FACTORS REGULATING ASEXUAL EMBRYOGENESIS IN VITRO IN LONGLEAF PINE

R. Nagmani[1], A.M. Diner[2], and G.C. Sharma[3]

Abstract.--Embryogenic cultures of longleaf pine (Pinus palustris Mill.) were established from female gametophytes with intact zygotic embryos (at developmental stages 1 and 2) cultured on MSG and DCR basal media in darkness. Induction of embryogenic tissue was observed from explants cultured on both basal media containing glucose, or maltose or sucrose as carbon source. All four levels of each carbon source (15-90 g/L) used supported production of embryogenic tissue. Both auxin (2,4-dichlorophenoxy acetic acid(2,4-D) and cytokinin (N6-benzyladenine (BA) at 2 and 1 mg/L; 3 and 0.5 mg/L; 5 and 2.5 mg/L and 2,4-D alone at 10mg/L were effective in producing embryogenic tissue.

Maintenance and proliferation of embryogenic tissue was achieved by transferring extruded embryogenic tissue to half-strength modified MSG medium supplemented with casein hydrolysate (lg/L). The level of 2,4-D was reduced to 0.5 mg/L and BA was eliminated from the medium. This transfer resulted in active proliferation of embryogenic tissue, and cultures were scaled up to 700 tissue masses each weighing approximately 250 mg. These embryogenic tissue masses have been subcultured 23 times to date for a period of 8 months.

Embryogenic tissue examined microscopically both at 3 or 4 weeks and 8 months after initiation revealed somatic embryos at several early stages of development. Somatic embryos from tissues on initiation medium were morphologically different from those on maintenance or proliferation medium. All somatic embryos were pre-cotyledonary and resembled their zygotic embryo counterparts at early stages of development.

Keywords: Pinus palustris(Mill.), longleaf pine, asexual embryogenesis, somatic embryogenesis, carbon source, glucose, maltose.

INTRODUCTION

Longleaf pine (Pinus palustris Mill.) is an extremely important softwood species in the southeastern United States, showing excellent form, good wood qualities, and resistance to fusiform rust. Substantial genetic variation in traits affecting survival, growth and disease resistance (Schmidtling and White 1989) , is seen in longleaf pine, which makes it an excellent candidate for artificial, clonal regeneration. Large scale micropropagation of desirable genotypes of longleaf pine via induction of asexual embryogenesis in vitro could offer not only selection of desirable genotypes but also efficient regeneration of genetically transformed plants.

. Research Scientist, Department of Plant and Soil Science, Alabama A & M University, Normal, AL 35762.
2. Plant Physiologist, U.S.D.A. Forest Service, Southern Forest Experiment Station, Alabama A & M University, Normal, AL 35762.
3. Professor, Department of Plant and Soil Science, Alabama A & M University, Normal, AL 35762.

Additionally, induction of somatic embryos at various developmental stages could become useful for mass production of thin layers of tissue for gene transfer via microprojectile bombardment technique (Sanford 1988).

Reports of somatic embryogenesis in conifers in general, and family Pinaceae in particular are mostly confined to the genera Pinus and Picea, although Larix, Pseudotsuga (Nagmani and Bonga 1985; Nagmani and Dinus 1991 and Nagmani et al. 1991), and Abies (Norgaard et al. 1992) can be reproduced via somatic embryogenesis. Tautorus et al.(1991) and Atree and Fowke (1991) have updated reports of somatic embryogenesis in conifers.

In this paper, we report the factors regulating initiation, maintenance, and multiplication of somatic embryos in longleaf pine and morphological variations of somatic embryos observed in cultures.

MATERIALS AND METHODS

Explants for initiation of embryogenic cultures were obtained from seed cones of longleaf pine collected on July 14 and 27, 1992 from open-pollinated tree growing in the Harrison Experimental Forest, Gulfport, MS. Aseptic techniques used for excision of explants and their culture were as described earlier for loblolly pine (Becwar et al. 1990) and Douglas-fir (Nagmani et al. 1991).

Female gametophytes containing zygotic embryos at stages 1 or 2 and isolated zygotic embryos primarily at stage 3 of development were cultured on modified Murashige and Skoog basal medium (1962) with glutamine (MSG medium) and DCR medium (Gupta and Durzan 1985).

