Laboratory Studies of Thermotolerance Acquisition During Seed Imbibition & Germination

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A common, yet often elusive, goal of teaching is to show that the specialized knowledge obtained from the various biological subdisciplines can be integrated to create a greater understanding of the whole of biology. This integration is important, not only because it helps to establish relevance for the student about seemingly obscure scientific data, but because it helps to show that all scientists (at least in theory) are part of a larger community held together with common, shared principles. This article will describe a series of experiments designed to investigate the ability of seeds from different species to acquire tolerance to thermal stress. The experiments are intended to be simple so that they can be undertaken in teaching environments with little in the way of supplies or equipment. Examples are presented and questions posed to illustrate how the data can be used in the classroom to discuss molecular biology, physiology, ecology and evolution. Additional experiments are posed to encourage students to actively participate in the scientific process by undertaking longer-term research projects.

What Is Acquired Thermotolerance?

Organisms displaying acquired thermotolerance are able to survive short-term exposure to what would normally be a lethal or extremely stressful temperature. This thermotolerance can be triggered by exposure to mildly stressful, but sublethal elevated temperature (Vierling 1991). The molecular causes for thermotolerance are a subject of intense research, but a great deal of circumstantial evidence and some direct evidence support the theory that thermotolerance is caused by environmental factors activating specific genes that encode molecular chaperones which act to protect sensitive proteins from denaturing under heat stress (Ellis 1991; Vierling 1991). These proteins are called heat shock proteins (hsp) and are induced during the mild heat stress period and remain in the organism for some time thereafter. Hsp were first discovered in the fruit fly, *Drosophila*, and have been subsequently identified in a wide variety of organisms including animals, plants, yeasts and bacteria (Vierling 1991). One of the big problems for researchers has been to specifically connect the synthesis of hsp with thermotolerance as there is a natural polymorphism of these proteins and it has been difficult to correlate qualitative differences in heat tolerance with quantitative differences in specific hsp (Coleman et al. 1995; Vierling 1991). Intriguingly, recent research with yeast mutants deficient in hsp 104 has shown that the synthesis of this protein (also found in higher plants) was directly correlated with the development of thermotolerance (Lindquist & Kim 1996). Whatever the specific connections between hsp and thermotolerance, heat stress appears to alter an organism's specific program of gene regulation inducing the synthesis of some proteins (including hsp) and inhibiting others.

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Thermotolerance Studies in Seedlings

A wide variety of work showing the acquisition of thermotolerance has been done in plants; the majority of the studies have looked at the effect of heat treatments on germinated seeds or seedlings (Vierling 1991). The quantification of whether thermotolerance has been acquired comes from comparing seeds pre-treated with mild stress (exposure to moderate temperature increases) to those directly stressed at high temperatures. Many parameters have been examined to indicate the degree of heat stress including: germination percentage, radicle (root) growth and the ability of whole seeds or seed parts to reduce 2, 3, 5-triphenyltetrazolium chloride (TZ) to formazan. Only a few studies have considered how thermal stress in the soil prior to germination might affect the germination success or subsequent survival of the seedling (Abernethy et al. 1989; Chikono & Choinski 1992; Van de Venter & Lock 1992). It is, however, interesting to note that this pre-germination period or imbibition period is of particular importance as the events that occur during these few first hours or days have a direct effect on whether that seedling survives.

The Thermotolerance Transition

Dry wheat seeds with short periods of imbibition (3 to 6 hours) have a substantial amount of high temperature tolerance, but are unable to acquire thermotolerance, whereas in wheat seeds given 9 to 12 hours of imbibition, the initial high temperature tolerance declines, but the seeds are able to become thermotolerant with moderate heat treatment. This necessary imbibition time is called the "thermotolerance transition" (Abernethy et al. 1989). Thus, for experiments designed to investigate the ability of

seeds to acquire thermotolerance, the seeds must be imbibed for at least 24 hours, or, if time permits, tested to determine the minimum time required for the thermotolerance transition.

