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SHORT COMMUNICATION

A new procedure for making observations of embryo morphology in dust-like seeds with rigid coats

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Abstract

Observing the embryo in dust-like seeds with a rigid coat presents a particular challenge, because it is not possible to use conventional techniques applied to normal-sized seeds. A new combination of softening and clearing techniques, which is remarkably effective for direct observations of embryos in minute seeds, is reported here. Moreover, no special seed treatment is required prior to application of this combination of techniques, and good results are obtained from living and preserved material. Dust-like seeds of *Cytinus hypocistis* and *Cytinus ruber* with a rigid coat were treated successively with Franklin's and with Jeffrey's softening fluids, and then with Herr's clearing fluid. The combined action of the two softening fluids caused sufficient seed coat rupture to allow Herr's fluid to clear seed tissues, permitting effective observation of the embryo. This technique should be applicable to a wide range of species with dust-like seeds, and its possibilities for biological studies are numerous.

Keywords: clearing technique, *Cytinus*, dust-like seeds, embryo visualization, holoparasitic plants, rigid-coated seeds

Introduction

The seed biology of dust-like seeds, such as those found in *Orchidaceae* and in some holoparasitic angiosperms, such as *Cytinaceae*, *Apodanthaceae*, *Mistratemonaceae*, *Hydnoraceae* and *Rafflesiaceae*, is not well known, because their minute size makes them difficult to study. All these families have fruits that contain thousands of seeds with undifferentiated embryos comprised of only a few cells (Kuijt, 1969; Rasmussen, 1995).

Moreover, embryo-less seeds have been found in different families with dust-like seeds (Kebreab and Murdoch, 1999; Matusova *et al.*, 2004). Selfing and different types of crosses can affect the proportion of seeds with an embryo (Wallace, 2003; Jersáková and Johnson, 2006; Noland *et al.*, 2006), such that self-pollinated (inbred) plants produce fruits with empty seeds (Ferdy *et al.*, 2001; Smithson, 2006). The fact that seeds with and without an embryo are frequently similar in size, makes it difficult to distinguish between them. Filled and empty seeds of taxa with normal-sized seeds have been separated by dissection (e.g. Johnson, 2000; Lennartsson *et al.*, 2000), flotation (e.g. Rajora *et al.*, 2002; Gleiser *et al.*, 2004), X-ray photographs (Noland *et al.*, 2006; Parker *et al.*, 2006), and near infrared spectroscopy (Tigabu and Odén, 2003). In other cases, germination tests provide valuable information about seed viability. However, germination studies with dust-like seeds are difficult due to their special requirements. For example, mycorrhizal fungi may be required for successful orchid seed germination and establishment of seedlings (e.g. Rasmussen, 1995), while parasitic plants have special physical and chemical requirements for germination related to host exudates (e.g. Boone *et al.*, 1995 and references therein). Except for *Orobanchaceae* and *Balanophoraceae*, little is known about seed germination in holoparasitic plants with dust-like seeds. Indeed, for the *Cytinaceae*, *Rafflesiaceae*, *Apodanthaceae*, *Hydnoraceae* and *Mistratemonaceae*, there is an almost total lack of information about germination requirements or other aspects of their seed biology.

A technique is needed that allows easy verification of the presence of an embryo in seeds for which

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conventional techniques cannot be used, due to their minute size and rigid coat. In this paper, we outline a combination of techniques that have proved to be particularly effective for direct observations of the embryo in dust-like seeds. No special treatment of the seeds is required prior to the application of this combination of techniques, and good results can be obtained from living, preserved or herbarium material.

Materials and methods

Fruits were collected from the holoparasitic species *Cytinus hypocistis* and *C. ruber* (*Cytinaceae*). These species have dust-like seeds with rigid coats that are approximately 190 × 140 μm in *C. hypocistis* and 210 × 115 μm in *C. ruber* (Bouman and Meijer, 1994). Seeds were removed from the fruits and preserved using two storage methods: 70% ethanol or paper bags at room temperature. These seeds were subjected to four techniques of combined softening and clearing. For each technique, aliquots of approximately 300 seeds were used, because some of them could be lost due to their small size.

To soften the seeds, we used (either singly or successively) two fluids: (1) Franklin's fluid (Franklin, 1945; modified by Kraus and Arduin, 1997), a mixture (1:1) of 30% of hydrogen peroxide and glacial acetic acid; and (2) Jeffrey's fluid (Johansen, 1940), which was composed of a mixture (1:1) of nitric acid and 10% chromic acid. Both fluids have been used traditionally for softening vascular tissues (e.g. Schneider and Carlquist, 1997; Chaffey *et al.*, 2002) and for fibre extraction or removal of other wood components (e.g. Ericsson and Fries, 2004, Kostiainen *et al.*, 2006). To our knowledge, these two softening fluids have not been used previously for seeds. Most researchers have heated or boiled plant material in Franklin's fluid, but we maintained *Cytinus* seeds in this reagent at room temperature, because heating was too aggressive for this material.

