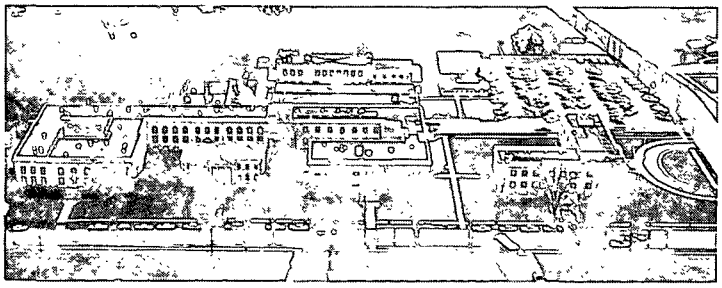

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A SURVEY OF IN VITRO INDUCTION FREQUENCY OF EMBRYOGENIC TISSUE FROM IMMATURE EMBRYOS OF TEN CLONES OF LOBLOLLY PINE (PINUS TAEDA)

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ABSTRACT

BECWAR, M.R., S.R. WANN, and R. NAGMANI. 1988. A survey of *in vitro* induction frequency of embryogenic tissue from immature embryos of ten clones of loblolly pine (*Pinus taeda*). Can. J. For. Res.

Explants from open pollinated seed cones of ten clones of loblolly pine (*Pinus taeda* L.); 7-34, 7-56, 11-9, 11-16, 11-25, 10-1003, 10-1007, 10-1011, 10-1018, and 10-1019 were collected at weekly intervals during the summer of 1986 and 1987 and cultured on three modified MS basal media. The three media contained no or low levels of ammonium (compared to MS medium), 2,4-D (0-5 $mg.l^{-1}$) and BA (0-0.5 $mg.l^{-1}$). Isolated zygotic embryos or female gametophytes with intact embryos were used as explants. A translucent to white, mucilaginous embryogenic tissue initiated from the suspensor region of zygotic embryos from both explants. Embryogenic tissue was induced from explants of 8 of the 10 clones surveyed; only explants from clones 11-25 and 10-1019 were not responsive. Over 70% of the embryogenic cell lines were derived from explants of three clones; 7-34, 11-9, and 10-1018. The highest induction frequency of embryogenic tissue was 5% from zygotic embryos on DCR medium with 3.0 $mg.l^{-1}$ 2,4-D and 0.5 $mg.l^{-1}$ BA. The optimum stage of explant development was precotyledonary zygotic embryos with embryo heads $\leq$ 0.5 mm.

INTRODUCTION

The developmental stage of the explant has proven to be a critical factor in establishment of totipotent plant cell cultures (Vasil 1987). Immature embryonic tissues have generally proven to be responsive explants for regeneration of plants. Embryogenic tissue in conifers was first reported from the culture of immature embryos of Picea abies (Hakman et al. 1985). The optimum stage of embryo (explant) development in Picea for induction of embryogenesis in vitro occurs at the time of cotyledon primordia formation (Lu and Thorpe 1987, Hakman and Fowke 1987, Becwar et al. 1988). Gupta and Durzan (1987) reported induction of embryogenic cultures in Pinus taeda from female gametophytes with intact zygotic embryos at 2 to 4 weeks after fertilization. The optimum window of zygotic embryo development in relation to induction of embryogenic tissue in Pinus species has not been determined. In view of this it has become necessary to determine the stage of zygotic embryo development that proves optimal for induction of embryogenic tissue.

Several authors have reported genotypic effects on somatic embryogenesis and regeneration in cereals (Tomes et al. 1985, Hodges et al. 1986, Luhrs and Lorz 1987). In some cases, media modifications extended regeneration to genotypes previously considered recalcitrant (Duncan et al. 1985), thereby demonstrating strong medium x genotype interactions. No reports to date have examined clonal effects on induction of somatic embryogenesis in conifers.

The approach used in our research has been to survey immature embryo explants from several open pollinated clones of loblolly pine in order to determine if clonal specificity existed for induction of embryogenic tissue and to examine medium x clone interactions. Explants were cultured during a three month period

of embryo maturation to determine the optimum stage of explant development for induction of embryogenic tissue.