Selection of Treatment Temperatures

The normal growth temperature of a plant is related to its genetic makeup and the characteristics of the environment in which it is found (Vierling 1991). Generally, the temperature for induction of thermotolerance is 5 to 10° C above the normal growth temperature. If the temperature were raised another 5 to 10° C, it would be substantially stressful or lethal to the seeds (Bewley & Black 1994; Vierling 1991). Depending on the time available, one can predict these temperatures based upon the above guidelines or design a class experiment to determine them. Be warned, however, that many imbibed seeds can be exposed to high temperatures for short periods without ill effects; that is, the high temperature stressed or lethal group will have results similar to the control groups. Thus, it is useful (perhaps before working with a class) to confirm that the highly stressful temperature chosen (e.g. 45° C) actually causes a substantial decline in germination or radicle growth without killing all of the seeds in the experimental group.

Imbibition protocols only require short-term incubations of imbibed seeds at the appropriate temperatures to allow for thermotolerance induction. Thus, if temperature-controlled incubators are not available, seeds can be incubated in beakers set in water baths maintained at the appropriate temperatures and then transferred to petri dishes for germination in the classroom.

Suggested Thermotolerance Induction Protocol

1. Prepare petri dishes of seeds as described under "germination methods." Divide the plates into four groups labeled "NT" for normal temperature control group, "IC" for induction temperature control group, "HSL" for highly stressed or lethal treatment group, and "TI" for thermotolerance induction group. Incubate all plates at 25° C for 24 hours.
2. Maintain the plates marked "NT" at 25° C for the duration of the experiment (normal temperature control group).
3. Incubate the plates marked "IC" for 2 hours at 35° C, then transfer them back to 25° C (induction temperature control group).
4. Incubate the plates marked "HSL" for 2 hours at 45° C, then transfer them back to 25° C (highly stressed or lethal treatment).
5. Incubate the plates marked "TI" for 2 hours at 35° C, followed by 25° C for 1 hour and then for 2 hours at 45° C (thermotolerance induction group), before transferring them back to 25° C for the remainder of the experiment.

Note that in the TI group, a 1-hour cool-down temperature is included prior to the high temperature stress treatment. It has been shown in soybean (Lin et al. 1984) and cotton (Abernethy et al. 1989) that this interim incubation enhances the thermotolerance induction response, possibly by providing time for the synthesis of heat shock proteins.

Seed Storage

Quercus palustris Muenchh. (pin oak) seeds were harvested from local trees and stored in plastic bags at 4° C until used. Seeds stored in this manner are viable for at least 1 year. Air dry the seeds before putting them in the plastic bags to prevent fungal contamination. *Pinus taeda* L. (loblolly pine) seeds were obtained from the Texas Forest Service (Texas A&M University, College Station, TX 77843-2585) and stored dry in paper envelopes at 4° C. Pine seeds required a period of stratification before they were germinable. This was accomplished as follows: the seeds were surface sterilized in 30% hydrogen peroxide for 30 minutes and rinsed well with distilled water, then the moist seeds were transferred to a 4° C incubator for 30 days to insure adequate stratification. *Gossypium hirsutum* L. var. Deltapine 33 (cotton) seeds obtained from a local grower were stored in plastic bags at 4° C until used.

Germination Methods

Q. palustris, *G. hirsutum* (20 seeds/plate) and *P. taeda* (10 seeds/plate) were all germinated in 9-cm petri dishes containing 1 sheet of Whatman #1 filter paper and 20, 20 and 4 ml of distilled water, respectively. Prior to plating, all the seeds were surface sterilized in 10% household bleach for 10 minutes followed by extensive washing with sterile distilled water. To speed up the germination rates of *Q. palustris*, the seed coats were manually removed prior to plating. The edges of the plates were wrapped with Parafilm™ to minimize water loss. Subsequent conditions for germination of each species are described in the legends to Tables 1 and 2.

Sample Results

Thermotolerance Induction Data

The following data obtained from plant physiology classes or independent

Table 1. Acquisition of thermotolerance in *G. hirsutum* or *Q. palustris* assessed by germination percentage and radicle growth. Seeds (*Q. palustris* seeds were decoated before use) were imbibed at 25° C for 24 hours prior to treatment. For *G. hirsutum*, all incubations were for 2 hours except for the 25° C treatment in the thermotolerance induction (TI) group which was for 1 hour. *Q. palustris* incubations were for 4 h except for the 25° C treatment in the thermotolerance induction (TI) group which was for 2 h. After heat treatments, the seeds were transferred back to incubators for 5 days at 25° C. Seeds were considered germinated if the radicles were at least 1 mm. Each data point represents the mean among three (for *Q. palustris*) or four independent experiments together with their standard deviations. Seeds are considered to have acquired thermotolerance if the value from the thermotolerance induction (TI) group is significantly different from the highly stressed or lethal (HSL) group (significant difference at P = 0.05 marked with an asterisk).