In addition to the customary use of sucrose as the carbon source, maltose and glucose were also used individually as carbon sources for initiation of embryogenic cultures. These 3 carbon sources were used at 4 levels each (15-90 g/L). Growth regulators used were 2,4dichlorophenoxy acetic acid (2,4-D) and N^6-benzyladenine (BA) at 0-10 mg/L both individually and in combination. Casein hydrolysate (lg/L) was used as a supplement in some experiments. Methods of culture and culture conditions were as described in detail previously (Nagmani et al. 1993, In press). Culture medium for maintenance and proliferation of embryogenic tissues were modified according to the methods described for Douglas-fir (Nagmani and Dinus 1991), to sustain growth of the embryogenic tissue.

For microscopic examination, embryogenic tissue was stained with 0.5% toluidine blue in glycerol and photographed under phase contrast microscope. Initiation and development of somatic embryos were followed and compared to zygotic embryos according to the system of zygotic embryo classification proposed by Owens and Molder (1984) and Buchholz and Steimert (1945).

Statistical analysis of the data comparing media effects on initiation frequency of embryogenic tissue was performed using the chi-square test (X^2)(Snedecor and Cochran 1967).

RESULTS AND DISCUSSION

Explant type, developmental stage and time of excision:

"Explant type" here refers to either the female gametophyte containing intact zygotic embryos or isolated zygotic embryos since both these explants were used. Developmental stage of the explant corresponds to the apparent morphological equivalent of the zygotic embryo both within the female gametophyte and in the isolated state. The "time of excision" refers to the time of the year during which the seed cones were collected from longleaf pine tree from which explants were extracted. Embryogenic

response was observed from female gametophytes containing zygotic embryos (33 cultures from a total of 944 explants cultured). None of the isolated zygotic embryos produced embryogenic tissue. Extrusion of the embryogenic tissue was observed from the micropylar region of the female gametophytes in all 33 responsive explants. Somatic embryos were observed in embryogenc tissue masses by 4 weeks in culture (Figure 1A). The developmental stage of the zygotic embryo within the female gametophyte was classified under stages G and H of (Owens and Molder 1984) lodgepole pine embryo classification. We have modified the classification to include stages G and H under stages 1 & 2. The isolated embryos cultured were at stage 3 of development according to the classification proposed by Buccholz and Stiemert (1945) for Pinus ponderosa. The time at which zygotic embryos reach certain stage of development is associated with the time of the year during which seed cones are collected for culture. Accordingly, in longleaf pine, explants excised from seed cones collected on July 14, 1992 proved to be generally responsive to initiation of embryogenic tissue. Isolated embryos cultured were at stage 3 of development, and were excised from seed cones collected on July 27. As the explant type, age and time of excision are interrelated, it is difficult to ascertain the role of each one of these three factors individually on initiation frequency of embryogenic cultures. However, longleaf pine female gametophytes containing embryos at stage 1 or 2 of development were responsive, in contrast to isolated embryos as explants. This may be partly due to nutritional support provided by gametophytic tissue. Reports of successful initiation of embryogenic cultures in other pine species such as P. strobus (Finer et al 1989), P. taeda (Becwar et al. 1990) P. radiata (Smith 1987) and P. elliottii (Jain et al. 1989) suggest that pre-cotyledonary zygotic embryos were most responsive to initiation of embryogenic tissue. However in Picea, cotyledonary embryos initiated embryogenic cultures (Hakman et al. 1985).

Effect of carbohydrates

Maltose, glucose and sucrose were equally effective in the induction of embryogenic tissue from the explants. Out of a total of 248 explants cultured on media containing glucose (at 4 levels) 4 explants produced embryogenic tissue. Maltose-containing media (at 4 levels) supported initiation of embryogenic tissue from 18 out of a total of 472 explants cultured. Media with sucrose as the carbon source, induced embryogenic tissue in 11 of 224 explants.

Effect of basal media

Only one of 192 explants cultured on growth regulator free basal media (modified MSG and DCR media) produced embryogenic tissue. There was no significant difference between MSG and DCR basal media used as far as embryogenic response of the explants. The addition of casein hydrolysate to the medium was not necessary for initiation of embryogenic tissue.

Effect of growth regulators

Combinations of 2,4-D and BA at 2 and 1 mg/L, 3 and 0.5 mg/L, 5 and 2.5 mg/L respectively were effective in the induction of embryogenic tissue. When 2,4-D was used alone at 5 and 10 mg/L, 4 explants out of 96 cultured produced embryogenic tissue at 10 mg/L of 2,4-D, while no response was seen at 5 mg/L.