MATERIALS AND METHODS

Plant materials. The use of the term "clone" hereafter refers to each source tree from which seed cones were obtained. Seed cones of Pinus taeda L. (loblolly pine) were collected from open pollinated trees in clonal (grafted) seed orchards of Westvaco Corp., Summerville, South Carolina (first generation clones; 7-34, 7-56, 11-9, 11-10, 11-16, and 11-25) and Union Camp Corp., Rincon, Georgia (second generation clones; 10-1003, 10-1007, 10-1011, 10-1018, and 10-1019) during the embryo development period and shipped to Appleton, Wisconsin in insulated containers. In 1986 cones collected at weekly intervals from July 14 to August 18 were stored in paper bags at 4°C for two months prior to use. In 1987 cones were collected at weekly intervals from June 15 to August 10 and used for experiments within two days of collection. Seeds were removed from cones and surface sterilized in commercial bleach (Hilex, 25% v/v) for 15 min. and rinsed three times in sterile distilled water. Seed coats were aseptically removed and the following explants were cultured: 1) female gametophytes with the intact developing zygotic embryos and 2) isolated zygotic embryos.

Media and culture conditions. Two basal media were used for induction and maintenance of embryogenic cultures: the MSG basal medium - a modification of the BLG medium of Amerson et al. (1988) and the DCR medium of Gupta and Durzan (1985). The inorganic, vitamin, and amino acid components of the basal media are listed in Table 1. Complete recipes of media used for induction and maintenance are listed in Table 2. The pH of media were adjusted to 5.8 with KOH and HCl prior to autoclaving at 121 psi for 15 min. L-glutamine was filter

sterilized and added to warm (≈ 50°C) medium. Eight to ten ml of medium was poured in each 50 mm Falcon plastic Petri plate. Five explants were cultured in each plate. The number of explants cultured in 1986 varied among the clones. One-hundred explants from each clone were cultured on each medium in 1987. Cultures were incubated in the dark at 23°C. After four weeks in culture, explants were transferred to new positions on the same plate. Two weeks later, embryogenic tissue was dissected away from responsive explants and transferred to fresh medium. Subsequently, embryogenic calli were maintained as separate cell lines derived from individual explants and subcultured every two weeks. Somatic embryo development was induced on the MSG4 medium described by Nagmani et al. (1988).

Data collection. When isolated zygotic embryos were used as explants, the length of embryos was measured (embryonic head, excluding suspensor) and the presence or absence of cotyledonary primordia was determined. These measurements were made on a dissecting microscope equipped with an eye-piece micrometer on randomly selected subsamples (n = 10-12) of embryo explants of clones 7-34, 7-56, 11-9, 11-16, and 11-25. After six weeks of culture, the number of explants per plate which formed embryogenic tissue was recorded. Data are presented as mean values among replicates (plates); the mean frequency or percentage of explants that formed embryogenic tissue.

RESULTS

Stage of explant development. Zygotic embryo development was tracked during the time of culture initiation to determine the optimum developmental stage for induction of embryogenic tissue. The exponential phase of zygotic embryo growth occurred between July 20 and August 3 in cones collected in 1986 and 1987 (Fig.

1). By August 4, 1986 the mean embryo length was about 1.5 mm and most embryos had formed cotyledonary primordia. Embryo development during 1987 lagged slightly behind 1986. On August 3, 1987 mean embryo length was about 1 mm and about 50% of the embryos had cotyledonary primordia.

1986 experiments. A translucent to white, mucilaginous tissue was initiated from nine of 1,665 explants cultured from nine clones (Table 3). The number of cones that were available and the number of viable seeds per cone varied considerably, and therefore the number of explants cultured varied from 450 of clone 11-9 to only 9 of clone 10-1011. Explants from three of the clones, 11-9, 7-34, and 10-1007, were responsive.

Embryogenic tissue from explants of clone 11-9 was extruded from the micropylar end of the female gametophyte which contained developing zygotic embryos (Fig. 2). Responsive explants were from seed cones collected July 14, at which time the exponential phase of zygotic embryo growth had not started (Fig. 1). Histological examination showed that extruded embryogenic tissue was composed of elongated highly vacuolated suspensor-like cells and small clusters of highly cytoplasmic cells (see Nagmani et al. 1988). To determine the origin of the extruded tissue, female gametophytes were slit open, and examined microscopically. In all cases the cell division and proliferation that produced extruded embryogenic tissue was confined to the suspensor region of the developing embryo, rather than from the embryo head.

