreated controls. Rupture strength of endosperm tissue excised from seeds pretreated with SHAM was 33% less than that of controls in the micropylar region. To determine if the embryo must be in contact with the endosperm for SHAM to weaken the endosperm, some endosperms were incubated with SHAM only after dissection from seeds. Rupture strength of SHAM-treated, isolated endosperms in the micropylar region was 25% less than that of untreated controls. There was no difference in rupture strength in the cotyledonary region of endosperm isolated from seeds treated with SHAM in buffer or buffer alone. SHAM therefore stimulates germination not by enhancing embryo growth potential, but by weakening the micropylar region of the endosperm enclosing the embryo.

Roberts (17) surveyed effects of respiratory inhibitors on dormant rice seeds and found terminal oxidase inhibitors such as KCN, NaN_3, NH_2OH, H_2S, and CO to stimulate germination. Further studies have shown release from dormancy by respiratory inhibitors to be of widespread occurrence. Barley (11), perennial ryegrass (11), cocklebur (6), winter oat (11), and lettuce seeds (3, 22) all have been stimulated to germinate by use of respiratory inhibitors. SHAM,[3] an inhibitor of alternative respiration, is known to stimulate lettuce seed germination (3). Alternative path inhibitors combined with KCN also have been shown to stimulate germination of lettuce and cocklebur (3, 4, 6, 22). Since germination of many species is stimulated by exposure to increased oxygen partial pressure as well as respiratory inhibitors (1, 11), it has been hypothesized that a shift in oxidative metabolism is the key factor in breaking seed dormancy. It also has been suggested that operation of the pentose phosphate pathway is important in release from dormancy (18, 19). In support of a role for altered oxidative metabolism, we have reported that SHAM alone or SHAM + CN will not stimulate germination in the absence of oxygen (4). Esashi *et al.* (6, 7) suggested that respiratory inhibitors break dormancy of cocklebur by enhancing alternative path capacity as well as the proportion of electron flux through the alternative relative to the CN-sensitive path. However, the studies cited have been conducted with intact seeds, which makes interpretation of experimental results difficult. Before proceeding with biochemical probes into the dormancy-breaking action of respiratory inhibitors, it is important to determine their site of action in the seed. We have chosen to work with a light-sensitive cultivar of lettuce that germinates in darkness when treated with certain respiratory inhibitors.

Lettuce seed dormancy is imposed naturally by covering structures that enclose the embryo (8–10). If the embryo is dissected from the dormant seed, the radicle will elongate. Thus, the embryo *per se* is not dormant. Covering structures of the embryo include the endosperm, remains of the integuments, and the pericarp (2). The endosperm is living tissue, about two cell layers thick, which envelops the embryo. The integuments and pericarp in turn surround the endosperm (2). Several studies indicate that the endosperm is the major barrier to radicle expansion (9, 10). Removal of, or injury to, the endosperm results in rapid germination, whereas germination does not occur if only the pericarp or integuments are punctured or removed (9, 10). The endosperm, then, imposes dormancy by mechanical restriction of radicle growth, presenting a barrier to oxygen diffusion, and/or by preventing leaching of endogenous germination inhibitors from the seed. For lettuce, available evidence indicates that seed dormancy is due to mechanical restriction. Thus, radicle emergence by lettuce should occur either by increasing capability of the enlarging radicle to penetrate the restraining endosperm or by weakening the endosperm. Scheibe and Lang (20) used an external osmoticum to mimic mechanical restraint of covering structures and demonstrated that red light increased growth potential of the embryonic axis of lettuce half-seeds. Pavlista and Valdovinos (13), on the other hand, observed microscopic holes in the micropylar end of endosperm from red-light-treated seeds.

The purpose of the present study was to determine whether SHAM stimulates lettuce seed germination by increasing growth potential of the embryo, by weakening the endosperm, or by some combination of these possibilities.

MATERIALS AND METHODS

General Incubation and Darkroom Procedures. Seeds were incubated in darkness at 22°C. All transfers and manipulations during incubation were conducted in a darkroom illuminated with green safelights. Unless otherwise stated, 50 seeds per Petri dish were incubated on two discs of Whatman No. 1 filter paper moistened with 1.8 ml of test solution. To ensure that seeds were

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[3] Abbreviations: SHAM, salicylhydroxamic acid; CN, cyanide.

dormant, all seeds were irradiated with dim far-red light for 10 min, 1 h after the start of imbibition. The far-red source consisted of a bank of six 60-W incandescent lamps with radiation filtered through an FRF 750 far-red filter (Carolina Biological Supply Co.).