Maintenance and proliferation

For maintenance and proliferation of embryogenic tissue, the concentrations of macro- and micro-nutrients in MSG medium were reduced to half-strength and casein hydrolysate (lg) was added as supplement. Further,the level of 2,4-D was reduced to 0.5 mg and BA was deleted. This resulted in proliferation of embryogenic tissue, and the cultures were scaled up to about 700 tissue masses each weighing approximately 250 mg.

Morphological variations of asexual embryogeny in vitro

Histological examination of embryogenic tissue at both 7-weeks and 8 months after initiation revealed somatic embryos at various stages of development (Figures 1B-I). Fig 1B-D shows somatic embryos from 7-week

-old embryogenic tissue. Figure 1B shows two somatic embryos with long, coiled suspensors and an embryonal mass (em) with 4, 12 or 24 cells which are densely cytoplasmic with prominent centrally located nuclei. These cells are compact and well defined. The cells of the suspensor (sc) are considerably enlarged and vacuolated with sparse peripheral cytoplasm (Figure 1C). The cells of the embryonal mass and suspensor are shown to have divided further resulting in multicellular embryonal head (eh) and multilayered suspensor (Figure 1D).

Figures 1E-I show somatic embryos from 8-month-old embryogenic cultures on maintenance and proliferation media. The embryogenic tissue masses were subcultured 23 times at 10-15 day intervals for a period of 8-months onto half-strength modified MSG medium with 2,4-D at 0.5 mg/L.

Figures 1E,F represent 2 stages of early somatic embryo development where an embryonal mass (em) of 2,4 and 6 cells with prominent nuclei and dense cytoplasm are subtended by 1 or 2 tiers of broad suspensor cells (sc). Figures 1G-I show morphological variations of early somatic embryos with loosely arranged cells in the embryonal mass (em) and elongated suspensor cells (sc). Early somatic embryos shown in Figures 1E,H resemble their zygotic embryo counterparts documented and illustrated for a conifer species (Konar and Nagmani 1980). Also, early somatic embryos documented in Figures 1B,C resemble in early somatic embryos of Norway spruce (Nagmani et al. 1987). Some embryogenic tissue masses of longleaf pine have been transferred to development and maturation media to further development of somatic embryos.

(Figures 1A-I here)

Early somatic embryogeny in longleaf pine differs from early zygotic embryogeny in pine species in that the former lacks the free nuclear division common to post-fertilization events in the zygote. In somatic embryogeny the early somatic embryos are cellular bipolar structures with embryonal mass subtended by suspensor cells.

Morphological variations are seen between somatic embryos on initiation, maintenance and proliferation media. Somatic embryos at pre-cotyledonary stages from embryogenic cultures on initiation media have long coiled massive suspensors at early stages of development (Figure 1B) as compared to somatic embryos from cultures on maintenance and proliferation media (Figures 1E-I). We speculate that early stages of embryo development observed in cultures on maintenance and proliferation media occur due to either continual de novo initiation from callus cells on maintenance and proliferation media or proliferation from pre-existing asexual embryos. Somatic embryos observed on initiation media (Figures 1B-D) represent rather advanced stages of early embryo development as compared to cultures on maintenance media (Figures 1E-I). This may be due to the relative age of embryogenic cultures where 7-week-old embryogenic tissue on initiation medium is exposed to auxin (2,4-D) in the medium for a shorter period of time (7-weeks) as compared to 8-month-old embryogenic tissue on maintenance medium (subcultured 23 times for a period of 8 months) exposed to auxin (2,4-D) for longer period of time. Transfer of these embryogenic cultures to a medium either free of auxin or to a medium with no auxin but with abscissic acid (ABA) might promote development of somatic embryos to cotyledonary stages of development. Experiments are underway to test this hypothesis.

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FIGURE CAPTIONS

Figure 1. Somatic embryos of longleaf pine.

A. 7-week-old embryogenic tissue on induction medium showing somatic embryo (se). Scale bar= 1mm.

B. Stage 1 somatic embryos showing an embryonal mass (em) and long coiled suspensor system (ss) on induction medium. Scale bar= 10)=.

