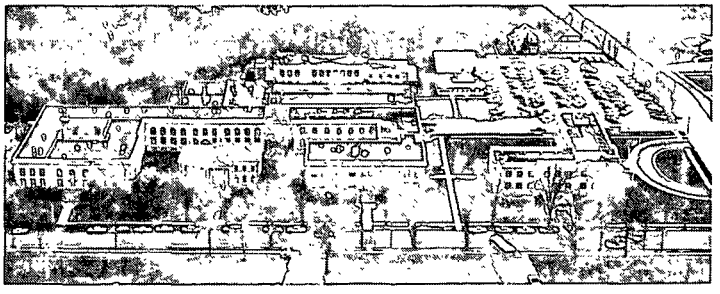

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CONIFER SOMATIC EMBRYOGENSIS AND CLONAL FORESTRY

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Conifer Somatic Embryogenesis and Clonal Forestry

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1. INTRODUCTION

Plants can be regenerated from cell and tissue cultures by two methods: organogenesis and somatic embryogenesis. Organogenesis is the production of unipolar bud primordia, either by the enhancement of axillary bud breaking or by production of adventitious buds. Regeneration via organogenesis is a sequential process: the induction of shoots with subsequent root formation. Somatic embryogenesis, by contrast, is a one-step induction process; bipolar embryos are formed with both root and shoot meristems (Thorpe 1988).

Somatic embryogenesis, the formation and development of embryos from somatic (vegetative) tissues under in vitro conditions, rather than maturation of the zygote, was first demonstrated with carrot (Steward et al. 1958). The list of plants that can be regenerated by somatic embryogenesis continues to grow (Tisserat et al. 1979, Ammirato 1983a, see references in this chapter). In fact, somatic embryogenesis has been demonstrated in essentially all major groups of higher plants, angiosperms (monocots and dicots) and gymnosperms (coniferous and nonconiferous).

The development of somatic embryogenesis in combination with encapsulated somatic or "artificial" seed technology holds immense potential for clonal forestry. This is especially true for coniferous trees that are currently propagated in seedling nurseries, because the potential exists for artificial seed technologies to easily interface with the existing seedling production systems.

Until recently most advances in somatic embryogenesis of woody plants had been with nonforest trees, e.g., citrus and palms (Litz 1985). Two recent reviews on somatic embryogenesis in woody plants thoroughly covered the considerable progress made with nonconiferous trees during the 1970s and 1980s

(Tulecke 1987, Wann 1989). Since the first report of somatic embryogenesis in conifers (Hakman et al. 1985), there has been a wealth of information published on conifer somatic embryogenesis. This chapter describes the process of regenerating plants from conifer somatic embryos with reference to current literature, and thereby synthesizes and summarizes the recent progress on conifer somatic embryogenesis.

2. HISTORICAL BACKGROUND

Many studies, primarily with herbaceous plants, have shown several factors to be important in the production of somatic embryos (Thorpe 1988, Brown 1988). These include the culture medium, the explant (starting tissue), and the genotype of the source plant. Carrot has served as an experimental model for in vitro embryogenesis in plants in many ways (Ammirato 1983a, Thorpe 1988). For instance, it was demonstrated with carrot that an auxin was critical for initiation of embryogenesis in vitro and that its removal induced embryo maturation (Halperin and Wetherell 1964). The importance of the level and form of nitrogen for somatic embryogenesis was also demonstrated with carrot tissue cultures (Halperin and Wetherell 1965, Wetherell and Dougall 1976). Considerable progress has been made recently on regenerating previously recalcitrant cereal crops via somatic embryogenesis. A key to this success has been the use of explants obtained at precise stages of development (Vasil 1987). The most responsive explants of grasses have been immature embryos, and young inflorescence and leaves. Genotypic effects on somatic embryogenesis and regeneration have been reported in several crop plants (Hodges et al. 1986, Brown 1988). In some cases, changes in culture conditions enabled regeneration to be extended to genotypes previously considered recalcitrant (Duncan et al. 1985), and thereby demonstrated a strong interaction between culture conditions and

genotype. The importance of developing efficient regeneration systems that, through modifications, are successful on several clones of diverse genetic background cannot be overemphasized for purposes of clonal forestry.

The application of tissue culture to clonal propagation of trees imposes unique problems that are not encountered with most herbaceous plants because of the long life cycle of trees and the need to regenerate from mature or genetically proven plants. Tissues from mature trees are often recalcitrant in terms of their in vitro regeneration capacity (Bonga 1987). Even so, several nonconiferous trees have been regenerated by somatic embryogenesis from tissues derived from mature trees (Litz 1985, 1987). The most progress has been made with tropical trees by using the nucellus tissue as an explant. The nucellus is maternal tissue within the seed which has not undergone meiosis and genetic recombination. Therefore, it is an ideal explant for clonal propagation. There has also been success with regeneration of tropical nonconiferous trees from young leaf tissue of mature trees (Sondahl and Sharp 1977, Litz 1988). Regeneration by somatic embryogenesis in temperate-zone trees has primarily been from juvenile explant tissue (Sommer and Brown 1980, Merkle and Sommer 1986). However, somatic embryogenesis was recently obtained from young leaves derived from a 20-year old Populus interspecific hybrid (Park and Son 1988). Two recent reviews by Tulecke (1987) and Wann (1989) contain references to numerous other studies and reviews on somatic embryogenesis in nonconiferous woody plants.

3. SOMATIC EMBRYOGENESIS IN CONIFERS

There were several reports of somatic embryogenesis in conifers in the 1970s and early 1980s, but these were not reproducible nor were plantlets regenerated. The first reproducible report of somatic embryogenesis was from immature embryos

of Picea abies (L.) Karst (Hakman et al. 1985). These cultures were also capable of plantlet regeneration (Hakman and von Arnold 1985). Nagmani and Bonga (1985) were the first to report somatic embryogenesis and plantlet regeneration from haploid explants; the megagametophyte of Larix decidua Mill. Since these three initial reports of somatic embryogenesis in 1985, there have been numerous reports to verify and extend the findings to other coniferous species (Table 1). With the exception of the recent report on Sequoia sempervirens (D. Don) Endl. (Bourgkard and Favre 1988), all studies have been on members of the Pinaceae. Clearly, the most progress has been made with the genus Picea.

[Table 1 here]

Figure 1 is a diagram of the regeneration process via somatic embryogenesis; its use for mass propagation and relationship to genetic modification (transformation). Starting with a piece of plant tissue referred to as the explant, an embryogenic culture is initiated and proliferated on a nutrient medium. The embryogenic culture can be maintained in a proliferative state as callus on solid medium or as a liquid suspension. Somatic embryo development is induced by transferring the embryogenic culture to a modified culture medium. Typically, the in vitro development process ends with the production of a plantlet which can then be transferred to nonaxenic conditions. The terminology used in this chapter to describe the sequential steps of the plant regeneration process via somatic embryogenesis are listed in Table 2. Each of these steps will be considered in detail in the following sections, with reference to the current findings from the literature on somatic embryogenesis.

[Fig. 1 and Table 2 here]

3.1. Explant

Conifer embryogenic cultures have only been initiated from juvenile explant tissues (Table 1) derived from developing seed, mature seed, or germinated seed. In general, immature embryos have proven to be the most responsive explants (Fig. 2 and 3), although several reports have used mature (fully developed) embryos. It is difficult to obtain sufficient immature embryos of some conifers for initiation of embryogenic cultures, e.g., Picea abies, because of the high percentage of embryo abortion and poor seed development. Prolonged storage of the immature seed cones, which contain immature embryos, is difficult due to fungal contamination. Therefore, the ability to initiate from mature, cold-stored seed is of considerable utility because it removes the narrow time restraints imposed by using immature embryos.

[Fig. 2 and 3 here]

The developing immature embryo with the intact megagametophyte was an effective explant for initiation of embryogenic cultures of Pinus radiata D. Don (Smith DR 1985 and 1988, unpublished). The megagametophyte surrounds the embryo and supplies nutrients to the developing zygotic embryo(s). A higher initiation frequency was obtained with the culture of immature embryos of Pinus lambertiana Dougl. with intact megagametophyte than with isolated immature embryos (Gupta and Durzan 1986b). The specific effect of the megagametophyte on culture initiation has not been determined. It most likely provides nutrients and/or endogenous phytohormones that are suboptimal in the culture medium (Mapes and Zaerr 1981).

