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Review of embryo culture in fruit trees

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ABSTRACT

Embryo culture, sometimes called embryo rescue, is an in vitro technique that has been used to save the hybrid products of fertilization when they might otherwise degenerate. Embryo culture involves isolating and growing an immature or mature zygotic embryo under sterile conditions on an aseptic nutrient medium with the goal of obtaining a viable plant. The basic premise for this technique is that the integrity of the hybrid genome is retained in a developmentally arrested or an abortive embryo and that its potential to resume normal growth may be realized if supplied with the proper growth substances. The technique depends on isolating the embryo without injury, formulating a suitable nutrient medium, and inducing continued embryogenic growth and seedling formation. The culture of immature embryos is used to rescue embryos that would normally abort or that would not undergo the progressive sequence of ontogeny. This process is difficult due to the tedious dissection necessary and the complex nutrient medium requirements. Success with this type of culture depends strongly on the developmental stage of the embryo when it is isolated. The culture of mature embryos from ripened seeds is used to eliminate seed germination inhibitors or to shorten the breeding cycle if, for example, dormancy is a problem. This culture is easy and only requires a simple nutrient medium with agar, sugar, and minerals.

Keywords: fruit trees, Embryo culture, embryo rescue

INTRODUCTION

The first successful embryo culture of fruit trees on an artificial medium was achieved by Tukey in 1933 who cultured cherry embryos and this was a milestone in embryo culture of fruit trees. His medium and procedure has been widely used and even applied to other crops. Using this procedure, Blake was the first to employ culture in a peach breeding program[6]. Larue successfully cultured and grew small (0.5 mm long) embryos of many genera as early as 1936 with the addition of inorganic salts, sugar, yeast extract, and indolebutyric acid to the medium[28]. However, embryos in the globular stage and smaller are very difficult to culture successfully. The successful growth of ovule-cultured embryos depends on their stage of maturity at the time of culture, genotype, culture medium and culture environment. The developing embryo is a dynamic system that has changing requirements as it matures. smaller embryos, required more complex medium. There are heterotrophic and autotrophic stages in embryo development. The embryo in the heterotrophic stage of development is smaller than in the autotrophic stage and usually requires the presence of growth regulators to allow for proper development. The autotrophic stage begins at about the late heart stage and development of such an embryo does not depend on exogenous sources of growth regulators, thus making it more amenable to in vitro culture.

Embryo culture is useful when there is poor embryo development or abortion. Embryo abortion occurs in early ripening genotypes of *Prunus* where the flesh matures before seed maturity, precluding their use as females in a breeding program[44, 48]. The poor germination of seeds from early ripening grapes is probably due to poor embryo

development. Embryos have been rescued from early ripening *Prunus* [2, 46] and *Vitis* [34] cultivars, thus allowing their use as females. Embryos have been rescued and grown into plants from stenospermocarpic seedless grapes [34, 41], allowing growth of progeny resulting from the hybridization of seedless with seedless grapes. The production of interspecific hybrids is useful for the transfer of desirable genes from wild to cultivated species. In many cases, wide crosses between species are difficult to produce because of many factors that act as barriers. Postzygotic barriers such as endosperm abortion are a common occurrence, but they have been overcome through the use of embryo rescue and many interspecific hybrids have been produced [9]. Embryo rescue has also been used to overcome postzygotic barriers during self- and cross-incompatibility [13,10,20]. Embryo culture has been used for rapid seed germination tests of peach [17,47], sweet cherry [15] and other woody plants. For production of virusfree citrus plants through culture of nucellar embryos [4,5] and for studying the developmental and growth requirements of embryos [43]. Embryo culture has also been used to shorten breeding cycles in apple [29], where effective germination was reduced from years to months by overcoming dormancy. Landa et al. in 1980 [9] investigated the possibility of selecting mutants with high amino acid content. Pollen might also be screened for temperature tolerance [57] before in vitro fertilization of ovules. Embryo culture has been reviewed by Sanders and Ziebur [37], Narayanaswami and Norstog [28], Raghavan [30,31], Bhojwani and Razdan [3], Collins and Grosser [9] and Bridgen [8]. One of the few reviews of ovule culture was by Rangan [35]. This review will cover the use of embryo culture for fruit crops.

