

GERMINATION OF *ACACIA* SEEDS

NB. Laboratory coats are required for this Practical

AIMS AND OBJECTIVES

At the end of this Practical, students should be able to:

- frame a question that can be answered using scientific method;
- design an experiment to answer a scientific question;
- execute the experiment;
- analyse the results;
- present the findings as a talk with PowerPoint slides.

2.1 INTRODUCTION

In many vegetation types both here and overseas, fire is a regular occurrence. It is common to find that germination of seeds of plant species present in these vegetation types is limited to the months immediately following a fire, with little or no germination in the inter-fire period. This flush of germination occurs when there is plenty of light at the soil surface; nutrient levels in the soil are usually elevated, as the fire has removed the previous vegetation cover, and nutrients present in the biomass are returned to the soil as ash. Further advantages to germinating in the immediate post-fire period may include reduced activity of soil-borne pathogens (killed by the fire), and satiation of seed/seedling predators with overwhelming numbers of seeds/seedling.

Observation of the flush of germination that follows a fire then leads to the following question: how do the seeds “sense” that a fire has occurred? There are a number of signals or “cues” that might operate to initiate germination: a partial list would include

- the pulse of heat (temperature achieved, for given time)
- chemicals released by the burning process
- elevated nutrient levels in the soil
- elevated light levels at soil surface

In order for us to know which one or ones of these (or other) factors might stimulate germination, we need to **conduct experiments** to test the effect of each factor (or combination of factors) on germination.

For one group of plants something is known about how the seeds “sense” a fire; this group is the members of the Pea Family (Fam. Fabaceae) and the related Wattle Family (Fam. Mimosaceae). The seeds of members of these two plant Families will not germinate readily when first released from the parent plant - they are said to be dormant (Auld & O’Connell 1991, Vleeshouwers *et al.* 1995). In this case, the dormancy is imposed by a “hard” seed coat which prevents the seed

taking up water, the critical first step in germination (Langkamp 1987, Morrison *et al.* 1992, 1998). Heating (or scarifying) the seed breaks this mechanical barrier to water uptake, and allows germination to proceed.

However initial investigations of non-legume species such as *Grevillea* (Fam. Proteaceae) showed that heat shock (by itself) had little effect on germination (Auld & Tozer 1995).

Interest recently has centred on smoke as a source of a germination signal. A positive germination response to smoke or smoke-derived extracts has been detected in 12 fynbos species from South Africa, including species in the Asteraceae, Ericaceae, Restionaceae and Proteaceae (Brown 1993, Brown *et al.* 1993), *Themeda triandra* from South Africa (Baxter *et al.* 1994), *Nicotiana attenuata* from America (Baldwin *et al.* 1994), and 45 species of Australian native plants from West Australia, including species in the Rutaceae, Dilleniaceae, Proteaceae, Myrtaceae and Cupressaceae (Dixon *et al.* 1995). This range of species from different continents suggests that stimulation of germination by smoke or smoke-derived compounds may be common in fire-prone vegetation (Baxter *et al.* 1994).

Work at UWS Hawkesbury (Morris 2000) demonstrated a positive germination response to smoke exposure in the laboratory, for some of the *Grevillea* species known to be unresponsive to heat (Auld & Tozer 1995). Some *Grevillea* species showed the highest germination if they received *both* smoke and heat shock treatments (Morris 2000). For one of these species (*G. linearifolia*), further work showed that dormancy was controlled by the seed coat, as removal of the seed coat resulted in 100% germination (Morris *et al.* 2000). Preliminary investigation of the seed coat of *Grevillea* identified at least three possible dormancy mechanisms in the seed coat (Briggs, Morris & Ashford 2005). Three of the same *Grevillea* species used by Morris (2000) showed positive germination responses to smoke and heat shock in experiments of Kenny (2000), with highest germination recorded when seeds received *both* smoke and heat treatments. Thus more recent work suggests that *Grevillea* seeds respond to smoke and heat shock, with most germination occurring if both cues are applied.

