Plant regeneration via embryogenic suspension cultures

JOHN J. FINER

1. Introduction

There was a time when plant regeneration from most of the agronomically important crop species was thought to be impossible. Cereals and small grains, as well as important dicotyledonous species such as soybean and cotton, appeared quite unresponsive to tissue culture manipulations. Some species such as tobacco (*Nicotiana tabacum*) and wild carrot (*Daucus carota* 'Queen Anne's Lace'), on the other hand, could be placed *in vitro*, manipulated in a number of ways, and then regenerated to form whole plants with a simple change in the supply of growth regulators (1, 2). These model species had one thing in common, the ability to form whole plants from a totally undifferentiated state (callus). Any species that did not respond to tissue culture manipulations in a manner similar to the model plants was termed 'recalcitrant'. Undifferentiated tissue or callus could be produced from the 'recalcitrant' species but regeneration from this tissue was virtually impossible. Although the model species have been and will continue to be valuable in plant cell biology research, it is unfortunate that such a large effort was placed in attempting to force many plants to respond to the same manipulations as did the model plants. In the end, the responses of the early model species *in vitro* may be the exceptions rather than the norm.

Due to the value of the agronomic species, there has been a strong effort to develop tissue culture systems for these crops. A new type of callus with a smooth, rounded surface and containing cells with a dense cytoplasm was observed. Although not completely undifferentiated, these meristem-like cells proliferated slowly and plants could be regenerated from this tissue. Indra Vasil (U. of Florida), Bob Conger (U. of Tennessee), and Ed Green (U. of Minnesota), along with their students and colleagues, were the main contributors to the early work elucidating the potential of this 'embryogenic callus' in grasses. Similarly, Jerry Ranch (Pioneer) and Norma Trolinder (Texas Tech) contributed much to the understanding of embryogenesis in the agronomic dicots soybean and cotton respectively. Based on theirs and
others' work, progress then followed on the development and refinement of embryogenic suspension culture systems.

Although the literature is full of reports of embryogenic callus and suspension cultures, it can be difficult to describe accurately a callus' phenotype, or, alternatively, to determine the fate of a piece of tissue based on the readings or photographs in a descriptive study. The best approach to gaining an understanding of embryogenesis is experimentation and experience. Once some experience is gained with one embryogenic system, it should not be difficult to perform parallel studies with another plant system. In fact, most embryogenic cultures look similar and respond to the same stimuli. These similarities become very apparent after working with many different systems.

This chapter describes the common features of embryogenesis systems and focuses on embryogenic suspension cultures. Protocols are given for initiation and regeneration of embryogenic suspensions of agronomically important monocot and dicot species; these protocols may be used in transformation studies with these crops.

2. Production of embryogenic suspension cultures

Embryogenesis is the process of embryo initiation and development. For zygotic embryos, embryogenesis starts at zygote formation, ends at seed maturation, and marks the beginning of the sporophytic generation of the life cycle. During embryogenesis, shoot and root meristems are initiated, the morphological pattern of the plant is determined, and carbohydrates, lipids, and proteins accumulate (3). During somatic embryogenesis, an embryo (similar to the zygotic embryo) containing both shoot and root axes, is formed from somatic plant tissue. An intact plant rather than a rooted shoot results from the germination of these somatic embryos.

For ease of explanation, the process of embryogenesis can be divided into four different stages. These are initiation or induction, proliferation, development or maturation, and finally germination (Figure 1). During initiation, cells are induced to form somatic embryos; during proliferation, these induced cells undergo multiplication with very little or no maturation of the embryogenic tissue. Although not entirely appropriate, germination is included in the process of somatic embryogenesis here because the dormancy associated with zygotic embryogenesis is often absent from somatic embryogenesis. In addition, many who work in the area of somatic embryogenesis also study germination of the somatic embryos. The proliferation stage will receive the greatest emphasis in this chapter. It is the least studied stage but is the most critical for embryogenic suspension culture work.

2.1 Initiation of embryogenic suspension cultures

For all tissue culture regeneration systems, there are numerous factors that affect tissue culture responses. These include the effects of media addenda
such as growth regulators, nitrogen sources, carbon sources, vitamins, and inorganic and complex organic addenda. In addition, the genetics and physiology of the starting or ‘explant’ material can have a major effect on the tissue culture response.

2.1.1 Explant status
Aside from the effects of various medium addenda, of equal or greater importance is the status of the explant. This includes the tissue type, physiological state of the tissue, and the genetic make-up of the donor plant. In most cases, the best explant for studies on induction of somatic embryogenesis is the immature zygotic embryo. This tissue is already embryogenic in nature and apparently requires less nurturing than other somatic tissues to elicit an embryogenic response. The zygotic embryo was not initially recognized as the best explant material for embryogenesis studies due to the relative plasticity of wild carrot (Daucus carota), the model for somatic embryogenesis studies. Almost any explant is adequate to induce embryogenesis in wild carrot.

The use of the zygotic embryo and description of a ‘response window’ for that zygotic embryo was first described for maize by Green and Phillips (4). Not only was it critical to use the zygotic embryo as the explant, but that embryo would only respond maximally if it were between 0.5 and 2 mm in length. The window for the zygotic embryo response was also observed with many cereals (wheat, rye, oats) as well as with other plants (5, 6). With some plants, explant tissue other than the proper staged zygotic embryo can produce somatic embryos, but the zygotic embryo is still considered to be the best overall explant for induction of somatic embryogenesis.

Beyond the physiological status of the explant, the next question is why are explants more responsive from some species than others? Even within a species, some cultivars are highly responsive while others do not give an embryogenic response. Are there ‘regeneration’ genes that can be isolated and studied? Ray and Bingham (7) developed alfalfa lines that were highly responsive to induction of embryogenesis using conventional breeding. The genetics of the embryogenic response was complex and multigenic. Recently, Armstrong et al. (8), using RFLP analysis, identified regions of the maize chromosomes that apparently carried genes that were associated with a high embryogenic response. A similar approach has been used for mapping genes
involved in androgenic embryogenesis in maize (9). Although some of the genes that are involved in the embryogenic response have been mapped in maize, these genes have not yet been cloned and studied.

