Simple Embryo Culture for Plant Breeders

A manual of technique for the extraction and in-vitro germination of mature plant embryos with emphasis on the rose

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Rose Hybridizers Association

Contents

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My wife Debbie supported my work and tolerated my eccentricities, not an easy thing to do.

Don Holeman
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Introduction

The seeds of many species exhibit orthodox dormancy, which is the inhibition of germination by the presence of certain plant hormones in the tissues that surround the embryo within the seed. Examples of such species include apples, pears, peaches, cedars, hemlocks, pines, firs, maples and roses[2]. Dormancy can be a vexatious problem for growers and hybridizers. Much effort has been made to overcome dormancy, and many techniques exist for the purpose[3].

Embryo culture is one such technique. Removing the pericarp and testa from around the embryo removes the source of the hormones that inhibit germination and (if all goes well) allows the embryo to germinate and grow. It is a well established technique, but (to my knowledge) all previously published protocols for embryo culture have complex and expensive culture requirements that make them inaccessible to mere mortals[4].

This manual presents a new and very simple protocol for the culture of mature embryos. The method outlined here has been used successfully to germinate embryos of apples, Asian pears, sassafras, sweet cherries, roses and other species, and to carry the embryos to the seedling stage. It requires no scientific training, no special equipment, no complex chemical solutions or culture media; nor does it require the burden of sterilization. The tools, equipment and supplies can usually be found around the home or else can be easily and inexpensively purchased.

The overall process is straightforward:

- Remove the embryo from the seed.
- Remove the testa from the embryo and place it in a solution of 0.1% hydrogen peroxide with a little surfactant added.
- Rinse it in a solution of 0.05% hydrogen peroxide.
- Place the embryo on a paper towel that is wetted with 0.05% hydrogen peroxide and contained in a re-sealable polyethylene sandwich baggie.
- Hang the baggie from a fluorescent light fixture. Wait about two weeks and pot-up the seedling.

If the embryo is extracted without injury, and if it is alive and healthy, it will germinate with a certainty approaching 100%, provided you give it a little tender loving care.

The devil is in the details, of course. In this manual we will walk through the entire process of extracting and germinating embryos step-by-step, using rose seeds as our example. My choice of rose seeds is due, in part, to these being my personal interest, but they are typical of many seeds in their size, morphology, dormancy and response to our selected treatment. There are some differences between rose seeds and other seeds, and these differences will be mentioned at the appropriate place in the manual.

The seeds of some species, such as apples, various citrus, and sassafras, have seeds that are very easily opened. The seeds of other species—grapes and walnuts come to mind—are quite atypical in their internal morphology and cannot be treated in the manner described here. Still other seeds contain immature embryos and so require culture media supplemented with nutrients in order to fully develop before they can germinate: the requirements of these seeds is outside the scope of this manual. However, the technique given here will perhaps suggest to the reader new strategies for dealing with these exceptional cases, and even for modifying other commonly used techniques for breaking seed dormancy.

Dormancy

Dormancy is a broad concept, defined in general terms as "any case in which a tissue predisposed to elongate does not do so" [5]. There are different types of dormancy which vary by degree and by their causes[6]. At one end of the spectrum is simple quiescence, a slowing or halting of plant growth due to unfavorable environmental conditions such as low or high temperatures and inadequate rainfall. At the other end of the spectrum is orthodox dormancy, which is also called internal or physiological dormancy, in which cell division (mitosis) is suppressed by the presence of certain chemical hormones produced by the plant. The most important of these hormones for our purposes is abscisic acid (ABA).

Nature has graced some plants with the ability to impose dormancy in coordination with the seasons, promoting growth and flowering at favorable times and suppressing it at unfavorable times, thus ensuring the survival of the plants and their offspring. Dormancy can affect an entire plant, such as occurs in "hardening off" in the autumn as a plant prepares itself to survive the extreme conditions of winter. It can also affect specific parts of a plant, particularly the buds (meristems) that will eventually grow to become new branches or flowers; and seeds, which hold embryos with the potential to become new plants.

Seed dormancy allows time for developing embryos to become fully formed and to mature—they need to build up a reserve of fats and oils that will provide a source of energy for them to grow into seedlings that can survive on their own through photosynthesis once they germinate. Dormancy also allows time for seeds to be dispersed and, for some plants, to survive the harsh conditions of winter or drought.

The exact mechanism by which seed dormancy is maintained and released (or 'broken') in nature is still not fully understood although certain features are well established. Most importantly for plant breeders is the knowledge that dormancy is mediated by ABA. The structure of seeds is significant in this respect because certain parts of a seed produce ABA and deliver it to the embryo to maintain dormancy. Understanding the structure of a seed is also important for understanding how to remove the embryo from it.

The structure of rose seeds is typical of many seeds that exhibit physiological dormancy.

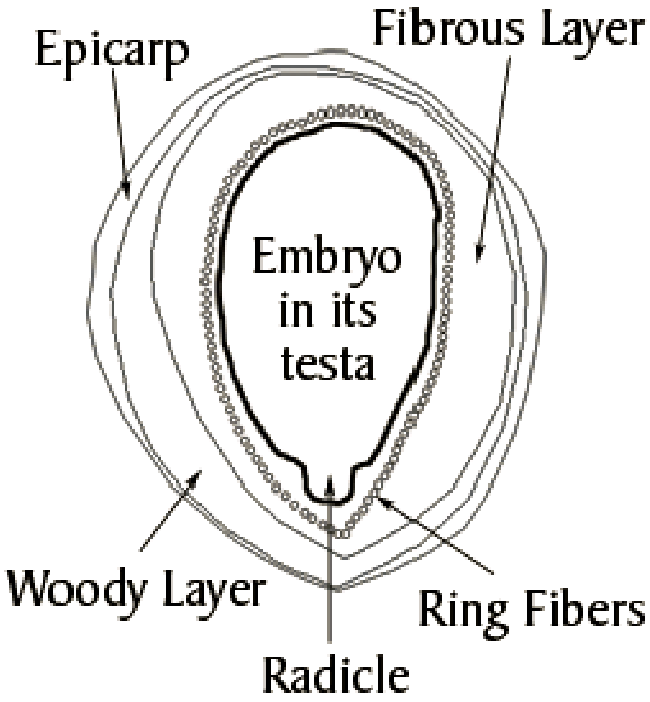

Figure 1. Diagram of a rose achene (seed).

Technically speaking, what we usually refer to as a rose seed is actually a rose *achene*. An achene is "a one seeded fruit, which consists of a mature embryo enclosed in a relatively thin seed coat (*testa*) to give [make up] the seed proper, which in turn is surrounded by a thick fruit wall (*pericarp*)" [7]. Figure 1 shows the structure of a rose seed. The pericarp is made of an epicarp and woody and fibrous layers. The ring fibers are the innermost part of the fibrous layer[8]. The pericarp surrounds the embryo, that part of the seed which will grow into a plant.

Inside the pericarp, the embryo is wrapped in a tough, thin sack called the testa (Figure 2). The testa is usually brown or tan. The embryo inside, if it is *viable* (alive and capable of germinating), is white. The testa actually has two layers: an outer layer that I theorize holds precursor chemicals (carotenoids) from which ABA is formed; and an inner layer that I theorize has cells which convert the carotenoids into ABA, as evidenced by a strong level of enzymatic (peroxidase) activity that you may see as you proceed through this protocol.

While the structure of most seeds is similar, their appearance, texture, composition, thickness and sizes of their pericarps and testae vary greatly among plant species.

Germination

In any case ABA, the hormone that imposes and maintains seed dormancy, is present in the tissues that surround the embryo, especially the testa. Remove this tissue, and the embryo springs to life—it is actually quite shocking to watch because it happens quickly, and more quickly still if the embryo is incubated with hydrogen peroxide. It is not enough to remove only the pericarp, however. Most of the inhibitor chemicals are present in the testa and they will not wash out quickly in hydrogen peroxide solution, so the testa must be removed as well in order to cause the embryo to germinate[9].

Figure 2. Rose embryo nested in one half of the pericarp. This seed has been split along its suture. The embryo is contained in its testa. Some ring fibers still surround the embryo at one end. The ring fibers are actually a single continuous fiber that spools neatly in a single layer around the embryo, along the axis of the hypocotyl and radicle.

There are several parts to a rose embryo. It has a pair of *cotyledons* and a stubby little 'tail' that contains the *radicle* and *hypocotyl*. When the embryo germinates, these parts start to grow:

- The cotyledons turn green, swell and grow. The force they exert splits the pericarp open along a natural seam call the *suture* (Figure 3). In roses the cotyledons contain food (mostly fats and oils) to nourish the growing plant until the cotyledons can emerge from the soil and begin the process of photosynthesis[10]. Cotyledons are not true leaves – those come later.

- The radicle emerges through the separating pericarps at the end of the seed that has a channel called a *micropyle*. The radicle becomes the root. The hypocotyl and the tissue above it, the epicotyl, become the stems and, eventually, the true leaves. The micropyle appears as a tiny dimple on the surface of the seed along the line of the sutures and is an important landmark for locating the radicle, although it is often difficult to see.

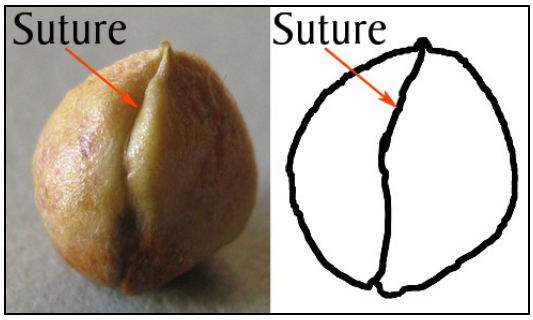

Figure 3. The suture of a rose seed.
Photo courtesy of Margit Schowalter.

Germination *in-vitro* (Latin for "within the glass", meaning "in a test-tube") differs from germination *in-vivo* ("within the living", that is to say, naturally) in two significant ways. Germination *in-vitro* occurs at a much faster rate than in nature; and all tissues of an embryo begin growing, greening and expanding simultaneously *in-vitro* whereas in nature the radicle and hypocotyl of an embryo extend first, followed by swelling of the cotyledons and then greening up and growth of the cotyledons. You may expect to encounter some spontaneously germinating embryos as you process your seeds using this protocol and these differences will be readily apparent.

Seeds

Seeds often contain no embryos, or have dried and shriveled embryos or even insect larvae inside them (for example, there may be larvae of the rose chalcid wasp and the rose seed megastigmus).

The size of a rose seed is not an indication of whether it contains an embryo. The smallest seeds from within a hip often contain viable embryos, while the largest seeds may have no embryos.