To investigate these phenomena we will use seeds from some species of wattle (*Acacia*), *Grevillea* (horticultural hybrid) and an agricultural cereal species for comparison. The wattle species may include some of the following:

- *Acacia melanoxylon* (Blackwood) - grows in Tall Forest to Rainforest on moist and fairly good soils in mountains from Queensland to Victoria; occurs in Blue Mountains as a tree.
- *Acacia decurrens* - the green wattle, from forest or open-forest vegetation on shale soils; occurs around Bargo and further south in NSW.

2.2 EXPERIMENTAL DESIGN

Rather than have the whole class follow the one experimental design, each group will discuss amongst themselves, and design and execute one experiment. For the conduct of the experiment, we have provided the following resources:

- seed of several *Acacia* species, *Grevillea* hybrid and one cereal (supplied by Southern Biological (<http://www.southernbiological.com/About/AboutUsFrameSet.htm>). Best results with *Acacia* are obtained if the seeds are placed in a desiccator for several days prior to the experiment. This keeps germination of the control seeds low).
- ovens set to cover a range of temperatures (60°C, 80°C, 100°C and 120°C);
- glass petri dishes for heating seeds;
- a smoking chamber (or smoke can be applied as ‘smoked water’);
- perspex petri dishes and sterilised filter paper for germination.

Some of the questions your experiment might answer are:

1. What is the best temperature of ‘heat shock’ to stimulate germination in the wattle species (for given duration of heat exposure)? Do different species show the same temperature optima?
2. How does time of heat exposure of the seeds (for a given temperature of heat exposure) affect the germination response in the wattle species?
3. How does heat affect the germination response of the cereal species compared to the wattle species?
4. Does the *Grevillea* species show a germination response to heat? To smoke? To smoke + heat?
5. Does the wattle species show a germination response to smoke, or to smoke + heat?

Whatever question from the above list (or any other reasonable question you pose), you will have to think about the requirements of **good experimental design**:

- your treatments
- your controls
- numbers of replicates (1 replicate = 1 petri dish with (say) 5 - 10 seeds in it).

READ BOX 2.1 *BEFORE* YOU DESIGN YOUR EXPERIMENT.

BOX 2.1 EXPERIMENTAL DESIGN

In order to answer the question or questions that you ask in this Practical, you will have to **design an experiment**. A brief word about some standard experimental designs, and the methods used to analyse them, is appropriate here. As we have already seen in this Subject, there is a **close link between the way data is gathered, and how it can be analysed**: if inferential statistics are to be used in analysis, the data must be obtained in a way that satisfies the assumptions of these tests.

One-way or Single Factor Design

Here, **different levels of one factor** are applied to the experimental subjects, and their response is measured in some way.

In the context of the seed germination experiment, an example of a Single Factor design would be to vary time of exposure of wattle seed to a given, fixed temperature (say 80°C).

Different levels of the Time of heat exposure factor are applied to batches of seeds - eg 1,2,5,10 and 20 minutes. Assume in each treatment there were four replicate petri dishes, each with 10 seeds.

Response is recorded as the number of seeds germinating in each replicate petri dish at the end of the experiment, in that time of heat exposure treatment. The data from such an experiment might look like Table 5.1

The results of such an experiment would be analysed by a One-Way Analysis of Variance (see *Banksia* Practical).

Table 1 Sample data from a Single Factor seed germination experiment.

TIME OF HEAT EXPOSURE	1 min	2 min	5 min	10 min	20 min
replicate 1	0	0	4	9	0
replicate 2	0	2	7	10	0
replicate 3	1	0	4	7	2
replicate 4	0	1	3	6	1

Two-way or Factorial Design

Here we add a **second Factor** to the experiment. If all levels of the second factor are tested with all levels of the first Factor, the design is said to be a **Factorial Design**.

Let's say that in addition to testing Time of heat exposure of the seed as a Factor, you wished to add Temperature of exposure as the second factor - say 60⁰C, 80⁰C, 100⁰C and 120⁰C. The combination of the two Factors can be seen in Table 5.2:

Table 2: Factorial combination of two experimental factors (1. time of heat exposure; 2. temperature of heat exposure). Every Temperature of exposure is tested at each Time of exposure.