2.1.2 Media addenda
For somatic embryo induction and proliferation, you must supply an auxin, (usually 2,4-D) to the tissue. The levels of auxin that are used for induction of embryogenesis are often fatal to the intact dicot plants used for somatic embryogenesis experiments. It is intriguing that a group of herbicides (2,4-D, picloram, dicamba) contains the most effective inducers of somatic embryogenesis. Although there has been some effort to understand the molecular biology of embryogenesis (10), and some auxin-inducible genes have been cloned and studied (11), the specific role of auxin in the somatic embryo induction process remains unknown. It is clear that auxin application results in production of ethylene, which in turn can inhibit induction of embryogenesis. Inhibitors of ethylene production (aminovinyl glycine, AVG) and ethylene action (AgNO₃) can be used in tissue culture to enhance the embryogenic response in maize (12, 13). Benefits of ethylene inhibitors have not been documented in dicot embryogenesis systems.

The importance of nitrogen in somatic embryogenesis is without question. Tissue culture media usually contain nitrate and ammonia; both are available to the plant tissue at very high concentrations. These compounds are usually not limiting for growth in culture. Effects of nitrogen on somatic embryogenesis are more often seen through the addition of certain amino acids (14). It is unclear if these amino acids are beneficial for embryo initiation, proliferation, or development. It is interesting that the levels of the amino acids that affect somatic embryogenesis (proline, glutamine, asparagine, alanine) are enhanced during times of plant stress and are a major component of seed and vegetative storage proteins.

2.2 Proliferation
Although an understanding of embryo proliferation is critical for manipulation of somatic embryogenesis, this phase has not been well studied. In the rush to claim recovery of plants from somatic embryos, the benefits of somatic embryogenesis are ignored. These benefits include generation of large amounts of uniform embryogenic tissue for development studies, and large scale plant recovery, as well as transformation research (protoplasts, electroporation, particle bombardment), and in vitro selection of mutants.

Proliferation of embryogenic tissue can be obtained and studied using either semi-solid or liquid media. Although one would assume that it is more difficult to work with proliferative liquid culture systems, this is not the case. The following are the benefits of using liquid media as opposed to solidified media:
more rapid growth
more efficient selection
fast and more uniform response to media manipulations
ey easy visualization of tissue quality

The first three benefits are based on the high tissue-to-medium contact in liquid medium. In a liquid medium, the embryogenic tissue is bathed in medium while with solid medium, an embryogenic callus 'sits' on top of the medium. With callus, all compounds used by the plant cells must diffuse through the medium and pass through the tissue that is in direct contact with the medium. Gradients are established and, if embryos are formed on the top of a callus, it is not known what concentration or ratio of growth regulators was responsible for that response in that tissue. If embryos or embryogenic tissues are buried in a callus, they cannot be observed easily. In a liquid medium, the callus will dissociate and the embryogenic tissue will be free-floating. Since embryos and embryogenic tissues are small, you need a good quality inverted microscope for proper observation and evaluation of embryogenic suspension cultures. Microscopes having condensers and light sources that can be raised and/or rotated so that there is sufficient space to place a flask on the microscope stage are preferred. Direct observation of embryogenic tissue in liquid culture is effective, rapid, and simple once the observer becomes familiar with the morphology of high quality embryogenic tissue.

2.2.1 Morphology of embryogenic suspension culture tissues
Embryogenic suspension culture tissue from most higher plants is very similar in morphology when observed using an inverted microscope. Embryogenic suspension cultures do not exist as cultures of proliferating single cells. Although there are a few cases where single, isolated cells from embryogenic suspension cultures can form embryos directly (15), embryogenic cultures for the most part proliferate as aggregates of embryogenic tissue. Single cells can slough from these aggregates but these cells are more likely non-embryogenic and probably have low survival rates.

The cells that make up the aggregates of embryogenic tissue are typically small (~20 μm diameter), isodiametric, and densely cytoplasmic with small vacuoles (Figures 2, 3, and 4). This type of description pervades the literature and refers not to free-living single cells but to the cells that make up the aggregates of embryogenic tissue. The embryogenic aggregates (not cells) are dense, yellow to brown in colour (when observed with an inverted microscope), and have a relatively smooth surface (Figures 2, 3, and 4). The surface characteristics of these clumps result from the isodiametric morphology of their cells. If the surface of a cell aggregate is rough and elongated cells (banana-shaped) protrude from the surface (Figure 3A, black-filled arrows), the aggregate is either non-embryogenic or contains non-embryogenic tissue.
Figure 2. (A) Relatively pure, high quality embryogenic suspension culture tissue of cotton (*Gossypium hirsutum*). (B) Newly established embryogenic suspension culture of bentgrass (*Agrostis palustris*). Note the presence of both dense embryogenic clusters and non-cytoplasmic, translucent non-embryogenic aggregates.
Figure 3. Embryogenic suspension culture tissue of maize (Zea mays). The white arrows show dark diffraction rings where the clusters are out-of-focus. (A) Black-filled arrows show non-embryogenic cells protruding from a cell aggregate. (B) Black-filled arrow shows a non-viable cell.
Cells that contain a reduced or shrunken, irregular cytoplasm are probably not viable (Figure 3B, black-filled arrow). Isodiamic cells (embryogenic) will form a clump that has a smoother surface. The exception to the observation of a smooth surface for embryos is in the gymnosperms (Figure 4B).
5: Plant regeneration via embryogenic suspension cultures

Suspensor cells (Figure 4B, arrows), which are always associated with the embryo-proper in embryogenic gymnosperm suspensions, are elongate and somewhat vacuolated. Suspensor cells appear to be very similar to non-embryogenic cells but the embryonal head or embryo-proper is morphologically identical to the embryogenic clumps described above.

The surface of embryos at late developmental stages is very smooth from the presence of an organized epidermal layer. Proliferative cultures can contain globular and possibly later-staged embryos if the cultures are newly initiated or if the medium composition is not adequate to prevent embryo development. With soybean (16), fine proliferative cultures were never obtained and the cultures proliferate as relatively large (up to 8 mm diameter) clumps or masses of globular embryos (Figure 4A).

If developing or larger embryos can be observed in liquid medium, they should always be attached basally. This means that the smooth apical surface should protrude out from a central area. Cultures that contain clumps of tissue that have roots protruding outwards from a central core are probably root cultures. Since root formation in tissue culture is fairly common, one should critically examine the reports of ‘embryo germination’ with root formation but no shoot elongation.