The size of the embryo itself can, however, be a clue to its ploidy level. The largest rose embryos that I have extracted have come from *R. moyesii*, a hexaploid species rose. Hybrid tea embryos, which are commonly either triploid or tetraploid, are usually larger than embryos from diploid species roses. Embryos from any given hip vary in size and hybridizers should be alert to the possibility that the (relatively) smallest ones may be lower in ploidy level than their siblings[11].

The age and condition of a seed affects the ability to extract its embryo and to remove the testa from that embryo, as well as the viability of the embryo. The older a seed is, the less likely it is to contain a viable embryo. Put another way, seeds die off as they age. Disease takes its toll, but simple aging does too.

Seeds that have been placed in soil (or even in so-called "soil-less" media) for even a short time will have higher numbers of dead and infected embryos than seeds stratified on moist paper towels. Nor are paper towels alone a guarantee against infection. Infection can be either fungal or bacterial, and it appears that oftentimes the contaminating agent is present on (or in) the seed coat before stratification.

In rose embryos, fungal infection is seen first as a ring surrounding the radicle where it joins with the hypocotyl, just at the point where it disappears under the base of the embryonic cotyledons. This implies that the fungal spores enter through the micropyle. As infection progresses it engulfs the entire radicle and the base of both cotyledons. Embryos that are visibly infected, even with only a spot or small ring of fungus on the radicle, fail to germinate. In one experiment, treatment of such embryos with the antifungal agent *tebuconazole* (Bayer Advanced® Disease Control for Roses, Flowers and Shrubs) failed to cure the infection. It is not known whether pre-treatment with fungicide prevents infection, though this seems likely. It seems reasonable that regular

application of systemic fungicide to breeding stock is probably the best insurance against fungus infecting seeds, but this remains to be proven.

Bacterially infected seeds can have testae that are empty or full of fluid. Sometimes the embryos are liquefied, and exude as a creamy paste as extraction is attempted. It is debatable whether bacteria kill embryos or simply devour them after they die of other causes. In any case, dead embryos can be assumed to harbor bacteria, and the presence of bacteria will quickly become apparent when they are put into culture.

Embryos can become infected through avenues other than the micropyle. Pericarps are composed of living tissue in freshly harvested seeds. As pericarps die and then decay, they become soft and porous and provide gateways for the entry of disease to the embryo. Seeds that have decaying pericarps are much less likely to contain viable embryos than seeds with pericarps that are intact. Embryos are actually more difficult to remove from a decaying seed because the sutures fail to split cleanly, and cutting actions are less controllable and their effects are less predictable[12].

Embryos can and do dry out in seeds that are left un-stratified. The moisture content of a seed affects embryo extraction and excision. Embryos extracted from dried seeds are waxy and hard, and are usually viable. While such embryos are less fragile than fully hydrated embryos, they require an intermediate period of soaking before the testae can be safely removed.

For many species, desiccation provides a good means of long-term storage of seeds. However, each species, or even each cultivar, will have its own optimal moisture content for storage. For example, while the seeds of most roses remain viable when dried, the seeds of *R. rubiginosa* and *R. rugosa* quickly lose viability when they are dried[13].

The viability of rose seeds that have been frozen depends on both the specific cultivar and the degree of desiccation of the seeds.

In general, the seeds of cold-hardy roses such as close hybrids of *R. acicularis*, *R. laxa*, Retzius and *R. spinosissima* are more resistant to freeze damage than those of more highly hybridized cultivars that have much *R. chinensis* in their ancestry, such as the hybrid teas. Seeds of 'Prairie Peace', a close hybrid of *R. laxa* and *R. spinosissima*, withstood -37° F (-38 ° C) in hips that overwintered on a plant in Rocky Mountain House, Alberta, with no loss of viability. Seeds of 'Mister Lincoln', 'Love', 'Last Tango' and 'Perfume Delight' all were killed by -7 ° F (-22 ° C) when overwintered in Hartford, Connecticut, but seeds of 'Veilchenblau', 'Applejack' and 'Mrs. A.C. James' survived.

Seeds that have been thoroughly desiccated are more likely to contain viable embryos after freezing than those with higher moisture content. For this reason, the smallest seeds from within a hip of a hybrid tea that has over-wintered on the plant are often found to be the only viable seeds from that hip. The larger seeds, having failed to dry completely, are more susceptible to freeze damage.

Intact pericarps and testae are more permeable to water than is generally recognized. Desiccation of embryos can occur quickly when seeds are stored dry or left in ripe fruit or (for roses) in ripe hips, even when the fruit or hips themselves retain enough moisture to remain pliable or pulpy. Rehydration of embryos occurs easily when dried seeds are soaked in water or dilute hydrogen peroxide solution.

Seeds intended for embryo culture should therefore be thoroughly cleaned with soap and water and then soaked overnight in 1000 ppm hydrogen peroxide (Solution A, below). If they are to be extracted within a couple of months of harvest then stratify them onto paper towels slightly dampened (not saturated) with 500 ppm

hydrogen peroxide (Solution B, below) and refrigerate them, with periodic changes of the towels, to help retard microbes and preserve their moisture[14]. Refrigeration itself is irrelevant to the success of germination by embryo culture but it helps to slow death and decay of the seeds. Drowning of seeds is possible, so care should be taken to provide only enough moisture to prevent desiccation.

Some breeders will want to wait until the spring following harvest before extracting and germinating their embryos. In this case, seeds can be stored dry to force dormancy and prevent decay after being thoroughly cleaned and soaked in 1000 ppm hydrogen peroxide (Solution A) overnight. The previously mentioned caveats notwithstanding, in my experience seeds stored dry seem to retain their viability well, at least through the first winter. Soak them in Solution B prior to extraction to begin the rehydration of the embryos.

Seeds that have already been sowed in soil but which have failed to germinate can be cleaned up in a similar manner but, as mentioned, will have fewer viable embryos. When the decision is taken to attempt embryo culture with such seeds they should be cleaned, dried, and refrigerated immediately, and their embryos should be cultured as soon thereafter as possible.

Embryo Culture

Plants require carbon dioxide, oxygen, water and certain mineral nutrients to create the sugars and other substances that they need to grow. They are usually able to extract these from their environment, absorbing water and nutrients through their roots and capturing carbon dioxide and oxygen from the air through their leaves. People wishing to grow only *parts* of plants must supply these requirements artificially, using a technique called plant tissue culture.

In plant tissue culture, cells are placed in a culture medium made of water and salts containing magnesium, calcium, nitrogen and other elements essential to plant growth. Lacking the ability to capture carbon dioxide, they must also be provided with a source of carbon, usually the sugar *sucrose*, which is the sugar that they would otherwise create by photosynthesis.

Embryo culture is a form of plant tissue culture that is often used to grow embryos that would might fail to live, such as those created by advanced laboratory techniques. It can also be used to *rescue* embryos that would normally fail to develop because of genetic incompatibilities or defects. In these cases the embryos are immature (not yet fully formed) so the culture medium must supply nutrients and sucrose.

Embryos which are already mature, such as those in seeds taken from ripened fruit or hips, do not need supplemental nutrients or carbon. They have built up a reserve of these substances in their endosperm or, in many plants such as roses, in their cotyledons. Culturing mature embryos therefore requires only that they be protected from contamination for long enough to allow them to germinate.

The instructions that follow will enable you to extract and culture mature embryos.

Tools

The most convenient tools to use for extracting embryos are fingernail clippers. You will need both a small and a large version. Revlon® brand clippers seem to work about the best. Avoid clippers that have pointed levers as these are uncomfortable to use. Another useful tool is the Lacross® brand cuticle trimmer, which has straight blades rather than curved.

Curved, finely pointed tweezers are extremely useful. A good and inexpensive example would be catalogue #624794 from Carolina Biological Supply, http://www.carolina.com.

You will also need a very finely pointed, hooked needle probe. Commercially these are known as micro-dissecting hooks, for instance catalogue #16-1150 from http://www.biomedinstr.com, but good ones tend to be very expensive. You can make a quite suitable one by gluing a very finely pointed sewing needle into the handle of an artist's paint brush. I recommend making one with the last eighth-inch of the tip curved back upon itself slightly. You can bend the tip of the needle with pliers by first heating it with a candle flame until it *just barely* glows in a darkened room. Quench it immediately in water to temper it. Take care not to burn yourself.

You will also need two spoon-type spatulas to lift the embryos as you move them about. One of these should have a fairly long handle so that you can reach inside a baggie with it. It is cost-effective to purchase spoon spatulas, for instance, from Ward Scientific, http://www.wardsci.com, catalogue #15 V 4330.

It also helps to have a scraping tool available to remove the waxy material that often covers the sutures of the seeds. For this you can use a small pen-knife or similar sharp item. I use a flat spatula, one blade of which I keep sharpened for such tasks. The tools are illustrated in Figure 4.

Figure 4. Tools. From bottom: curved, fine pointed tweezers; home-made hooked needle probes; professional dissecting hook; sharpened flat blade spatula; small spoon spatula; large spoon spatula; Lacross® cuticle trimmer (left); small and large Revlon® nail clippers.

These tools are all that are needed to extract embryos from their pericarps. However, I have invented a useful little gadget that greatly speeds up the process, especially for medium and large size seeds (Figure 5).

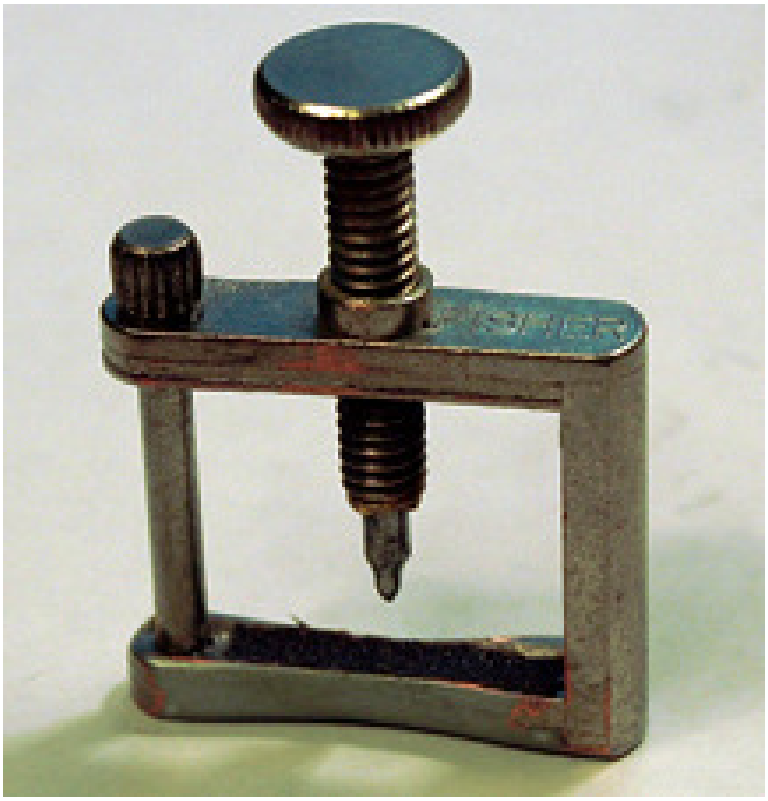

Figure 5. Don's handy, dandy, do-it-yourself seed splitter.