APPLICATIONS

Embryo culture is one of the earliest forms of in vitro culture applied to practical problems and is probably the tissue culture technique that has proven of greatest value to breeders [11]. Its major application in plant breeding has been for interspecific hybridization. Early embryo abortion occurs primarily because the endosperm fails to develop properly [23]. With interspecific crosses, intergeneric crosses, and crosses between diploids and tetraploids, the endosperm often develops poorly or not at all. By aseptically culturing the embryo in a nutrient medium, this problem may be overcome.

Embryos of some nonviable hybrids may possess the potential for initiating development by avoiding postzygotic barriers within the mother plant. Several successful cases have been documented with embryos arising from interspecific hybrids and intergeneric hybrids [32, 40, 53, 54, 55, 39, 2]. Embryo culture can be successfully used as a tool in an apricot [24] and sweet cherry [2,39] breeding program to obtain higher percentages of seedling from planned hybridization or to overcome a lack of seed germination.

Embryo culture can shorten the breeding cycle by overcoming dormancy in seeds. Dormancy may be caused by endogenous inhibitors, light requirements, low temperatures, dry storage requirements, and embryo immaturity [56]. Seed dormancy factors may be localized in the seedcoat, the endosperm, or both. By removing the embryos from the influences of these factors, the embryos germinate and grow quickly and the breeding cycle is shortened. Isolated embryos can also be vernalized and may, in some instances, reduce the generation time by 40 days [40]. Germination of mature seeds in early-ripening sweet cherry cultivars such as 'Silej-Delamarka' is 0% by conventional seed culture methods, the embryo culture successfully increased germination up to 30 and 60 percent on the embryos sampled 21 and 28 days after full bloom respectively [14, 16]. In addition to the applied uses of embryo culture, the procedure is useful in basic studies. Growing embryos outside the ovule (ex ovulo) is an excellent way to study the nutrition and metabolism of the embryos at various stages of development. The technique can also be used to examine the growth requirements of embryos, the effects of phytohormones and environmental conditions on zygotic embryogenesis, and the regeneration potentials of whole embryos and their segments [56]. Embryo culture can be used to localize sites of germination promoters and inhibitors, for studies of embryogenesis, and for cryopreservation [22]. Germination of early ripening apricot has been improved by using an agar-sucrose medium [24].

Embryo culture can be used to propagate plants vegetatively. Embryos from genera that have both juvenile and adult characteristics are used as starting material for vegetative propagation. Embryos are responsive because they are juvenile. Embryo culture can be used to study precocious germination, the germination of embryos before the completion of normal embryo development. Usually, precocious germination causes the formation of weak seedlings. To understand the factors that regulate the orderly development of embryos in nature, embryos can be cultured under various conditions to determine what simulates embryological development. Precocious germination occurs because inhibitors are lost when the testa is removed or because the negative osmotic potential is a higher value in vivo. Precocious germination has been prevented in *Prunus* through ovule culture, where the integument acts as a natural inhibitor [33]. Embryo culture has been very useful in determining seed viability. This use arose out of early findings that there was a good correlation between the growth of excised embryos of non-after-ripened peach [*Prunus persica* (L.) Batsch.] seeds and germination of the afterripened seeds [47]. Embryo culture allows the rapid testing of seed viability when seed dormancy can be circumvented.

TECHNIQUES

In most situations, embryos are located in the sterile environment of the ovule and surface sterilization of embryos is not necessary. Instead, entire ovules or ovaries are surface-sterilized and then embryos are removed aseptically from the surrounding tissues. Since the embryo is often well-protected by surrounding tissues, harsh procedures may be used in surface disinfection. Thus, axenic cultures of embryos are often easily established. Direct disinfection of embryos is needed if seedcoats are cracked or if endophytic pathogens exist inside the seedcoats. The dissection of the embryos can produce problems. Large embryos are not difficult to excise. However, small embryos require the use of microdissecting tools and a dissecting microscope to excise without injury. Embryos are easily damaged when the seedcoat is cut, it is also important that the excised embryo does not become desiccated during culture [35]. The process of excising immature embryos varies with species. However, many times an incision can be made at the micropylar end of the young ovule and pressure applied at the opposite end to force the embryo out through the opening. If liquid endosperm surrounds the embryo, the pressure it exerts may injure the fragile embryonic tissue if caution is not exercised. When heart-stage and younger embryos are excised, it is important to keep the suspensors intact [23].