The most significant progress on initiating embryogenic cultures from more mature explants (but still juvenile) has been made with Picea abies. Krogstrup (1986) and Lelu et al. (1987) initiated embryogenic cultures from cotyledons derived from 7-day-old germinated embryos (Table 1). Besides the importance of extending initiation to more mature explants, perhaps the most immediate usefulness of the cotyledonary initiation technique would be to maintain a true genotype control for embryogenesis studies (Wann SR 1987, personal communication). This could be done by removing a few cotyledons for regeneration via embryogenesis and growing out the remaining germinant to serve as the true genotype control. With these techniques it may be possible to compare regenerates derived from different morphogenic pathways (embryogenesis and organogenesis) to the original genotype of the explant.

3.2. Medium and Culture Conditions

The nutrient medium for growth of plant tissue cultures is comprised of five groups of ingredients: inorganic nutrients, a carbon source, vitamins, phytohormones, and organic supplements (Gamborg et al. 1976).

Studies on somatic embryogenesis with Picea species have most frequently used the basal medium (prior to addition of phytohormones) of von Arnold and Eriksson (1981) at full- or half-strength. Although this medium is frequently coded LP, in this chapter it will be referred to as AE to avoid confusion with other media coded LP (Aitken-Christie and Thorpe 1984, Durzan and Gupta 1987). AE medium is a modification of the standard Murashige and Skoog (1962) medium. Although full-strength AE has been effective for initiation of embryogenic cultures from immature embryos of P. abies, studies have shown that modifications in the basal medium and culture conditions were necessary to initiate efficiently from mature embryo explants. For instance, half-strength AE with

30 mM sucrose and 15 mM NH_4NO_3 was optimal for initiation from mature P. abies zygotic embryos (von Arnold 1987). By using half-strength BLG medium (Amerson et al. 1985) which had no NH_4NO_3, 1 mM KNO_3, and 10 mM glutamine, Verhagen and Wann (1989) obtained efficient initiation of embryogenic callus from mature P. abies zygotic embryos cultured in 16 hr light. These reports point out the importance of adjusting several factors, including the level of medium components and culture conditions, in order to extend initiation to mature zygotic embryos. They also serve to demonstrate the potential for complex interactions among these factors.

The level and form of reduced nitrogen in the basal medium appears to be important for initiation of somatic embryogenesis in Pinus species, but less so among Picea species. Three basal media, DCR (Gupta and Durzan 1985), BM (Gupta and Durzan 1987a), and MSG (a modification of the BLG medium of Amerson et al. 1985, Becwar et al. 1989b) have been effective for initiation in Pinus species. These media contain either no ammonium or much lower levels of ammonium (20 to 33%) compared to AE medium which contains 15 mM. By comparison, embryogenic callus has been initiated in Picea species on several basal media with ammonium levels ranging from 0 to 21 mM (Hakman and von Arnold 1985, Gupta and Durzan 1986a, Hakman and Fowke 1987b, Verhagen and Wann 1989). Collectively, these results suggest that initiation in Pinus species is more sensitive to the level of inorganic reduced nitrogen (ammonium) than in Picea species.

All reports of initiation in Picea species have included both auxin and cytokinin; the most commonly used forms are 2,4-dichlorophenoxyacetic acid (2,4-D) and N6-benzyladenine (BA), respectively. With the auxin naphthalene-1-acetic acid (NAA) there was an absolute requirement for cytokinin (BA) for induction of embryogenic callus from mature embryos of P. abies (von Arnold

1987). Von Aderkas et al. (1987) reported optimum initiation from megagametophytes of Larix decidua on basal medium supplemented with only 2,4-D. Embryogenic cultures were also initiated on basal medium with no auxin. Thus, there does not appear to be an obligatory requirement for both auxin and cytokinin for initiation of embryogenesis from megagametophytes of L. decidua.

Similarly, auxin and cytokinin were used to initiate somatic embryogenesis in Pinus taeda L. (Gupta and Durzan 1987a). But, initiation did not require exogenous phytohormones when immature embryos with intact megagametophytes of P. taeda were used as explants (Becwar et al. 1989b). However, the highest initiation frequency was obtained when isolated immature embryo explants were cultured on media containing auxin and cytokinin. Somatic embryogenesis was also initiated in Pinus radiata (Smith DR 1985 and 1988, unpublished) and Pinus serotina (Becwar et al. 1988) from immature zygotic embryos within the intact megagametophyte on basal medium with no exogenous phytohormones. As previously indicated, the megagametophyte may supply the necessary phytohormones necessary for initiation from zygotic embryos.

3.3. Initiation of Embryogenic Cultures

Embryogenic cultures of conifers have a distinctly different phenotype than previously reported conifer callus. All reports have described embryogenic callus as translucent to white (even when grown in light), and moist or mucilaginous; quite different than opaque (green when grown in light) and friable nonembryogenic conifer callus. Embryogenic conifer callus contained proplastids which lacked the internal organization (grana) typical of mature chloroplasts but closely resembled those found in early zygotic embryos (Feirer 1988). In contrast, the chloroplasts in nonembryogenic callus were more typical of mature chloroplasts found in vivo in light grown plants.

Conifer embryogenic callus has been characterized biochemically (Wann et al. 1987, 1988). The embryogenic callus evolved less ethylene, contained lower amounts of glutathione and other reducing agents, and synthesized or turned-over protein at a faster rate relative to nonembryogenic callus. These results suggest that conifer embryogenic callus is biochemically and metabolically similar, but distinctly different from nonembryogenic callus.

3.3.1. Terminology and Early Embryogeny

In the strictest sense, the term callus refers to unorganized growth of plant cells. Often the term is used in a more general way to refer to cell and tissue cultures grown on solid medium. Embryogenic cultures of conifers have been referred to as callus (Hakman et al. 1985, Nagmani and Bonga 1985) even though they contained very early stage somatic embryos. In this chapter embryogenic cultures maintained on solid medium are referred to as embryogenic callus.

Gupta and Durzan (1986a, 1987a) used the term "embryonal suspensor masses" to refer to proliferating embryogenic cultures obtained in Picea abies and Pinus taeda. They also referred to the in vitro embryo formation process as "somatic polyembryogenesis". This was based on its similarity to in vivo cleavage polyembryony which occurs in Pinus (Gupta and Durzan 1987a). They also observed the free nuclear proembryonic stage followed by early somatic embryogeny. Thus, they suggested a repetitive embryo formation process in vitro that reflects the in vivo cleavage process. Their observations support the hypothesis that there is no true callus or unorganized phase of proliferation in embryogenic cultures of P. taeda, and thus their use of the term embryonal suspensor mass. Further detailed studies are needed to determine the validity of this hypothesis.

Hakman et al. (1987) suggested three possible mechanisms of somatic embryo formation in conifer embryogenic cultures: 1) development from single cells by an initial asymmetric division; 2) development from small meristematic cells within the suspensor or embryonic region; and 3) development of new embryos by cleavage of the embryonic region, similar to in vivo cleavage polyembryony. Although these deductions were based on observations of early stages of embryogeny in established cultures, they may also apply to how somatic embryos are initially formed from explants. In vitro embryo formation in Picea abies and Picea glauca (Moench) Voss. occurred via an asymmetric division of a single cell (Hakman and Fowke 1987a, Nagmani et al. 1987). Free nuclear divisions, which are characteristic of early conifer zygotic embryos, were not observed. Furthermore, there was evidence for an unorganized (callus) phase preceding embryo formation (Nagmani et al. 1987). A callus phase also preceded somatic embryo formation from megagametophytes of L. decidua (Von Aderkas and Bonga 1988) and from immature zygotic embryos of Pseudotsuga menziesii (Webb DT 1989, personal communication).