This gadget is easy to make from a laboratory tubing clamp[15]. It is not essential, however, and clippers often need to be used along with it. Mastery of the use of clippers should therefore be your first priority.

Extraction Technique

By far, the most difficult part of our task is removing (extracting) the embryo from the pericarp. Be prepared for the fact that you will be squishing a lot of embryos until you get some practice. Once you get a handle on the technique, though, you will find that such losses will be minimal.

Practice on seeds you don't care about losing, without regard for sanitary conditions or any intent to keep the embryos in order to germinate them. Your objective will be to develop confidence in your ability to remove the embryos so that you can move on to seeds that you do care about. When you become proficient at extracting embryos you will be able to focus on coordinating all the other things you will need to do to get them through to become seedlings.

A few tips will help you get started, but you will need to work out your own technique by trial and error as you would with any other manual art.

First and foremost, never touch a tool to the radicle of an embryo. In fact, don't even come close to the radicle. It is never necessary and always results in irreversible damage to the embryo. Without a radicle an embryo may appear to germinate but it will never be able to grow roots or even a stem. This means that you must also avoid cutting or probing the area of a pericarp that covers the radicle.

It is necessary, then, to understand the topography of a rose seed, to be able to 'read' the seed and know where the radicle lies beneath the pericarp. This can only come with experience, by opening many seeds and examining them closely as you do.

Rose seeds come in two basic shapes and these reflect the shape of the embryos they contain. These basic shapes are: 1 - more globular than cylindrical, and 2 - more cylindrical than globular. The shape is consistent among seeds from a specific 'mother' plant – seed morphology is a maternal characteristic – and so it is predictable. Once you open one seed from a plant you will know what to expect of the others.

For roses, large rose seeds often contain larger embryos than small seeds, but not always. The difference between the sizes of embryos is usually not as great as the difference between the overall sizes of the pericarps that contain them. That is to say, big seeds usually have thicker pericarps than small seeds, and will take more abuse as you try to open them. Small seeds generally have much thinner, weaker pericarps and the embryos they contain can be more easily damaged than larger seeds.

Some small seeds (such as *R. beggeriana*) are nearly impossible to open by any technique without squashing the embryo because their pericarps are very flexible, even though the pericarps can be easily removed. Other small seeds (particularly *R. canina* seeds) are nearly impossible to open because their pericarps are highly fibrous and their sutures are strongly bonded together, very much like Brazil nuts in miniature.

Your objective is to remove enough of the pericarp to expose at least half of the embryo, lengthwise along the axis of the radicle. When the embryo is thus exposed it can easily be lifted away from the remaining pericarp - there is no 'glue' that keeps it seated.

Ideally, you would simply split the seed in half along the suture, which is the natural way that seeds open when they germinate. This is sometimes possible to do with small seeds by using large clippers. Position the suture between the blades of the clippers and apply gentle pressure until the suture pops open. You can then use your fingernails or your clippers to grip the two halves and separate them. Resist the temptation to insert a blade or spatula between the pericarps to pry them apart –you will just slice the embryo, not a good thing to do.

Splitting a seed along its suture is more easily done (especially on medium and large seeds) by using my gadget because it allows you to apply pressure precisely on the suture. Using the gadget, you can sometimes pop seeds right open with only a minimal amount of additional fuss needed to remove their embryos.

Otherwise, simply chip away gradually at the pericarp. This is often necessary even when the sutures are split with the gadget because they often do not split evenly or completely. Gradually chipping away the pericarp is also necessary on seeds that have weak, soft or decaying pericarps. Once you understand the topography of a seed, try to chip away parallel to the axis that runs through the radicle (Figure 5).

Figure 5. Chip away around the seed, avoiding the radicle. Expose half the embryo then tease it from the remaining pericarp with a probe or tweezers, taking care not to puncture the embryo. Note that the clippers illustrated here have a pointed lever. Avoid such clippers as they are uncomfortable to use.

For medium and large seeds, start with the large clippers and round off ribs and lobes on the pericarps. The flat face and the pitch of the clipper blades help to prevent removing too much tissue and biting into the embryo. As you work toward the depth of the embryo you will expose small sections of it, usually without breaking through the testa.

When you start to see the embryo becoming exposed, switch to the small clippers and try to position them so as to lift the pericarp away from the embryo with one blade as you snip. Work around the embryo as before, but focus on revealing at least half of the embryo. Do not cut near the radicle.

When you have exposed about half of the embryo you should be able to tease it out using your tweezers. Lay a blade of the tweezers alongside the length of the embryo and gently apply pressure in a rolling and lifting fashion to free it. If the top of the embryo is exposed (the end opposite the radicle) then apply gentle lifting pressure to that end. Do not poke the embryo with the point of the tweezers.

Some seeds, such as apple and pear seeds, have a flat profile with thin, flexible pericarps. These are best opened by clipping the periphery of the seeds then teasing the pericarp away from the embryo with a thin, sharp and flat spatula. The hulls (pericarps) of very large seeds such as those from peaches and other stone fruit can sometimes be split usefully with a pair of carefully adjusted 6” Vise-Grip® pliers. Apply the jaws of the pliers directly on the sutures, incrementing the force applied by adjusting the screw on the pliers until the sides of the hulls split. Rotate the seed and repeat the process until enough of the hull is broken that you can pry the pieces apart with needle-nosed pliers, avoiding injury to the embryo as you do. A standard lever style nutcracker can also be used on large seeds in a similar manner but the risk of damaging the embryos is greater than with adjustable-force pliers.

When you are ready to begin extracting embryos for germination you will need to have your workspace prepared to deal with them. The following sections will detail how to:

- prepare the necessary hydrogen peroxide solutions, various paper towelettes, and incubation baggies;
- lay out and prepare your work space
- process your extracted embryos using appropriate sanitary technique

Equipment and materials

Corelle® brand dinner ware is ideally suited to processing rose embryos because it is easily cleaned, lightweight and nearly unbreakable. You can also mark it with a permanent marker and the marks will wash off with soap and water. In any case, you will need

- three small, flat plates
- one large plate
- five small, shallow bowls
- two wide, flat-bottomed bowls or deep dinner plates
- Bounty® brand paper towels
- scissors
- re-sealable polyethylene sandwich bags (5 ½" x 6 7/8").
- a clean, dry cloth, preferably cotton
- a fine-point, black permanent marker
- a bottle of 3% hydrogen peroxide solution from your local pharmacy or grocery store
- liquid dishwashing detergent

Hydrogen peroxide solutions

> ⓘ **A word of caution**: if you spill any 3% hydrogen peroxide on yourself stop what you are doing and rinse it off with water or a damp cloth right away. It is not particularly dangerous but it is a strong enough oxidizing agent that it can give you a superficial and annoying burn if you ignore it for a few minutes. Read the label on the bottle for proper precautions.
>
> The solutions you will be making are very dilute and you can get these on your skin without too much worry.

Our objective is to clean up the embryos and keep them clean long enough for them to germinate and grow into respectable seedlings that can stand a fighting chance on their own in soil. We do not need to sterilize everything; we just need to keep things clean. Soap, water, hydrogen peroxide and a little discipline are the tools we use to do this, just as our mothers taught us.

Well, OK, your mother may not have mentioned hydrogen peroxide. She did tell you to wash your hands with soap and water, and clean under your fingernails. This is good advice. Do it often while culturing plant embryos.

The purpose of using hydrogen peroxide is two-fold. First, it cleans bacteria and fungi off the embryos after extraction and helps maintain a fairly clean (though not sterile) environment in the baggies as the embryos germinate. Second, it promotes germination. Embryos will germinate without it but the speed of germination and numbers of germinations are far better if hydrogen peroxide is used[16].

We need two different strengths of hydrogen peroxide:

- Solution A will be 1,000 ppm (parts-per-million) of peroxide, plus a little soap.
- Solution B will be 500 ppm of peroxide (but no soap).

We will make twice as much Solution B as Solution A because we will be using more of it. We will also be using a little bit of 3% hydrogen peroxide to clean our tools and equipment.

- Solution A will be used to clean and hydrate the embryos, still in their testae, as you extract them from their pericarps.
- Solution B will be used to keep naked embryos clean and moist after you remove their testae. This solution will also be used to wet down paper towelettes needed for the removal of the testae (excision beds) and for incubation of the embryos (incubation beds).

You will also use some 3% peroxide for cleaning your tools as you go along. You can pour this into a bowl directly from the bottle.

The directions given (Figure 6) here are in metric units. If you don't have metric measuring cups and spoons, substitute a ¼ teaspoon measure for your 1 ml measure and a ¼ cup measure for your 60 ml measure.

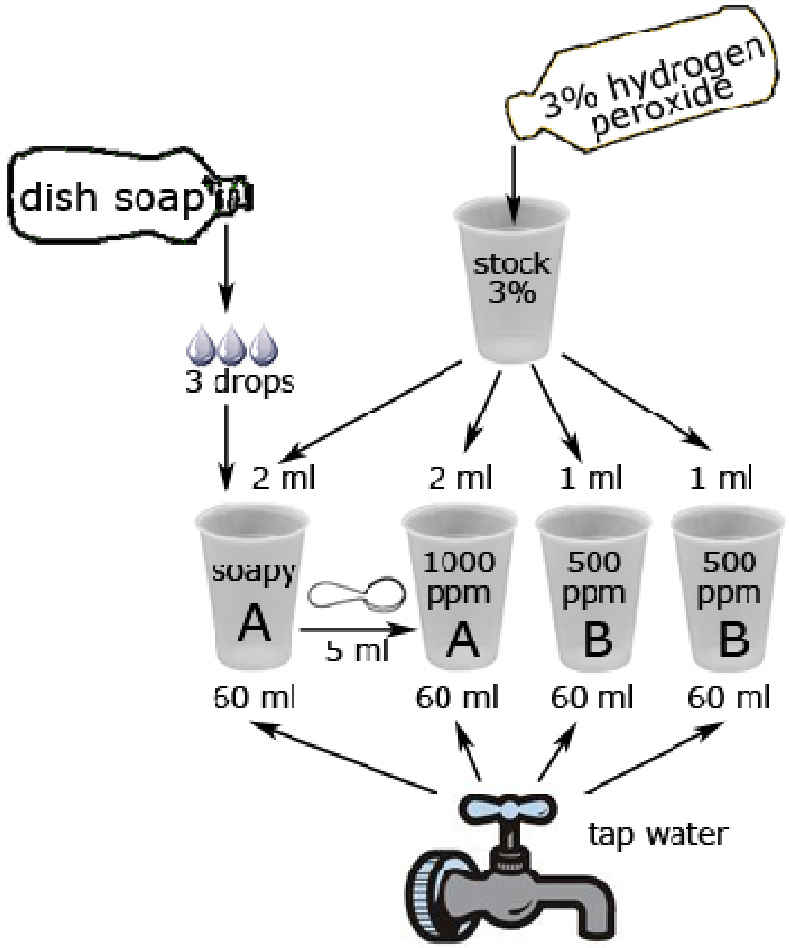

Figure 6. Preparing hydrogen peroxide solutions.