We used Herr's fluid (Herr, 1971), which is comprised of lactic acid (85%), chloral hydrate, phenol, clove oil and xylene (2:2:2:2:1, by weight) as the clearing technique. Traditionally, Herr's clearing technique has been useful for the study of ovule development, megasporogenesis and megagametogenesis (using phase-contrast microscopy) (e.g. Herr, 1971; González and Rudall, 2003; Heo *et al.*, 2004), and it has been used only occasionally with seeds (e.g. Czakó *et al.*, 1992; Wang *et al.*, 2004).

Treatment 1. Clearing (Herr's fluid)

The effect of the clearing fluid without softening pretreatment was tested by adding 1.5 ml of Herr's clearing fluid to the seeds. Seeds remained in Herr's fluid for 15 d at room temperature.

Treatment 2. Softening and clearing (Franklin's + Herr's fluids)

Seeds were soaked with 2 ml of modified Franklin's fluid and left in this solution at room temperature for about 36–48 h. The softening solution was carefully removed with a Pasteur pipette, and the seeds were washed in four changes of distilled water. It is important to remove all of the water before placing the seeds in the oily Herr's clearing fluid, so after the last washing the seeds were dried at 35°C for 2–4 h. Herr's clearing fluid (1.5 ml) was added, and seeds were maintained in this solution for 48–72 h at room temperature.

Treatment 3. Softening and clearing (Jeffrey's + Herr's fluids)

Seeds were soaked in 2 ml of Jeffrey's fluid, placed in a heating chamber for 1 h at 40°C and then left at room temperature for 24 h. The softening solution was removed, and seeds were washed as described above. Herr's clearing fluid (1.5 ml) was added, and the seeds remained in this solution for 48–72 h at room temperature.

Treatment 4. Softening and clearing (Franklin's + Jeffrey's + Herr's fluids)

Seeds were soaked with 2 ml of the modified Franklin's fluid and maintained in this solution at room temperature for 36–48 h. The softening solution was removed, and seeds were washed. Once the seeds were dried, 2 ml of Jeffrey's fluid was added, and seeds were incubated at 40°C for 1 h, and then at room temperature for 24 h. Jeffrey's solution was removed, and seeds were dried at 35°C for 2–4 h. Finally, 1.5 ml of Herr's fluid was added, and the samples were left in the solution for 48–72 h at room temperature.

For microscopic examinations, seeds subjected to the four aforementioned treatments were transferred to Raj-type chambered slides with a small amount of Herr's clearing fluid (Herr, 1971).

Anatomical technique

Finally, we wanted to know if the embryo features visible by means of these combinations of fluids were similar to those observed using anatomical techniques. Hence, a group of seeds was dehydrated in an ethanol series, embedded in Historesin (glycol methacrylate, Leica, Heidelberg) and serially sec-

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tioned at 10–15 μm using a rotary microtome. Sections were stained with 0.12% toluidine blue and 0.05% basic fuschin and mounted with Eukitt (Kindler, Freiburg, Germany). All images were taken using a Leica DC300 camera attached to a Zeiss Axiophot light microscope.

Observations and discussion

Similar results were obtained for both species of *Cytinus* subjected to each of the four treatments. Likewise, there were no differences between seeds from different storage methods.

Cytinus seeds subjected only to Herr's clearing fluid (Treatment 1) were opaque under light microscopy, and therefore this technique does not allow embryo visualization (Fig. 1a). In seeds given this treatment, only the exotegmic cells with simple pits were observed (Fig. 1a). It is likely that it was not possible to observe the embryos because the marked thickness of the seed coat (Guzowska, 1964; Bouman and Meijer, 1994) impeded entry of the clearing fluid into the seed. Good fixations of fully developed embryos of *Cytinus* for ultrastructural observations have been impossible due to the same problem (Ponzi and Pizzolongo, 1982). Likewise, neither Treatment 2 nor Treatment 3 allowed embryo visualization. Franklin's and Jeffrey's fluids used independently were not capable of breaking the *Cytinus* seed coat to allow the successful penetration of Herr's clearing fluid.

Only the combination of techniques described in Treatment 4 (Franklin's + Jeffrey's + Herr's fluids) permitted the observation of the presence and morphology of *Cytinus* embryos (Fig. 1b, c). The first two steps of this technique, successively treating the seeds with Franklin's and Jeffrey's fluids, produced a rupture of the seed coat that was essential for the penetration of Herr's clearing fluid.