The density of the embryogenic aggregates results from the cytoplasmic nature of the constituent cells. Non-embryogenic cells are more vacuolated and clusters of non-embryogenic cells are much less dense (Figures 2B, 3A and B). The density and colour of the aggregates reflect the growth rate or status of the tissue. Embryogenic aggregates that are rapidly proliferating are yellow to light brown while aggregates that have reduced growth appear very dark and are no longer translucent. If rapidly proliferating embryogenic aggregates are observed under the microscope and taken out-of-focus, they will exhibit a dark ring of diffraction and will appear almost ‘oily’ in appearance (Figure 3, white arrows). The colour and density of embryogenic aggregates can be used rapidly and precisely to gauge the quality of the embryogenic material in suspension culture. The effects of various media on the quality of embryogenic tissue can be accurately determined and liquid cultures that contain the highest amount of embryogenic material can be preferentially subcultured.

2.2.2 Preferential or selective subculture

Through preferential subculture, it should be possible to maintain embryogenic cultures for long periods of time (years) without a loss in embryo-forming capability. Plant regeneration capacity and fertility of regenerated plants usually decline in long-term embryogenic suspension cultures but the cultures do not turn non-embryogenic. Rather, the non-embryogenic tissue simply out-competes embryogenic tissue without selective subculture practices.

Selective or preferential subculture here has two meanings. The first refers to the selection of whole cultures that have the greatest proportion of
proliferating, high quality embryogenic aggregates compared to non-embryogenic cells and debris. Flasks that contain the highest quality tissue are preferentially subcultured. With repeated subculture, this may narrow the genetic base of the suspension culture, but the quality of the suspension culture can be rapidly improved. The second type of selective subculture involves the isolation and purification of embryogenic tissue from liquid cultures. Embryogenic tissues can be identified with the aid of a microscope and subcultured into fresh medium. You can be extremely selective using this method by picking only a few pieces of the highest quality tissue for subculture. This type of selective subculture is not too different from isolation and maintenance of embryogenic callus cultures but you can subculture smaller pieces of tissue and this tissue can be more critically evaluated for subculture using the characteristics outlined above.

The 'low inoculum rule' for initiating and maintaining embryogenic suspension cultures is based on the ability of embryogenic tissue to survive and proliferate at very low inoculum densities in liquid culture. The main benefit of low inoculum culture is that very small amounts of tissue are required to initiate and maintain an embryogenic suspension culture. If you need to evaluate many different liquid media and high quality embryogenic callus tissue is limiting, you can use extremely small amounts of tissue (from 10 to 50 mg) to initiate liquid cultures. In addition, a very small amount (one or two aggregates) of proembryonic tissue from a mixed embryogenic and non-embryogenic suspension culture can be used for subculture.

A second benefit of low inoculum subculture of embryogenic tissue is that the non-embryogenic tissue does not survive subculture whereas the embryogenic tissue continues to proliferate regardless of cell density. In a pure embryogenic culture, non-embryogenic tissue can come from embryogenic tissue as sloughed cells and cell clusters. At high inoculum, this non-embryogenic tissue may interfere with embryogenic cell growth or proliferate but, at low density, these cells and cell aggregates have little effect and proliferate slowly if at all.

A third advantage of low inoculum culture is that the subculture period is extended. With rapidly growing cells in culture, the doubling time may be around three days, therefore cultures can be divided or split every three days if desired. A three day subculture regime will provide large amounts of rapidly proliferating tissue, but this is labour intensive and is quite inconvenient. If you use less tissue for initiation and maintenance of embryogenic suspension cultures, the subculture period can be lengthened to every one, two, or even four weeks. Subculture of approximately 100 mg (approx. 100 μl packed cell volume) of tissue every one to two weeks is both convenient and beneficial, providing adequate amounts of rapidly growing, high quality, embryogenic suspension culture materials.

The low inoculum rule can be easily verified. Take a small amount of high quality embryogenic tissue for initiation or maintenance of suspension cul-
ture. If the medium is not adequate for growth of embryogenic tissue (low or high inoculum) or if the tissue is poor quality (non-embryogenic or large embryos), you will not see a low inoculum effect. Perhaps the best place to start experimenting with low inoculum cultures is with newly established cultures and a medium that is known to support embryogenic growth. A series of subcultures comparing low and high inoculum will be a small effort and can save much time and effort in the long-term.

The quality of the embryogenic callus used for initiating suspension cultures cannot be overemphasized. Simply put, the higher the quality of the callus, the faster the suspension will become established. Without the proper starting material, it can be difficult to impossible to establish a high quality embryogenic suspension culture.

2.3 Methods for establishing an embryogenic suspension culture

Establishment of an embryogenic suspension culture may take from one to 12 months. Any callus which contains embryogenic sectors can be utilized but tissue that contains rapidly growing proembryonic material is best. The general methods used to initiate and maintain embryogenic suspension cultures are similar across genera, and there are some guidelines that can be followed regardless of plant type. As with most procedures, there are exceptions. Below, a general procedure for initiation and maintenance of embryogenic suspension cultures is presented. This is followed by a description of five different systems that should provide information on most of the different situations that may be encountered with induction of liquid cultures.

In most cases, tissue that is already embryogenic (embryogenesis has been induced) is used to initiate liquid cultures (specific details on the induction of embryogenic callus are given in Protocols 1–5). You can use either small (100 mg) or larger amounts (1–2 g) of tissue to initiate suspension cultures. However, when using large amounts of tissue, you must closely watch cultures to prevent overgrowth of non-embryogenic or embryogenic tissue. At high density, either non-embryogenic tissue out-competes or outgrows embryogenic tissue or embryogenic tissue will start to senesce, a process that is sometimes difficult to reverse. You must also be cautious in distinguishing between embryo proliferation and development. A new culture may appear to be proliferating but the new tissue may be embryos that arise from pre-existing proembryos. Once embryo development is triggered, it is also a difficult process to reverse. Embryo development is distinguished by the presence of a smooth surface on the developing embryos when viewed using an inverted microscope.

For induction and maintenance of liquid cultures, 125 ml baffled deLong flasks are preferred. These flasks are relatively expensive but well worth the investment. The baffles aid in agitation of cultures resulting in superior tissue
dissociation and good culture aeration. It is possible to get sufficient mixing in
non-baffled flasks if the agitation speed is increased and/or the medium
volume is reduced. Place 33–50 ml of medium in a 125 ml deLong flask and
agitare at 130–150 r.p.m. A 125 ml Erlenmeyer flask agitating at 150 r.p.m.
using 25 ml of medium may also be satisfactory. If, during culture with
agitation, the cell clumps are not evenly distributed in the flask but remain in
the centre of the flask, the agitation may be insufficient.