You will need five plastic (not paper) cups. I use small (three-ounce) bathroom cups because these hold 60 ml of liquid conveniently. Mark one cup "stock 3%"; mark another "soapy A"; mark a third cup "A"; and mark the last two cups "B".

Pour about half an ounce of 3% hydrogen peroxide from the bottle into the cup marked "3%", then cap the bottle tightly and put it aside. Use the peroxide in this cup to make up the rest of your solutions.

You will need to be able to measure out 1 milliliter (ml) doses of peroxide. I use a plastic syringe that came with a refill kit for my printer ink cartridges because it is marked in ml, but you can use a 1 ml measuring spoon.

Measure out 2 ml of 3% peroxide into each of the two cups marked “soapy A” and “A”.

Measure out 1 ml of 3% peroxide into each of the two cups marked “B”.

Put the cup with the remaining 3% peroxide aside. Do not pour it back into the bottle from which it came. You can use it for up to six hours or so after you take it from the bottle. Discard it after six hours because it loses strength when exposed to light.

Add 60 ml of cold tap water[17] to each of the four remaining cups.

Carefully add three drops of liquid dishwashing detergent (such as Dawn® or Palmolive®) to the cup marked “soapy A”. Stir this cup with a teaspoon to dissolve all the detergent, then use the spoon to measure out 5 ml (about one teaspoon) of this solution into the cup marked “A”. Put the cup marked “soapy A” aside for later use. It can be used for up to six hours.

You will need to replenish your solutions as you work. Allow them to stand for ten minutes after you make them up and before you use them so that the hydrogen peroxide has a chance to eliminate any microbes that may be present in the tap water. I usually make up two extra cups of Solution B when I am preparing to work on a large number of seeds.

Towelettes

You will be using a lot of paper towels for the processing and culture of your embryos. I use, and recommend, Bounty® brand papers because they maintain their physical integrity when wet. Make up three different sizes of towelettes by cutting standard 12” square sheets as described below.

- *Sanitary towelettes* are used to wipe down tools and dishes with peroxide solution.
- *Excision beds* are used to hold each embryo in place as you excise it from the testa.
- *Incubation beds* hold embryos in place and bath them in Solution B within the incubation baggie.

It is a good idea to make up a supply of extra towelettes and store them in a small baggie to minimize disruptions to your workflow.

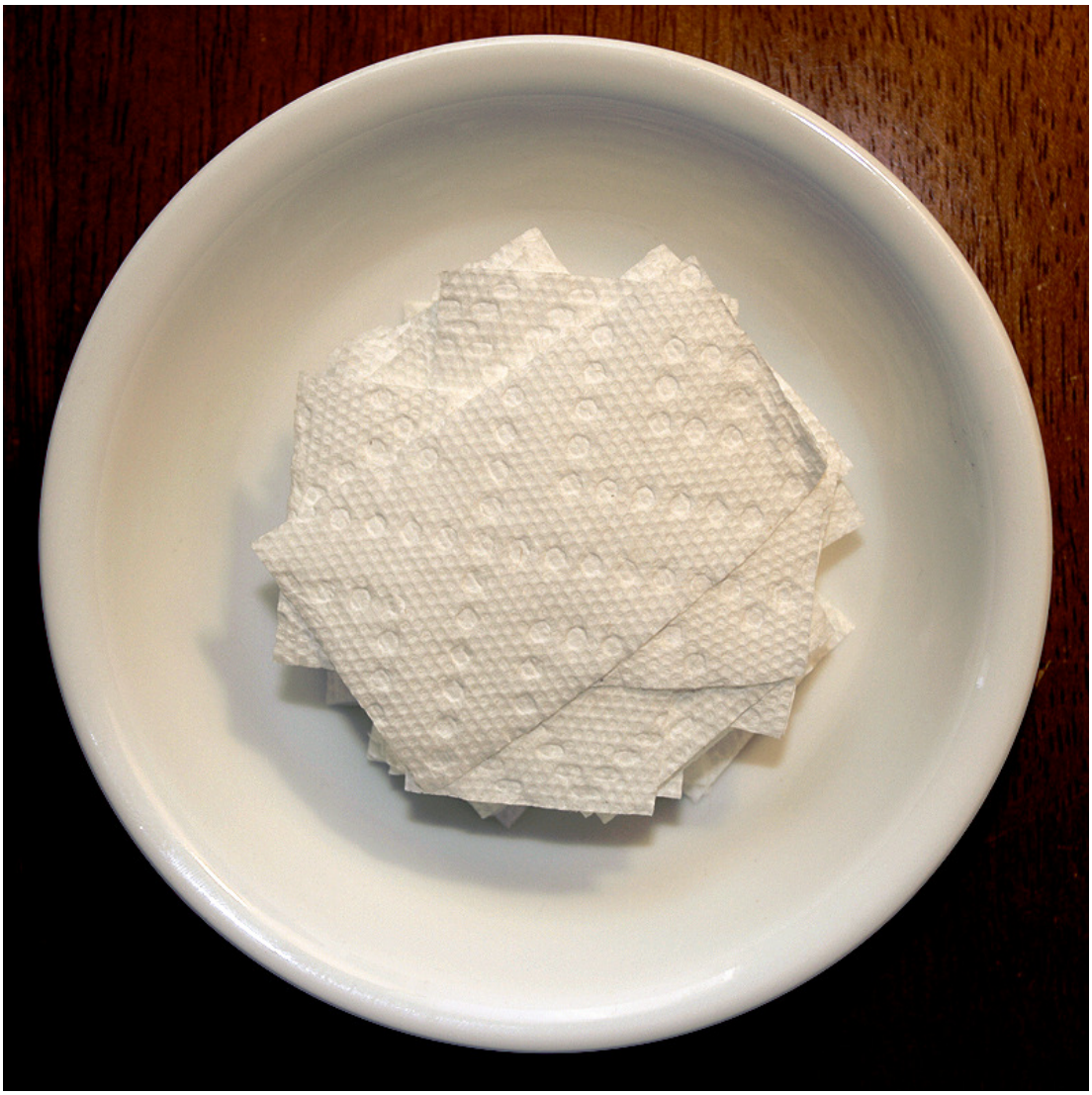

Figure 7. Stack sanitary towelettes so that they can be picked up easily and quickly.

Preparing the Sanitary towelettes

Make small sanitary towelettes by cutting a paper towel into 1 ½" x 3" rectangles. Do this by cutting the towel in half five times in succession. Place these in one of the small bowls for later use. Alternate them lengthwise in a stack such that each is offset from the one beneath and above it to make them easy to pick up as you work (Figure 7). Make five or six sanitary towelettes for each embryo you plan to extract. One sheet of paper towel makes 32 sanitary towelettes.

Figure 8. Lay out excision bed towelettes in a clockwise, overlapping fashion so that the may be taken as needed with a minimum of effort. Mark the topmost towelette so that it can be found easily when wet; take towelettes from the stack in a counterclockwise manner.

Preparing the Excision beds

Make some square, 3” x 3”, towelettes by cutting several paper towels in half four times in succession. Pour a small amount (about a tablespoon) of Solution B into one of the small plates. Overlap the excision beds sequentially in a clockwise fashion on one of the small plates, about 25 towelettes to a plate (figure 8). The solution will keep the beds in place as you lay them out. Mark the outside corner of the topmost towelette with a black marker so you can identify it easily when the excision beds are fully wet. Finish by pouring enough Solution B onto the plate to completely saturate the excision beds.

Cover the plate with a bit of plastic wrap to prevent the towelettes from drying out. If the towelettes do dry out then simply re-wet them with solution B.

If you are planning to be extracting a lot of embryos, make up several plates of towelettes. Plan to use one towelette per embryo until you gain experience.

Preparing the Incubation beds

Make some square (6" x 6") towelettes by cutting several paper towels in half twice in succession. Make one towelette for each embryo you plan to extract.

The embryos will be incubated on these paper towelettes for many days so it is necessary to remove certain processing chemicals ("rewetting agents") from the towels to deter the growth of mold.

Fold each towelette in half twice lengthwise so that each measures about 1 ½" x 6". Stack the towelettes squarely on top of each other. Place the stack in a deep dinner plate or flat-bottomed bowl. Fill the bowl with very hot water so that the towelettes absorb the water completely. Squeeze the water out by placing a second, matching plate or bowl on top and pressing hard to remove as much water as possible. Repeat the hot water wash for a total of five washes. Finish the process with five additional, cold water rinses. Squeeze as much water as possible out of the towels each time.

Divide the towels into stacks no more than ten towelettes high. Place the stacks in a clean, dry dish or bowl. Saturate each stack of towelettes with Solution B by pouring the solution on them slowly enough so that you can see it being absorbed as you pour. Move the cup from side to side as you pour so that the solution absorbs evenly. Keep pouring until a little bit of excess solution runs out onto the plate all around the towels.

Let the towelettes sit for two minutes, then squeeze the solution out of them into a sink. Saturate them again with Solution B as before. Drain off the excess solution into a sink but do not squeeze the towelettes.

Place each folded towelette squarely into its own baggie so that the bottom edge of the towelette touches the bottom of the baggie. Zip each baggie closed while holding the baggie taut to minimize trapped air, and place them together in another baggie in the refrigerator until you are ready to use them. The hydrogen peroxide contained in the baggies will begin to break down into oxygen and water quickly if left at room temperature.