Embryogenic tissue from isolated embryos of clones 7-34 and 10-1007 also originated by cell divisions at the interface of the suspensor and embryonal cells (Fig. 3). Embryogenic tissue was only initiated from zygotic embryo explants collected on July 21 and 28. Zygotic embryos at this time were precotyledonary

and less than 0.5 mm in length (Fig. 1). After six weeks in culture the embryo heads of responsive explants were swollen and necrotic, and could easily be separated from the rapidly proliferating embryogenic tissue (Fig. 4). Embryogenic tissue originating from isolated zygotic embryos was similar in cellular composition to embryogenic tissue extruded from female gametophyte explants - elongated suspensor-like cells and small clusters of highly cytoplasmic cells. At the time of initiation and after repeated subcultures on maintenance media, the embryogenic tissue did not have well-formed somatic embryos. There was a differential response among embryogenic cell lines when transferred to development medium (MSG4, see Nagmani et al. 1988). Two of the cell lines efficiently developed well-formed (stage-2) somatic embryos (Fig. 7). Both of these cell lines originated from isolated precotyledonary embryo explants cultured on DCR1 medium.

1987 experiments. During the summer of 1987 a total of 12,434 explants from seed cones of ten clones of loblolly pine were cultured (Table 4). Forty-four embryogenic cell lines were initiated and 80% of these were maintained in culture for at least 8 months. The remainder (20% of the cell lines) grew slowly and became necrotic even after several subcultures. Some of the vigorous embryogenic cell lines of 1987 were phenotypically similar (Fig. 5) to those initiated in 1986, whereas other cell lines had numerous stage-2 somatic embryos while on maintenance medium (Fig. 8). Similar to results with 1986 cell lines, there was a differential response among embryogenic cell lines when transferred to development medium. Some embryogenic cell lines which did not have stage-2 somatic embryos while on maintenance medium (Fig. 5) efficiently developed well-formed stage-2 somatic embryos when transferred to MSG4 development medium containing ABA (Fig. 6).

From July 6 to August 3 explants of all ten clones were cultured on an equivalent basis, making a large scale comparison of induction frequency of embryogenic tissue from explants of clones possible (Table 5). During this time 36 of 9714 explants cultured were responsive. Embryogenic tissue was induced from explants of 8 of the 10 clones; only explants from clones 11-25 and 10-1019 were not responsive. Explants from the three most responsive clones, 7-34, 11-9, and 10-1018, accounted for 25 of the 36 embryogenic cell lines (≈ 70%) induced during this time period. The highest induction frequency occurred on July 27 on DCR1 medium. At this time the mean length of zygotic embryos was less than 0.5 mm, the exponential phase of embryo growth had just commenced, and very few embryos had initiated cotyledonary primordia (Fig. 1).

The effect of medium and explant on induction of embryogenic tissue during the July 6 to August 3 time period is summarized in Fig. 9. The most effective explant-medium combination was the culture of isolated embryos on DCR1 medium (Fig. 9A). About 1% of the zygotic embryo explants (averaged across clones and collection dates) were responsive on DCR1. Culture treatments that used female gametophytes with developing zygotic embryos as explants were less effective. Only about 0.1 and 0.3% of the explants cultured on MSG1 and MSG2, respectively, were responsive (Fig. 9A).

There was evidence for an interaction of the clonal source of explants and culture medium (Fig. 9B). Explants from a larger number of clones were responsive on the DCR1 medium than on either MSG1 or MSG2 medium. When isolated embryos were used as explants, seven of the eight clones were responsive on DCR1 medium, whereas when female gametophytes were used as explants only two clones were responsive on MSG1 or MSG2 medium. The highest induction frequency on the DCR1 medium was 4% (averaged across collection dates) from explants of clone 7-34

which was more than twice the level of induction from explants of other responsive clones. Explants of clone 7-34 were only responsive on the DCR1 medium and not on MSG1 or MSG2 medium (Fig. 9B).

DISCUSSION

This is the first report to quantify differences of induction of embryogenic tissue from embryonic explants of seed orchard derived clones of a conifer. Several of the loblolly pine clones included in this survey are used for seed production for reforestation purposes. The results demonstrated that embryogenic tissue can be initiated at low frequencies from immature embryo explants of most of the clones surveyed. Even so, differences were found in induction frequency among clones, and there was evidence for an interaction among clones and medium. Similar interactions have been reported for induction in many plant tissue cultures (Vasil 1987). This suggests that further modification of medium components may significantly increase induction and/or extend it to explants of recalcitrant clones, e.g., 11-25 and 10-1019. The most effective medium, explant, and clone combination was culture of isolated immature embryos of clone 7-34 on the DCR1 medium.