Temperature treatment	*Germination (%)*	*Radicle length (cm)*
	G. hirsutum:	
25° C (NT)	93 ± 3	8.5 ± 0.1
35° C (IC)	81 ± 3	3.1 ± 0.1
45° C (HSL)	2 ± 1	0.5 ± 1
35° C → 25° C → 45° C (TI)	76 ± 3*	3.2 ± 0.1*
	Q. palustris:	
25° C (NT)	99 ± 1	15.4 ± 7.9
35° C (IC)	75 ± 3	8.8 ± 5.6
50° C (HSL)	20 ± 0	1.2 ± 0.2
35° C → 25° C → 50° C (TI)	60 ± 3*	7.4 ± 4.4*

Table 2. Acquisition of thermotolerance in different populations of *P. taeda* assessed by germination percentage. Pre-stratified seeds were imbibed at 25° C for 16 h prior to heat treatments. All incubations were for 2 h except the 25° C in the thermotolerance induction (TI) group which was for 1 h. After heat treatments, the seeds were transferred back to incubators for 7 days at 25° C. Seeds were considered germinated if the radicles were at least 1 mm. Each data point represents the mean among 3 independent experiments together with their standard deviations. Seeds are considered to have acquired thermotolerance if the value from the thermotolerance induction (TI) group is significantly different from the highly stressed or lethal (HSL) group (significant difference at P = 0.05 marked with an asterisk).

	Temperature Treatment			
Population	*25° C (NT)*	*37° C (IC)*	*50° C (HSL)*	*37° C → 25° C → 50° C (TI)*
1	88 ± 13	71 ± 15	46 ± 7	83 ± 14*
2	67 ± 11	63 ± 6	48 ± 6	78 ± 22*
3	95 ± 9	95 ± 5	36 ± 4	95 ± 9*
4	76 ± 7	48 ± 8	33 ± 16	90 ± 17*
5	91 ± 9	56 ± 9	12 ± 5	33 ± 14*
6	72 ± 12	85 ± 15	15 ± 0	8 ± 0
7	93 ± 12	89 ± 19	40 ± 9	29 ± 19
8	83 ± 15	86 ± 17	25 ± 23	38 ± 6
9	90 ± 9	59 ± 16	27 ± 5	43 ± 4
10	91 ± 8	78 ± 10	23 ± 5	47 ± 9*

research projects illustrate a variety of questions that can be discussed by students. Most thermotolerance studies have been conducted on agronomic crops such as soybean, millet, barley and cotton (Vierling 1991). Cotton seeds demonstrated a consistent ability to acquire thermotolerance with both germination and radicle length increasing from 2% to 76% and 0.5 to 3.2 cm, respectively, when 45° C incubations were preceded by a 35° C treatment compared with a 45° C treatment alone (Table 1). These data can be discussed from a wide variety of physiological standpoints including morphological, cellular and biochemical aspects of germination. Topics such as germination, hormone and enzyme levels, storage product utilization and shoot/root growth ratios can also be addressed. Examples of questions for discussion include:

- What effect does high soil temperature have on the survival of seedlings in the farmer's field?
- Is the farmer's field a special selective environment for acquired thermotolerance?
- What is the role of root length in seedling survival?
- How does the rate of root growth affect the food supply in the seed during the heterotrophic period before the seedling breaks through the ground?

An interesting question might be to consider the advantages and disadvantages of planting with seed sources produced as a result of extended genetic selection programs.

Oak seeds from a wild population also showed the ability to acquire thermotolerance as assessed by germination percentages and root extension, although considerable variation was seen in radicle length in all treatments (Table 1). In addition to the physiological implications discussed above, students could consider the possible role(s) of thermotolerance acquisition in the natural environment. Does this process have any role in seedling survival? What factors affect conditions in the field during the germination period? The students could be asked (when possible) to do a survey of seedlings found in the vicinity of mature trees and to discuss the factors that limit their survival.