3.3.2. Site of Origin on Explant

Hakman et al. (1985) first reported that embryogenic callus originated from just beneath the cotyledons (near the attachment of the cotyledons to the hypocotyl) of Picea abies zygotic embryos (Fig. 4). They also indicated that the suspensor cells of the most immature embryos may have given rise to embryogenic callus. Subsequent studies verified the hypocotyl and/or the interface of the hypocotyl and cotyledons was the site of embryogenic callus formation from both immature and mature embryos of several Picea species (Krogstrup 1986, Nagmani et al. 1987, Lu and Thorpe 1987, von Arnold and Woodward 1988, Verhagen and Wann 1989, Webb et al. 1989). Embryogenic callus also originated from cotyledons

excised from germinated P. abies zygotic embryos (Krogstrup 1986, Lelu et al. 1987). Nagmani et al. (1987) provided histological evidence that the embryogenic callus resulted from mitotic activity of the outer cell layers of the hypocotyl of both P. abies and Picea glauca. Furthermore, they found that the callus which formed at the radicle of the immature embryos was nonembryogenic. While Gupta and Durzan (1986a) reported that embryogenic callus originated from the radicle of mature P. abies embryos, results with immature and mature embryo explants of P. abies and P. glauca have not corroborated their observation (Verhagen and Wann 1989; Webb et al. 1989; Becwar MR 1987, unpublished; Amerson HV 1987, personal communication). In vitro embryogenesis has been reported from the radicle of Picea sitchensis (Bong.) Carr. (Krogstrup et al. 1988).

[Fig. 4-6 here]

Whereas most evidence in Picea species indicates that embryogenic callus originated from the outer cell layers of the hypocotyl and cotyledons of zygotic embryos, there have not been similar reports for Pinus species. Embryogenic callus from immature and mature zygotic embryos of Pinus lambertiana (Gupta and Durzan 1986b) and immature embryos of Pinus taeda (Becwar et al. 1989b) originated from the suspensor region (Fig. 5). The embryogenic callus extruded from P. taeda megagametophytes containing immature zygotic embryos also originated from the suspensor region (Fig. 6). It should be emphasized that origin of the embryogenic callus from the suspensor region of the zygotic embryo does not necessarily imply origin from suspensor cells, per se. The embryogenic proliferation may be initiated from small cells of the embryo at the interface of the suspensor and embryo (the second mechanism cited in previous section, suggested by Hakman et al. 1987).

The observed differences between Picea and Pinus species in the site of origin of embryogenic callus support the hypothesis suggested by Becwar et al. (1988) that differences in how in vitro embryogenesis is initiated from zygotic embryos reflects the in vivo occurrence (in the case of Pinus) or the absence (in the case of Picea) of cleavage polyembryony (CPE). In other words, in Pinus species it appears that somatic embryogenesis either initiates de novo from the suspensor region or is a continuation of embryo cleavage in vitro. By contrast, in Picea species (which do not undergo CPE in vivo) the suspensor region is less responsive and the initiation of embryogenesis from the hypocotyl and cotyledons cannot be due to cleavage, because it occurs via a callus phase (Nagmani et al. 1987). This hypothesis relates only to how embryogenesis is initiated from the zygotic embryo explant, since in vitro formation of somatic embryos by cleavage has been reported in established cultures of species which do not undergo CPE in vivo, e.g., Picea glauca and Pseudotsuga menziesii (Hakman et al. 1987, Durzan and Gupta 1987). Differences observed in the optimum stage of zygotic embryo development between Picea and Pinus species for initiation of embryogenic callus also support the hypothesis.

3.3.3. Optimum Stage of Explant Development

In Pinus taeda the optimum stage for initiation of embryogenic callus occurred at the precotyledonary stage (Fig. 2) of zygotic embryo development (Becwar et al. 1989b). As the embryos matured and formed cotyledon primordia (Fig. 3), the frequency of embryogenic callus decreased. Other studies with Pinus radiata (Smith DR 1988, personal communication) and Pinus strobus L. (Becwar et al. 1988, Finer et al. 1989) also suggested that in Pinus species the optimum stage of immature zygotic embryo development for initiation was precotyledonary. In Picea species the optimum stage of zygotic embryo development

occurred just when cotyledon primordia formed (Hakman and Fowke 1987b, Lu and Thorpe 1987, Becwar et al. 1988, Webb et al. 1989). A comparison of initiation of embryogenic callus in Picea glauca and P. taeda is shown in Fig. 7.

[Fig. 7 here]

In contrast to the differences in optimum initiation windows observed between Picea and Pinus species, embryogenic callus was obtained from both pre-cotyledonary and cotyledonary zygotic embryos of Pseudotsuga menziesii (Mirb.) Franco (Webb DT 1989, personal communication).

3.3.4. Effect of Genotype on Initiation

Unfortunately, most reports have not indicated if qualitative or quantitative differences were observed in somatic embryogenesis either among source trees from which the zygotic embryos were collected, or among the resulting embryogenic callus lines. Significant differences in the level of somatic embryogenesis have been reported among callus lines derived from different immature zygotic embryo explants (different genotypes) collected from a Picea abies tree (Becwar et al. 1987b). The level of embryogenesis ranged from 100 to 1500 immature somatic embryos per gram of embryogenic callus. Callus lines with either a low or a high density of somatic embryos grew vigorously and were phenotypically similar. Genotypic differences have also been observed among embryogenic callus lines derived from different megagametophyte explants of Larix decidua (von Aderkas and Bonga 1988). In a survey of ten clones of Pinus taeda, embryogenic cultures were initiated from immature zygotic embryos of eight of the ten clones (Becwar et al. 1989b) (Fig. 8). There were differences in the mean initiation frequency from the explants derived from different clones. Over 70% of the embryogenic cultures were derived from explants of

three clones. In a survey of five Picea glauca trees and one Picea engelmannii (Parry) Engelm. tree, embryogenic cultures were initiated from zygotic embryos of all trees, with the overall initiation frequencies ranging from 6 to 15% among different trees (Webb et al. 1989). Collectively, these results provide evidence that both the genotype of the source tree and individual explant can play an important role in establishing an efficient somatic embryo regeneration system.

[Fig. 8 here]

3.4. Maintenance of Embryogenic Cultures

In general, embryogenic cultures can be maintained on the same basal medium and phytohormones on which they are initiated. The medium is solidified with agar or other gelling agents. Alternatively, they can be maintained as a liquid suspension on the same medium without the gelling agent.

Embryogenic cultures of Picea abies initiated from immature zygotic embryos in July, 1985 (Becwar et al. 1987b) have been maintained for over three years with no apparent loss of vigor, embryogenic, or plant regeneration capacity (Becwar MR 1988, unpublished). Embryogenic cultures of Picea glauca have been maintained on solid medium for 16 months (Hakman and von Arnold 1988). Subsequently, this callus was used to initiate liquid suspension cultures that maintained plantlet regeneration capacity for over 6 months. Thus, embryogenic cultures grown on either solid or liquid medium can be maintained for prolonged periods of time without losing regenerative capacity. Embryogenic suspensions of Pseudotsuga menziesii and Picea abies capable of plantlet regeneration (Durzan and Gupta 1987, Boulay et al. 1988) and of Pinus taeda capable of immature embryo formation (Gupta and Durzan 1987a) have also been reported. The

establishment of efficient liquid embryogenic culture systems and the verification of long-term regeneration capacity are essential for purposes of mass propagation and clonal forestry.

3.5. Somatic Embryo Development and Plant Regeneration

The process of somatic embryo development and plant regeneration can be divided into the following steps according to the stage of embryo development and the culture conditions used: induction, maturation, germination, and conversion (Table 2). Furthermore, Hakman and von Arnold (1988) proposed a system for identification of the stage of conifer somatic embryo development as follows: Stage 1, small embryos consisting of an embryonic region of small, densely cytoplasmic cells subtended by a suspensor comprised of long and highly vacuolated cells. Stage 2, embryos with a prominent embryonic region that is more opaque and with a more smooth and glossy surface than stage 1 embryos. Stage 3, embryos with small cotyledons. This system of identification of stages of embryo development will be used in this chapter.

3.5.1. Induction of Embryo Development

In most conifer embryogenic cultures, the very early stages of embryo formation (stage 1) occur on a medium containing auxin and cytokinin (Fig. 9 and 10). However, some embryogenic cultures of Pinus taeda had a mixture of long suspensor-like cells and small dense clusters of embryogenic cells (proembryogenic masses) while on maintenance medium. Well formed somatic embryos were only observed after the culture was transferred to medium devoid of auxin (Becwar et al. 1989b). Regardless, auxin or auxin and cytokinin in combination promote proliferation of the culture and arrest embryo development. Further development of somatic embryos to stage 2 (Fig. 11) is induced by transferring the embryogenic culture to a modified medium.