TIME OF HEAT EXPOSURE	1 min	2 min	5 min	10 min	20 min
TEMP. OF HEAT EXPOSURE	60 ⁰ C	60 ⁰ C	60 ⁰ C	60 ⁰ C	60 ⁰ C
	80 ⁰ C	80 ⁰ C	80 ⁰ C	80 ⁰ C	80 ⁰ C
	100 ⁰ C	100 ⁰ C	100 ⁰ C	100 ⁰ C	100 ⁰ C
	120 ⁰ C	120 ⁰ C	120 ⁰ C	120 ⁰ C	120 ⁰ C

In this case, replicate petri dishes would be set up as above, and the number of seeds germinated in each one scored at the end of the observation period.

These data would then be analysed by a **Two-Way Factorial ANOVA**, which would let you judge the significance of each of the two Factors used (Time of exposure, Temperature of exposure), and any **interaction** between the two Factors (if one exists).

To do a Two-Way ANOVA, and interpret it, see Appendix IV and your Demonstrator.

Replication, Randomness and Independence in experimental design

We have already met the requirements for data to be **random** and **independent** when we have used *t*-tests and ANOVA in earlier Practicals. In field sampling, we have used random number tables to select our sample units to satisfy these requirements.

In experiments, there are other steps to take to ensure that the measurements we take on our experimental subjects are replicated, random and independent.

Replication: the treatment is applied not merely to a single experimental subject, but to a number of them, and the average response of subjects to the treatment is calculated. The response of a single subject to an experimental treatment, while interesting, cannot be taken as indicative as the general response of subjects to the treatment. Subjects differ from each other in all sorts of ways (size, health, previous history) and all of these affect the response of the subjects to the treatment. What we are trying to see is whether there is any general response of subjects to the treatment, given this background level of variability in the population.

Randomness: this is required in experiments so that any effects of treatments we detect can be clearly inferred to be the result of the treatment, and not some artefact of the way we have run the experiment. Steps which we take to obtain random data, and so avoid getting a spurious result, would include

- **random allocation of subjects into treatments.** If for example in our seed experiment, we put all the big, healthy-looking seeds into one treatment, and all the small, unhealthy-looking seeds into another treatment, any differences between the treatments could simply reflect this initial non-random allocation of seeds into treatments. So **subjects must be randomly allocated into treatments** to avoid **bias** in the estimation of treatment effects.
- **random arrangement of petri dishes after treatment.** The petri dishes need to be left for several weeks, to allow germination to occur. It is important to randomly arrange the petri dishes in a stack to be left in the growth cabinet - if all the replicates of one treatment are put together, and there is some peculiarity of the growth cabinet where they sit (warmer, colder, lighter, darker than the rest of the cabinet) germination of the seeds in these replicates may be affected by the particular growth conditions in that part of the cabinet. This would be confused with a treatment effect. The requirement for random arrangement of experimental subjects in a growth area is referred to as **random interspersion**. So random interspersion of the experimental subjects in the incubation area guards against spurious effects due to peculiarities of the growth cabinet.

Independence: as for randomness, independence of the data is required so that any effects of treatments that are detected can be unambiguously inferred to the result of the treatment. Many of the steps taken to ensure randomness will also ensure independence, but there are some additional procedures involved for independence of data from experiments:

- **independent application of the treatment to replicates.** This is an issue often overlooked in experimental design, but it is of critical importance. We have already seen that replication of subjects is required, to estimate the average response of subjects to an experimental treatment, against the background of variability due to other sources present in the population. Given that replication of subjects is required, replication of applications of the treatment are also required for the same reason - a single application of the treatment is not adequate to estimate the general effect of the treatment on the population. If only a single application of the treatment is made, even if to replicate subjects, any peculiarity of that single application would affect all the subjects to which it was applied, and this could be confused with a treatment effect. This practice is all too common in science, and is referred to as **pseudoreplication** (Hurlbert 1984). Thus, there must be repeated and independent applications of any experimental treatment to experimental subjects. See the discussion of this problem in seed germination experiments in Morrison & Morris (2000). A true replicate is thus the smallest experimental unit to which a treatment is independently applied. If the treatment is independently applied to replicate experimental units, the deviations of the responses of these replicates from the treatment mean are independent of each other, and are independent estimates of the background variability (or “error”) in the system. If the treatment is only applied once, to replicate subjects, the deviations of the responses of these replicates from the treatment mean are not independent of each other - they are linked by being part of the single application of the treatment.