The results of a population study of thermotolerance acquisition done using different populations of pine seeds obtained from a commercial growers cooperative is shown in Table 2. The seed-producing generation of each population had been previously tested and had different growth rates and yields. Some populations acquired thermotolerance, while other populations did not. The diversity of responses opens up questions as to how this polymorphism might affect the selection of seedlings, particularly under changing environmental conditions. Interesting discussions include possible environmental scenarios where seeds are subjected to dangerously high soil temperatures, or the role of seed polymorphism in evolution in natural populations.

Additional Experiments

Greenhouse or Field Trial Experiments

Does seed-acquired thermotolerance pass to seedlings? Does heat-acquired thermotolerance allow seedlings to survive exposure to other stresses such as a lack of water or high soil salt concentrations? These are interesting questions not addressed in the laboratory study and, thus, provide students with an opportunity for further work in the greenhouse or field. Seeds that have been heat treated can be planted in the greenhouse or out in a common garden under reduced water, stressed with salt solutions, or grown at higher temperature to determine if any differences were seen with control (non-heat stressed) populations. An interesting study done with sorghum showed that heat pretreatments led to increased yields under conditions of reduced rainfall (Van de Venter & Lock 1992).

Formazan Estimation

An easy method to determine if heat treatments have affected the process of respiration in the seedlings involves the use of 2, 3, 5-triphenyltetrazolium chloride (TZ) (Abernethy et al. 1989; Chikono & Choinski 1992). TZ in the oxidized state is colorless, but when reduced is converted to a product called formazan. Formazan absorbs light at 480 nm and appears visually as dark red or purple in color. An actively respiring seed will readily reduce TZ to formazan, thus a reduction in the ability of heat-treated seedlings to reduce TZ indicates that components of the respiratory process (especially enzymes) were damaged by the heat treatments. Thermotolerance is thus measured by how the ability to reduce TZ is preserved by preheat treatments.

Heat Shock Protein Profiles

If the equipment and facilities are available, profiles of proteins induced by heat treatments can be examined using one or two-dimensional SDS-polyacrylamide gel electrophoresis (Abernethy et al. 1989). Briefly, the seeds are incubated in a solution containing a radioactive amino acid (usually [^{35}S] methionine), total proteins are extracted, and then subjected to electrophoresis and autoradiography of the dried gel. This technique allows the student to differentiate proteins that are present in the seeds from those that are newly synthesized in response to the heat treatments.

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References

Abernethy, R.H., Theil, D.S., Petersen, N.S. & Helm, K. (1989). Thermotolerance is developmentally dependent in germinating wheat seed. *Plant Physiology, 89,* 569–576.

Bewley, J.D. & Black, M. (1994). *Seeds: Physiology of Development and Germination.* New York: Plenum Press.

Chikono, C. & Choinski, J.S., Jr. (1992). Thermotolerance characteristics of seeds from geographically isolated populations of the African tree, *Combretum apiculatum* Sonder. *African Journal of Ecology, 29,* 65–73.

Coleman, J.S., Heckathorn, S.A. & Hallberg, R.L. (1995). Heat-shock proteins and thermotolerance: Linking ecological and molecular perspectives. *Trends in Ecology and Evolution, 10,* 305–306.

Ellis, J.R. (1991). Molecular chaperones. *Annual Review of Biochemistry, 60,* 321–347.

Lin, C.Y., Roberts, J.K. & Key, J.L. (1984). Acquisition of thermotolerance in soybean seedlings. Synthesis and accumulation of heat shock proteins and their cellular components. *Plant Physiology, 74,* 152–160.

Lindquist, S. & Kim, G. (1996). Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. *Proceedings of the National Academy of Sciences, 93,* 5301–5306.

Van de Venter, H.A. & Lock, W.H. (1992). An investigation of heat shock and thermotolerance responses as indices of seed vigour in grain sorghum [*Sorghum bicolor* (L.) Moench.]. *Plant Var. Seeds, 5,* 13–18.

Vierling, E. (1991). The roles of heat shock proteins in plants. *Annual Review of Plant Physiology, 42,* 579–620.