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PROTOCOL NOTE

Applications in Plant Sciences

Embryo excision in Compositae, with implications for combating biodiversity loss

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Abstract

Premise: Embryo excision is an effective, under-described means of promoting germination in the sunflower family and may help to ensure the survival of endangered taxa or lineages with limited seed availability.

Methods and Results: We describe and illustrate a detailed method of embryo excision used successfully to stimulate germination in a diversity of composites and that requires minimal materials and expense, using *Layia platyglossa* as an example. We show how this procedure greatly increases germination compared to control treatments in *Madia elegans*, a close relative of Hawaiian silverswords that exhibits physiological dormancy.

Conclusions: This technique can be learned quickly and is highly effective. Embryo excision can aid conservation efforts dependent on minimal seed resources by enhancing germination and allowing evaluation of seed quality before or after storage, as well as synchronizing seedling development, thereby allowing for refinement of ex situ seed bank conditions and efficient use of horticultural resources.

KEYWORDS

Asteraceae, endangered plants, ex situ conservation, germination, *Layia platyglossa*, *Madia elegans*, Madiinae, seed banks

Resumen

Premisa: La escisión de embriones es un medio eficaz y poco descrito para promover la germinación en la familia Asteraceae y puede ayudar a garantizar la supervivencia de taxones o linajes en peligro de extinción con disponibilidad limitada de semillas.

Métodos y Resultados: Describimos e ilustramos un método detallado de escisión de embriones utilizado con éxito para estimular la germinación en una diversidad de asteráceas y que requiere materiales y gastos mínimos, utilizando *Layia platyglossa* como ejemplo. Mostramos cómo este procedimiento aumenta en gran medida la germinación en comparación con los tratamientos de control en *Madia elegans*, un pariente cercano de las silverswords hawaianas que exhibe latencia fisiológica.

Conclusiones: Esta técnica se puede aprender rápidamente y es muy eficaz. La escisión de embriones puede ayudar a los esfuerzos de conservación que dependen de recursos mínimos de semillas al mejorar la germinación y permitir la evaluación de la calidad de las semillas antes o después del almacenamiento, así como sincronizar el desarrollo de las plántulas, permitiendo así el refinamiento de las condiciones del banco de semillas ex situ y el uso eficiente de los recursos hortícolas.

The sunflower family (Compositae or Asteraceae) represents about one-tenth of flowering plant species globally (Mandel et al., 2019), with an extensive diversity of rare taxa, including >1000 species considered Vulnerable, Endangered, or Critically Endangered by the International Union for Conservation of Nature (IUCN, 2023). On the world's oceanic islands, for example, where plant endangerment and extinction are exceptionally high (see Rønsted et al., 2022), Compositae are better represented than other flowering plant families by species that are endemic to only one island (Lenzner et al., 2017; also see Crawford et al., 2023). Such species include representatives of major adaptive or ecological radiations (e.g., Cho et al., 2020; Fernández-Mazuecos et al., 2020; Knope et al., 2020; Baldwin et al., 2021; Mort et al., 2022; also see Schenk, 2021; Cerca et al., 2023), yet many are in decline, if not already extinct, as victims of direct or indirect impacts of human activities (IUCN, 2023). The pace of loss of native plant diversity on islands and elsewhere often warrants active intervention that may include facilitation of sexual reproduction by such direct means as hand pollination (see, e.g., Walsh et al., 2019) or ex situ cultivation and propagation (e.g., Mounce et al., 2017; Werden et al., 2020). Seed collection and storage under conditions favoring long-term embryo survival is a crucial component of endangered plant conservation (Havens et al., 2004; Chau et al., 2019; Walsh et al., 2024), as is refinement in our ability to assess the status of such collections over time and to maximize, accelerate, and synchronize germination of limited seed resources. Here, we detail a simple but effective technique for achieving such refinement of germination from Compositae fruits that we have used in a broad diversity of species, including mainland taxa and endangered members of the Hawaiian silversword alliance (*Argyroxiphium* DC., *Dubautia* Gaudich., *Wilkesia* A.Gray; Madiinae).