The results provided information on the optimum time or "window" for induction of embryogenic tissue in Pinus taeda: July 20 to 28, during both 1986 and 1987. The highest induction frequencies occurred when embryos entered their exponential phase of growth and were less than about 0.5 mm in length. This finding is significant because it provides an easy way to determine the optimum stage of explant development that is based on the morphological stage of the zygotic embryo and is independent of phenological factors that might vary significantly with season. Gupta and Durzan (1986) used the number of weeks post-fertilization as an indicator of the time of induction in Pinus lambertiana.

Although a potentially effective marker, the time of fertilization is more difficult to determine than embryo length and stage of cotyledonary development. Our results also demonstrate the optimum window for induction from P. taeda (precotyledonary embryos) differs from that in Picea species (post-cotyledonary embryos) (Lu and Thorpe 1987, Hakman and Fowke 1987, Becwar et al. 1988).

Smith et al. (1985) were the first to report that culture of whole female gametophytes of Pinus radiata on medium containing activated charcoal extruded proembryonic tissue at the archegonial (micropylar) end of the gametophyte. Some proembryonic tissue proliferated to form embryogenic tissue, and plantlets have been regenerated from these embryogenic cultures (Smith, personal communication 1988). In the present investigation the MSG1-female gametophyte culture treatment was designed to test the effectiveness of the Smith et al. technique. Results showed that an embryogenically competent tissue was extruded from female gametophytes of loblolly pine. Results with Pinus serotina (Wann unpublished 1986, see Becwar et al. 1988) also showed induction of embryogenesis without exogenous auxin and cytokinin on the MSG1-female gametophyte culture treatment. Collectively, these results show that embryogenesis can be initiated in vitro in Pinus species without an obligatory requirement for exogenous auxin and cytokinin. This is in contrast to induction in Picea species, which have an apparent obligatory requirement for auxin and cytokinin (Hakman and von Arnold 1985). Regardless, in this study induction on MSG1 medium devoid of auxin and cytokinin was the least effective of the three induction protocols tested in loblolly pine (Fig. 9A). Induction frequencies on MSG2 and DCR1 media (both contained auxin and the DCR1 medium contained BA) were about five and sixteen times higher, respectively, than on the MSG1 medium (no auxin or cytokinin) (Fig. 9A).

Gupta and Durzan (1987) reported induction of embryogenesis *in vitro* from the culture of female gametophytes with intact developing zygotic embryos of loblolly pine. Our results are consistent with their suggestion that somatic embryogensis in loblolly pine reflects the cleavage polyembryony process that occurs *in vivo*. We have not referred to the cell lines as embryo suspensor masses, because several did not contain well developed somatic embryos until transfer to development medium (Nagmani *et al.* 1988). Gupta and Durzan (1987) reported that 9 to 10% of the explants initiated an embryogenic tissue 4 to 5 weeks after fertilization, although the clone(s) of loblolly pine used was not identified. In light of the evidence for an interaction of medium and clone in the current study, it is possible that their higher induction frequency was due to obtaining explants from a more responsive clone.

The DCR basal medium (Gupta and Durzan 1985) was effective for induction of somatic embryogenesis in *Pinus lambertiana* (Gupta and Durzan 1986). All mediums that have been effective for induction of embryogenesis *in vitro* in *Pinus* species have contained either no ammonium or reduced levels compared to MS medium, which contains 21 mM ammonium (Murashige and Skoog, 1962). The DCR medium contained 5 mM ammonium, the modified MS basal medium used by Gupta and Durzan (1987) for induction in *P. taeda* contained 3 mM, and MSG used in the current study contained no ammonium (Table 1). Other studies of micropropagation from embryonic explants of *Pinus* species also yielded best results when ammonium was omitted or reduced (Perez-Bermudez and Sommer 1987, Flinn *et al.* 1986). Somatic embryogenesis from immature embryos of *Picea abies* and *P. glauca*, in contrast, occurs efficiently on medium with higher levels of ammonium (15 mM) (Becwar *et al.* 1987, Hakman and von Arnold 1988). Collectively, these results suggest that induction of embryogenic tissue in *Pinus* species is more sensitive to the level of ammonium than in *Picea* species.

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TABLE 1. Inorganic, vitamin, and amino acid components of basal media.