Do not use incubation baggies stored for more than 12 hours in the refrigerator. Prepare only as many as you will use at any one time. You can recycle unused towelettes on a subsequent day by squeezing the solution out of them (between two dishes) and rewetting them with fresh solution, without going through the process of washing them again. You can also place the refreshed towelettes back into their original baggies.

Preparing the Workspace

Make yourself comfortable. I work at a kitchen table while seated in my computer chair. I adjust the chair low so that I don't have to bend my shoulders while I work. I find this helps prevent finger cramps. I'm also old enough that I need lots and lots of light so I keep a desk lamp on the table very close to me and a floor lamp just over my shoulder, in addition to the overhead room light.

You do not need to use a microscope but if you are an aging baby-boomer like me you might find a pair of extra-strong reading glasses to be helpful. These are also a good means for preventing bits of pericarp from getting into your eyes, which happens often enough to warrant this warning:

ⓘ **A word of caution**: Wear protective eyewear when extracting embryos.

Sweep the floor and remove extra chairs and anything else in the vicinity that a seed can roll under. Get out a strong flashlight, too. You'll appreciate these measures when you are on your hands and knees groping about for a dropped seed.

Place a trash barrel next to you so that you can easily dispose of the litter you will generate as you work.

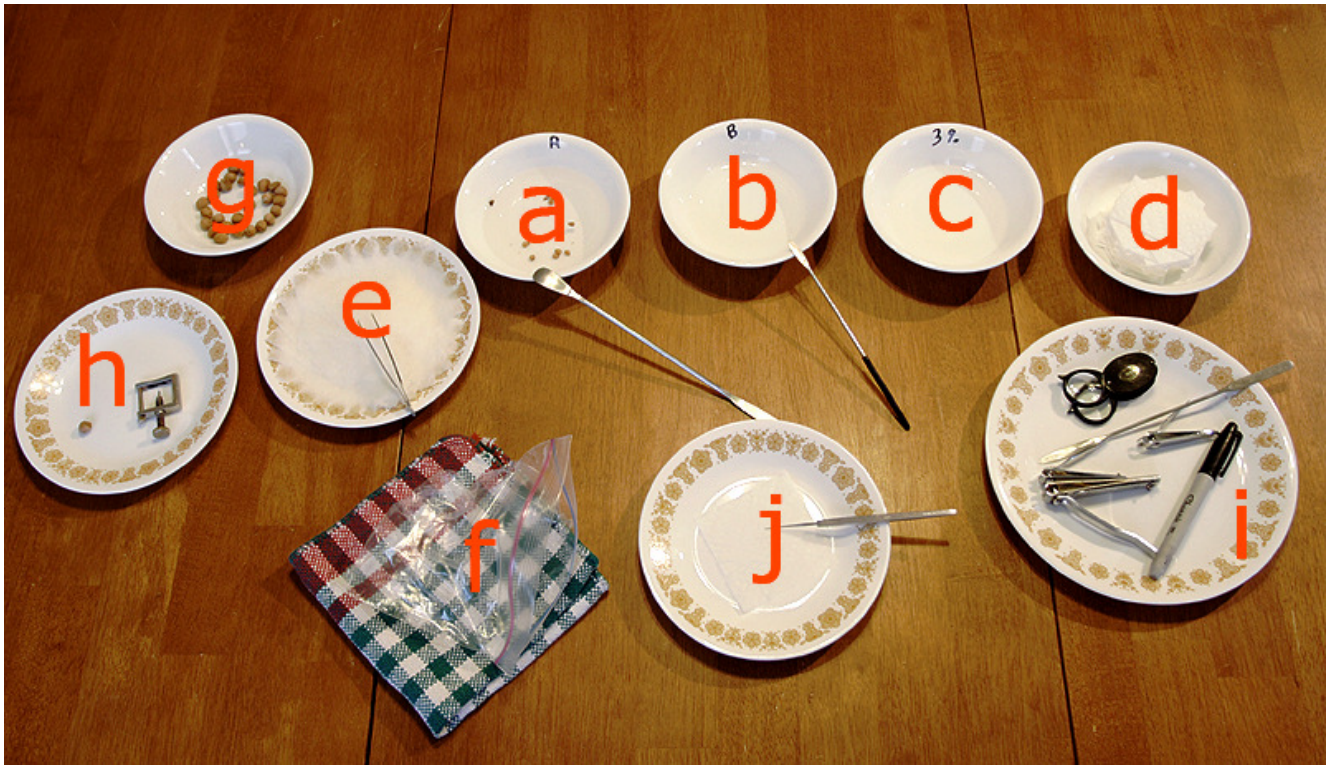

Figure 9. Extraction theatre: **a**. bowl with Solution A for receiving and holding extracted embryos; **b**. bowl with Solution B for receiving and holding embryos after removing their testae. **c**. bowl with 3% hydrogen peroxide for wetting sanitary towelettes; **d**. bowl with sanitary towelettes; **e**. plate of prepared excision beds wetted with Solution B; **f**. cloth and baggie to be used as a 'glove' for manipulating sanitary towelettes wetted with 3% hydrogen peroxide; **g**. bowl for holding seeds to be extracted. **h**. plate for catching bits of pericarp and dropped seeds; **i**. plate with extraction tools; **j**. plate with excision bed and hooked needle probe.

Preparing the Extraction Theatre

Clean the surface of your table with soap and water. Lay out your equipment and materials to create an organized theatre for processing embryos as follows (Figure 9).

Mark three small bowls that will contain the working solutions of hydrogen peroxide: A, B and 3%. Lay the bowls out side by side in front of you, set back so that you can reach them easily but still leaving room to work directly in front of you.

Place a small flat plate on top of the bowl marked "B". The bowl and the plate will be used while excising embryos from their testae but, until that step, the plate will keep bits of pericarp from flying into the bowl.

Place the small bowl containing the sanitary towelettes where you can reach them easily while seated.

Place the plate of excision beds within easy reach off to one side on the table. Leave it covered until the excision step.

Place a small wash cloth on the table next to your 'helper' hand (if you are right-handed, your helper hand is your left hand). Open a fresh baggie and place it on the cloth with the open end facing you. You will use this as a makeshift glove to hold the sanitary towelettes from time to time as you work, as these will be wetted with 3% peroxide which would otherwise irritate your fingertips. The small wash cloth will absorb any peroxide solution that drips from the baggie after you've used it.

Place a small bowl and a small plate directly in front of you. The bowl will hold your seeds and the plate will catch dropped seeds and bits of pericarp as you work.

Gather your tools together and wash them in hot, soapy water, rinse and dry them and place them together on a clean plate within easy reach.

Pour some of Solution A into the bowl marked "A". The solution should fill the bowl only about ¼" deep, just enough to allow the embryos to be completely immersed.

Pour 3% peroxide from the bottle into the bowl marked "3%". This will be used to wet the sanitary towelettes as needed by dipping them into it. Fill the bowl about 3/8" deep, and replenish as needed.

Sanitary technique

We won't be using sterile technique but we will be keeping things very clean.

Most of the microbes that we are worried about come from the seeds themselves. The overall goal of using sanitary technique is to prevent the transfer of microbes from the seed coats to the embryos and from one embryo to another. We are not trying to sterilize everything; we just want to keep the bugs from hopping around the table.

Wipe down the lip of the bowl containing Solution A by using a sanitary towelette as follows. Put your helper hand into the baggie reserved for this purpose—this measure prevents repeated contact with peroxide which would otherwise lead to skin irritation. With your other hand, pick up a sanitary towelette and dip it about 1/3 of the way into the bowl of 3% peroxide. Hold it by the dry end and work quickly before the solution soaks the towelette. Place the towelette on the rim of the bowl containing Solution A and release it. Hold the bowl with your fingers placed exactly on the mark you made to label the bowl, and nowhere else. Use your 'gloved' hand to wipe the rest of the rim with the towelette, lift the towelette off and discard it in the trash. From this point on grip the bowl only at the place where it is marked to prevent contamination of the clean portion of the rim.

Keep the 'glove' on.

Wipe down the needle probe as follows. Wet another sanitary towelette as before but this time place it on the palm of your 'gloved' hand, with that hand held palm upwards. Lay the needle-end of the probe on the towelette then draw it across, rotating it as you do to wipe the entire needle. Set the probe down on a clean plate within easy reach, taking care not to let the needle touch anything. Discard the used sanitary towelette.

Repeat this process with any other probes, spatulas, tweezers and any other tools that may contact an embryo. It is not necessary to wipe down the clipper tools because, hopefully, these won't actually touch an embryo.

However, you should wash the clipper tools with soap and water whenever you start working on a different group of seeds, or before going on to the next seed if you have worked on a seed that is oozing microbes.

Workflow

The sequence of tasks we will be performing is as follows.

- Extract all the embryos from their seeds and place them together in the bowl with Solution A.
- Move each embryo to the excision bed one by one, remove its testa, and then place it in the bowl with Solution B. Repeat this for all the embryos.
- Move the embryos from the bowl of Solution B into a prepared incubation baggie, six embryos per baggie.
- Tape the incubation baggies together and suspend them from the side of a fluorescent light fixture.

Extracting the embryos

Place the seeds in a small bowl. Remove them one by one and work on them as you hold them over a small plate. This will help to collect the bits of debris that you generate, and—more often than not—catch the seed when you drop it.

As you extract embryos place them gently into the bowl with Solution A using a spoon spatula, or drop them from the pericarp—whatever works: just treat them gently. Leave their testae on; we will remove those in the next step. Swirl the bowl from time to time to make sure the embryos are wet with solution and not floating, but do not touch the embryos in an effort to immerse them because you will crush them. If necessary, blow puffs of air onto the embryos to immerse them. Don't be concerned with contaminating them with your breath: the solution will prevent this.

You may notice tiny bubbles rising from the embryos and forming little clouds above them on the surface of the solution. These are bubbles of oxygen being formed by enzymes (peroxidases) present in the embryos. They are a good sign because they indicate that the embryos are alive. Active peroxidases are present both in the testae and the embryos. You will see more of them in Solution A than in Solution B because there is soap in Solution A and because the concentration of hydrogen peroxide is greater in solution A.

Leave the embryos in the solution for at least fifteen minutes after extracting the last embryo to give the peroxide time to do its job. It is an excellent disinfectant but it needs time to work, unlike isopropyl alcohol which works pretty much instantly.

This brings up the question of why we are not using alcohol instead of peroxide to disinfect our tools and dishes, or even the embryos. This is because alcohol is toxic to embryos—and we need to incubate them in peroxide solution to promote germination. Nor do we want to use alcohol on our tools because alcohol and hydrogen peroxide will react with each other, possibly explosively – when combined they make an excellent rocket fuel, for instance.