REQUIREMENTS FOR SUCCESS

Successful development of an embryo depends on many factors. As with most other processes, the plant genotype greatly influences success. Embryos of some species are easier to grow in culture than are others, and differences sometimes occur between closely related cultivars [9, 35]. As already indicated, small embryos are difficult to grow in vitro. Specialized techniques can be used to improve success. The use of “nurse” endosperm involves inserting a hybrid embryo into an endosperm dissected from a normally developing, self-pollinated ovule from one of the parents or a third species. The embryo and endosperm are transferred together to the surface of the culture medium [55]. Modified versions of the nurse endosperm, such as embryo implantation or transplantation, have been adapted to other species [53, 54]. By using embryo rescue, one can achieve a 30% to 40% success rate with intergeneric crosses compared to a 1% success rate when embryo–nurse endosperm transplants are not used. Small or young embryos that abort at early stages of development are often difficult to isolate. Embryos of apricot between 5 and 9 mm germinated and developed into plants in a significantly higher percentage than in the other two more mature stages [24].

The nutritional requirements of young embryos vary greatly and the chances of damaging the embryos are great. In such situations, it may be possible to rescue embryos by ovary or ovule culture methods [35]. Ovaries are excised after pollination and the calyx, corolla, and stamens are removed. The ovary is surface-sterilized and cultured with the cut end of the pedicel inserted into the nutrient medium. If all goes well, the ovary then develops into a fruit with fully developed seeds. For ovule culture, the sterilized ovary is opened and the fertilized ovules are scooped out and transferred to the surface of the culture medium. The reasons for the successful recovery of hybrids from ovary or ovule culture rather than through embryo culture are probably related to nutritional and physical factors and protection of the embryo by the maternal or sporophytic tissues.

Light and temperature are two environmental factors that are of major concern in embryo culture. Embryos sometimes grow best when maintained in darkness for the first 1 to 2 weeks of culture and then transferred to light to allow chlorophyll formation. Isolated embryos frequently germinate in a wider temperature range than intact seeds. Some embryos require a cold treatment of 4°C to break dormancy. The growth of sweet cherry embryos was suitable when embryos were treated with 40 days and 60 days cold treatment (4°C) on the immature and mature embryos respectively [14]. Balla and Brozik, stratified sweet cherry embryos at 4°C for 120 days [2]. The optimum temperature for growth of embryos depends on plant species, but normally a high range of 25 to 30°C is used [28]. The growth conditions of the mother plant are also a consideration in embryo culture. The endosperm and the cotyledons will develop more if the mother plant is grown under well-controlled conditions; embryo growth will consequently be promoted [7].

MEDIA

Many scientists believe that the most important aspect of embryo culture is medium selection. Several formulations of mineral salts have been used for embryo culture without much critical evaluation of the role of individual elements [3]. Murashige and Skoog [27], and Gamborg's B5 medium [21], with certain degrees of modification, are the most widely used basal media in embryo culture. (Rizzo *et al.*, 1998) have used three media [MS, Knopp and woody plant (WP) media] on peach embryo culture, embryos cultured on WP media with 3% sucrose had better germination [36].