Controls

In order to tell whether a given treatment has had an effect, we compare the response of subjects receiving the treatment, to the same response of subjects not receiving the treatment. Subjects not receiving the treatment are called **controls**. Sometimes, when applying the treatment involves a number of steps, we will use **procedural controls** (handled in same way as treated subjects, without receiving the actual treatment. Thus in trials of a new drug, control subjects receive a dummy pill, called the placebo, to imitate the effects of taking a substance on the subject). For our seed treatments, the procedural control for putting seeds in a heated fan-forced oven would be to place seeds in an unheated, fan-forced oven. If a significant germination response is detected in the heat-treated seeds, it can be safely concluded that the heat produced the effect (and not the convection oven by itself).

2.3 METHOD

Once you have designed an experiment, talk over the details of it with a demonstrator. After the discussion, execute the experiment (as originally designed, or modified after discussion) using the methods below.

Constraints on your design: please use no more than 24 petri dishes for germination trials.

As noted in Box 2.1, a **replicate** in an experiment is the **smallest unit to which a treatment is independently applied**. In our case, treatments will be applied separately to a seed lot of 6 - 10 seeds in a petri dish, and the response measured as the percent germination of the seeds at the end of the experiment. Thus the replicate is the seed lot (and not the individual seeds in it).

To satisfy the requirement **for independent application of smoke and heat**, seed lots must be put in the oven or the smoking chamber **one after another** (and not all put in simultaneously). This ensures repeated, independent application of the smoke or heat treatment. (Putting all the seed lots in the oven or smoking chamber simultaneously means that the treatment has been applied once, albeit to replicate seed lots).

Treating seed samples with heat

1. place an individual seed lot in a **glass** petri dish, and leave it in the oven (with the **lid off**) for specified period of time.
2. Repeat for your remaining replicates.

N.B. The glass petri dishes are for heating or smoking seed lots in only - they are not to be used for germination.

Use the perspex petri dishes for germination tests.

Treating seed samples with smoke

1. place an individual seed lot in a **glass** petri dish, and leave it in the smoking chamber (with the **lid off**) for specified period of time.
2. Repeat for your remaining replicates

Germinating seed lots

Germination trials are often affected by fungal infection - to minimise fungal infection,

- Seeds will be **surface sterilized** 1 day prior to the experiments, by soaking them in 1% sodium hypochlorite solution (bleach) for 10 minutes. The bleach will be rinsed off, and the seeds allowed to dry.
- **DO NOT HANDLE SEEDS WITH YOUR BARE FINGERS** - wear disposable gloves, and handle seeds with sterilised forceps.
- use **sterile technique** (from Microbiology) to place a **sterilised filter paper** inside the **sterile petri dish**

To set a petri dish up for a germination trial,

1. place a filter paper inside the base of a petri dish using sterile technique
2. arrange 6 - 10 seeds around the dish (wear gloves, use sterile forceps)
3. **label the lid** with your group number, experimental details (temperature, time), species used, and date
4. **wet the filter paper up with distilled water** - leave a surface film of about 1 mm depth of water
5. replace lid
6. repeat procedure for your remaining replicates
7. place labeled petri dishes inside a plastic bag, label the bag, and leave where directed.

YOU WILL NEED TO EXAMINE YOUR PETRI DISHES FOR GERMINATION IN SUBSEQUENT WEEKS.

KEEP A RECORD OF THE NUMBER OF SEEDS THAT HAVE GERMINATED (EXTRUDED A RADICLE) IN EACH PETRI DISH, ON EACH OCCASION.