If you damage an embryo during extraction, do not assume it has been killed. I have plenty of seedlings with nicks in their cotyledons. However, if the cotyledons split apart then retain only the cotyledon to which the radicle remains attached, if it does at all. There is a chance that this will form a proper seedling. If you damage the radicle or any tissue very close to the radicle then discard the embryo and move on.

Excising the testae

Wash your hands with soap and water and dry them with a clean towel.

Empty the seed bowl and dissection plate of debris and set them aside, out of your way. Clean the table top in front of you with a damp cloth, removing the bits of pericarp that inevitably fly about.

Lift the plate that is covering the bowl labeled "B" and place the plate directly in front of you. Fill that bowl with solution B to about ¼" deep. This will be used to receive the naked embryos after you remove their testae. Wipe down the rim of the bowl with a sanitary towelette as you did for the first bowl, and clean any tools you used during the extraction step by wiping them down with 3% hydrogen peroxide as before, using the plastic baggie as a glove.

Rest the spoon-end of one spatula face down on the rim of the bowl containing the embryos. Rest the spoon-end of a second spatula on the rim of the bowl that will receive the naked embryos (see figure 9). After each transfer of an embryo in the next step, replace the spoon in the same place on the same bowl. Don't despair if you drop the spoon on the table, just put it back again. The idea here is to keep the working parts of your tools clean and under control at all times so that you do not mix fluids from one bowl to the other. This will become a convenient habit as you start to pick up momentum.

Using your tweezers, pick up the marked towelette from the dish of extraction bed towelettes, flip the towelette over and place it flat on the center of the small plate in front of you. This exposes the underside of the towelette, which is free from dust and particles that may have settled on it. Place your tweezers on the plate of prepared excision beds such that the points sit on the towelettes and the other end sits on the edge of the plate. You will mostly be using the tweezers to change towelettes, and this will keep them clean for that purpose.

As you proceed from embryo to embryo, take fresh excision beds from the plate in a counter-clockwise fashion. They were laid out with this purpose in mind, so as to minimize the effort required to change excision beds.

Place a cleaned needle probe on the excision bed in the plate in front of you such that the point lies more or less in the center of the towelette.

Carefully retrieve an embryo from the bowl of extracted embryos, using the spatula lying on that bowl. Once you have the embryo in the spoon, tilt the bowl *away* from you slightly and touch the spoon to the inside surface of the bowl nearest you. This allows any solution it contains to run back into the bowl while retaining the embryo in the spoon. The objective is to transfer the embryo to the excision bed with a minimum of fluid transfer. By tilting the bowl you can keep the spoon relatively level as you drain solution from it, so that the embryo does not also spill back into the bowl.

Place the embryo on the excision bed (the towelette on the small plate). Orient the embryo so that you can easily touch your curved needle probe to the end opposite the radicle. Brace the end with the radicle very gently under the index finger of your other hand. This is the only time you will ever touch that end of the embryo. You are very big, the embryo is very small, so work very slowly and deliberately until you get used to this technique. Apply only enough pressure to the embryo to keep it positioned firmly under your finger as you work.

With the embryo braced by your index finger, apply the rounded portion of the hooked needle probe to the other end of the embryo and gently abrade the testa. It will begin to come apart slowly (Figure 10).

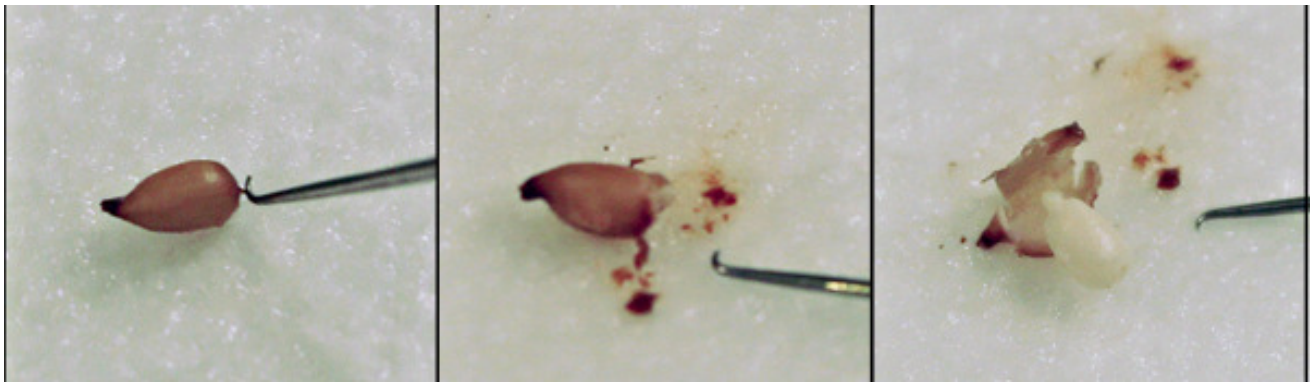

Figure 10. The testa is removed from an embryo by abrading the end opposite the radicle to create a small tear, then expanding the tear in a circular fashion until the embryo slips out.

The testa has two layers, which will become evident as you work to remove it. The outer layer is very thin, is usually dark-colored and will come apart easily. The inner layer is much like cellophane tape or a tiny plastic bag wrapped around the embryo and will take a little more work to tear. Continued gentle abrasion will be enough to tear it. Once it begins to tear, work to expose the embryo completely at that end. Eventually you will remove enough of the testa so that the embryo will slide from the remaining testa and lie naked on the excision bed.

> If the testa sticks to the embryo such that it does not slip out and the only way to remove it completely is to peel it, then the embryo is not fully hydrated. In this case, leave the remaining embryos to soak in Solution A long enough period to become fully hydrated. Embryos may be left in Solution A for several hours without harm. If overnight soaking is necessary, then pour 60 mls of Solution B into the bowl of embryos to dilute the concentration of hydrogen peroxide. In this case, embryos can be left for up to 24 hours to hydrate. Unusually large or dry embryos may need longer than 24 hours to hydrate. In such cases, pour off most of the solution after 24 hours and replace it with fresh Solution B.

Using the spatula that is lying on the receiving bowl filled with Solution B, lift the naked embryo from the excision bed and place it gently into the solution in that receiving bowl. Embryos without their testa are very fragile so minimize handling of them and treat them with extreme care.

If you must work with only one spatula, then clean it with 3% peroxide after every time you move an embryo so that you do not transfer microbes from one bowl to the other.

After you have excised all the embryos from their testae, inspect them for viability. Fully hydrated, viable embryos are usually pearl-white and glisten like a wet, hard boiled egg. Embryos that appear dull but are white may still germinate, but embryos that are translucent, dark or discolored are probably dead. Discard any dead embryos as they would only serve as a free lunch for microbes in the incubation baggies. If you wish to try to germinate suspect embryos, incubate them together in a separate incubation baggie.

While most viable embryos are white, there are exceptions. For example, the embryos of Sassafras tzumu, a Japanese variety of Sassafras, are stained brown by a pigment in their thin pericarps and appear mottled.

Culturing the embryos

Wash your hands with soap and water and dry them with a clean towel.

Remove an incubation baggie from the refrigerator and label it using the permanent marker. Open the baggie and reach into it with your fingers to spread the sides apart and lift one side of the baggie away from the wetted towel that it contains.

Bring the bowl of naked embryos forward and use the spatula lying on that bowl to retrieve an embryo. As before, tilt the bowl away from you and touch the spoon to the bowl to allow any solution contained in the spoon to run back into the bowl while retaining the embryo.

Hold the incubation baggie open with one hand and insert the embryo into the baggie. Deposit it gently on the incubation bed (the towelette inside the baggie) about 3/8 inches (1 cm) from the top of the bed. Using the spatula, gently rotate the embryo so that the radicle is pointed toward the bottom of the baggie. This will help keep the radicle from twisting as it emerges because it will want to grow downward (due to *gravitropism*).

Repeat this with all the embryos, placing up to six embryos in a single incubation baggie. Space them evenly on the paper towel. Six embryos per baggie allows for spacing adequate to prevent cross contamination from one embryo to another. Close the baggie while holding it taut to minimize trapped air. Place the baggie aside in a safe place until you have finished extracting embryos for the day.

Clean your workspace and all your tools before proceeding to the next batch of seeds. Empty the solutions from the two receiving bowls. Wash them with soap and water and let them air-dry. Replenish your peroxide solutions, towelettes and other supplies if necessary. Wipe down your tools and bowls as before with 3% hydrogen peroxide.

Incubation

When you have finished extracting embryos for the day, collect the incubation baggies containing your embryos and tape them together. Do this by first laying the baggies embryo-side down on the table and stacking them so that the bed in each one is positioned below the bed of the one beneath it (Figure 11). Place tape horizontally across the top of the upper baggie. Tape no more than six incubation baggies together – this will ensure that the embryos are suspended close enough to the light bulb to receive adequate illumination for germination.

The embryos need light to germinate and ‘green up’ – there is a step in the biosynthesis of chlorophyll that requires light. As the embryos become seedlings, they will begin the process of photosynthesis using carbon dioxide that diffuses into the baggie from the atmosphere.

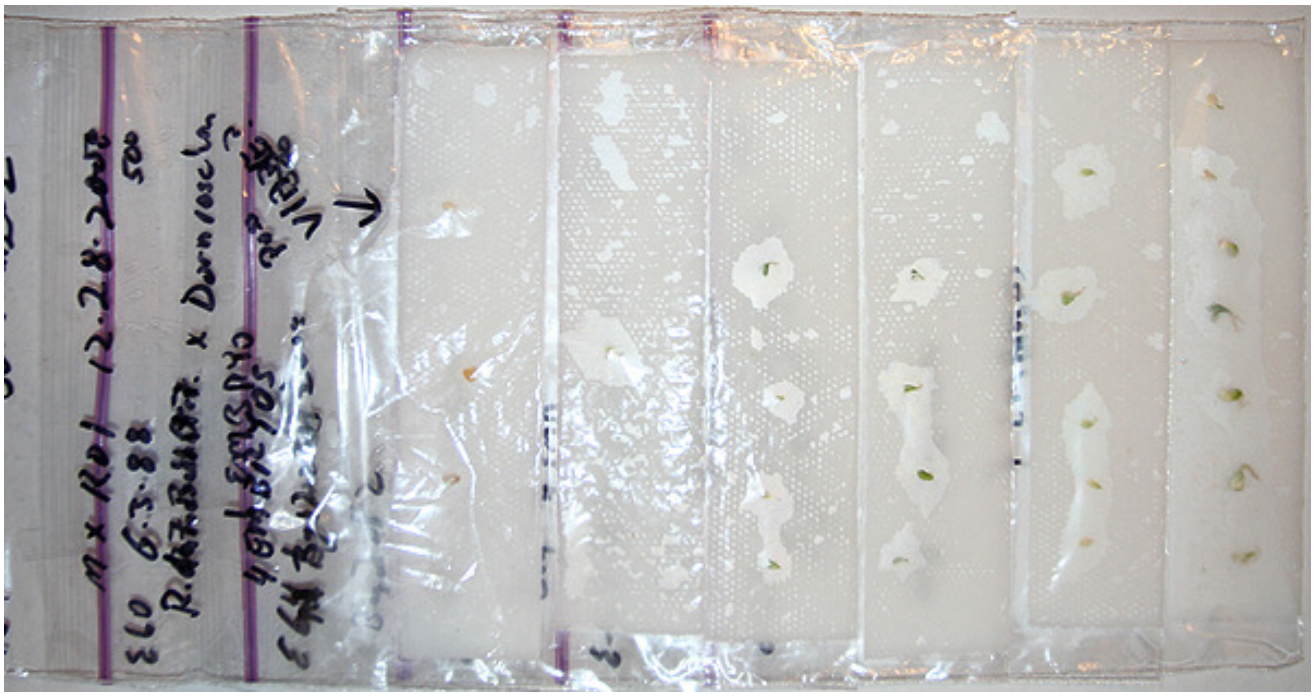

Figure 11. Tape the incubation baggies together to optimize the space required to suspend them under lights. Avoid pressing on the embryos as you place them face down on a table to tape them.