[Fig. 9 and 10 and Fig. 11-18 here]

Several different protocols have been used to induce development of somatic embryos. The protocols can be grouped as follows according to changes made in the phytohormones in the culture medium: 1) complete elimination of auxin with the same or reduced level of cytokinin (Hakman and von Arnold 1985, von Arnold 1987); 2) reduction in the level of auxin or change to a less potent auxin, with the same or lower level of cytokinin (Gupta and Durzan 1986b, Hakman and Fowke 1987b, Lu and Thorpe 1987, Gupta and Durzan 1986a, Gupta and Durzan 1987a, Gupta et al. 1987); 3) elimination of cytokinin, reduction in the level of auxin or change to a less potent auxin, and addition of abscisic acid (ABA) (Becwar et al. 1987a & b, Mohan Jain et al. 1988, Verhagen and Wann 1989, Becwar et al. 1989a); 4) reduction in the levels of auxin and cytokinin and addition of ABA (Durzan and Gupta 1987); and 5) elimination of auxin and cytokinin, and addition of ABA (Boulay et al. 1988, von Arnold and Hakman 1988, Hakman and von Arnold 1988, Dunstan et al. 1988). These studies, conducted on a variety of basal media, demonstrate that embryogenic cultures can progress from a proliferative to an embryo development mode after several quite different changes in phytohormones. Common to all of these changes is the removal or reduction of auxin from the medium. Activated charcoal, which acts as an absorbent, has also been used in media during induction of embryo development (Hakman et al. 1985, Becwar et al. 1987a & b, Verhagen and Wann 1989).

3.5.2. Maturation and Use of ABA

There have been numerous reports of somatic embryo maturation (Table 1). Maturation as used here (development of embryos to stage 3) does not necessarily imply that the embryos are physiologically mature.

Two initial reports found that ABA did not improve Picea abies and Picea glauca somatic embryo development and maturation (Hakman and von Arnold 1985, Lu and Thorpe 1987). Becwar et al. (1987a) first reported the use of ABA in combination with indole-3-butyric acid (IBA) for maturation of P. abies somatic embryos (Fig. 12). Since then numerous studies have verified the effectiveness of ABA for improving somatic embryo development and maturation (see numerous references listed in previous section on induction of development). ABA is not required, per se, for somatic embryo development, but quantitative studies have shown that it increases 1) the frequency of calli which form stage 2 somatic embryos (von Arnold and Hakman 1988); 2) the number of plantlets regenerated per ml of embryogenic suspension (Hakman and von Arnold 1988); and 3) both the density of immature somatic embryos (Becwar et al. 1989a) and their maturation frequency (Dunstan et al. 1988, Becwar et al. 1989a).

Hakman and von Arnold (1988) provided histological evidence that lipids and storage proteins accumulated during treatment of somatic embryos with ABA. They noted the similarity of in vitro accumulation to in vivo accumulation during zygotic embryo maturation. ABA plays a key role in the accumulation of nutrient reserves in angiosperm embryos (Walton 1980). Furthermore, the addition of ABA inhibited accessory embryo formation, decreased the frequency of abnormal embryo formation, and prevented precocious germination of somatic embryos of angiosperms (Ammirato 1974 and 1983b). Durzan and Gupta (1987) suggested that ABA inhibited somatic embryo cleavage in embryogenic cultures of Pseudotsuga menziesii.

There is evidence that the level of nutrients in the basal medium influences the effectiveness of ABA (von Arnold and Hakman 1988). They found that half-strength AE medium with 90 mM sucrose (3%) resulted in the highest frequency of

calli forming stage 2 somatic embryos. This result may explain why several previous studies using differing protocols did not find positive effects of ABA on somatic embryo maturation.

Only two reports have provided quantitative data on the frequency that immature somatic embryos developed to the cotyledonary stage (> stage 3). Two techniques were used to count somatic embryos: dispersion of an aliquot of suspension culture onto black filter paper discs (Dunstan et al. 1988) and dispersion of embryogenic callus in liquid and plating it in a thin layer solidified with agarose (Becwar et al. 1989a). The maturation frequencies were: 4% for somatic embryos of Picea glauca cultured on AE medium supplemented with 8 to 12 μM ABA (Dunstan et al. 1988) and 3% for somatic embryos of P. abies cultured on AE medium supplemented with 1 μM each ABA and IBA (Becwar et al. 1989a). Such quantitative techniques provide ways to evaluate the effect of cultural, biochemical, and genetic factors on embryo maturation and to optimize maturation frequency. For instance, Dunstan et al. (1988) used these techniques to demonstrate that ABA more effectively induced maturation than three ABA analogues.

3.5.3. Germination

Nearly all studies which obtained mature conifer somatic embryos also obtained embryo germination (Table 1). Reports have frequently referred to germination as "plantlet" formation; growth of a primary root and enlargement of the cotyledons (Fig. 13). The wide variety of basal media and culture conditions used suggest that requirements for germination are less critical than earlier stages of regeneration. In general, conifer somatic embryo germination media have not contained phytohormones, although germination of Picea abies somatic embryos was reported on media with cytokinin (Hakman and von Arnold

1985, von Arnold 1987). Frequently, the concentration of the basal media used during germination was reduced to one-fourth to one-half the levels used during embryo maturation (Becwar et al. 1987b, Lu and Thorpe 1987, Gupta and Durzan 1987a, Verhagen and Wann 1989). Several studies have obtained germination on filter-paper supports in liquid media (Gupta et al. 1987, Durzan and Gupta 1987, Boulay et al. 1988). Unfortunately, in most of these studies germination levels on appropriate control media were not presented in order to verify the beneficial effect of the protocol modification. Thus, it is not clear to what extent the modified protocol improved germination.

Several reports have indicated the frequency at which embryogenic cultures produced germination stage plantlets. For instance, 15 to 24% of embryogenic callus pieces of Picea abies produced plantlets (von Arnold 1987, Hakman and von Arnold 1985). Twelve percent of the immature Larix decidua somatic embryos formed plantlets (Nagmani and Bonga 1985). Reports that have expressed regeneration levels per callus weight or relative to the number of cotyledonary stage somatic embryos allow for comparisons to be made among studies and protocols. Embryogenic callus of P. abies, which produced 80 cotyledonary stage somatic embryos per gram of callus, yielded 4-8 plantlets per gram callus; a 5 to 10% germination frequency (Gupta and Durzan 1986a). Boulay et al. (1988) reported 10 to 15% germination of the cotyledonary stage P. abies somatic embryos derived from liquid suspension cultures. von Arnold and Hakman (1988) reported that the highest level of P. abies somatic embryo germination (35%) occurred from embryos which had matured in dark culture conditions. The only report of germination of somatic embryos of a Pinus species indicated that 1 to 5 plantlets were produced per gram of P. taeda embryogenic culture (Gupta and Durzan 1987a).

Germination of Picea abies somatic embryos was improved on culture configurations which avoided immersion of the radicle in agar solidified medium (Becwar et al. 1989a). Germination ranged from 27% for somatic embryos germinated with radicles immersed in medium, 45% when placed on the surface of the medium, and 56% when cotyledons were immersed in the medium and the culture vessel inverted. Previous studies with zygotic conifer embryos have demonstrated a beneficial effect of sucrose on root growth, when supplied via the cotyledons rather than via the roots (Brown and Gifford 1958). Several other studies have used germination protocols for somatic embryos which avoided immersion of the radicle in agar solidified medium by supporting embryos on filter paper (Gupta et al. 1987, Durzan and Gupta 1987) or cheese cloth (Boulay et al. 1988).

3.5.4. Conversion

The process of embryo-to-plant development has been referred to as "embryo conversion" to distinguish such development from somatic embryo germination (Redenbaugh et al. 1986, 1987). Conversion, therefore, refers to development of "seedling-quality" plants capable of growth and development beyond the initial stages of germination (Fig. 14). Although conversion has been used to refer to both in vitro and ex vitro (nonaxenic) plant development from somatic embryos, it is more applicable for production purposes to evaluate conversion under nonaxenic conditions. Use of the term conversion in this sense includes 1) the acclimation process that in vitro derived plantlets undergo in order to survive in nonaxenic conditions and 2) subsequent continued growth and development under nonaxenic conditions (Becwar et al. 1989a). In vitro germination or plantlet growth is much more easily achieved with conifer somatic embryos than subsequent survival and continued growth under nonaxenic conditions. Although there have

been numerous reports of conifer somatic embryo maturation and germination under in vitro conditions (Table 1), only three reports have verified longer-term (> 2 months) growth and development (i.e., conversion) under nonaxenic conditions (Durzan and Gupta 1987, von Arnold and Hakman 1988, Becwar et al. 1989a).