WITH ACACIA SEEDS, TAKE CARE TO DISTINGUISH BETWEEN THE

- **RADICLE (EMERGING ROOT)**
- **ELAISOME* OR ARIL - POINTED EXTERNAL STRUCTURE AT ONE END OF THE SEED.**

EXAMINE GERMINATING WATTLE SEEDS UNDER A DISSECTING MICROSCOPE TO DISTINGUISH BETWEEN THE RADICLE AND THE ELAISOME.

*The elaisome or aril is a lipid or protein-rich body often found on the exterior of many seeds, including wattle seeds. Its function is to attract ants, which 'handle' the seed, trying to obtain the

elaisome. The seed is moved as a consequence, and this aids dispersal of the seeds (Augee and Fox 2000 p.69).

2.4 DATA COLLATION AND ANALYSIS

Calculate the

- **mean no. of seeds germinating in each treatment**
- **Standard Error or Confidence Limits around the mean.**

This is the basic step of summarizing your results, as we have done throughout the Unit.

Do not graph results for individual replicates within treatments.

Decide what type of graph best presents the results you have obtained.

Since each group performed its own experiment, it is difficult to specify in advance what will be the most appropriate method of analysis for your data.

Consult with a demonstrator to see what (if any) statistical analysis can be used for your data.

This goes back to your

- original hypothesis, and the
 - experimental design
- you used to test it.

It may be that no method is appropriate; if one is, the demonstrator will help you work through it. You may be able to use

- *t*-test (for comparing 2 samples)
- 1-Way ANOVAR (>2 samples, single factor design)
- 2-Way ANOVAR (two factors varied in design)

You may need to check **Homogeneity of Variances** amongst samples being compared; transform data if variances are heterogeneous.

Final comments

Acacia seeds show a germination response to heat shock, generally in the range 60 – 120 °C (depending on length of exposure, and the seed lot in question). They do not respond to smoke. The *Grevillea* hybrid seeds have quite thin seed coats, and may show a positive response to short bursts of heat (1 – 2 mins), and to smoke exposure. Longer exposures to heat shock kills them. If using a cereal, these are intolerant of heat shock altogether (this makes a nice contrast with the *Acacia*, which require heat).