Photosynthesis requires light. Artificial lighting is barely sufficient to support photosynthesis. Leave the light turned on continuously to compensate for this. There are no biological requirements for a dark period for germinating *mature* embryos from seeds exhibiting orthodox dormancy (however, see [3a] regarding a dark requirement for *immature* embryos). Some plants do require a dark period to induce flowering; roses are not among these.

Suspend the baggies all together from the side of a fluorescent light fixture using transparent tape. Hang the baggies so that the top-most excision bed is about two inches/five centimeters below the level of the light bulb. At an ambient temperature of 65° F/18° C the temperature at the surface of a 40 watt F12 cool white fluorescent tube is about 85° F/29° C. F8 tubes are somewhat warmer. Incubation beds (and embryos) in baggies placed too close to the bulb will dry out from evaporation.

Optimal temperatures for germination have not yet been determined for this method. To date, all embryos tested have germinated readily at a temperature of 65° F/16° C. Warmer temperatures increase the rate of germination (and growth of microbes) and lower temperatures retard the rate of germination. Rose seeds incubated at 80° F /27° C have germinated and have been ready to pot up in as few as six days. For rose seeds at 65° F/16° C germination usually takes about 10 to 14 days, though there is a lot of variability.

The efficiency of photosynthesis drops off dramatically as temperatures approach 90° F/32° C, so manage your incubation baggies (and seedlings) to keep them well below this temperature[18].

In general, germinations should begin within three to five days. The first sign of germination will be greening of the cotyledons, followed by swelling, then extension, of the radicle and hypocotyl. Cultivars differ in the rate at which they germinate and grow, and individual embryos can differ quite a lot from each other even within the same cultivar or cross. Germination occurs regardless of whether the seeds from which the embryos originate have been stratified under refrigeration.

The size of an embryo affects the time required for germination to begin. Large embryos such as citrus and sassafras can take longer to metabolize (or leach) residual ABA, and so germinate less quickly than small embryos. Sometimes this delay can be shortened by soaking the excised embryos in Solution B for a day or two, provided that the solution is poured off and replaced every 12 hours and monitored closely for contamination. Be aware, however, that prolonged soaking in dilute hydrogen peroxide solution can eventually 'burn out' the embryos, leading to their death.

Watch your incubation baggies for signs of contamination. The hydrogen peroxide in each baggie will break down into oxygen and water after only a few days but so long as the baggies remain closed they will be remain relatively free of contamination. Certain fungi, notably *Aspergillis niger*, can subsist quite well by digesting paper and this is the most common form of contamination that first appears as time goes by.

Dead embryos will begin to decay very quickly, usually in a day or two. They become discolored and begin to disintegrate. Often they are quickly enveloped in microbial growth, indicating that they were already heavily contaminated at the time they were cultured. Small embryos can even disappear, melting into the paper towelette and leaving only a discolored spot.

If you are worried that your embryos are threatened by visible microbe colonies, simply transfer them to a freshly prepared incubation baggie (see below for instructions on removing seedlings from their baggie). If a particular seedling or embryo is contaminated but alive and you want to try to save it, remove it and soak it in Solution A for about 30 minutes, rinse it in Solution B and place it alone in a freshly prepared incubation baggie, transferring the other embryos to a separate fresh incubation baggie. No matter what you do some embryos will be contaminated beyond recovery. Remarkably, though, these often disintegrate while the embryos next to them carry on, oblivious to the demise of their neighbor.

Seedlings

Not all embryo germinations result in viable seedlings. If the radicle of an embryo has been damaged, the seedling may germinate without extending a root or the root may be truncated. This can happen even when an embryo is undamaged, and often does. Rootless seedlings have been the most common abnormality seen with roses. Another common abnormality is seedlings that fail to develop true leaves.

Sometimes incomplete germination occurs. The radicle may extend somewhat, and the cotyledons may expand and may even turn green or partially green. The embryo remains alive but stops growing while other seedlings from the same source develop normally and thrive.

When the radicle has developed enough to have root-hairs, the seedling can be transferred to soil. Do not be in a hurry to transfer seedlings to soil. I often wait until the first set of true leaves has emerged.

The roots of seedlings can dry out very quickly as you remove them for potting or transfer. Make preparations so that seedlings can be moved directly from the incubation baggie into soil or into new baggies, if necessary, without delay. If you will be processing many seedlings at once you can collect them into a bowl filled either with cold, clean water or with Solution B, depending on whether they are being transferred to soil or to fresh incubation baggies, respectively.

If you will be transferring seedlings to a fresh incubation baggie, clean your spatula and finely pointed, curved tweezers by wiping them with a sanitary towelette wetted with 3% hydrogen peroxide as before. Dip the spatula and the blades of your tweezers into freshly prepared Solution B to rinse off the 3% hydrogen peroxide and place the tools on a clean plate.

Transferring seedlings

Lay the baggie flat on a cutting board. Use a clean, sharp razor blade to slice through the baggie along the top of the incubation bed, taking care not to cut through any taller seedlings that may be sticking up above the bed. Next, slice through the baggie along both sides of the incubation bed. Fold back the plastic covering the embryos on the incubation bed and the slice it off along the bottom of the bed to completely expose the bed.

Examine the roots of your seedlings to identify any that are growing into the paper towel. These will require careful removal of the towel covering the roots to free them from the bed, using a spatula and your finely pointed tweezers. Do not tug on the seedling to try to pull it free from the incubation bed, as you will likely break the root off. Free any roots that are growing into the paper towel as follows.

With one hand, place the tip of the spatula against the towel about 3/16”/3 mm to the side of the root where it enters the paper towel and hold the towel firmly against the cutting board. With the tweezers held in your other hand, gently pinch the paper towel where it covers the root and lift it away and to the side, tearing the paper towel a bit at a time and teasing it off the root. Take care not to poke the root with the tweezers or break the root as you tease away the towel. Repeat this action along the root until it is completely freed from the paper towel.

The roots of the remaining seedlings (those not growing into the paper towel) will usually adhere to the paper towel because their root hairs grow into the towel a little bit. Separate the root of each seedling from the towel as follows.

Squeeze your curved, finely pointed tweezers closed (blades touching together). Slip the blades of the tweezers under the stem of the seedling, between the seedling and the towel. Slowly slide the tweezers between the root and the towel, moving toward the tip of the root. As you proceed, watch closely to be sure that the root has not grown into the paper towel somewhere along its length.

Potting seedlings

Select a high quality potting soil that suits your particular needs and situation. I use Miracle Gro® Organic Choice brand potting soil which I steam-sterilize it to eradicate bugs (spider mites have been a problem for me with this product) then pass through a 3/16” mesh screen and mix with perlite, about 2/3 soil and 1/3 perlite.

Wean your seedlings from their incubation baggie gradually by transferring them each to a small cup of soil and then covering the cup with a bit of polyethylene cut from a baggie and held in place with a rubber band. The same 3 oz. plastic cups used to prepare the hydrogen peroxide solutions work nicely if you poke six or seven evenly spaced holes in their sides along the bottom edge for drainage. A small soldering iron can be used for the purpose.

Water the seedlings from the bottom by floating the cup in a pan or bowl of water for several minutes as needed. Remove the plastic covers when the seedlings grow tall enough to be pressing against them. As a final tip, I find that cups of this size (3 oz./90 ml) require watering at a rate of one tablespoon per day, on a three day watering interval.

The care and feeding of your seedlings from this stage onward is up to you.

Conclusion

All things being equal, it is better to germinate your seeds the old fashioned way by stratification, refrigeration and incantation. Embryo culture is a labor intensive technique that most folks will reserve for their 'hard case' seeds. For these seeds it can make all the difference in the world.

For example, this technique has been used to germinate embryos from *Prairie Peace*, a rose by Robert Erskine that has seeds that had never been germinated before. The same is true for some of Walter Schowalter's *Ross Rambler* seedlings and for Percy Wright's *Yellow Altai* and *Kilwinning*, all of which now have seedlings by this method.

Further refinements of the method are needed. Optimizing such variables as temperature and hydrogen peroxide concentration and exposure time would benefit people wishing to use the technique routinely or for particular species or cultivars. The use of sucrose and hormone supplements during incubation may be found to enable the development and germination of immature embryos, possibly extending the usefulness of this technique of embryo *culture* to embryo *rescue*[19].

Another refinement would be to identify a suitable inorganic material for use as incubation beds to replace paper towels. Molds feed on paper, and often times the contamination that occurs in incubation baggies is because of this rather than because of infected embryos. Fiberglass cloth is an obvious but expensive alternative relative to paper towels. Another possibility is to dispense with incubation beds altogether, following the example of an RHA messageboard participant who uses empty jars instead[20].

The ability to germinate seeds by this method immediately after harvest offers some tantalizing prospects for accelerating the hybridization cycle. In principle, at least, such embryos can become plants ready to breed the following season where a greenhouse and supplemental lighting are available. For some plants, the breeding cycle could even be multiplied to two or more generations per year. This is quite feasible with seedlings that have the juvenile remontancy trait and so flower within weeks of germination.