Embryo rescue has been long used by plant breeders to cultivate weak or otherwise inviable offspring, such as hybrids between distantly related taxa (reviewed by Rogo et al., 2023). The technique typically involves excising embryos at the most delicate early stages of development and growing them to maturity on sterile nutritive media. In contrast, the method described below involves excising Compositae embryos from mature, viable fruit under non-sterile conditions, to be planted directly into potting soil shortly after germination. It is a simple and accessible method that can be used with minimal tools and limited investment in time and resources, in part because it does not require sterile technique or use of a laminar flow hood if a broad-spectrum antimicrobial is used, as described below. Embryo excision has been found to be an effective means of releasing Compositae from non-deep and intermediate physiological dormancy (Nikolaeva, 1977; Nur et al., 2014), but methodological details about how to excise Compositae embryos effectively have been lacking in the literature. We have found that Compositae embryo excision is a valuable approach for conservation as this method facilitates cultivation and enhances the chances of survival of viable seedlings from precious limited material, as well as allows a careful assessment of seed condition before or after long-term storage. For example, the technique has been used with seed collections made from small populations or isolated individuals with few opportunities to outcross (e.g., *Dubautia kalalauensis* B.G.Baldwin & G.D.Carr, *D. latifolia* (A.Gray) D.D.Keck), in which a large majority of fruits collected were empty, as well as older seed collections nearing the end of their potential viability, with weakened embryos of reduced germinability (B. G. Baldwin, unpublished).

The technique detailed here was taught to one of the authors (B.G.B.) by the late Donald W. Kyhos, who used it for ensuring germination of F_1 hybrids in cytogenetic studies of various Compositae, including the Hawaiian silversword alliance (Carr and Kyhos, 1986), and in overcoming dormancy of primarily dry-adapted composite lineages in southwestern North America (e.g., *Encelia* Adans.; Kyhos et al., 1981). The first author has used the method to elicit germination of many taxa within the tarweed tribe Madieae and other composites, including taxa with strongly dormant ray fruits (e.g., in the tarweed genera *Calycadenia* DC., *Centromadia* Greene, *Deinandra* Greene, *Holocarpha* Greene; also see Tanowitz et al., 1987). Although this technique is time consuming, especially in comparison to sowing whole fruits, the enhancement of germination success for weakly viable embryos, as well as reduction of generation times and synchronization of seedling development, allows for maximizing efficient use of limited conservation and horticultural resources (Anderson et al., 1990).

METHODS AND RESULTS

Fruit preparation

Mature fruits were first sorted from any associated material (e.g., paleae, floral debris) using forceps and dissecting needles. Empty or abortive fruits often (not always) can be detected visually or by gently applying pressure to the fruit wall, as full fruits are firm and resist compression or bending (but fruit wall thickness varies across species, and some species develop normal fruit walls even if fertilization fails to occur, e.g., in *Deinandra increscens* (H.M.Hall ex D.D.Keck) B.G.Baldwin [Tanowitz et al., 1987] and *Dubautia latifolia* [B. G. Baldwin, personal observation]). In some taxa, full fruits can be distinguished from empty fruits with various levels of success by immersion in distilled water (plus a detergent, such as Tween 20 [Thermo Fisher Scientific, Waltham, Massachusetts, USA], to promote wetting of the outer fruit wall), with full fruits tending to sink and empty fruits floating. Screening out empty fruits is generally easier after imbibition, so this fruit-evaluation step can be delayed until then.

Imbibition by mature fruits was achieved by placing them in a 90-mm Petri dish on blotter paper (Anchor Paper,

St. Paul, Minnesota, USA) using a 0.1% antimicrobial solution of a plant preservative mixture (PPM; Plant Cell Technology, Washington, D.C., USA) in distilled water to inhibit fungal and bacterial growth without affecting germination, then sealing the dish with plastic paraffin film (Wolkis et al., 2022). If fruits have been stored under desiccating conditions, then an overnight humidification step (i.e., exposure to high humidity but not standing water) should precede imbibition to prevent cellular damage that may occur because of overly rapid uptake of water by the embryo (Perán et al., 2004). Such hydration can be achieved by placing the fruits (in an unsealed Petri dish) in a sealed chamber with a small wet sponge or paper towel, ensuring the wet material is not in physical contact with the fruits.

After wetting the fruits, the sealed Petri dish was placed in an incubator (Hoffman Manufacturing, Corvallis, Oregon, USA; or Percival Scientific, Perry, Iowa, USA) for approximately 24 h, with 12 h at 25°C in light and 12 h at 15°C in dark. We recommend beginning with no more than 15 fruits at a time to allow for ample working space within the Petri dish and to make it less likely that the fruits will desiccate while excising embryos under the microscope lights (see below). Alternatively, if no incubator is available, the sealed Petri dishes may be kept at ambient temperature overnight. For seeds of species with physiological dormancy and especially those with a stratification requirement for germination, better results often can be obtained by 1–2(3+) weeks of refrigeration at this stage. For species without dormancy, overnight imbibition at room temperature is often sufficient.

