18 Embryo Rescue

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CHAPTER 18 CONCEPTS

- Embryo rescue procedures have been widely used for producing interspecific and intergeneric hybrids.
- Depending on the organ cultured, embryo rescue is referred to as embryo, ovule, or ovary culture.
- Ovule and ovary culture are more suitable than embryo culture for small-seeded species or very young embryos.
- Cultures must be initiated before embryo abortion occurs.
- Media requirements depend on the stage of embryo development.
- Young embryos (proembryos) require a medium with a high osmotic potential.

INTRODUCTION

The term "embryo rescue" refers to a number of *in vitro* techniques whose purpose is to promote the development of an immature or weak embryo into a viable plant. Embryo rescue has been widely used for producing plants from hybridizations in which failure of endosperm to properly develop causes embryo abortion. In embryo rescue procedures, the artificial nutrient medium serves as a substitute for the endosperm, thereby allowing the embryo to continue its development. Embryo rescue techniques are among the oldest and most successful *in vitro* procedures.

One of the primary uses of embryo rescue has been to produce interspecific and intergeneric hybrids. While interspecific incompatibility can occur for a wide variety of reasons, one common cause is embryo abortion. The production of small, shrunken seed following wide hybridization is indicative of a cross in which fertilization occurred but seed development was disrupted. Embryo rescue procedures have been very successful in overcoming this barrier to wide hybridization in a wide range of plant materials (Collins and Grosser, 1984). In addition, embryo rescue has been used to recover maternal haploids that have developed as a result of chromosome elimination following interspecific hybridization.

Embryo rescue techniques also have been utilized to obtain progeny from intraspecific hybridizations that do not normally produce viable seed. For example, triploids have been recovered from crosses between diploid and tetraploid members of the same species, and progeny have been obtained from crosses utilizing early-ripening and "seedless," or stenospermacarpic, fruit genotypes as maternal parents. Embryo rescue techniques have also been used in situations in which embryo abortion is not a concern, such as for overcoming seed dormancy and studying seed development and germination. The various applications of embryo rescue to both applied and basic plant research

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have been reviewed by Bridgen (1994), Collins and Grosser (1984), Ramming (1990) and Sharma et al. (1996).

Depending on the organ cultured, embryo rescue may be referred to as embryo, ovule, or ovary culture. While the disinfestation and explant excision processes differ for these three techniques, many of the factors that contribute to the successful recovery of viable plants are similar. This chapter will begin with a discussion of general factors that should be considered when utilizing embryo rescue and then turn to techniques specific to each type of embryo rescue procedure.

FACTORS INVOLVED IN EMBRYO RESCUE

Media

Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Gamborg's B-5 (Gamborg et al., 1968) media are the most commonly used basal media for embryo rescue studies (Bridgen, 1994). Types and concentrations of media supplements required depend greatly on the stage of development of the embryo.

Raghavan (1976) identified two phases of embryo development. In the heterotropic phase, the young embryo, which is often referred to as a proembryo, is dependent on the endosperm. Embryos initiated at this stage require a complex medium. Amino acids, particularly glutamine and aspargine, are often added to the medium. Various vitamins may also be included. Natural extracts, such as coconut milk and casein hydrolysate, have sometimes been used instead of specific amino acids. Young embryos require a medium of high osmotic potential. Sucrose often serves both as a carbon source and osmoticum. High osmotic concentration in the medium prevents precocious germination and supports normal embryonic development. For heterotropic embryos, 232 to 352 m*M* (8–12%) sucrose is commonly used. Other sugars have been successfully used instead of or in addition to sucrose; however, sucrose has been by far the most commonly utilized sugar for embryo rescue.

The second stage of embryo development is the autotrophic phase, which usually begins in the late heart-shaped embryo stage (Raghavan, 1976). At this time the embryo is capable of synthesizing substances required for its growth from salts and sugar. Germination will usually occur on a simple inorganic medium, supplemented with 58 to 88 m*M* (2–3%) sucrose.

Growth regulators have been extensively used in embryo rescue studies, especially for heterotropic embryos; however, their effects have been highly inconsistent. In general, low concentrations of auxins have promoted normal growth, gibberellic acid has caused embryo enlargement, and cytokinins have inhibited growth (Sharma, 1996). In addition to supplying vitamins and amino acids to the medium, natural extracts often also supply growth regulators.

As stated earlier, media requirements differ depending on stage of embryo development. For cultures initiated using very young embryos, more than one media formulation may be needed. For example, embryos of *Trifolium* interspecific hybrids were first cultured on a high sucrose medium containing a moderate level of auxin and a low level of cytokinin. After 1 to 2 weeks on this medium, embryos stopped growing. Growth resumed after they were transferred to a medium with a lower sucrose concentration, a low level of auxin, and a moderate level of cytokinin (Collins and Grosser, 1984).

For interspecific hybrids, it may be useful to develop media that can nurture embryos of one or both parental species. While the nutritional needs of the hybrid may be different from the parents, the parental media formulations will serve as a good starting point for the hybrid.

Temperature and Light

Temperature and light requirements vary among species. According to Sharma (1996), the growth requirements of embryos often mimic those of their parents, with embryos of cool-season crops requiring lower temperatures than those of warm-season crops. Cultures are often incubated at 25

to 30° C, although considerably lower temperatures are needed for some species. In species that normally exhibit seed dormancy, a cold treatment may be required.

Cultures are usually initially cultured in the dark to prevent precocious germination, but are moved to a lighted environment to allow chlorophyll development after 1 to 2 weeks in the dark.