The exact nutritional requirement depends on the stage of embryo development. Raghavan identified two phases of embryo development. In the heterotrophic phase, the young embryo depends on the endosperm and the surrounding maternal tissues, and requires a more complex medium and higher osmotic pressure than older embryos. The

continued development of young embryos requires complex media supplemented with combinations of vitamins, amino acids, growth hormones, and in some cases, natural extracts, such as tomato juice and coconut milk, to support development. During the autotrophic phase, the second stage of embryo growth, the embryo is metabolically capable of synthesizing substances required for its growth from the salts and sugar. In this phase, embryos can germinate and grow on a simple inorganic medium supplemented with a carbon source, such as sucrose[30]. Ammonium nitrate and potassium nitrate are the most frequently used sources of inorganic N in embryo culture. Ammonium in the medium is essential or preferential for proper growth and differentiation of immature embryos [25]. Ammonium usually is combined with an organic acid, particularly with malate or citrate anions. Among various amino acids, glutamine and asparagine are the most effective [38]. Casein hydrolysate is a complex mixture of amino acids and is commonly used in embryo culture media to stimulate growth. Vitamins, such as biotin, thiamine, pantothenic acid, nicotinic acid, ascorbic acid, inositol, and pyroxidine, are commonly added, but have not been proven to be essential. Adding amino acids to the culture medium may stimulate embryo growth [3]. Glutamine is the most effective amino acid for cultured embryo growth [26]. Asparagine may also enhance embryo growth but it can be inhibitory [25]. Casein hydrolysate is a complex mixture of 18 amino acids that has been widely used as an additive to embryo culture media. When added alone to a medium, none of the amino acids match the beneficial effect of casein hydrolysate [38]. However, work with the induction and maturation of somatic embryos demonstrates that amino acids such as proline, serine, and glutamine can replace casein hydrolysate. The landmark work of van Overbeek *et al.* demonstrated that embryos younger than the post-torpedo stage could be cultured by adding the liquid endosperm of coconut to the culture medium. By modifying a medium to mimic the endosperm that surrounds immature embryos in the ovule, success was obtained where it had not been possible previously. By using coconut milk in media for young embryos, precocious germination can be avoided[50]. Other natural substances, such as skim milk, dried brewer's yeast (malt extract), casein hydrolysate, and diffusates from the seeds of several plant species, can substitute for coconut milk, depending on the species under investigation [3]. Although synthetic media are used, these natural plant extracts are still suitable amino acid sources for culturing immature embryos. Sucrose is the most commonly used C energy source for embryo culture. Sucrose is primarily an energy source, although it also plays an important role in maintaining suitable osmotic potential of nutrient media. Mature embryos are usually grown on media with 2% to 3% sucrose, whereas immature embryos grow better at 8% to 12%, which mimic the high osmotic potential within the young embryo sac. Generally, the younger the excised embryo, the higher the medium osmolarity required. Raghavan, believes that this high osmolarity prevents precocious germination and keeps cells that are in a state of division from going into a state of elongation[30, 31].

Agar is the most commonly used agent to solidify culture media. Concentrations of 0.5% to 1.5% are generally used for embryo culture [23]. High concentrations of agar may inhibit growth due to reduced water availability, quality of agar, or contaminating salts. Instead of agar Using the vermiculite support system for small embryos of early maturing peach cultivars, obtained at fruit maturity, were cultured successfully and used as maternal parents in breeding programs [1].

Plant growth regulators generally play a small role in embryo culture. Exogenous auxins do not seem to be required for plant embryo growth in vitro and This observation supports reports that somatic embryo induction is inhibited by high concentrations of exogenous auxin in the medium and stimulated by low concentrations or in its absence. Cytokinins, when used as the sole hormone, are ineffective or only slightly promote young embryo growth. However, they promote growth and differentiation of embryos when they are combined with some auxins [51]. Monnier suggests that hormones should not be added to embryo culture media as they cause structural abnormalities[26]. Auxins and cytokinins are not generally used for embryo culture unless callus induction is needed. Gibberellins sometimes stimulate precocious germination or are used to overcome dormancy [26, 14].

CONCLUSION

Embryo culture is a valuable in vitro tool for breeding. It is most often used to rescue embryos from interspecific and intergeneric crosses and from embryos that do not fully develop naturally (as in early ripening and seedless fruit where the embryo aborts). The method also can be used to rescue seedless triploid embryos, produce haploids, overcome seed dormancy, or determine seed viability. It is useful in understanding embryo morphogenesis and precocious germination. As research continues with this technique, new and valuable uses will be developed to assist the biotechnological breeding of plants.

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