Embryo excision

Embryo excisions were made using #4 size stainless steel insect pins, designed for mounting beetles. They are 0.55 mm wide and 38 mm long, and taper to a sharp point. The tips of the pins should be routinely checked under the dissecting microscope to see if they have become bent or damaged. A finely pointed tip is most effective for piercing the fruit wall and manipulating membranes without scarring the cotyledons. Because this technique involves long periods at the microscope using fine-motor skills, an ergonomically optimized workstation is an important consideration.

The methods for excision presented here are based on commercially obtained fruits of the California tarweed *Layia platyglossa* (Fisch. & C.A.Mey.) A.Gray (coastal tidytips), which has physical fruit characteristics that roughly approximate those of some members of the closely related Hawaiian silversword alliance and many other composites (see, e.g., Carlquist, 1965, 1980; Robinson, 1981). The fruit of Compositae is a cypsela (plural: cypselas or cypselae)—a dry, indehiscent, one-seeded fruit derived from a bicarpellate but unilocular inferior ovary (see Bremer, 1994). The composite fruit is often associated with a persistent calyx, represented by the pappus, which may be absent or, in most species, is evident at the fruit summit, as in *L. platyglossa* (Figure 1A). A single ovule is attached at the base of the ovary and the ovules are anatropous. The embryo is epitropous, with a straight, upright, vertical orientation within the fruit. Inside the fruit wall is the seed coat, represented by papery or membranous

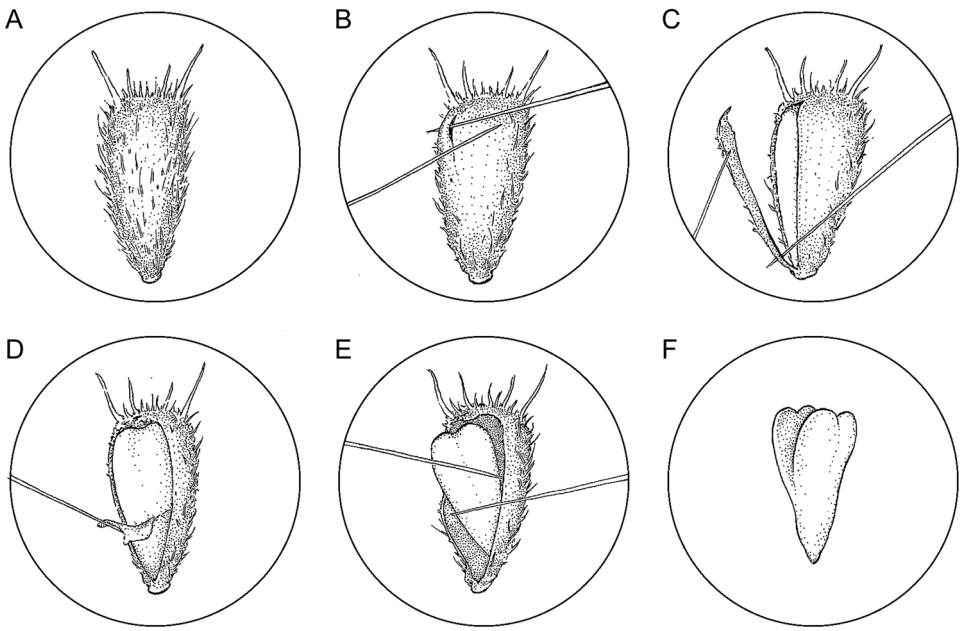

FIGURE 1 Embryo excision of *Layia platyglossa*. (A) Mature fruit from commercially available seed. (B) Indument is scraped away from the fruit wall to improve visibility of the fruit surface; one pin is used to stabilize the fruit, while the second pin pierces the fruit wall just below the ovary summit at an oblique angle revealing the embryo. (C) A strip of fruit wall is pried away from the apex towards the base. (D) Half of the fruit wall has been removed, and the membranous seed coat is gently removed. (E) Pins are used to pry open the fruit wall to remove the embryo. (F) A mature, healthy, well-hydrated embryo spreads its cotyledons apart shortly after the membranous seed coat is removed. Illustration by S. Fawcett.