Components	MSG	DCR
Inorganics, $mg.l^{-1}$		
NH_4NO_3	--	400
KNO_3	100	340
$Ca(NO_3)_2.4H_2O$	--	556
$MgSO_4.7H_2O$	370	370
KH_2PO_4	170	170
$CaCl_2.2H_2O$	440	85
KCl	745	--
KI	0.83	0.83
H_3BO_3	6.2	6.2
$MnSO_4.H_2O$	16.9	22.3
$ZnSO_4.7H_2O$	8.6	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.25
$CoCl_2.6H_2O$	0.025	0.025
$NiCl_2.6H_2O$	--	0.025
$FeSO_4.7H_2O$	27.8	27.8
Na_2EDTA	37.3	37.3
Vitamins and amino acid, $mg.l^{-1}$		
Nicotinic acid	0.5	0.5
Pyridoxine.HCl	0.1	0.5
Thiamine.HCl	0.1	1.0
Glycine	--	2.0

TABLE 2. Variations in media used for induction and maintenance of embryogenic tissue.

Components	MSG1	MSG2	MSG3	DCR1
Basal medium[a]	MSG	MSG	MSG	DCR
Supplements, $g.l^{-1}$				
Inositol	0.1	0.1	0.1	0.2
Casein hydrolysate	--	1.0	1.0	0.5
L-glutamine	1.5	0.5	0.5	0.5
Sucrose	30	30	30	30
Activated charcoal	10	--	--	--
Agar	8	8	8	8
Growth regulators, $mg.l^{-1}$				
2,4-D	--	5.0	2.0	3.0
BA	--	--	1.0	0.5

[a]Refer to Table 1.

TABLE 3. Survey of the induction frequency of embryogenic tissue from explants of nine clones of loblolly pine collected during the summer of 1986.

Clone	No. of explants		Responsive	
	Plated	Forming embryogenic tissue (%)	Explant[a]	Medium
11-9	450	3 (0.7)	FG	MSG1
11-16	325	0	--	--
7-56	280	0	--	--
7-34	275	5 (1.8)	E	DCR1 or MSG3
10-1019	137	0	--	--
10-1003	114	0	--	--
10-1007	50	1 (2.0)	E	DCR1
10-1018	25	0	--	--
10-1011	9	0	--	--
totals:	1,665	9		

[a]FG = female gametophyte with intact developing embryo,
E = isolate embryo.

TABLE 4. Summary of the induction frequency of embryogenic tissue *in vitro* from embryonic explants of ten clones of loblolly pine collected during the summer of 1987.

Clone	No. of embryogenic explants/ total no. of explants[a] collected on									
	June 15	June 22	June 29	July 6	July 13	July 20	July 27	Aug 3	Aug 10	totals
10-1003	--	--	--	1/160	2/200	1/200	0/190	0/200	--	4/ 950
10-1007	--	--	--	0/185	0/200	1/190	0/185	0/200	--	1/ 960
10-1011	--	--	--	0/200	0/200	1/190	1/185	0/190	--	2/ 965
10-1018	--	--	--	1/200	0/200	4/200	2/195	0/200	--	7/ 995
10-1019	--	--	--	0/195	0/200	0/185	0/200	0/200	--	0/ 980
7-34	0/189	0/177	0/191	0/195	0/200	4/180	5/195	2/190	1/200	12/1717
7-56	--	1/192	0/190	0/190	0/200	0/195	3/200	0/190	--	4/1357
11-9	0/200	4/195	2/189	2/198	1/198	1/200	2/193	1/178	0/200	13/1751
11-16	--	0/200	0/200	0/194	0/200	0/200	0/200	1/190	--	1/1384
11-25	--	0/198	0/199	0/199	0/199	0/190	0/200	0/190	--	0/1375
totals:	0/389	5/962	2/969	4/1916	3/1997	12/1930	13/1943	4/1928	1/400	44/12,434

[a]Explants were either female gametophytes with intact zygotic embryos or isolated zygotic embryos.

TABLE 5. Induction frequency of embryogenic tissue *in vitro* from embryonic explants of ten clones of loblolly pine cultured on three media from July 6 to August 3, 1987.