In addition to improved aeration, the deLong flasks are much easier to
work with aseptically. The necks of deLong flasks are straight as opposed to
those of Erlenmeyer flasks, which have slightly fluted neck openings. The
advantage of the straight necks is that rigid plastic or metal caps can be used
to close the flasks. These caps allow one-handed, one-step manipulations. To
prevent any potential contamination problems during culture, seal the rigid
caps to the flask using laboratory film. The film can be placed in such a
position as to allow some gas exchange while keeping the cap firmly in place
and sealing all large gaps.

2.3.1 Newly established suspension cultures
When embryogenic tissue is initially placed into liquid medium, non-
embryogenic cells will be present. These cells are either present in the starting
material or are sloughed from embryogenic material. Some cell cultures will
also produce polysaccharides, which, together with the non-embryogenic
cells, give the initiated suspension culture a cloudy appearance. It is important
to make some initial observations (directly following initiation of liquid
cultures) and follow the embryogenic cultures through their ‘maturation’. The
embryos and embryogenic tissue, if present, will first appear yellow to light
brown under the inverted microscope but may become very dense or darken
within a few days. Once the tissue has become adjusted to the liquid medium,
the embryogenic tissue will start to proliferate and the new growth will appear
yellow to light brown with the other visual characteristics described earlier.
Some embryogenic tissue starts growth immediately following initiation of
suspension cultures while in other cases, it can take one to four weeks for
embryogenic tissue to resume growth in liquid culture. If the tissue remains
dark and very dense for an extended period (when observed using an inverted
microscope), this is an indication that the tissue is not rapidly proliferating.
You can rapidly evaluate suspension culture media and response of embry-
ogenic tissue to limited media by comparing clump density during the first few
weeks following initiation of the suspension culture.

2.3.2 Purification of the suspension culture
If the media/tissue combination supports growth of embryogenic tissue, the
‘young’ embryogenic suspension culture will still contain a mixture of different
cell types and debris. Before embryogenic cultures can be used for
physiological studies or transformation work, the culture needs to be purified
of the non-embryogenic tissue. There are at least four basic means of ‘cleaning’ a suspension culture of non-embryogenic cells and debris. Cleaning can be a continual process with some systems and these procedures must be used throughout the maintenance or subculture period. The choice of methods is dependent on the nature of the suspension cultures and suspension culture tissue. If the clumps of tissue are large, it certainly would not be appropriate to use a narrow-mouth pipette to transfer the tissue. If there is a lot of debris in the suspension, it may be difficult to separate clusters by filtration. Three general methods which can be used to purify embryogenic suspension cultures are described below.

i. Density transfer
This method uses density to separate physically the lighter non-embryogenic cell clusters, cells, and debris from the denser embryogenic clumps. Pipette 1–5 ml of a young embryogenic culture using a 10 or 25 ml wide-mouth glass or plastic pipette. Gently agitate the flask either just prior to (5–10 sec) or during the pipetting to resuspend the cell clusters. Then remove the pipette from the flask and place the tip in fresh medium in a new flask (without actively discharging the pipette). The denser embryogenic clusters will fall out of the pipette while the non-embryogenic cells and debris will remain in the pipette. Sometimes, if there is a lot of embryogenic tissue or the operator is not rapid enough, the embryogenic tissue will collect in the tip and temporarily clog the pipette. If this happens, take up a small amount of fresh medium (0.5 ml) into the pipette to resuspend the ‘pellet’. Alternatively, gently tap the pipette to loosen the embryogenic tissue. This simple technique is very efficient for the separation of embryogenic and non-embryogenic tissue. You can selectively subculture small clumps by allowing the larger clumps to settle to the bottom of the flask prior to removal of embryogenic tissue using a pipette. This procedure, as with most clean-up protocols, does not result in instant success. From four to seven repetitive transfers are required before the culture is purified. Carefully watch the subculture period so that the cultures are not permitted to become dense with cell clusters and debris. As stated before, embryogenic suspension cultures that become overgrown will start to senesce and produce non-embryogenic tissue.

ii. Repetitive frequent transfer
In this method, frequently remove and transfer aliquots of the young suspen-
sion, usually at weekly intervals. At low density subculture, embryogenic tissue can survive this rapid subculture whereas non-embryogenic tissue does not survive. With frequent transfer, cellular debris and non-embryogenic tissue are simply diluted with each subculture.

iii. Manual selective transfer
You can physically select the aggregates of choice using either fine forceps and a good dissecting microscope or a pipette and an inverted microscope;

111
both methods must be performed in a sterile environment. This technique requires proper identification of embryogenic material and a steady hand to transfer small pieces of tissue in liquid without damaging that tissue. When using forceps to transfer a small piece of tissue, use one hand to hold the forceps as you normally would while the other hand can be used to stabilize the instrument. To stabilize the forceps properly, the index finger of the stabilizing hand should be touching the other index finger, thumb, and two prongs of the forceps at the same time. It should be possible to maintain a 0.5 mm gap between the forceps prongs using this technique. You can use a 1 ml pipette to transfer small pieces of tissue from a Petri dish using an inverted microscope. The small opening of the pipette is very large when viewed under an inverted microscope and, with some practice, it is possible to pick out single clumps of tissue less than 100 μm in diameter. It is difficult to obtain large amounts of proliferative embryogenic tissue using physical selection. Therefore, this method takes advantage of low inoculum subculture of embryogenic tissue.

2.4 Development and maturation of somatic embryos
For embryo development, the proliferative embryogenic tissue is transferred to a medium containing either no or low auxin and sometimes ABA, high sugar, or amino acids (see Protocols 1–5). The effect of ABA on somatic embryogenesis has received much attention in recent years. Ammirato (26) was the first to report ‘normalizing’ effects of ABA on somatic embryogenesis in caraway. He noticed that ABA suppressed embryo development and allowed more normal cotyledon formation. Crouch (27) demonstrated an accumulation of seed storage protein in Brassica napus following exposure of somatic embryos to ABA. Without this exposure to ABA, the somatic embryos failed to accumulate seed storage protein or develop normally. High sugar (6–18% sugar) or amino acids may substitute for this ABA effect as interactions between osmotic stress, ABA production, and accumulation of certain amino acids (glutamine, proline) are well documented.

Germination of developed somatic embryos can occur sporadically, with some embryos never germinating to form roots and shoots. Embryos may not germinate if they have not reached physiological maturity. Although embryos can be morphologically similar, they may be quite different physiologically. In most cases, it is beneficial to allow for an extended embryo development period to assure physiological maturity. With soybean, the somatic embryos turn from green to yellow (similar to zygotic embryos) as they mature. Embryos that develop over one to two months will be more responsive to desiccation and other dormancy-breaking treatments.