Plantlets transferred to nonaxenic conditions have been grown in soil/peat (1:1) (Nagmani and Bonga 1985), peat/vermiculite/perlite (1:2:1) (Gupta and Durzan 1987a, Durzan and Gupta 1987, Boulay et al. 1988), and Jiffy potting mix (Jiffy Products, West Chicago, IL, USA) (Becwar et al. 1989a).

Plants derived from somatic embryos of Picea abies responded to environmental changes in a manner similar to seedlings grown and over-wintered under identical conditions (Becwar et al. 1989a). They set dormant terminal buds (Fig. 15), survived over-wintering to -5 C, and renewed vegetative growth synchronously with control seedlings (Fig. 16 and 17). The somatic plants continued to grow and appeared phenotypically normal relative to seedlings (Fig. 18). These plants were examined by isozyme analysis (Johnson et al. 1988). Subsequently, the somatic plants and seedlings set dormant buds, over-wintered, and produced new vegetative growth during their second growing season (Fig. 19 and 20).

[Fig. 19 and 20 here]

3.6. Regeneration from Protoplasts

The ability to induce protoplasts (single cells with cell walls removed) to form cell colonies capable of somatic embryogenesis and regeneration could be of value to 1) enable somatic cell hybridization studies and 2) utilize gene transfer techniques that are only effective with protoplasts, e.g., electroporation (Boston et al. 1987) and passive DNA uptake (Negrutiu et al. 1987). The first report of regenerating cell colonies from conifer protoplasts capable of

forming somatic embryos was with Pinus taeda (Gupta and Durzan 1987b). This study utilized an embryogenic suspension culture as the source of cells for protoplast isolation. Similarly, Bekkaoui et al. (1987) reported the isolation of protoplasts capable of forming microcalli from an embryogenic cell suspension culture of Picea glauca, and Attree et al. (1987, 1989) reported formation of somatic embryos from the protoplast derived cell colonies. Transient expression of a foreign gene has been reported in protoplasts derived from embryogenic cultures of P. taeda, Pseudotsuga menziesii, and P. glauca (Gupta et al. 1988, Bekkaoui et al. 1988).

Although regeneration from protoplasts is not a prerequisite to development of clonal forestry, it demonstrates the rapid successes that have resulted once embryogenic cultures were used as the source tissue. This rapid progress reflects the rapid advancements that were made with regeneration of cereal crops from protoplasts derived from embryogenic suspensions (Vasil 1987). For stable integration of foreign genes into embryogenic cultures, Agrobacterium mediated transformation (Sederoff and Loopstra 1989) and particle-accelerator systems (Christou et al. 1988) that do not require protoplasts will likely be preferred techniques.

4. UTILITY OF SOMATIC EMBRYOGENESIS FOR CLONAL FORESTRY

One of the most promising applications of somatic embryogenesis, and of most importance to clonal forestry, is the potential to efficiently and economically produce large numbers of propagules (Ammirato and Styer 1985). For example, a one liter liquid conifer embryogenic culture contains about 100,000 somatic embryos (Becwar et al. 1988). The fact that the cultures can be grown in liquid makes possible the development of large scale bioreactors, similar to those used

for microbial culture. Thus, many of the manipulations required to produce propagules can be automated. Furthermore, somatic embryos can be encapsulated (Redenbaugh et al. 1986, 1987) to form artificial seeds making possible the development of an efficient propagule delivery technology.

The potential also exists for combining readily available gene transfer techniques with somatic embryogenesis systems to mass produce forest trees with economically useful traits, e.g., increased pest, disease, and stress resistance, which are difficult or impossible to obtain by conventional breeding. The first steps have been taken in this direction with the expression of a marker gene in coniferous embryogenic cultures (Gupta et al. 1988, Bekkaoui et al. 1988) and the regeneration of transgenic plants via somatic embryogenesis of a nonconiferous tree species (McGranahan et al. 1988).

The full potential of conifer somatic embryogenesis for clonal forestry cannot be achieved until regeneration is from mature or genetically proven trees. Even so, there appears to be potential for micropropagation of Pinus radiata from juvenile explants (cotyledons) of controlled crosses of elite trees to be competitive with seedlings if a significant genetic gain can be realized (Smith 1986). Similarly, it may be possible to implement a somatic embryo regeneration system from zygotic embryos derived from controlled crosses of elite conifers in the near future.

The utility of a somatic embryogenesis system from juvenile (embryonic) tissue is also evident for species in which natural seed production is difficult due to embryo abortion. Species that fit into this category include two of major economic importance, Picea abies and Pseudotsuga menziesii, which have been regenerated via somatic embryogenesis. Thus, there appears to be potential

for applying somatic embryogenesis to clonal forestry even before it is possible to regenerate from mature tissues.

5. SUMMARY AND CONCLUSIONS

Because of the considerable amount of research and development that needs to be completed to implement somatic embryogenesis to clonal forestry, it is easy to lose sight of the enormous amount of progress that has been made since 1985 toward achieving this goal. It is worthwhile to emphasize that nearly all integral parts of the envisioned protocol outlined in Fig. 1 have been achieved with coniferous trees. However, this has not been done completely or with high efficiency with any one species. Although initiation of embryogenic cultures from mature explants has not been achieved in conifers, recent progress made with mature nonconiferous trees may serve as useful experimental models (Wann 1989). High frequency embryogenic cultures capable of long-term embryo production have been maintained in both coniferous and nonconiferous species. Somatic embryo maturation, germination, and conversion have been achieved in several forestry species. In summary, therefore, there is reason to believe that the problems of developing an efficient somatic embryo regeneration system in forest trees can be surmounted.

Perhaps the weakest link in the steps for achieving an efficient regeneration system outlined in Table 2 is somatic embryo maturation. But, recent results in conifer somatic embryogenesis indicate a likely pivotal role for the phytohormone ABA to improve maturation frequencies. Furthermore, these encouraging empirical results serve to emphasize the need for more basic and mechanistically oriented research on the physiology and biochemistry of zygotic and somatic embryo development. As indicated recently (Thorpe 1988), there are

numerous research laboratories worldwide concentrating on developing an *in vitro* embryogenesis protocol for a particular species, but there are relatively few scientists specifically studying the basic process of somatic embryogenesis.

Verification of the genetic fidelity of plants regenerated via somatic embryogenesis from individual explants is of paramount importance for clonal forestry. For the first time, it is now possible to do so with plants regenerated from conifer embryogenic cultures. Because the most progress has been made in regeneration of *Picea abies* and *P. glauca* (Table 1), these species can serve as model systems for the evaluation of genetic fidelity. As further improvements are realized in the conversion frequency of conifer somatic embryos, it should be possible in the near future to implement large scale evaluations of field performance and genetic fidelity.

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Table 1. Reports of somatic embryogenesis in conifers. Species listed alphabetically with the explant used, site of origin of embryogenic callus (EC), and the stage of somatic embryo development obtained. [1st of 3 sections]

Species	Explant[a]	Site of origin of EC[b]	Somatic Embryo[c]			Reference
			Maturation	Germination	Conversion	
Larix decidua	MG	nr	+	+	-	Nagmani & Bonga 1985
"	MG	nr	-	-	-	von Aderkas et al. 1987
"	MG	CC	+	+	-	von Aderkas & Bonga 1988
Picea abies	IE	S, H/C	+	-	-	Hakman et al. 1985
"	IE	nr	+	+	-	Hakman & von Arnold 1985
"	ME	R	+	+	-	Gupta & Durzan 1986a
"	COT	E & SE	-	-	-	Krogstrup 1986
"	ME	nr	-	-	-	von Arnold & Hakman 1986
"	IE	nr	+	+	-	Becwar et al. 1987a
"	IE	H	-	-	-	Wann et al. 1987
"	IE	nr	+	+	-	Becwar et al. 1987b
"	IE	H	+	-	-	Nagmani et al. 1987
"	COT	E & SE	-	-	-	Lelu et al. 1987
"	ME	nr	+	+	-	von Arnold 1987

Table 1. continued [2 of 3]