C. Somatic embryo enlarged showing compact embryonal mass (em) and broad suspensor cells (sc). Scale bar= 0.1mm.

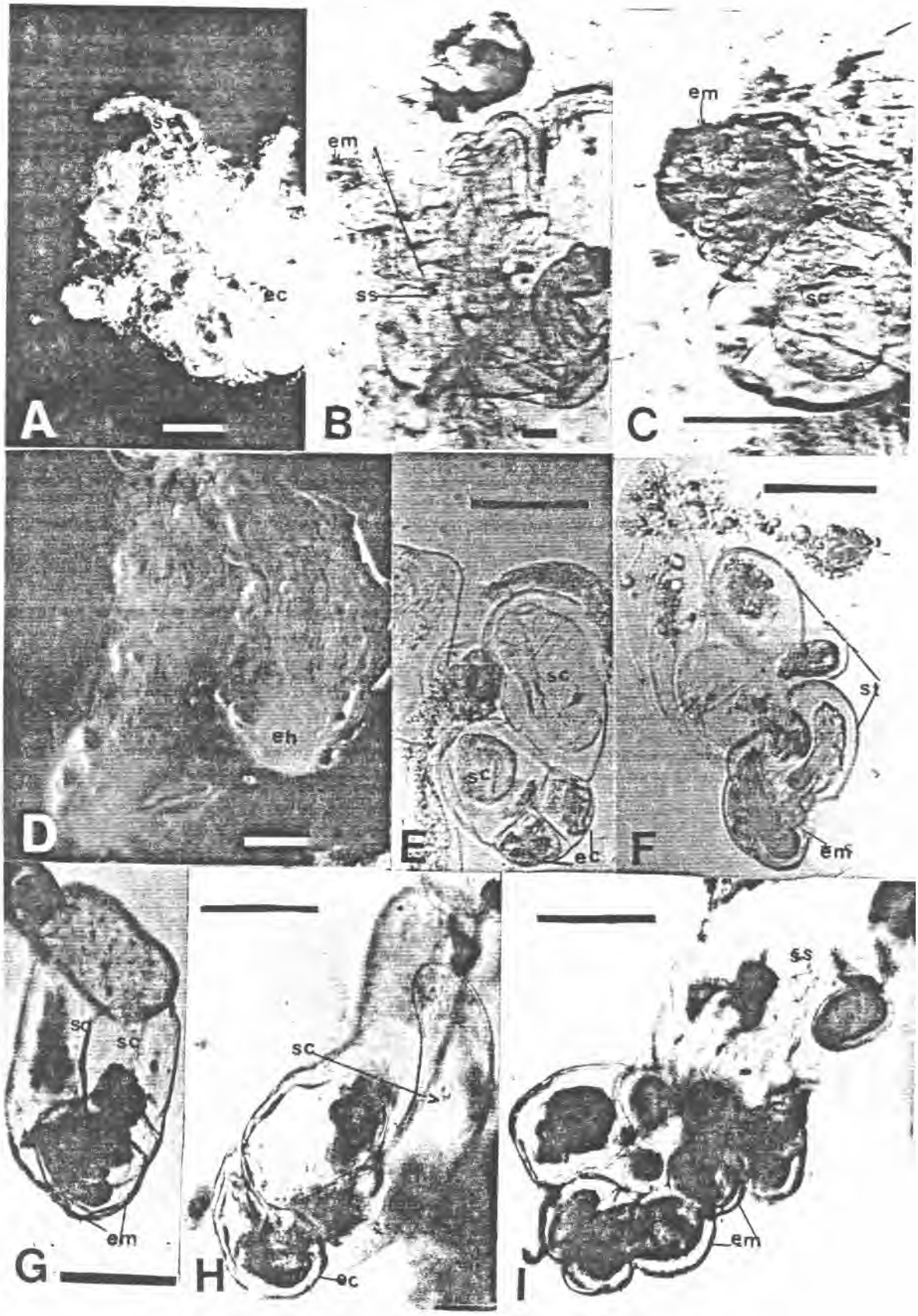
D. Stage 2 somatic embryos with an embryonal head (eh) and multilayered suspensor system (ss). Scale bar= 0.5mm.

E. 4 or 8-celled somatic proembryo with 2-4 embryonal cells (ec) subtended by 2-4 suspensor cells (sc). Scale bar= 0.1mm.

F. Somatic proembryo with 2 tiers of suspensor cells (st) and an embryonal mass (em). Scale bar= 0.1mm.

G,H. Morphological variations of 4 or 8 celled somatic proembryo eith an embryonal mass (em) subtended by 2 or 4 broad or elongated suspensor cells (sc). Scale bar= 0.1mm.

I. Variation of stage 1 somatic embryo with an embryonal mass of loosely arranged cells (em) and long suspensor system (ss). Scale bar= 0.1mm.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