REFERENCES AND BIBLIOGRAPHY

- Auld, TD, Bradstock RA (1996) Soil temperatures after the passage of a fire: do they influence the germination of buried seeds? *Aust. J. Ecol.* 21, 106 - 9.
- Auld, TD, and O'Connor MA (1991) Predicting patterns of post-fire germination in 35 eastern Australian Fabaceae. *Australian Journal of Ecology* 16, 53 - 70.
- Auld, T. & M. Tozer (1995) Patterns in emergence of *Acacia* and *Grevillea* seedlings after fire. *Proc. Linn. Soc. N.S.W.* 115, 5-15.
- Baldwin, I.T., L. Staszak-Kozinski & R. Davidson (1994) Up in smoke: I. Smoke-derived germination cues for postfire annual, *Nicotiana attenuata* Torr. ex. Watson. *J. Chem. Ecol.* 20, 2345-2371.
- Baskin C.C., Baskin J. M. (1998) *Seeds: ecology, biogeography and evolution of dormancy and germination*. Academic Press, Sydney. Chapter 3.
- Baxter, J.M., J. van Staden, J.E. Granger & N.A.C. Brown (1994) Plant-derived smoke and smoke extracts stimulate seed germination of the fire-climax grass *Themeda triandra*. *Environ. Exper. Bot.* 34, 217 -223.
- Bradstock RA, Auld TD (1995) Soil temperatures during bushfires in relation to fire intensity. *J. Appl. Ecol.* 32, 76 - 84.
- Briggs C. L., Morris E. C., Ashford A.E. (2005) Investigations into seed dormancy in *Grevillea linearifolia*, *G. buxifolia* and *G. sericea*: anatomy and histochemistry of the seed coat. *Ann. Bot.* 96, 965-980.
- Briggs C.L., Morris E.C. (2008) Seed-coat dormancy in *Grevillea linearifolia*: little change in permeability to an apoplastic tracer after treatment with smoke and heat. *Ann. Bot.* 101, 623-632.
- Brown, N.A.C. (1993) Promotion of germination of fynbos seeds by plant-derived smoke. *New Phytol.* 123, 575-83.
- Brown, N.A.C., G. Kotze & P.A. Botha (1993) Stimulation of seed germination in South African species of Restionaceae by plant-derived smoke. *Seed Sci. & Technol.* 21, 573-580.
- Burrows, G. E., Virgona, J.M., Heady, R.D. (2009) Effect of boiling water, seed coat structure and provenance on the germination of *Acacia melanoxylon* seeds. *Australian Journal of Botany* 57, 139-147.
- Dixon, K.W., S. Roche & J.S. Pate (1995) The promotive effect of smoke derived from burnt native vegetation on seed germination of Western Australian plants. *Oecologia* 101, 185-192.
- Enright, N.J., Goldblum D, & Pita J.M. (1997) The independent effects of heat, smoke and ash on emergence of seedlings from the soil seed bank of a healthy Eucalyptus woodland in Grampians (Gariwerd) National Park, western Victoria. *Aust. J. Ecol.* 22, 81 - 88.
- Hurlbert, S. H. (1984) Pseudoreplication and the design of ecological field experiments *Ecol. Mon.* 54, 187 - 211.
- Keeley, J.E. & Fotheringham, C.J. (1998) Smoke-induced seed germination in California chaparral. *Ecology* 79, 2320 - 2336.
- Kenny, B. (2000) Influence of multiple fire-related germination cues on three Sydney *Grevillea* (Proteaceae) species. *Austral Ecology* 25, 664-669.
- Langkamp P (ed.) (1987) *Germination of Australian Native Plant Seed*. Inkata Press, Sydney.

- Morris, E.C. (2000) Germination response of six East Australian *Grevillea* species (Proteaceae) to smoke, heat exposure and scarification. *Aust. J. Bot.* 48, 179-189.
- Morris, E.C., Tieu A.L., Dixon K.D.W. (2000) Seed coat dormancy in two species of *Grevillea* (Proteaceae). *Annals of Botany* 86, 771-775.
- Morrison DA *et al.* (1992) Patterns of testa-imposed dormancy in native Australian legumes. *Annals of Botany* 70, 157-163.
- Morrison DA *et al.* (1998) The role of the lens in controlling heat-induced breakdown of testa-imposed dormancy in native Australian legumes. *Annals of Botany* 82, 35-40.
- Morrison DA, Morris EC (2000) Pseudoreplication in experimental designs for the manipulation of seed germination treatments. *Austral Ecology* 25, 292-296.
- Roche, S., Dixon K.W., & Pate J.S. (1997) Seed ageing and smoke: partner cues in amelioration of seed dormancy in selected Australian native species. *Aust. J. Bot.* 45, 783 - 815.
- Thomas, P.B., Morris E.C., Auld T.D. (2003) Interactive effects of heat shock and smoke on the germination of nine species forming soil seed banks within the Sydney region. *Austral Ecol.* 28, 674-83.
- Thomas, P.B., Morris E.C., Auld T.D. (2007) Response surfaces for the combined effects of heat shock and smoke on the germination of 16 species forming soil seed banks in south-eastern Australia. *Austral Ecol.* 32, 605-616.
- Thomas, P.B., Morris E.C., Auld T.D., Haigh A.M. (2009) The interaction of temperature, water availability and fire cues regulates seed germination in a fire-prone landscape. *Oecologia* 162, 293-302.
- Shea SR, McCormick J, Portlock CC (1979) The effect of fires on regeneration of leguminous species in the northern jarrah (*Eucalyptus marginata* Sm) forest of Western Australia. *Australian Journal of Ecology* 4, 195-205.
- Vleeshouwers LM, Bouwmeester HJ, and Karssen CM (1995) Redefining seed dormancy: an attempt to integrate physiology and ecology. *Journal of Ecology* 83, 1031 - 1037.