2.5 Specific protocols for plant regeneration via somatic embryogenesis
Protocols 1–5 describe the establishment of embryogenic cell suspensions and subsequent regeneration in orchardgrass, rice, maize, soybean, and cotton.
5: Plant regeneration via embryogenic suspension cultures

Establishment of an embryogenic callus culture of alfalfa is described in Chapter 6C, Protocol 1, and regeneration of caucasian bluestem through embryogenic cultures in Chapter 1, Protocol 7.

**Protocol 1.** Production and regeneration of embryogenic cultures of orchardgrass* (Dactylis glomerata L.) (17)

A. Establishment of embryogenic cultures

1. Grow Dactylis clones selected for high regeneration capability in the greenhouse.
2. Cut out several tillers including the root mass. Rinse off residual soil.
3. Cut off root mass leaving the basal meristem intact. Discard the root mass.
4. Split the youngest (innermost) leaves, longer than 4 cm, longitudinally along the midrib.
5. Sterilize the leaf tissue in 50% ethanol for 2 min and then rinse two or three times with sterile distilled water.
6. Slice the leaves into five 3 mm segments starting at the base.
7. Plate the leaf segments on to Schenk and Hildebrandt (SH, ref. 19) basal medium containing 30 μM (6.6 mg/litre) dicamba and 0.25% (w/w) Gelrite.
8. Culture the leaf segments in the dark at 25°C. Embryogenic callus should form within one and a half to two weeks.
9. After three or four weeks of callus growth, remove approximately 0.5 g and inoculate into 50 ml of liquid SH medium containing 45 μM (10 mg/litre) dicamba and 4 g/litre casein hydrolysate in a 125 ml baffled flask.
10. Grow cultures in the dark on a gyratory shaker at 130 r.p.m. for four weeks.
11. Transfer to fresh medium using the 'sedimentation method' (20).b
12. Repeat step 11 every three to four weeks using the best quality flasks as judged by the abundance and quality of the embryogenic material (see Section 2.2).

B. Regeneration of plants

1. Plate 1 ml of the embryogenic suspension on SH medium containing 30 μM (6.6 mg/litre) dicamba.
2. Place cultures in the dark for two to three weeks.
3. Isolate and culture single somatic embryos (identified as solid, very pale structures against the cream coloured callus) and plate on growth regulator-free SH medium.
4. Place somatic embryos in low intensity light at 25°C.
Protocol 1. Continued

5. When plantlets are about 3 cm tall, transfer to culture tubes containing growth regulator-free SH medium. When plants are 8–10 cm tall, transfer to soil, acclimate, and move to the greenhouse.

* Orchardgrass is one of the most responsive of the Gramineae in production of embryogenic callus. Establishment of suspension cultures is routine but requires some experience. Best results will be obtained when using clones selected for high regenerative capacity, such as those developed by Conger et al. at the University of Tennessee (18).  

b The sedimentation method is similar to the density transfer method (Section 2.3.2) but the larger cell clumps are allowed to settle prior to removal of the pipette from the stock culture. The result is the transfer of smaller clumps of embryogenic tissue.

Protocol 2. Production and regeneration of embryogenic cultures of rice (*Oryza sativa* L.) (from Horn, personal communication)

A. Establishment of embryogenic cultures

1. Surface sterilize mature, dehusked rice seeds by treating with bleach (5.25% NaHClO₄) for 30 min under constant agitation.
2. Rinse seeds three times with sterile distilled water.
3. Repeat steps 1 and 2 two more times to ensure disinfestation (mature seeds are able to tolerate harsh disinfestation treatments compared to other plant materials).
4. Plate seeds embryo side up on N6 medium (21) containing 12 µM proline, 20 µM 2,4-D (4.4 mg/litre) (N6–20 medium), and 0.25% Gelrite.
5. After three days, transfer the embryos that are not contaminated to fresh N6–20 medium.
6. After eight days, dissect the scutellar callus dome from the endosperm, root, and shoot. If non-embryogenic (NE) callus is present on the callus dome, remove it and discard.
7. Transfer the embryogenic callus to solid N6 medium for two to four weeks.
8. For initiation of embryogenic suspension cultures, inoculate 0.5–1.0 g fresh weight of pure embryogenic callus into 50 ml of liquid N6 medium containing 12 mM proline, 20 µM (4.4 mg/litre) 2,4-D, and 0.34 mM glutamine.
9. Examine the suspension cultures for quality. If necessary, perform selective subculture on the suspension culture material.

B. Regeneration of plants

1. For embryo development, collect the suspension culture material and rinse with liquid growth regulator-free N6 medium. Plate tissue on solid
N6 medium containing 12 mM proline, 50 μM ABA, 6% sucrose, and 2–5 μM (0.4–1.1 mg/litre) 2,4-D, and place in the dark for two weeks.

2. Transfer the callus to N6 medium containing 12 mM proline and 6% sucrose and place back in darkness for two to four weeks. Examine the cultures periodically for the appearance of somatic embryos. If few or no somatic embryos appear after four weeks, repeat steps 8 and 9.

3. Remove mature somatic embryos and plate on MS medium containing 100 mg/litre inositol, 5 mg/litre thiamine HCl, 2 g/litre casein hydrolysate, 2.3 μM kinetin, 8 g/litre agar for germination.

4. Transfer plantlets containing shoots and roots to soil after they reach a height of 10 cm. Grow initially in the shade with moderate misting.

**Protocol 3. Production and regeneration of embryogenic cultures of maize***(Zea mays L.)*

A. **Establishment of embryogenic cultures**

1. Harvest ears from plants about 12–14 days after pollination or when the embryos are 1–1.5 mm long and remove husks. Wash the ear thoroughly to remove silks, insects, and other large surface contaminants.

2. Insert pipette or long forceps into the basal end of the cob and put the cob into a sterile 500 ml beaker. Tall beakers usually work best. The pipette or forceps will serve as a cob handle for the rest of the procedure.

3. Pour 50% bleach into the beaker to cover the cob and soak for 30 min. Make sure that the cob does not float out of the bleach.

4. Pour off the bleach into a waste container and rinse the cob three times with sterile water to remove the bleach.

5. Cut off the top half of the kernels using a sterile scalpel. Kernels are not removed from the cob and the tops of 20–30 kernels are removed at a time. Change blades after every cob as they dull easily. The zygotic embryos are at the base of the kernel on the side wall toward the tip of the cob.