		No. of embryogenic explants/ total no. of explants cultured[a]										
		Clone										
Date	Medium	10-1003	10-1007	10-1011	10-1018	10-1019	7-34	7-56	11-9	11-16	11-25	totals
July 06	MSG1	0/100	0/100	0/100	1/100	0/100	0/100	0/100	0/100	0/100	0/100	1/1000
	MSG2	1/60	0/85	0/100	0/100	0/95	0/95	0/90	2/98	0/94	0/99	3/916
July 13	MSG1	0/100	0/100	0/100	0/100	0/100	0/100	0/100	0/98	0/100	0/100	0/998
	MSG2	2/100	0/100	0/100	0/100	0/100	0/100	0/100	1/100	0/100	0/99	3/999
July 20	MSG1	0/100	1/100	0/100	1/100	0/100	0/90	0/100	0/100	0/100	0/90	2/980
	DCR1	1/100	0/90	1/90	3/100	0/85	4/90	0/95	1/100	0/100	0/100	10/950
July 27	MSG1	0/100	0/100	0/100	0/100	0/100	0/100	0/100	0/93	0/100	0/100	0/993
	DCR1	0/90	0/85	1/85	2/95	0/100	5/95	3/100	2/100	0/100	0/100	13/950
Aug 03	MSG1	0/100	0/100	0/100	0/100	0/100	0/100	0/100	0/93	0/100	0/100	0/993
	DCR1	0/100	0/100	0/90	0/100	0/100	2/90	0/90	1/85	1/90	0/90	4/935
	totals	4/950	1/960	2/965	7/995	0/980	11/960	3/975	7/967	1/984	0/978	36/9714

[a]Explants were female gametophytes with developing embryos on MSG1 and MSG2 media, and isolated embryos on DCR1.

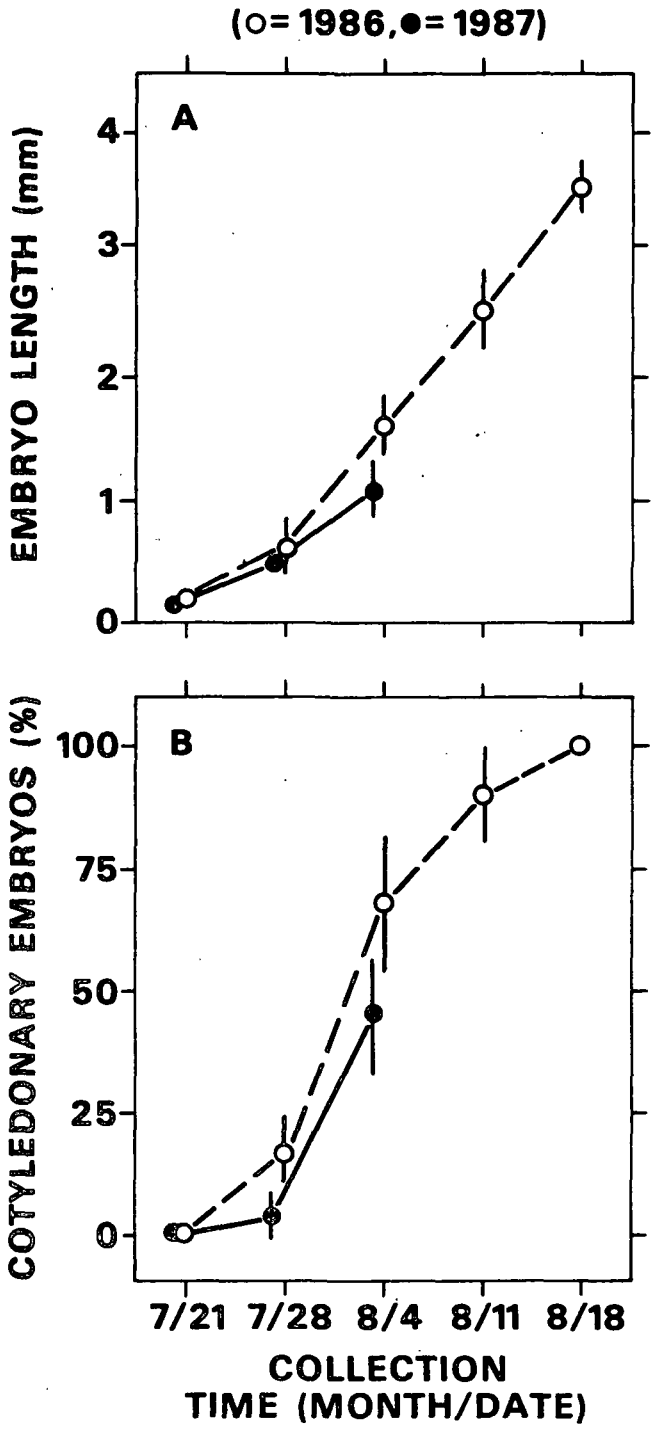
FIGURE CAPTIONS

FIG. 1. Time course of zygotic embryo development in loblolly pine during 1986 (open circles) and 1987 (closed circles). A: Embryo length. B: Percentage of embryos with cotyledon primordia. Each data point is the mean value among embryos collected from five clones in Westvaco's Summerville, SC seed orchard, with 10-12 observations per clone. Error bars are ± standard error of the mean.