For cultivars that have seeds which normally require more than a single season of stratification to germinate, that long wait can be eliminated. For instance, *R. canina* embryos (when they can be excised without damage) begin to germinate as well as any other, in only a few days.

Viability testing is another application for this method. Such testing is already done by embryo extraction but usually requires sacrifice of the embryo for staining[21]. A useful goal in this respect would be to determine the best storage conditions (moisture and temperature) for rose seeds, something that is still not well established. It can also be used to establish the best stratification procedure, one which promotes spontaneous germination while minimizing losses to disease.

Experimentalists may be enticed by the prospect of working with embryos, knowing that they can reasonably be expected to grow into plants if they survive the experimental treatment. One example might be polyploidy induction of diploid and triploid cultivars using colchicine expressed from crocus bulbs or oryzalin applied to the embryos[22].

Hybridizers can look forward to answering some old questions, such as whether seeds that have been frozen remain viable; whether particular pesticides affect the ability of seeds to germinate; and whether particular treatments enhance or diminish embryo viability and germinability.

We have been using the term 'mature' to mean the point at which an embryo is morphologically and physiologically fully formed and capable of germinating and then surviving on its own. The age of an embryo at which maturity is reached—as measured from the time of fertilization—is not well established, at least for roses. This method has an obvious use for determining the minimum time and temperature ('degree-days') required for seeds to mature.

Likewise, breeders can test the validity of certain long-held beliefs about seed germination. One of these is that viable seeds sink in water while non-viable seeds float (the basis for the so-called *float-sink* test). Another is that particular colors (wavelengths) of light, especially red light, promote germination.

However you decide to apply this technique of embryo culture, I wish you the best of success. If you find this manual to be useful, and especially if this technique helps you to accomplish something new and interesting, I would welcome learning about it. If you can improve on the method, or find something that needs correction or clarification, I would welcome your comments. My current contact information can always be found on my website, http://www.holeman.org. Feel free to drop me a line.

All the Best
Don Holeman

References

1. Current contact information may be found at http://www.holeman.org, my email address at the time of publication being don@holeman.org.
2. Hartmann H, Kester D, Davies F. *Hartmann and Kester's Plant Propagation Principles and Practices 7th edition.* p. 141 (1995) Prentice-Hall.
3. For an assessment of some techniques for roses see Zhou, Z, Bao, W, Wu, N. "Dormancy and germination in Rosa multibracteata Hemsl. & E. H. Wilson". *Scientia Horticulturae* 119 4, pp 434-441 (2009); See also [5].
4. See, for instance, a. Asen, S. "Embryo culture of rose seeds" in *The American Rose Society Annual* p.151-152 (1948); b. Pierik, R. *In Vitro Culture of Higher Plants: 4th Edition*. (1998) Springer; c. Mohapatra, A, Rout, G. "Study of embryo rescue in floribunda rose" *Journal of Plant Cell, Tissue and Organ Culture* 81:113–117 (2005); d. Zhou Z, Bao W, Wu N. "Dormancy and germination in Rosa multibracteata Hemsl. & E. H. Wilson". *Scientia Horticulturae* 119 4, 434-441 (2009).
5. Doorenbos, J. "Review of the literature on dormancy in buds of woody plants". *Medelingen van de Landbouwhogeschool to Wageningen/Nederland* 53 1-24 (1953).
6. The most comprehensive review of germination to date is Baskin, C. and Baskin, J. *Seeds: ecology, biogeography, and evolution of dormancy and germination*. Academic Press. 1998.
7. Buckley, F. *Germination of Rose Achenes*. p. 4. (1985) Amateur Rose Breeders Association.
8. I found no mention of *ring fibers* in the scientific literature although my reading was limited to a few commonly available texts on plant anatomy. Careful dissection shows them to actually be a single, continuous fiber originating and terminating at the axial apices of the embryo and spooling in a single layer around the embryo. A more appropriate name might therefore be *spool fiber.* It seems likely that this spool fiber originates as a modified stem during seed formation. If so, one wonders whether it is a conduit for photosynthate to feed the developing embryo.
9. The mystique surrounding seed germination, and rose seed germination in particular, starts to evaporate when you get into embryo culture. From my reading and my own observations I have concluded that germination is a balancing act between two enzyme systems - one in the testa (and possibly also the pericarp) that produces abscisic acid and the other in the embryo that degrades abscisic acid. There is a threshold level of abscisic acid below which an embryo will germinate. Temperature influences these

enzyme systems, higher temperatures favoring the formation of abscisic acid and lower temperatures favoring degradation of abscisic acid.

The testa and pericarp are living tissues and can continue to produce abscisic acid long after the seed has been removed from the hip. Thus, for an embryo to emerge from dormancy the seed has to be kept humid enough for the enzyme systems within the embryo to degrade abscisic acid; and cold enough so that the testa doesn't keep on making abscisic acid to replace that which is degraded by the embryo. Rose seeds can drown, and they go completely dormant when desiccated, so stratification is really the art of providing just enough moisture at just the right temperature to allow the embryo to continue to metabolize and thus rid itself of the abscisic acid holding it in dormancy.

Furthermore, all this metabolic activity in the embryo, testa and pericarp takes energy and that energy has to come from somewhere. The only possible source of energy for all this metabolism is that which is stored in the embryo itself, in the form of fats and oils. I believe this is the reason that I find that embryos that have been stratified for lengthy periods - four or more months - often appear to be emaciated and that more of them are simply dead than are embryos extracted sooner after harvest.

10. Some seeds such as have a separate storage organ called the *endosperm.*
11. Zlesak, D. Personal communication.
12. My sense is that thick pericarps (hence, large seeds) protect embryos far longer than thin pericarps. I have made some observations on very old rose seeds that support this hypothesis. The seeds of certain cultivars, *R. acicularis* Dornroschen, in particular, maintain embryos intact (that is, not rotted, molded or otherwise disintegrated) for exceptionally long periods of time. The seed pictured in Figure 2, from a Jackson and Perkins cultivar (74-3045) that was never released commercially, was over 20 years old. The embryo failed to germinate but did turn slightly green.
13. Zlesak, D C. “The effects of short term drying on seed germination in Rosa”. *HortScience* 40 6 1931-2 (2005).
14. It seems clear to me that seeds intended for sowing should also be treated in this manner to suppress microbial growth.
15. Fischer Scientific catalogue number 05-847Q (search at http://www.fisher.com) or Troemner (the actual manufacturer) catalogue number 916062 (see http://www.troemner.com/equip_labjaws_flow.php, downloaded February 4, 2008).
    Separate the upper jaw of the clamp from the thumbscrew. Retain both pieces.
    Turn down the lower 3/16” (4.8 mm) of the shaft of the thumbscrew to a diameter of 1/16” (1.5 mm).
    Make a rounded convex point on the tip of the shaft with a nominal angle of about 30º.
    Polish the point to minimum fineness of 600-grit.
    Grind the middle portion of the base of the clamp to a width of 7/32” (5.6 mm) to make it easier to manipulate seeds.
    Round off any rough edges and polish the base to a minimum fineness of 600-grit so that it is comfortable to hold during use and slides easily in your fingers.
    Use gel-type Krazy®-Glue or Super-Glue® or a quick-setting epoxy to attach a piece of high quality, durable and waterproof 80-grit silicon carbide sandpaper to the concave face of the lower jaw, grit-side up. The sandpaper can be held in place until the glue sets with the upper jaw that was removed earlier, secured with a standard screw that matches the thread pattern of the thumbscrew. Replacement of the sandpaper will be an ongoing maintenance task. Sanding disks, meant for mounting on a rotary sander or drill motor, are thicker and more durable and last longer in the gadget.
    A similar device can be made from a less expensive Hoffman tubing clamp, http://sciencekit.com/hoffman-screw-tubing-clamp/p/IG0026560/ (downloaded February 4, 2009), SKU WW61130M02 or see the manufacturers website http://humboldtmfg.com/lab/screw-compressor.html (downloaded February 4, 2009), catalogue number H-8720. If using the less expensive Hoffman clamp

be sure to align the base exactly perpendicular to the thumb-screw to prevent clutching-off of the seeds as you work on them. With that in mind, protective eyewear is recommended with the use of this device, as it is with fingernail clippers. To use the device most effectively, position the point on a suture of the seed with the thumbscrew aligned with the plane of the suture. Drive the screw slowly into the suture, being careful not to penetrate the embryo. It is often necessary to work the point at several places along the suture before it yields.

16. Hydrogen peroxide jump-starts germination processes in the embryos by fueling enzymatic (peroxidase) reactions in certain biochemical pathways. See Ching T.M. "Activation of germination in Douglas fir seed by hydrogen peroxide" *Plant Physiology* 34:557-563 (1959) for an early paper on the subject, downloaded October 3, 2009 from http://www.plantphysiol.org/cgi/reprint/34/5/557 . Hydrogen peroxide has other practical horticultural uses as well. See Davis L. "The value of peroxide" Vol. XL No. 3 (Fall 2009) *Rose Hybridizers Association Newsletter* for advice on using hydrogen peroxide to improve the rooting of roses.
17. The quality of tap water quality varies widely, although if you use tap water successfully for germinating your seeds naturally then you should have no trouble using it to germinate embryos using this method. Filtered or bottled drinking water (without preservatives) or distilled or de-ionized water can be used to make up your hydrogen peroxide solutions if necessary.
18. See Figure 13.14 and the accompanying discussion about photorespiration in Mohr H, Schopfer, P, Lawlor G. *Plant Physiology* p.200 (1995) Springer. This is an excellent textbook on plant physiology.
19. Mohapatra & Rout [3a] found that hybrid-tea rose embryos were immature when harvested 40 days after pollination and that they required about four additional weeks to reach maturity and germinate *in-vitro* on standard plant tissue culture media supplemented with plant growth hormones and 3% sucrose.
20. See the discussion at http://www.rosehybridizers.org/forum/message.php?topid=23111&rc=59&ui=2406478589 . George also dispenses with the use of hydrogen peroxide and extracts embryos using a box-cutter style razor blade, neither being a practice I endorse but which do illustrate the fundamental simplicity and effectiveness of embryo culture.
21. Moore, R. "Tetrazolium as a universally acceptable quality test of viable seed". *Proc. Int. Seed Testing Assoc*. 27 795–805 (1962).
22. Numerous protocols exist for the purpose. For a compendium see http://members.tripod.com/~h_syriacus/tetraploidy.htm, downloaded February 6, 2009. A quite suitable method for inducing polyploidy in roses that involves apical meristems rather than embryos has been published by David Zlesak, see "Trifluralin-mediated polyploidization of Rosa chinensis minima (Sims) Voss seedlings", *Euphytica* (2005) 141: 281–290.

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