6. With a spatula, scoop out and discard the endosperm. Gently push on the side of the kernel wall to push the embryo up and out of the kernel.

7. Place the embryos, axes up (flat side down), on callus induction media.³

8. Allow embryogenic callus to form in the dark or low light at 25 °C for one to two weeks. Subculture high quality callus, as judged by friability and colour, on the same callus induction medium for two to four weeks with weekly transfers. These transfers require examination of the callus using a dissecting microscope and selective subculture.

9. Initiate embryogenic suspension cultures by inoculating 100–200 mg fresh weight of high quality embryogenic callus into 35 ml of liquid proliferation medium.
Protocol 3. Continued

10. Subculture selected suspension cultures containing the highest proportion of high quality embryogenic material (Figure 3) using the density method (see Section 2.3.2). Subculture at low density is quite beneficial for these newly established cultures.

B. Plant regeneration

1. For somatic embryo development, plate the suspension culture on solidified induction medium for two to four weeks until an embryogenic callus is again formed. Then transfer the embryogenic callus to a growth regulator-free medium containing 6% sucrose with or without 12 mM proline. Somatic embryo formation and maturation can require from two to six weeks depending on the genotype and age of the suspension culture.

2. For germination of the mature somatic embryos, transfer tissue to a growth regulator-free medium with 2% sucrose.

3. Transfer plantlets to soil after they reach a height of 8–10 cm. Gradually expose plantlets to lower humidity in a growth chamber before moving to a greenhouse.

*a The most responsive maize tissue for embryogenic callus and suspension culture work is obtained from the hybrid immature embryo from an A188 × B73 cross. Embryogenic callus and suspension cultures from this hybrid callus can be maintained on a simple medium containing MS salts, B5 (22) vitamins, 1–1.5 mg/litre 2,4-D, and 2% sucrose. Other lines have different media requirements such as inclusion of proline or casein hydrolysate, preference for other auxins such as dicamba, or increased sucrose concentration.

*b The optimum callus induction medium depends on the genotype (23–25). Most of these media are based on N6 or MS salts and vitamins, 2% or more sucrose, and an auxin such as 2,4-D or dicamba.

*c The liquid medium can be the callus induction medium without the gelling agent or may be substantially modified, again depending on the genotype.

---

Protocol 4. Production and regeneration of embryogenic cultures of soybean (Glycine max L. Merrill)

Soybean has proven to be the most unusual embryogenesis system of those described here. Initiation of proliferative embryogenic tissue requires use of extremely high 2,4-D levels and the embryogenic suspension culture is very clumpy.

A. Establishment of embryogenic cultures

1. Harvest pods from greenhouse grown soybean plants approximately 14 days post-pollination. The immature seeds should be about 4 mm in length and can be observed through the pod by back-lighting.

2. Wash the pods with warm soapy water and surface disinfect with 20% bleach for 20 min. Wash the pods five times with sterile distilled water.

3. Cut 4 mm off the end of the pod and peel back the pod to expose the immature seeds.*
4. Cut and discard the embryo axis by slicing 1–1.5 mm off the pointed end of the immature seed.

5. Put gentle pressure on the remaining portion of the seed to remove the two cotyledons. Place the cotyledons, flat surface-down, on the callus induction medium containing MS salts, B5 vitamins (22), 40 mg/litre 2,4-D, 6% sucrose, and 0.8% Nobel agar.

6. After six to eight weeks, remove the proliferative embryogenic material for initiation of suspension cultures.\(^b\)

7. Place one small piece of tissue (≤ 1 mm in diameter) in 35 ml of suspension culture medium containing modified MS salts (10 mM NH\(_4\), 40 mM NO\(_3\), ref. 16), B5 vitamins, 6% sucrose, and 5 mg/litre 2,4-D. Make an initial observation and observe weekly thereafter.

8. Monthly, remove only the high quality embryogenic tissue with forceps for subculture.\(^c\)

9. For plant recovery, plate the clumps of embryogenic tissue on development medium containing MS salts, B5 vitamins, 6–12% maltose, and 0.2% Gelrite at 23°C. After one month, isolate single embryos and plate on the development medium for one additional month.

B. Plant regeneration

1. After the embryos start turning yellow, place nine embryos in a dry 100 mm Petri dish for desiccation treatment.

2. Place the dish at 25°C for two to three days.\(^d\)

3. Transfer the embryos to a medium containing MS salts, B5 vitamins, 3% sucrose, and 0.2% Gelrite at 27°C for germination.

4. After germination (one to two weeks), transfer the plantlets to a larger container such as a Magenta GA7 for further growth.

5. After the plant reaches the top of the container, transfer to a soil : sand : peat (1:1:1) mix and acclimate for two weeks before gradual exposure to ambient humidity and transfer to the greenhouse.\(^e\)

\(^a\) An immature soybean seed has a rounded and a more pointed end. The embryo axis is located at the pointed end.

\(^b\) The tissue becomes proliferative when secondary somatic embryos grow from the apical surface of the primary somatic embryos. In cases where proliferation is vigorous, a whorl or rosette of secondary embryos is formed.

\(^c\) The clumps of tissue will remain large (average size of 4 mm, Figure 4A) and a fine suspension culture will never be formed. Tissue that is spherical and consists of bright green, compact lobes is best.

\(^d\) The embryos should wilt but should not lose too much water; smaller embryos will desiccate faster than larger embryos and severe desiccation is not beneficial.

\(^e\) Unlike other germinating somatic embryos, soybean somatic embryos from suspension culture do not survive rapid transfer to a soil mix directly following germination. Harden them off in vitro as they increase in size prior to transfer to soil.
Protocol 5. Production and regeneration of embryogenic cultures of cotton (*Gossypium hirsutum*)

A. Establishment of embryogenic cultures

1. Sterilize seed material; the sterilization technique is dependent on the status of the starting seed material.
   
   (a) If seeds are delinted, sterilize the seed with a 1–2 min dip in 70% ethanol, followed by treatment with 20% bleach for 20 min.
   
   (b) If seeds are delinted and coated with fungicide, rinse them four times with 95% ethanol prior to immersion in 20% bleach solution.
   
   (c) For seeds that are not delinted, place the seeds in concentrated H$_2$SO$_4$ for approximately 5 min or until the residual fibres are visibly removed and the seed turns black. Rinse the seeds carefully ten times with sterile water before bleach disinfection.