layers that can be associated with physiological dormancy as a source of chemical inhibitors (e.g., abscisic acid), and it is necessary to remove these layers to stimulate germination in some species (Nikolaeva, 1977; Nur et al., 2014). The cotyledons are positioned towards the broad apex of the fruit, whereas the embryonic root (radicle) is positioned at the narrowed proximal end where the ovule attaches to the fruit, and the fruit attaches to the receptacle of the head. The shoot apical meristem is protected between the cotyledons, whereas the root tip is more exposed, although the root apical meristem is protected by a root cap. Any damage to the radicle and hypocotyl diminishes the chances of embryo survival, while the cotyledons may be more robust to minor damage. To reduce the possibility of damaging the delicate tissues of the hypocotyl and radicle, removal of the fruit wall and membranes should be done by manipulating the apical portion of the fruit. Removing the fruit wall reduces the push power required for germination, which may be especially beneficial for weak embryos, or those nearing the limits of viability.

In species with hairy fruits, it may be easier to see the surface of the fruit wall by gently scraping the surface with the shaft of the needle to remove excess indument (Figure 1B). The fruit summit is typically indurated and relatively difficult to penetrate with pins, so the fruit wall may be best penetrated just below this point (Figure 1B). To begin excising the embryo from the fruit, one pin may be used to stabilize the fruit by applying pressure with the shaft (not tip) of the pin, while the second pin can be used to pierce the fruit wall at a highly oblique angle to avoid piercing the embryo. Once the fruit wall has been pierced, it can be gently pried away, and will usually break into elongate strips running parallel to the length of the fruit (Figure 1C). These may be pinned down with one needle, as the other is used to pry the fruit away. Once the first strip of fruit wall is removed, the subsequent pieces may be removed in a similar manner, inserting the needle between the interior of the fruit wall and the embryo to gently pry the ovary wall away.

Once about half of the fruit wall is removed, the membranes can begin to be removed (Figure 1D). It is critical to maintain hydration of the fruits, especially under the lights of the microscope. Once the seed coat membranes begin to dry, they may adhere to the embryo and become much more difficult to manipulate or remove. In some cases, the membranes adhere to the inside of the fruit wall as the embryo is excised. It is also possible to remove the entire fruit wall before removing the membranes, but we have often found it easier to protect the embryo by maintaining some of the fruit wall in position as the membranes are removed. The membranes may appear translucent, barely distinguishable from the tissue of the embryo, or may be pigmented. The membranes may be most easily pierced and eroded by focusing on the narrow gap between the cotyledons, allowing the spreading force of the hydrated cotyledons to help breach the membranes. One needle may be used to pin the fruit wall to the filter paper in the bottom of the Petri dish while the other is used to gently pry the embryo free (Figure 1E). Any remaining membranes may be removed by very gently peeling them away towards the radicle with the shaft of the needle parallel to the embryo axis.

Once the membranes are removed, the cotyledons tend to spread, indicating a healthy, viable embryo that appears pearlescent white, with the surface of the radicle sometimes slightly darker (Figure 1F). The spreading of the cotyledons may happen immediately, or it may take several minutes. Part of the value of embryo excision is to allow for this evaluation of the condition of the embryo. Indications of low or no embryo viability include failure of the cotyledons to spread (even when the surface tension holding the cotyledons together is released by gently sliding a pin between them), discoloration or lack of turgor of the embryo, incomplete embryo development, or aberrant embryo morphology. These considerations may be particularly important in assessing the status of stored fruits and in evaluating the longevity of embryos under particular storage conditions. See Video 1 for a demonstration of embryo excision; a step-by-step protocol for embryo excision and germination is provided in Appendix 1.

Germination

Excised embryos kept under the same conditions used above for embryo imbibition may begin germinating within 24 h. Delayed germination warrants rinsing of embryos daily with the 0.1% PPM solution. Embryos of some taxa (e.g., those with a light requirement for germination) may be stimulated to germinate by adding 150–200 ppm gibberellic acid to the rinse solution (Plummer and Bell, 1995).