Optimization of SHAM-Stimulated Dark Germination. To maximize dark germination of SHAM-treated seeds, SHAM was supplied either in 50 mM sodium phosphate buffer (specific pH treatments ranging from 5–8) or in citrate- phosphate buffer (pH 3 or 4). Seeds were imbibed for 24 h in 1.8 ml buffered SHAM solution or in buffer alone, rinsed six times with deionized-distilled water (hereafter referred to only as water), and gently transferred to Petri dishes containing filter paper moistened only with water. Seeds were returned to the incubator for the remainder of the 72-h incubation period, and germination was scored as protrusion of the radicle from the coat.

Embryo Growth Potential Studies. Intact seeds were imbibed in 4 mM SHAM in 50 mM citrate-phosphate buffer (pH 4) or in buffer alone for 24 h. Seeds then were rinsed six times with water. Embryos were isolated by gently pressing the cotyledonary end of the seed with a fingertip until the embryo popped out. Isolated embryos were placed on filter paper moistened with 2.0 ml of either water or 0.03, 0.04, or 0.05 molal PEG 3400 and incubated for 24 h. Radicle length was determined after dyeing radicles with crystal violet and measuring their length with the aid of a dissecting microscope equipped with an occular ruler.

General Endosperm Strength Measurements. Endosperm strength was measured with a penetrometer device specifically designed and constructed for that purpose. The essential components included a holder for tissue samples (Fig. 1), a vertically movable platform created from a motorized unislide, a force transducer with attached probe, and a chart recorder. The holder was mounted on the movable platform and the tissue sample moved upward toward the transducer probe by slowly advancing the platform of the unislide. When the probe contacted the endosperm tissue, a voltage signal was generated proportional to the physical resistance of the endosperm until it ruptured. When the endosperm tissue ruptured, the signal disappeared immediately.

Endosperm Strength in the Micropylar Region. Seeds were treated with 4 mM SHAM as described. Twelve h after transfer to water, the pericarp and integuments were dissected from the seeds. A transverse cut was made through the endosperm and embryo at a point one-fourth embryo length from the cotyledonary end of the seed. To separate the endosperm from the embryo, gentle pressure was applied with a fingertip at the radicle end of the seed. Longitudinal cuts were made along both sides of the endosperm extending from the transverse cut to one-fourth embryo length from the radicle end. The dissected endosperm tissue was then placed in the endosperm holder with the radicle zone aligned over the central opening and the lateral, cotyledonary region of the endosperm lying on a layer of moistened filter paper. To hold the endosperm firmly in place during probing, the tissue and filter paper were enclosed between two Plexiglas discs with center holes to allow passage of the probe. The force required to rupture the endosperm was measured by moving the holder slowly into contact with the probe.

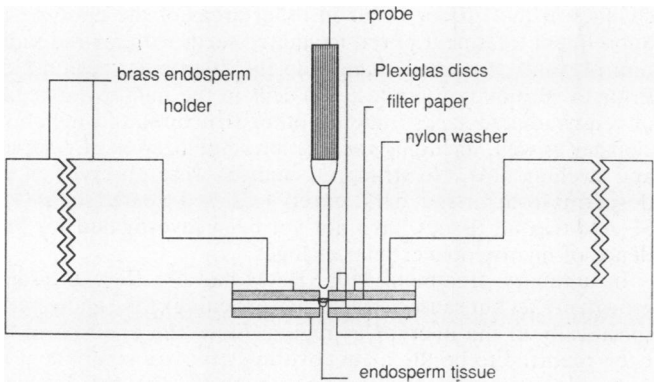

FIG. 1. Schematic of the specimen holder for endosperm strength measurements. In the center of the holder is a hole through which the probe passes while rupturing endosperm tissue. Two Plexiglas discs and a filter paper disc fit tightly into the holder. Holes in the center of the Plexiglas and filter paper discs are aligned with the central hole in the specimen holder. The endosperm tissue is placed on the filter paper disc over the hole. A nylon washer fits on top of the Plexiglas to keep the Plexiglas from turning when the disc is threaded snugly into the main body of the holder.

Endosperm Strength in the Lateral Cotyledonary Region. Seeds were treated as described except that a different portion of the endosperm was dissected. A transverse cut was made 25% of the distance from the radicle end of the endosperm, and a longitudinal cut was made along the entire length of one side of the endosperm. The endosperm was unfolded into a single layer and the lateral cotyledonary region was placed over the central hole of the holder for rupture strength measurement.