2. Rinse the seeds four times and place on growth regulator-free MS medium containing 3% sucrose and 0.8% agar. Germinate the seeds for one week at 27–31°C with a light intensity of 30 μE·m$^{-2}$·s$^{-1}$.

3. Excise cotyledon sections and place pieces (approximately 3 × 3 mm) on medium containing MS salts, B5 vitamins (22), 3% glucose, 2 mg/litre NAA (or 0.5 mg/litre 2,4-D), 1 mg/litre kinetin, and 0.8% agar. Transfer the cotyledon pieces to fresh induction medium every week to prevent accumulation of phenolics in the medium.

4. After one month, transfer callus tissue to a medium similar to the induction medium but containing 3% sucrose.

5. After one additional month, transfer one piece of callus (250 mg) to 35 ml of suspension culture medium containing MS salts, B5 vitamins, 3% sucrose, and either 0.5 mg/litre picloram or 0.1 mg/litre 2,4-D.

6. Observe cultures using an inverted microscope for the presence of embryogenic cotton tissue (*Figure 2A*). Subculture those flasks that contain embryogenic tissue using the suspension culture medium.

7. After one additional month of proliferation, transfer the tissue to a medium containing MS salts, B5 vitamins, 3% sucrose, and 5 mg/litre 2,4-D. In this medium, the embryogenic tissues proliferate rapidly and the cluster size is reduced.

B. Plant regeneration

1. For embryo development, transfer the suspension culture tissue to the seed germination medium but containing 15 mM glutamine.

2. After one to two months of development, transfer the embryos to a
medium containing modified MS salts (no NH₄, two times NO₃), 1% sucrose, and 0.2% Gelrite. Nitrate treatment can be used to break dormancy in some seeds.

3. Transfer the germinating embryos to a 1:1:1 mix of vermiculite:perlite:peat under high humidity.

4. Gradually expose the plantlets to ambient humidity and move to the greenhouse.

*a This procedure was developed for Coker lines of cotton. Other lines may respond to the manipulations but use of Coker 310, 312, or 315 is preferred.

*b The callus used for initiation of cotton suspension cultures is the only tissue in this chapter that does not contain large, recognizable embryos. Callus should be yellow to cream coloured and friable.

*c Over the first month of culture in liquid medium, it is not unusual for the culture medium to darken considerably.

3. Uses of embryogenic suspension cultures

Embryogenic suspension cultures have been utilized for many purposes. Their mitotic indices are quite high compared to callus cultures and most plant parts. This rapid division is important in the scale-up for cloning of elite germplasm. The suspension cultures are a good source for physiological studies and artificial seed production (see Chapter 6C) because the population of cells is more uniform than in callus, and somatic embryo development can be partially synchronized. The small size of the clumps makes penetration of growth regulators, inhibitors, and selective agents faster and more uniform, and makes scientific results less variable since gradients are less likely to complicate physiological studies. In many species of the Gramineae, embryogenic suspension cultures are the only source of protoplasts capable of cell division and subsequent plant regeneration. The relatively thin wall and high cytoplasmic density makes embryogenic suspension tissue ideal for cryopreservation work (see Chapter 7).

3.1 Desiccation and artificial seeds (see Chapter 6C)

Desiccation of somatic embryos serves two related purposes. First, in some species, desiccation breaks the dormancy associated with the somatic embryos. Secondly, desiccation promotes the accumulation of nutrients, such as storage protein, which will benefit the embryo when germination is allowed to proceed. Grape (29) and soybean (30) are two examples where the beneficial effects of desiccation on germination frequency have been shown.

The production of artificial seed (28) requires a large supply of normal mature somatic embryos. Embryogenic suspension cultures can provide the proper starting material for this type of work. As a mass expansion step, embryogenic suspension cultures are clearly superior to other tissue types.
with regards to growth rate. Although it is possible in some species (e.g. carrot) to produce artificial seeds from embryos developing while still in the liquid suspension phase, maturation for most somatic embryos requires plating on to a solid embryo development/maturation medium and then, at some later time, hand picking and processing (encapsulating) the somatic embryos.

3.2 Transformation

3.2.1 Protoplasts

Undoubtedly the most visible use of embryogenic suspension cultures has been for transformation in the economically important group of plants known as the Gramineae. I. K. Vasil and colleagues at the University of Florida showed that in the Gramineae, embryogenic suspension cultures were unique in their ability to release protoplasts capable of dividing at a reasonable frequency. Initially, this work met with much scepticism but it soon became apparent that embryogenic suspension cultures from a broad array of species such as pearl millet (31), Napier Grass (32), guinea grass (33), sugarcane (34), maize (35), orchardgrass (20), rice (36), and wheat (37) were capable of producing totipotent protoplasts.

These protoplast systems led to the first reports of recovery of transgenic Gramineae species such as maize (38), orchardgrass (39), and rice (40). Protoplast transformation is accomplished by direct gene transfer using one of two methods (see Chapter 3, Section 2):

- electroporation (41)
- polyethylene glycol (42)

Each of these protoplast transformation methodologies opens pores in the protoplasts allowing DNA to enter the cytoplasm. The difficulty with protoplast transformation systems is that protoplast isolation and culture is required (see Chapter 2). This is a laborious process and plant recovery from protoplasts may be extremely difficult in some cases.

3.2.2 Particle bombardment

The last several years have seen reports of alternatives to protoplasts for genetic transformation of monocot species. Recovery of transgenic plants via particle bombardment of embryogenic cells (43), apical meristems (44), and whole zygotic embryos (45, 46) is routine in certain laboratories. Whole tissue electroporation has also been reported as a viable alternative to particle bombardment (47). For transformation via particle bombardment and whole tissue electroporation to be successful, the target tissue must be competent to form germ-line tissues. It appears that cells that are actively dividing are more receptive to DNA introduction. Because apical meristems and whole embryos have been successfully used for some of these studies, it has been falsely assumed that the meristems simply elongate and the embryos germinate.
directly to form transgenic plants. This is far from the truth. To enhance recovery of transgenic tissue/sectors, the meristems and whole embryos proliferate, and any potentially transformed tissue can be either selected and/or multiplied. This proliferation takes the form of a shoot multiplication system for meristem transformation and embryogenic callus formation for whole embryo transformation.