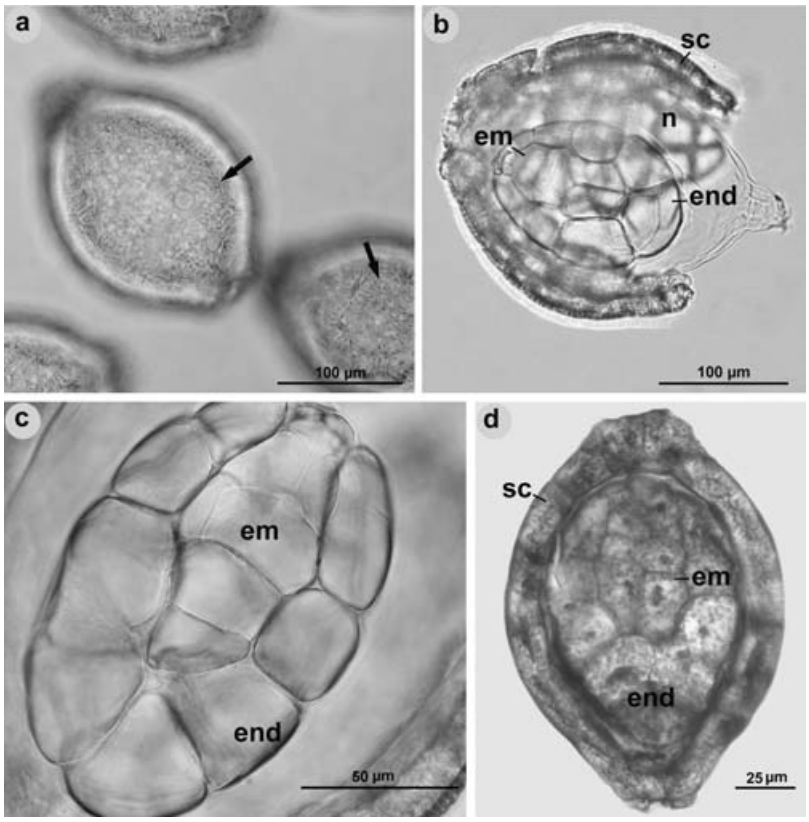

Figure 1. Mature seeds of *Cytinus hypocistis*. (a) Treatment 1, 15 d in Herr's clearing fluid. Seeds appear opaque under light microscopy. Arrows indicate exotegmic cells with simple pits. (b) Treatment 4, softening and clearing successively with Franklin's, Jeffrey's and Herr's fluids. The seed coat has ruptured and the nucellar tissue has swelled, allowing visualization of the undifferentiated embryo surrounded by the cellular endosperm. (c) Seed from Treatment 4: detail of the minute embryo composed of only a few cells surrounded by the cellular endosperm. (d) A longitudinal thin section of a seed showing the thick seed coat and undifferentiated embryo embedded in endosperm. end, Endosperm; em, embryo; n, nucellus; sc, seed coat.

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Consequently, seeds became transparent, and features of the embryo and endosperm could be observed (Fig. 1b, c). The embryos of *Cytinus hypocistis* and *C. ruber* were similar; they are very small, composed of about ten cells, and surrounded by an endosperm of a few much larger cells (Fig. 1b, c), as has been described previously (Guzowska, 1964; Ponzi and Pizzolongo, 1982; Bouman and Meijer, 1994). Similar features were observed in the anatomical sections (Fig. 1d), but the anatomical technique was more time consuming, and moreover, with *Cytinus* seeds, it was not always successful, since resin penetration was frequently impeded by the thick seed coat. Consequently, part or all of the contents of the seed were frequently detached by the microtome knife.

A sac-like structure surrounding the embryo was also observed (Fig. 1b), probably derived from the nucellus, which had swelled following hydration, thus allowing a better visualization of the embryo. This sac-like structure was rigidly perceptible in seed anatomical sections (Fig. 1d), since the remains of the nucellus are compressed in the mature seed (Guzowska, 1964; Bouman and Meijer, 1994). Thus, the combination of Franklin's, Jeffrey's and Herr's fluids yielded preparations that, in some cases, rival the details observed in conventional resin sections. Additionally, our combination of Franklin's and Jeffrey's fluids is applicable as a pretreatment to small seeds before being embedded in resin, facilitating the penetration of plastic resin. Recently this procedure has been used successfully with seeds of *Miconia*, *Tococa*, *Leandra* and *Clidemia* (Daniela G. Simão, personal communication).

The application of the technique described here can provide useful information by allowing rapid visualization of the embryo stage. Seeds from other holoparasitic angiosperms, such as *Rafflesiaceae*, *Mistratemonaceae*, *Hydnoraceae* and *Apodanthaceae*, also have a mechanical layer of cells with strongly thickened walls (Bouman and Meijer, 1994), and application of the technique described here could be useful in visualizing embryos in seeds of these taxa. In addition, this technique could be used to facilitate studies on different aspects of the reproductive biology of species with dust seeds, to compare seeds from different pollination treatments and quickly classify them into those with viable (with a normal embryo), non-viable (with an abnormal embryo) and empty (without embryo) seeds. This would provide insight into the influence of self- versus cross-pollination on fitness of these plants. Furthermore, most holoparasitic plants have many-seeded, fleshy fruits dispersed by mammals and birds (Gómez, 1983; Emmons *et al.*, 1991; García-Franco and Rico-Gray, 1997; de Vega, unpublished observations). This technique will make it possible to observe embryo damage after consumption by animals and to discriminate between dispersers and predators.

The efficacy of the combination of softening and clearing techniques described in this paper is based on seed coat rupture that facilitates clearing of seed tissues with Herr's fluid. The technique developed here for *Cytinus* should be applicable to a wide range of species with dust-like and/or thick-walled seeds, and its possibilities for biological studies are numerous.

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