FIGS. 2-8. Somatic embryogenesis from the culture of female gametophytes with intact zygotic embryos and isolated zygotic embryos of loblolly pine. FIG. 2. Embryogenic tissue (et) extruded from the micropylar end of female gametophyte (fg) four weeks after culture of female gametophyte with intact zygotic embryo. FIG. 3. Origin of embryogenic tissue from cell division in the suspensor region (arrow) of isolated precotyledonary zygotic embryo. FIG. 4. Proliferation of transluent to white, mucilaginous embryogenic tissue. Arrow indicates swollen zygotic embryo head of original explant. FIG. 5. Embryogenic tissue from zygotic embryo explant of clone 7-34 after 8 months of subculture on maintenance medium (DCR1). FIG. 6. Development of somatic embryos with dense embryonal heads and elongated suspensors after culture of embryogenic cell line shown in Fig. 5 on MSG4 medium. FIG. 7. Somatic embryo development on MSG4 medium. FIG. 8. Embryogenic tissue from zygotic embryo explant of clone 10-1011 with numerous stage-2 somatic embryos while on maintenance medium, DCR1. Scale bars in FIG. 2 = 1 mm, FIG. 3 = 0.5 mm, and FIGS. 4 to 8 = 1 mm.

FIG. 9. Frequency of induction of embryogenic tissue of loblolly pine on three culture treatments (medium-explant combinations). Solid bars are female gametophyte explants. Open bars are isolated zygotic embryo explants. A: Induction frequency on three culture treatments (number of responsive explants over total number cultured in parentheses). B: Induction frequency of responsive clones on three culture treatments. Clone identification numbers listed.

Fig. 1.


(○= 1986, ●= 1987)
A
EMBRYO LENGTH (mm)
0
1
2
3
4
B
COTYLEDONARY EMBRYOS (%)
0
25
50
75
100
7/21
7/28
8/4
8/11
8/18
COLLECTION
TIME (MONTH/DATE)