Strength of the Micropylar Region of Isolated Endosperm. To prevent injury to the endosperm, seeds were imbibed in water for 8 h before dissecting the pericarp and integuments. Endosperm tissue was isolated and then incubated in Petri dishes containing either 1.8 ml of pH 4-buffered SHAM or buffer alone for 24 h at 22°C in darkness. Tissue samples were then gently rinsed six times with water and transferred to Petri dishes containing filter paper moistened with 1.8 ml of water for another 12 h. Isolated endosperms were dissected as described and the force required to rupture the endosperm was measured in the micropylar region.

RESULTS AND DISCUSSION

The pH at which dormant seeds are exposed to respiratory inhibitors may affect their germination (5). To optimize SHAM stimulation of germination, lettuce seeds were treated with SHAM in buffer solutions ranging in pH from 3 to 8. Germination of SHAM-treated seeds was greatest when supplied in pH 4 citrate-phosphate buffer (data not shown). During studies of the effect of SHAM on endosperm strength, concurrent experiments were done to measure the effect of buffered SHAM on germination. Mean dark germination of seeds treated with pH 4 SHAM was 81%, whereas mean dark germination of seeds treated with pH 4 buffer alone was 9% after 72 h of incubation.

Growth potential of excised embryos was determined by mimicking mechanical restraint of the seed coat with osmotic concentration of the incubation medium, and by measuring radicle length after an appropriate incubation period. Pretreatment of intact seeds with SHAM had no significant effect on radicle length of decoated embryos incubated in water or 0.03, 0.04, or 0.05 molal PEG, compared with appropriate nonpretreated controls (Fig. 2), indicating that treating seeds with SHAM did not enhance growth potential of embryos *per se*. In contrast, red light increases growth potential of lettuce half-seeds containing embryonic axes (20). Nabors and Lang (12) quantified this increased growth potential in terms of water potential changes. Red-irradiated embryos developed a lower water potential than nonirradiated embryos, with the difference equivalent to 0.3 molal mannitol. Physical strength of the seed coat was estimated to be equivalent to 0.16 to 0.38 molal mannitol (12). Nabors and Lang (12) thereby concluded that red light stimulates germination by lowering the water potential of embryos sufficiently to allow radicles to imbibe water, expand, and overcome the physical restraint of the seed coat.

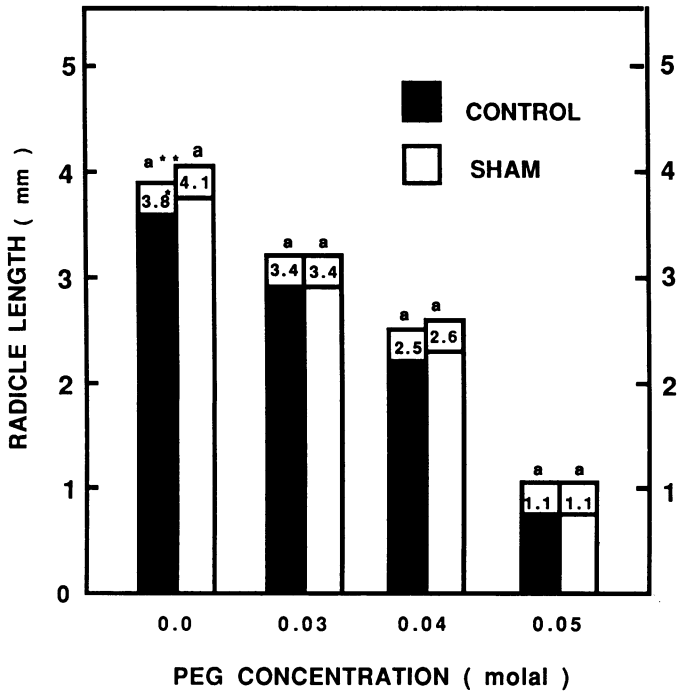

FIG. 2. Effect of SHAM on radicle length of decoated embryos incubated in PEG 3400. Intact seeds were imbibed with or without 4 mM SHAM for 24 h and rinsed with water. Embryos were removed and placed on filter paper moistened either with water or 0.03, 0.04, or 0.05 molal PEG. Radicle length was measured after incubating for another 24 h in one of these test solutions. Means represent data from three separate experiments with a minimum of 100 observations per treatment. Different letters within bar pairs indicate significant differences at the 5% level according to F test.