Species	Explant[a]	Site of origin of EC[b]	Somatic Embryo[c] Maturation	Germination	Conversion	Reference
Picea abies	ME	nr	+	+	-	Boulay et al. 1988
"	IE & ME	nr	+	+	+	von Arnold & Hakman 1988
"	ME	H	+	+	-	Mohan Jain et al. 1988
"	ME	H/C	+	+	-	Verhagen & Wann 1989
"	IE	nr	+	+	+	Becwar et al. 1989a
Picea engelmannii	ME	nr	-	-	-	Lu & Thorpe 1988
"	IE	R	+	+	-	Krogstrup et al. 1988
"	IE & ME	H/C	+	+	-	Webb et al. 1989
Picea glauca	IE	nr	-	-	-	Hakman Fowke 1987a
"	IE	H	+	-	-	Nagmani et al. 1987
"	IE	nr	+	+	-	Hakman & Fowke 1987b
"	IE	H	+	+	-	Lu & Thorpe 1987
"	IE	nr	-	-	-	Hakman et al. 1987
"	IE	nr	+	+	-	Hakman & von Arnold 1988
"	IE	nr	+	+	-	Dunstan et al. 1988

Table 1. continued [3 of 3]

Species	Explant[a]	Site of origin of EC[b]	Somatic Embryo[c] Maturation	Germination	Conversion	Reference
Picea glauca	IE & ME	H/C	+	+	-	Webb et al. 1989
Picea mariana	IE	nr	+	+	-	Hakman & Fowke 1987b
Picea sitchensis	ME	H/C	-	-	-	von Arnold & Woodward 1988
Pinus lambertiana	IE, IE*, ME	S	+	+	-	Gupta & Durzan 1986b
Pinus strobus	IE*	S	-	-	-	Finer et al. 1989
Pinus taeda	IE*	nr	+	+	-	Gupta & Durzan 1987a
"	IE*	nr	+	+	-	Gupta et al. 1987
"	IE & IE*	S/E	-	-	-	Becwar et al. 1989b
Pseudotsuga menziesii	IE	nr	+	+	+	Durzan & Gupta 1987
Sequoia sempervirens	ME, COT	H/C	+	+	-	Bourgkard & Favre 1988

a Megagametophyte (MG), isolated immature embryo (IE), immature embryo with intact megagametophyte (IE*), isolated mature embryo (ME), and cotyledons (COT) excised from germinated mature embryo.

b Not reported (nr), corrosion cavity cells (CC), suspensor region (S), between hypocotyl and cotyledons (H/C), epidermal and subepidermal cells (E & SE), hypocotyl (H), cells at interface of suspensor and embryonal head (S/E), and radicle (R).

c Stage of embryo development reported (+): Maturation, development of embryos to cotyledonary stage; germination, growth of primary root (in vitro "plantlet" formation); and conversion, survival and continued growth (> 2 months) in nonaxenic conditions.

Table 2. Terminology used to describe the steps in the plant regeneration process via somatic embryogenesis.

Regeneration Process	Terminology	Description
Culture Establishment	Initiation	*In vitro* formation of embryogenic callus (EC)
	Maintenance	Proliferation of EC
Somatic Embryo (SE) Development	Induction	Change from proliferative growth to SE development
	Maturation	Development of immature SE to cotyledonary stage
	Germination	Growth of primary root (*in vitro* "plantlet")
Plant Growth	Conversion	*Ex vitro* survival and growth of *in vitro* derived plantlet

LIST OF FIGURE CAPTIONS

Fig. 1. The conifer somatic embryogenesis process for use in mass propagation and genetic transformation (from Becwar *et al.* 1987b).

Fig. 2 & 3. Immature zygotic embryo explants of *Pinus taeda*. **Fig. 2:** Precotyledonary stage of embryo development. Note elongated suspensor cells (s) attached to embryonal head (e). **Fig. 3:** Cotyledonary stage of embryo development. Note cotyledon primordia (c). Scale bar, 100 µm (Becwar MR 1988, unpublished).

Fig. 4-6. Initiation of embryogenic callus in conifers. **Fig. 4:** Embryogenic callus initiated from the hypocotyl region of an immature zygotic embryo explant of *Picea abies*. Note numerous somatic embryos (se) on surface of embryogenic callus (ec) and swollen cotyledons (c) of explant. **Fig. 5:** Embryogenic callus initiated from a precotyledonary immature embryo of *Pinus taeda*. **Fig. 6:** Embryogenic callus extruded from the micropylar end of a whole megagametophyte (mg) of *P. taeda*. The ec originates from the immature embryo contained within the mg. Scale bars: Fig. 4 & 6, 2 mm; Fig. 5, 1 mm (Becwar MR 1988, unpublished).

Fig. 7. The relationship of the stage of immature embryo development as measured by embryo length (top graphs) to the frequency of initiation of embryogenic callus (bottom graphs) in *Picea glauca* and *Pinus taeda*. The shaded region corresponds to when ≥ 50% of the embryos formed cotyledons. The solid lines in the bottom graphs are initiation from isolated immature embryo explants and dashed lines from immature embryos with intact megagametophyte (Becwar MR 1988, unpublished data).

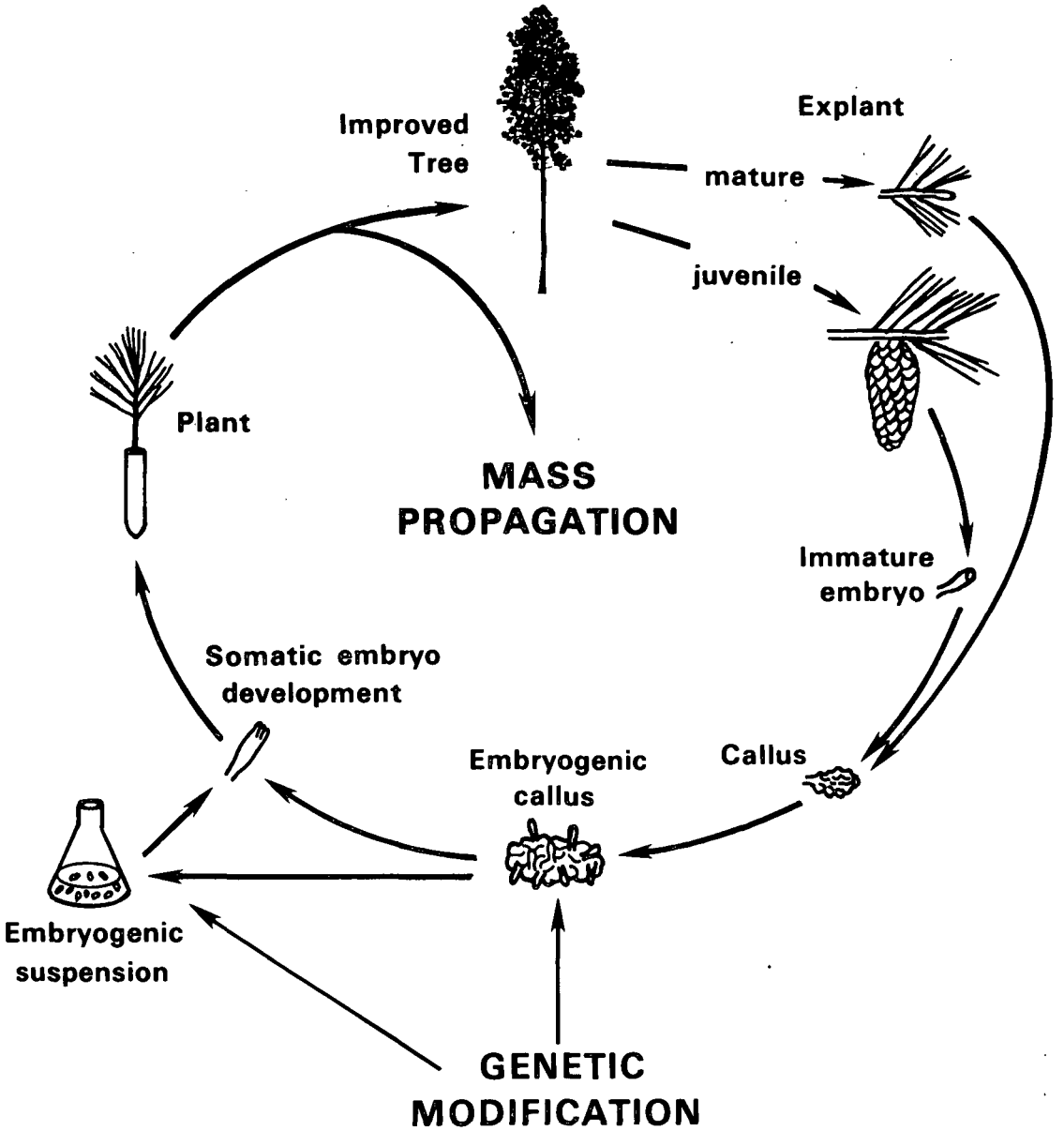
Fig. 8. Comparison of initiation frequency of embryogenic callus from immature embryos derived from ten clones of Pinus taeda. Approximately 1000 explants cultured per clone (from Becwar et al. 1989a).