Time of Culture

When attempting to rescue embryos of incompatible crosses, it is critical that the cultures be initiated prior to embryo abortion. However, because it is more difficult to rear young embryos than those that have reached the autotrophic phase of development, chances of success are maximized by allowing the embryo to develop *in vivo* as long as possible. Histological examinations can be used to determine the time of endosperm failure and embryo abortion; however, these evaluations can be very laborious. Cultures are often initiated at various intervals following pollination to maximize chances of recovering viable plants. Since an interaction between media and time of culture is expected, it is important to test a range of media ranging from complex with high sucrose to simple with low sucrose at the various culture times.

GENERAL EMBRYO RESCUE PROCEDURES

Embryo Culture

The most commonly used embryo rescue procedure is embryo culture, in which embryos are excised and placed directly onto culture medium. Fruit from controlled pollination of greenhouse- or field-grown plants is collected prior to the time at which embryo abortion is thought to occur. Since embryos are located in a sterile environment, disinfestation of the embryo itself is not required. In some cases, the entire ovary is surface-sterilized. In other cases, ovules are removed from the ovary under nonaseptic conditions and then disinfested. In either instance, a harsh disinfestation procedure can usually be applied, since the embryo is protected by the surrounding tissue.

Careful excision of the embryo is critical to the success of embryo culture. A stereomicroscope is usually required, and must be placed in the laminar flow hood in such a manner as not to restrict airflow. The best point of incision into the ovule differs among species. In some cases, embryos can be extracted by cutting off the micropylar end of the ovule and then applying gentle pressure at the opposite end of the ovule. This results in the embryo being pushed out through the opening. It is crucial that the embryo be placed directly into culture after its excision so that it does not become dry. For heart-shaped and younger embryos, the embryo should be excised with the suspensor intact (Hu and Wang, 1986). Because of the extreme importance and frequent difficulty of excising embryos without causing damage, it may be helpful to develop and practice an excision technique under nonaseptic conditions.

Embryo culture is sometimes preceded by ovule or ovary culture. One advantage of this technique (sometimes termed ovule-embryo or ovary-embryo culture) is that embryo excision is delayed until the embryo becomes large enough to remove without damage. Also, the presence of the integument during the ovule or ovary culture phase has been found to reduce the possibility of precocious germination (Ramming, 1990). Once excised, the embryo may benefit from being in direct contact with the medium. Also, for those species affected by dormancy, removing the embryo may overcome any inhibitory effects imposed by the surrounding ovular tissues.

Nurse cultures have been used for rescuing embryos (Williams et al., 1982). This technique involves inserting the embryo from an incompatible cross into endosperm removed from a related compatible cross. For example, the embryo of an interspecific hybrid may be inserted into endosperm from an intraspecific cross involving one of the parental species. The embryo and endosperm are then placed into culture together.

Ovule Culture

Embryos are difficult to excise when very young or from small-seeded species. To prevent damaging embryos during the excision process, they are sometimes cultured while still inside the ovule. This technique is referred to as ovule culture or *in ovolo* embryo culture. As with embryo culture, ovaries are collected prior to the time at which embryo abortion is thought to occur. The ovary is surface-sterilized and the ovules removed and placed into culture. This step ranges from extremely easy to accomplish, for large-seeded species in which only a single ovule is present, to time-consuming and difficult, for small-seeded polyovulate species. Excision of the ovules may require the use of a stereomicroscope. Including placental tissue in ovule cultures has been found to be beneficial in some species (Rangan, 1984).

Recent modifications of the standard ovule culture technique have been developed for use in peach (*Prunus persica* [L.] Batsch) (Pinto et al., 1994). One technique, ovule perforation, requires making small holes in each ovule just prior to its placement on the culture medium. These perforations, which should be made with care not to damage embryos, increase water and nutrient uptake. Two types of ovule support systems have been developed. The filter paper support system involves culturing ovules on top of filter paper placed over liquid medium, whereas the vermiculite support technique entails placing ovules micropylar side down into a sterile vermiculite/liquid media mixture (vermiculite support). While the ovule perforation and vermiculite support systems may not be feasible in small-seeded species, ovule size should not pose a limitation for testing the filter paper support system in species other than peach.

Ovary Culture

In ovary or pod culture, the entire ovary is placed into culture. Ovaries are collected and any remaining flower parts removed. Disinfestation protocols must remove surface contaminants without damaging the ovary. The ovary is placed into culture so that the cut end of the pedicel is in the medium. At the end of the experiment, seed are removed from the fruit that develop in culture.

A technique known as ovary-slice culture has been utilized for rescuing *Tulipa* interspecific hybrid embryos (Van Creij et al., 1999). Ovaries were cut transversely into sections and the basal cut end of the sections placed on the culture medium. In *Tulipa*, ovule culture and ovary-slice culture produced similar germination rates; however, the ovary-slice culture procedure was considered to be the superior of the two techniques because it was less time consuming.

SUMMARY

Embryo rescue procedures have been successfully used for many years for producing interspecific and intergeneric hybrids and progeny of other incompatible crosses. While embryo culture is the most widely used embryo rescue procedure, ovule and ovary culture are more suitable for small-seeded species or very young embryos. For all three procedures, the probability of success increases with maturity of the embryos; however, cultures must be initiated before embryos abort. The type of medium needed for rescuing embryos is strongly dependent on the stage of embryo development. Young embryos require a complex medium with high sucrose concentrations, while more mature embryos can usually develop on a simple medium with low levels of sucrose. Continued investigations into nutritional requirements of young embryos, along with modifications of existing embryo rescue techniques, should lead to successful application of this highly valuable *in vitro* procedure to additional crop species.

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