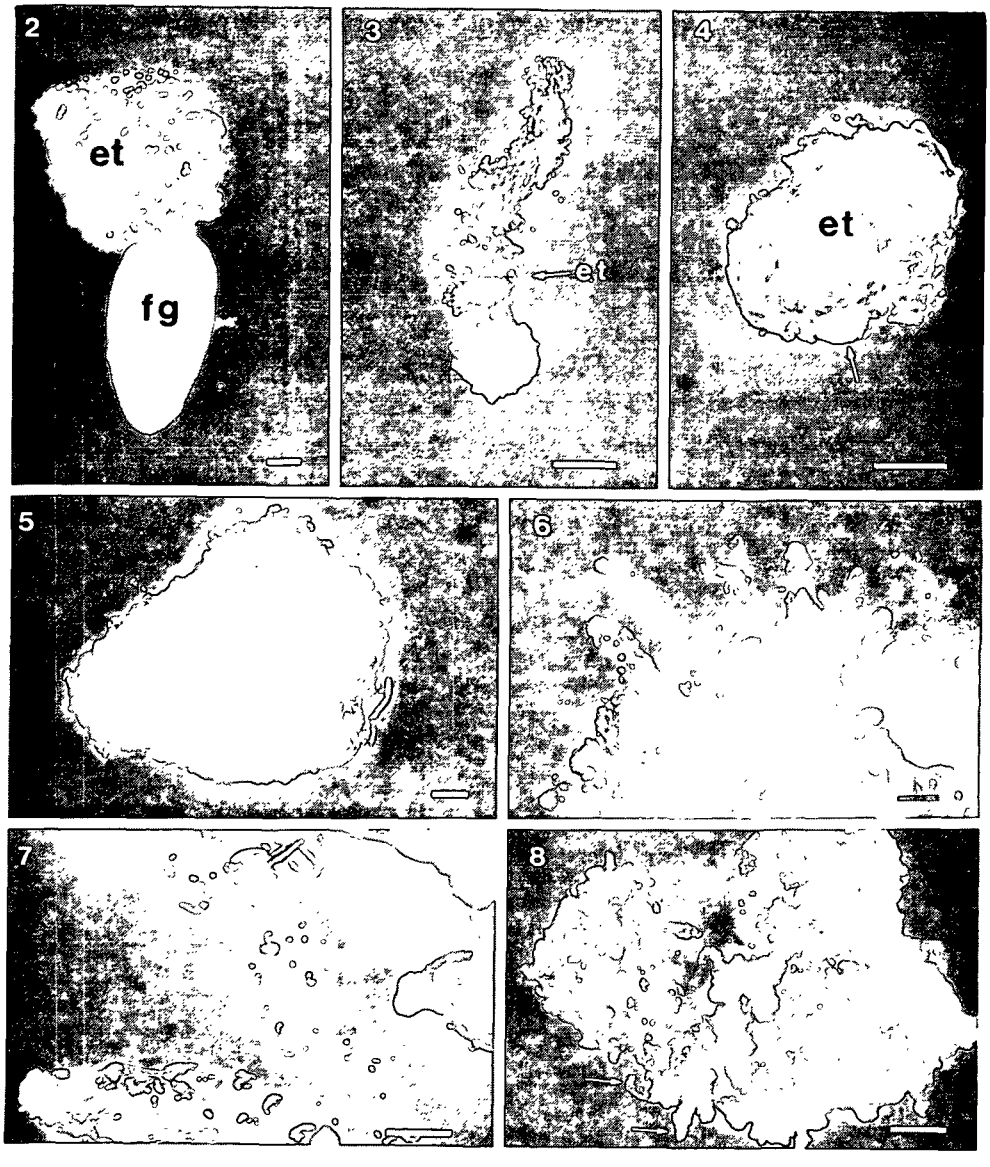
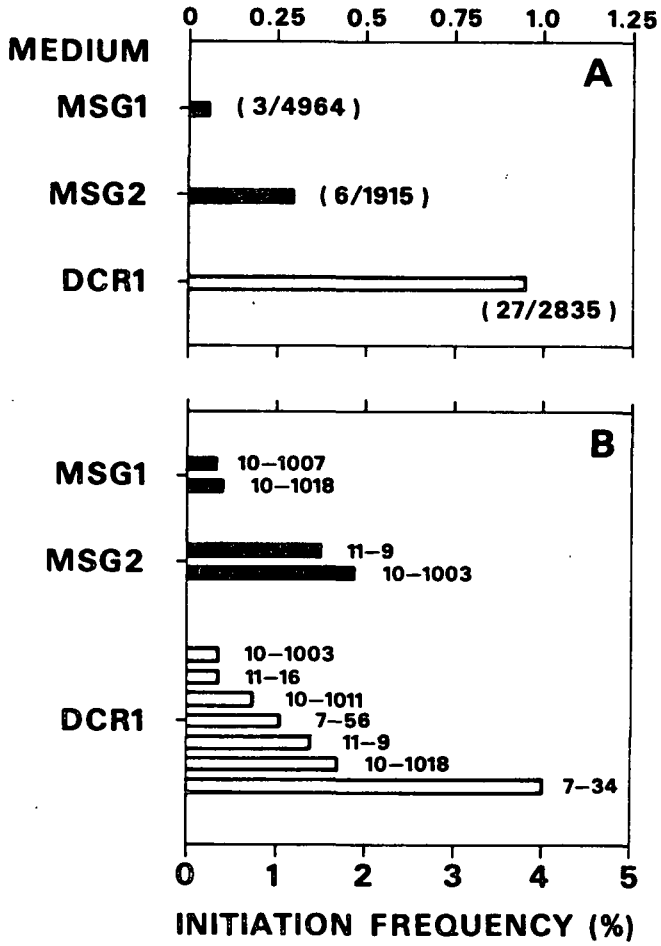

2
et
fg
3
et
4
et
5
6
7
8

Fig. 9.


0
0.25
0.5
0.75
1.0
1.25
MEDIUM
A
MSG1
(3/4964)
MSG2
(6/1915)
DCR1
(27/2835)
B
MSG1
10–1007
10–1018
MSG2
11–9
10–1003
DCR1
10–1003
11–16
10–1011
7–56
11–9
10–1018
7–34
0
1
2
3
4
5
INITIATION FREQUENCY (%)