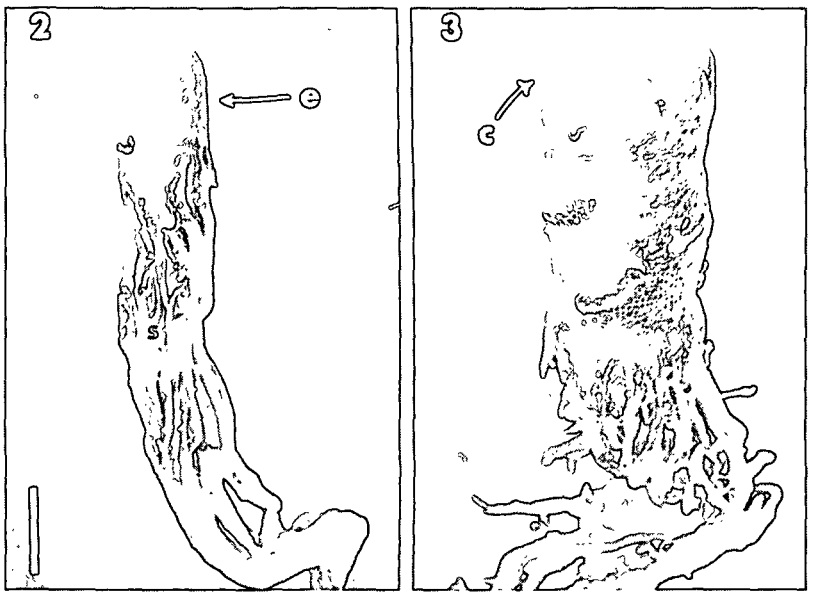
Fig. 9 & 10. Stage 1 somatic embryos (se) of Pinus taeda. **Fig. 9:** Micrograph of embryogenic callus with somatic embryo visible (scale bar = 250 μm). **Fig. 10:** Somatic embryo on surface of embryogenic callus (scale bar = 500 μm) (Becwar MR and Nagmani R 1988, unpublished).

Fig. 11-18. Regeneration of Picea abies plants from embryogenic callus and comparison of plants grown from somatic embryos and seedlings grown from zygotic embryos. **Fig. 11:** Immature somatic embryos (mostly stage 2) after 21 days on development medium. **Fig. 12:** Cotyledonary somatic embryos (mostly stage 3). **Fig. 13:** Somatic embryo germination, primary root (pr) growth. **Fig. 14:** Conversion, survival and growth of somatic plantlet in potting soil mix. **Fig. 15:** Somatic plant with dormant vegetative bud (db) prior to overwintering. **Fig. 16:** Renewed vegetative growth (arrow) of control seedling (ze) after overwintering. **Fig. 17:** Renewed vegetative growth of somatic plant (se). Fig. 16 and Fig. 17 photos taken on same date. **Fig. 18:** Seedling plant (left) and somatic plant (right). Scale bars: Fig. 11-13, 1 mm; Fig. 14-17, 1 cm; and Fig. 18, 5 cm (from Becwar et al. 1989b).

Fig. 19 & 20. Plants derived from zygotic embryo (**Fig. 19**) and from somatic embryo (**Fig. 20**) after resumption of vegetative growth during the second growing season. Scale bar, 10 cm (Becwar MR 1988, unpublished).

FIG. 1


Improved
Tree
Explant
mature
juvenile
Plant
MASS
PROPAGATION
Immature
embryo
Somatic embryo
development
Embryogenic
callus
Callus
Embryogenic
suspension
GENETIC
MODIFICATION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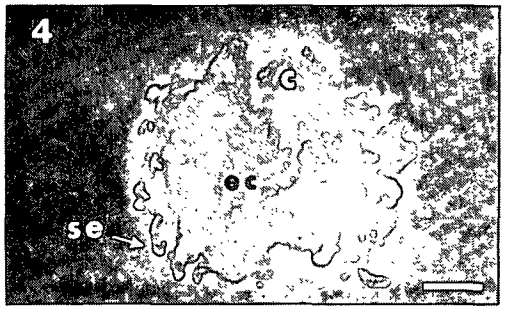
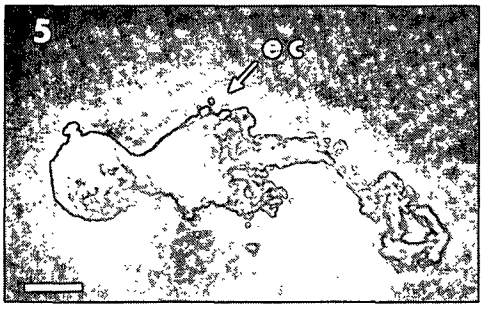
3
P


4
G
ec
se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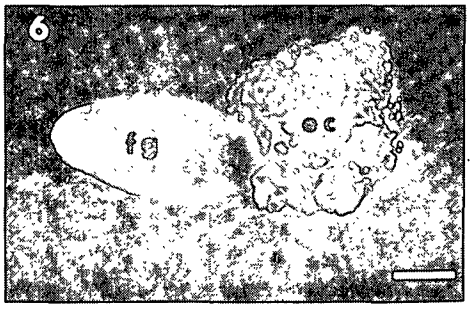
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fg
ec