Virtually all transformation methodologies show higher transformation efficiencies with suspension cultured cells than with callus, as judged by transient β-glucuronidase positive (GUS+) events. This holds for transfection in rice (48), use of SiC fibres in maize (49), and particle bombardment in maize (43), soybean (50), and cotton (51). Franks and Birch (52) bombarded regenerable suspension cultures, non-regenerable suspension cultures, and callus cultures of sugarcane and found that the regenerable suspension cultures showed over five and a half times the number of GUS+ events than was obtained from the callus tissue. The non-regenerable suspension culture tissue showed more than 41 times the number of GUS+ events than was obtained from the bombarded callus. The higher transient transformation rates are probably related to the higher growth rate of the suspension cultured cells compared to callus. Although embryogenic suspension cultures provide the most suitable starting tissue for most transformation work, use of this tissue has been hampered by a poor understanding of embryogenic suspension culture systems. Hopefully, this chapter will alleviate some of these concerns.

3.2.3 Fate of introduced DNAs

With all naked DNA transformation systems (e.g. direct DNA uptake with protoplasts, particle bombardment, whole tissue electroporation, SiC fibres), the fate of the introduced DNAs are similar. Once inside, the DNA somehow travels to the nucleus where it is incorporated into a site on one or more of the chromosomes. This DNA integration step is poorly controlled and a great deal of variability exists in terms of the level of gene expression and number of integrated gene copies. When plasmid DNA is introduced into plant cells, it recombines with both itself and plant DNA. Plasmid concatenation which is indicative of extrachromosomal homologous recombination has been reported with electroporated tobacco protoplasts (53) and bombarded cotton and soybean tissues (50, 51). Homologous recombination of plasmid DNA with plant DNA has been reported but the efficiency is very low (54). If the recombination process could be better controlled, copy number and therefore expression levels could be more accurately regulated. The variability in levels of gene expression in transgenic plant tissues has been somewhat reduced using scaffold attachment regions or SARs (55). These regions, which integrate with other transforming DNAs, may act by buffering the introduced DNA from repression by the native DNA. SARs appear to reduce variability and raise the level of gene expression between transformation events.
3.3 Cloning of elite germplasm

The inherent objective of cloning elite germplasm is to mass produce exact copies in as little time as possible. Somaclonal variation is not considered desirable in this type of endeavour. Since more clones can usually be produced with more time and tissue mass, it is useful to utilize a 'mass balance' calculation. This calculation details the steps of the system and provides the time needed as well as the mass expansion expected at each step. An example of such a calculation is shown in Table 1 for rice. The primary stage of mass expansion is an embryogenic suspension culture initiated soon after callus induction and continued until the desired mass is achieved. Knowledge of the expected yield of somatic embryos per gram of tissue and the expected germination frequency of the somatic embryo is essential for making the decision as to the length of the suspension culture step. Mass expansion via the suspension culture step is clearly preferable to simple callus expansion since the growth rate is much faster in suspension cultures. Culture of tissue for relatively brief periods of time (≤ 15 weeks) in suspension culture is not detrimental to subsequent embryo formation and plant germination. Beyond 15 weeks, some species have a tendency to produce fewer somatic embryos (see Section 2.3). Rice has been an excellent example of the potential that mass cloning brings to agriculture.

Table 1. Mass balance calculation for a typical rice cultivar (Horn et al. unpublished)

<table>
<thead>
<tr>
<th>Time elapsed</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryogenic callus initiation</td>
<td>22 days</td>
</tr>
<tr>
<td>Tissue proliferation</td>
<td>13 weeks</td>
</tr>
<tr>
<td>Embryo production</td>
<td>25 weeks</td>
</tr>
<tr>
<td>Plants</td>
<td>7 months</td>
</tr>
</tbody>
</table>

*Per 20 seeds.

3.4 Physiological studies

Aside from carrot which appears to be a special case, there has not been a large number of basic physiological studies involving embryogenic cultures. Of particular interest would be studies comparing embryogenic and non-embryogenic cultures with regard to respiration, secondary metabolism, and phytohormone metabolism. There have been several such studies conducted with an emphasis on somatic embryo development and artificial seed. These have generally shown that ABA promotes embryo maturation with a concomitant increase in storage protein accumulation (56, 57).

Molecular analysis of embryogenic and non-embryogenic cultures have generated cDNA clones or protein profiles that are unique to embryogenic
cultures. In many cases, the justification for this type of work has been to isolate a ‘marker’ for embryogenesis *in vitro*. Unfortunately, the physiological functions of these cDNAs and proteins have, in most cases, not yet been determined. In addition, the necessity for the isolation of markers (for identification of embryogenic versus non-embryogenic tissues) is questionable based on the information in this chapter. It is simply much easier to identify embryogenic tissue in suspension culture than to perform molecular analysis of that tissue to come to the same (or maybe a different) conclusion. The problem with a marker is that it may be difficult to determine at the molecular or biochemical level if a cluster of cells has become embryogenic. With visual selection however, it is possible to observe a rare event in a population of cells.

The value of studying gene expression during embryogenesis to understand and possibly control zygotic and somatic embryogenesis is without question. There has been some progress in understanding the molecular biology of embryogenesis using *Arabidopsis* embryogenesis mutants (58). This zygotic system should also provide some very valuable and exciting information relevant to the molecular biology of somatic embryogenesis.

3.5 Cryopreservation (see Chapter 7)

Cryopreservation is important for embryogenic suspension culture work for a number of reasons. In many cases, establishment of an embryogenic suspension culture can be a large effort, requiring input of much time and expertise.

(a) Once established, the suspension culture may be genetically stable for a short period of time.

(b) Embryogenic cells are highly cytoplasmic and hence contain less water for ice crystal formation.

(c) The small size of the embryogenic clumps allows quick and uniform penetration of the cryoprotectant solution. Penetration is not a problem with DMSO, which readily traverses membranes, but it is a factor for the osmoticum(s) such as sucrose or trehalose which are common constituents of the cryoprotectant solutions.

(b) The rapid cell divisions of embryogenic suspension culture material allows viable cells in a post-freeze environment to re-establish the culture in a short period of time.

Acknowledgements

The author wishes to gratefully acknowledge Dr Michael Horn, a good scientist and friend, for his numerous and unselfish contributions to this chapter. His input in this and other pursuits has been invaluable. Salaries and research support were provided by State and Federal funds appropriated to
OSU/OARDC. Mention of trademark or proprietary products does not constitute a guarantee or warranty of the product by OSU/OARDC, and also does not imply approval to the exclusion of other products that may also be suitable. Journal Article No. 210–93.

References

5: Plant regeneration via embryogenic suspension cultures