Planting of the resultant seedlings, with green cotyledons and root hairs evident, requires carefully creating a soil opening (e.g., with a glass rod or sharpened pencil), placing the seedling root completely into the soil opening, and gently packing soil around the root, with care to leave the young stem unburied. To allow for the root system to

VIDEO 1 Demonstration of embryo excision from disk fruit of *Layia platyglossa*. The full-text HTML version of this article includes video content. To view this version, please visit https://doi.org/10.1002/aps3.11608.

become established, the seedling should be kept in a cool humid air space that receives at least filtered light. This can be achieved by placing the pot in a tray covered by a transparent plastic lid, with adequate cooling of the outer environment to prevent heat build-up inside the covered tray, or by draping a durable white tissue (e.g., KimWipes, Kimberley-Clark, Texas, USA) over the pot, using the pot label to support the tissue like a tent pole, and gently spraying water over the tissue and molding it around the outer edges of the pot. The covering can be removed from the pot once the first foliage leaves are evident, by which time the root system should be well established.

Experimental results

Using recently harvested ray fruits of cultivated *Madia elegans* D.Don (common madia), which produces no disk fruit, we conducted two trials using different germination conditions. The California tarweed *M. elegans* was chosen for this purpose because tarweed ray fruits generally exhibit more dormancy than disk fruits (Palmer, 1982; Tanowitz et al., 1987; Gregory et al., 2001), which are conveniently lacking in this species. In each trial, both sets of fruits underwent imbibition using the methods outlined above (see Fruit Preparation), followed by embryo excision for one set of fruits and no manipulation of the fruit walls in the other set. Subsequent incubation conditions differed between the two trials in 12-h dark and 12-h light period temperatures, with 10°C dark/20°C light in one trial and 15°C dark/25°C light in the other. Incubation proceeded for 10 days in both trials.

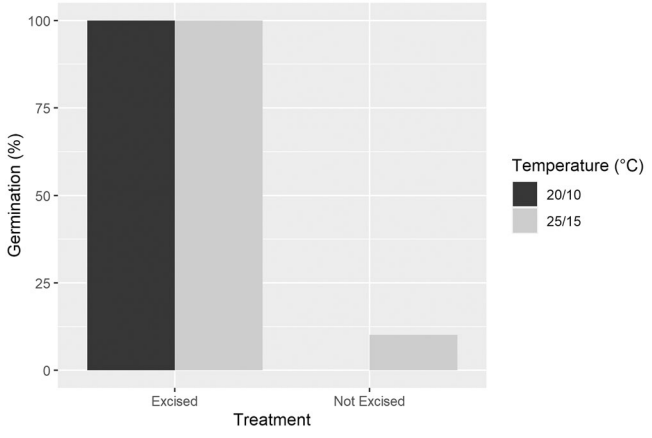

FIGURE 2 Bar chart showing final germination (20/10°C) or mean germination (25/15°C) of *Madia elegans* fruits incubated at a 20/10°C or 25/15°C temperature regime and a 12-h light/12-h dark daily alternating photoperiod for 10 days. Germination was 100% for excised embryos under both temperature regimes ($n = 1$ replicate of nine total excised embryos at 20/10°C, and $n = 2$ replicates of 30 total excised embryos at 25/15°C). None of the intact (i.e., non-excised; control) fruits germinated under the 20/10°C temperature regime ($n = 1$ replicate of five total fruits), but 10.1% of the intact (i.e., non-excised; control) fruits germinated ($n = 2$ replicates of 29 total fruits) under the 25/15°C temperature regime.

In the first trial, at relatively cold dark (10°C) and light (20°C) conditions, no fruits germinated without excision ($n = 5$) and all excised embryos germinated to produce normal seedlings ($n = 9$) (Figure 2). In the second trial, at relatively warm dark (15°C) and light (25°C) conditions, 10.3% of seeds germinated without excision ($n = 29$) and 100% of excised embryos germinated ($n = 30$) (Figure 2). Based on the second trial, the excision procedure significantly enhanced germination within the 10-day timeframe using an independent *t*-test ($t = 15.84886$, $P < 0.00001$).