FIG. 7

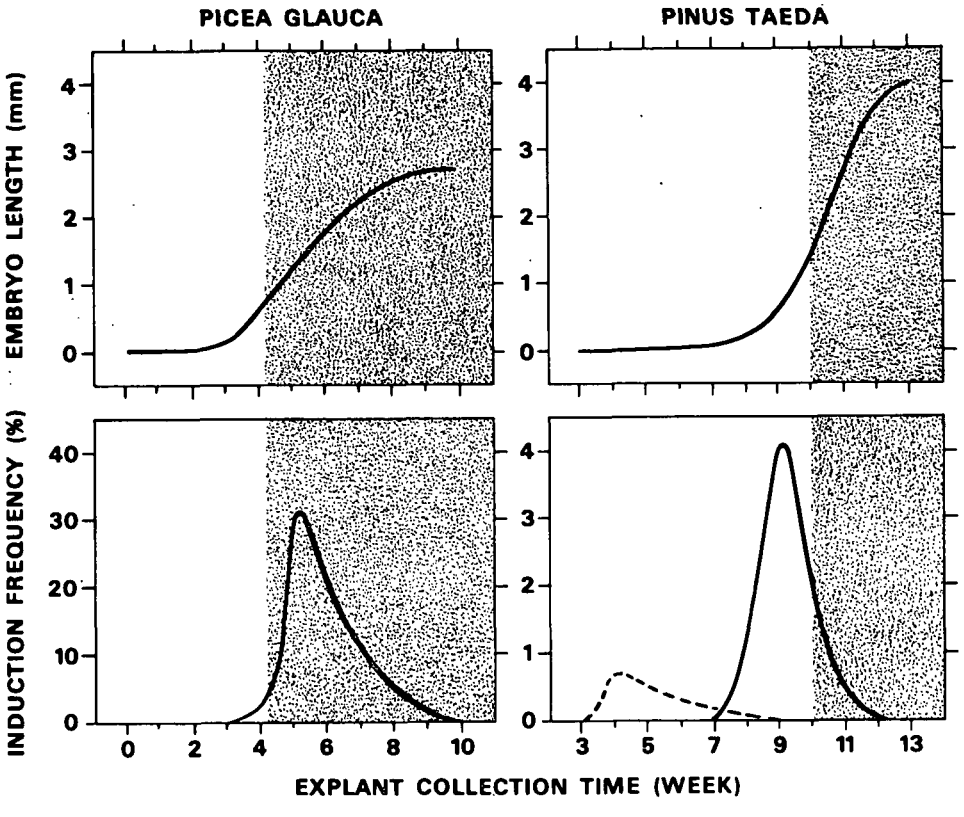
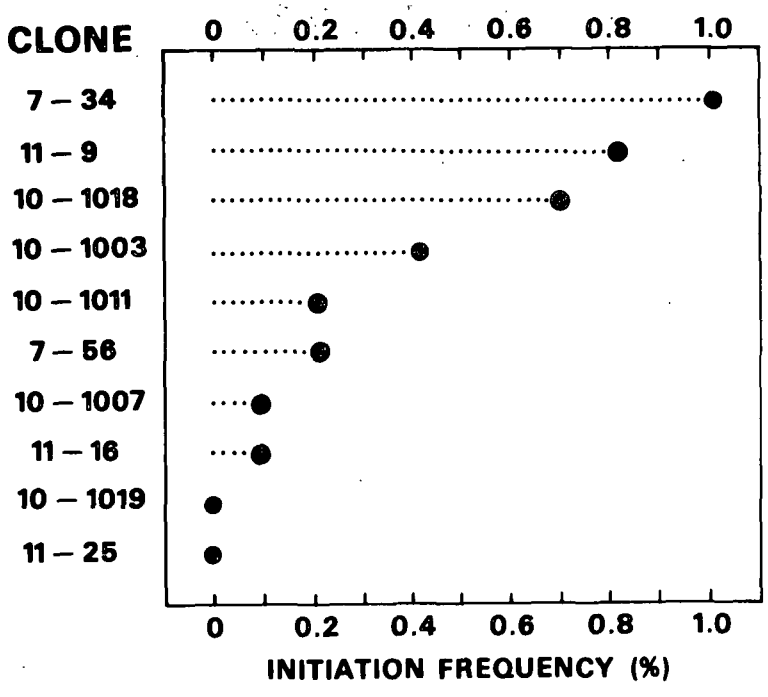
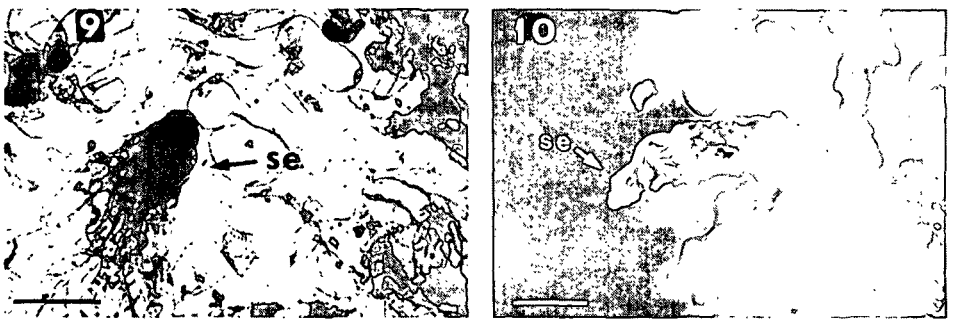

PICEA GLAUCA
PINUS TAEDA
EMBRYO LENGTH (mm)
INDUCTION FREQUENCY (%)
EXPLANT COLLECTION TIME (WEEK)

FIG. 8


CLONE
0
0.2
0.4
0.6
0.8
1.0
7 – 34
11 – 9
10 – 1018
10 – 1003
10 – 1011
7 – 56
10 – 1007
11 – 16
10 – 1019
11 – 25
INITIATION FREQUENCY (%)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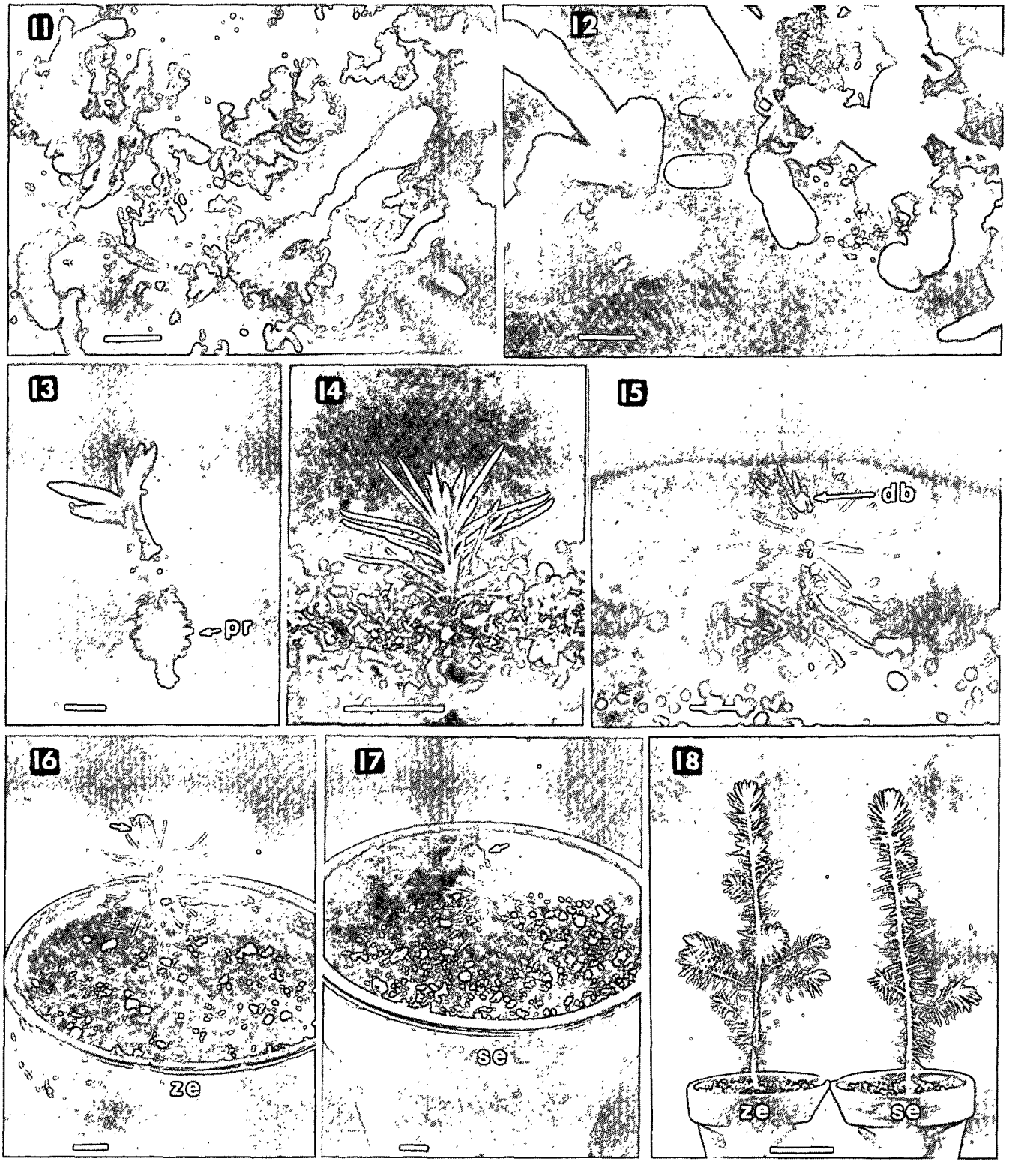
se
10
se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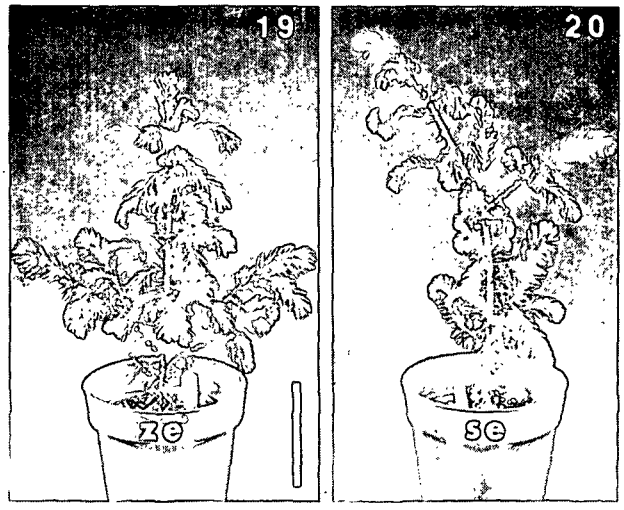
19
20