Table I. *Effect of SHAM on Strength of Endosperm Tissue*

Seeds were imbibed in test solutions for 24 h, then rinsed with water, and incubated for another 12 h. After 36 h from the start of imbibition, endosperms were dissected from intact seeds and the force required to rupture the endosperm measured.

Tissue Type	Endosperm Strength	
	Control	SHAM 4 mM
	g	
Micropylar region	10.2 a*	7.2 b
Cotyledonary region	15.6 a	15.8 a
Micropylar region[a]	7.2 A***	5.4 B

[a] Endosperm tissue was isolated from the embryo 8 h after the start of imbibition, then treated with SHAM for 24 h, rinsed, and imbibed in water for 12 h more prior to measurement of endosperm strength. * Means represent at least two separate experiments and those means within rows followed by different lowercase letters are significantly different from one another at the 5% level as determined by F test. *** Means represent three separate experiments and those means within rows followed by different uppercase letters are significantly different from one another at the 1% level as determined by F test.

The micropylar region of endosperm tissue from SHAM-treated seeds was 33% weaker than that from control seeds (Table I). Differences in rupture strength of the micropylar region did not become apparent until 33 h after the start of incubation with SHAM (data not shown). At 36 h, SHAM-treated seeds were at the incipient germination stage (50% germinating). Two lines of evidence suggest that internal radicle pressure is not the basis for SHAM-induced weakening of the micropylar region of the endosperm: First, pretreatment of intact seeds with SHAM had no effect on radicle growth (Fig. 2). Second, SHAM induced endosperm weakening in the absence of the embryo (Table I).

The micropylar end of endosperms excised from embryos and then incubated with SHAM was significantly weaker than that of excised endosperms not receiving SHAM treatment (Table I). There was a 25% reduction in the force required to rupture the micropylar end of SHAM-treated, isolated endosperms compared with that of controls. SHAM-induced weakening of isolated endosperms indicates that the presence of an embryo is not required for SHAM action. An interaction between embryo and endosperm in the intact seed is not ruled out in germination responses to SHAM treatment, but may not be necessary because SHAM weakens isolated endosperms to approximately the same extent as endosperms from intact seeds. However, there could be a prerequisite for embryo-endosperm contact in the imbibed state prior to SHAM treatment of the endosperm. For example, lettuce seeds had to imbibe water for at least 8 h prior to dissection of the endosperm to initiate structural changes in the micropylar region of isolated endosperms in response to gibberellic acid (16).

The failure of SHAM to affect rupture strength of the lateral cotyledonary region of the endosperm (Table I) indicates that SHAM action is specific to the micropylar region of the endosperm. It is notable that the lateral cotyledonary region also is appreciably stronger than the micropylar region. We have no explanation for this difference, but perhaps the micropylar region is inherently weaker to accommodate radicle penetration once dormancy has been broken. Unlike SHAM, red light and gibberellic acid have been reported to weaken endosperm tissue in the lateral cotyledonary region (21).

Similar to SHAM, light also has been shown to cause changes specific to the micropylar region of the endosperm (13–15). As early as 8 h after the start of imbibition, microscopic cracks and pits appear in the micropylar region of the endosperm (13). Although germination occurs shortly after their appearance, it is not clear whether endosperm disruption is a direct action of red light or is due to pressure from growth of the radicle. However, thermodormant seeds, which do not germinate when exposed to red light, still develop cracks and pits in the endosperm when irradiated, whereas thermodormant seeds that remain in darkness exhibit no disruption of the endosperm. No microscopic changes in the surface occur in other areas of the endosperm. Subsequent treatment of red-irradiated seeds with far-red radiation prevents structural changes in the micropylar region (15). Prior to germination, endosperm cells in the micropylar region of red-irradiated seeds undergo other structural and metabolic changes as well, including vacuolation, mobilization of reserves, and swelling (14). No structural changes were observed in endosperms from far-red, dark, or red + far-red-treated seeds (15). SHAM-treated tissues have not yet been investigated for incidence of microscopic cellular changes.

In summary, treatment with SHAM had no effect on radicle growth *per se*, but caused structural weakening of the endosperm, specifically in the micropylar region. Since the endosperm has been reported to be the main covering structure component responsible for restraining embryo growth (9, 10), this localized weakening appears to be the physiological basis for SHAM-promoted germination of lettuce seeds.

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