CONCLUSIONS

The embryo excision method described here is simple to learn and has proven to be valuable for enhancing germination under controlled conditions, when care is taken to prevent extensive physical damage to the embryo surface and to remove all membranes enveloping the embryo. The results for *Madia elegans* are consistent with unpublished observations by B.G.B. for many species of composites and provide a conservative demonstration of the value of embryo excision for promoting germination, given the relatively weak dormancy of fruits of *M. elegans* compared to those of other taxa, such as ray fruits of *Deinandra increscens* (Tanowitz et al., 1987). We have trained students to become proficient in this technique in a few hours, with increased proficiency expected with practice and experience. As it is a delicate and time-consuming procedure, embryo excision is best employed for particularly valuable or germination-resistant seeds. Although the protocol presented here takes advantage of a laboratory setting, it could easily be adapted for use by non-professionals. The value of this method in aid of recovery efforts for rare and endangered composites warrants its wider application in ex situ conservation strategies.

AUTHOR CONTRIBUTIONS

B.G.B., S.F., and D.W. conceived and conducted the study. B.G.B. and S.F. drafted the manuscript. All authors contributed to and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

All data used in this study are available within this article.

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Appendix 1. Detailed procedure for embryo excision and germination from Compositae fruits (cypselae). See Video 1 for a demonstration of embryo excision.

Materials list

1. Mature fruits
2. Dissecting microscope with illumination
3. 0.1% plant preservative mixture (PPM) in distilled water
4. Pipette (or dropper)
5. Petri dishes (we find 90-mm diameter works well)
6. Blotter paper, round
7. Paraffin film
8. Forceps
9. Dissecting needles
10. Insect mounting pins (#4 size)
11. Marking pen
12. Incubator or grow lights with a timer

Step-by-step protocol

1. Sort mature fruits and remove excess material with forceps and dissecting needles (e.g., phyllaries, paleae).
2. Prepare Petri dish by placing one piece of blotter paper into it and labeling the dish with the taxon, collection or accession number, date, etc.
3. Place up to 15 mature fruits on the blotter paper, more or less evenly spaced.
4. Hydrate the plate with the PPM, wetting all fruits and saturating the filter paper (but not allowing excess water to pool).
5. Seal the Petri dish with paraffin film.
6. Allow the seeds to imbibe water overnight.
7. A second Petri dish may be prepared for excised embryos as described in step 2.
8. Open the Petri dish and place it on the stage of the dissecting microscope, ensuring that the fruits, embryos, and blotter paper remain well-hydrated throughout the remaining steps.
9. With an insect pin in each hand and your hands resting comfortably on the stage of the microscope and the Petri dish rim, use one insect pin to stabilize the fruit (with left hand if right-handed or vice versa), then pierce the fruit wall with the tip of the second pin just below the summit of the cypsela at an oblique angle, gently prying away a strip of the ovary wall (Figure 1B).
10. Using the shaft of the first pin to brace the ovary against the blotter paper, the second pin may be carefully inserted between the fruit wall and the embryo, gently prying the fruit wall away from apex to base (Figure 1C).
11. Once about half of the fruit wall has been removed, exposing one side of the embryo, the remaining fruit wall can serve as a cradle, allowing for peeling away of the seed-coat membranes from the embryo with the shaft of the needle, ideally first piercing and eroding the membranes between the cotyledons, while pinning the remaining fruit wall against the filter paper for stability (Figure 1D). Alternatively, the membranes around the ovary can be removed after the embryo is freed completely from the fruit wall, using the shaft of one pin to stabilize the embryo (distal to the hypocotyl).
12. Once sufficient fruit wall has been removed, the embryo may be gently pried from the remaining fruit wall (Figure 1E). Check for any remaining seed-coat membranes on the embryo and gently remove them with the pin.
13. The moistened embryo may be gently lifted into the second Petri dish, using surface tension to adhere it to the shaft of the pin.
14. Repeat the process from step 9 with the remaining fruits, ensuring that the embryos, fruits, and blotter paper remain well hydrated with the 0.1% PPM solution.
15. Seal the Petri dish containing the excised embryos with paraffin film and move to the incubator or growth lights, with a 12-h light period (under controlled temperature conditions to prevent overheating).
16. Check the embryos daily until the cotyledons are fully photosynthetic and abundant root hairs are visible. Prior to germination, embryos can be washed daily with PPM solution using a pipette.
17. The seedlings may now be planted in the desired growth medium, with care to gently but firmly pack soil or media around the young root. Reduce transpiration by maintaining high humidity until the first foliage leaves are clearly visible (when the root system is well established) by covering with a transparent tray lid or, if overheating is possible, by draping a thin but durable permeable tissue (e.g., KimWipe) over the pot, using the pot label tag as a support for the tissue, and spraying the tissue with water to allow for molding it around the outer edges of the pot.