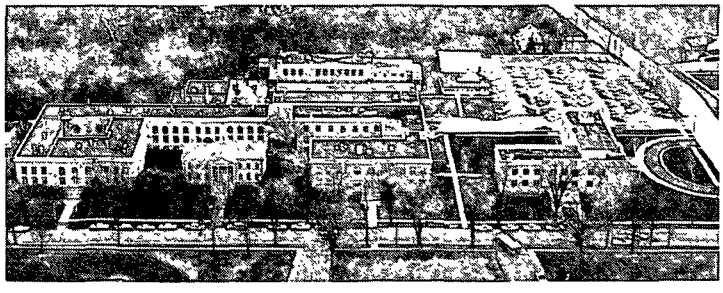
263



THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

IPC TECHNICAL PAPER SERIES
NUMBER 263

FACTORS INFLUENCING THE INITIATION OF SOMATIC EMBRYOGENESIS IN *PINUS STROBUS* L.

S. R. WANN, M. R. BECWAR, L. F. KROLL, R. NAGMANI, AND H. B. KRIEBEL

OCTOBER, 1987

Factors Influencing the Initiation of Somatic Embryogenesis in Pinus strobus L.

S. R. Wann, M. R. Becwar, L. F. Kroll, R. Nagmani, and H. B. Kriebel

This manuscript is based on results obtained in IPC Project 3223 and has been submitted for consideration for publication in Plant, Cell, Tissue and Organ Culture

Abstract. Embryogenic callus was initiated from immature embryos of Pinus strobus L. (eastern white pine) on defined medium containing 2,4-D and BA. Under these conditions, the developmental stage of the embryo explant was the determining factor for successful initiation of embryogenic callus. Embryogenic callus could be initiated at a low, but reproducible frequency from precotyledonary embryos but not from cotyledonary embryos. Both the site of origin of the embryogenic callus and the phenotypic similarity between the callus and zygotic cleavage embryos of Pinus suggest suspensor tissue was embryogenically competent. Embryogenic callus could be maintained on a variety of basal media supplemented with cytokinin but with or without auxin. Embryo development progressed to near the point of cotyledon formation on transfer of embryogenic callus to a medium supplemented with ABA.

FACTORS INFLUENCING THE INITIATION OF SOMATIC EMBRYOGENESIS IN PINUS STROBUS L.

S. R. Wann,[1] M. R. Becwar,[1] L. F. Kroll,[1] R. Nagmani,[1] and H. B. Kriebel[2]
[1]Forest Biology Division, The Institute of Paper Chemistry
Appleton, WI 54912
[2]School of Natural Resources, Division of Forestry,
Ohio State University, Wooster, OH, 44691

INTRODUCTION

Somatic embryogenesis has been only recently described in conifers. In the genera in which it has been reported (Picea, Pinus, and Larix) little in the way of detail has been reported on factors affecting the initiation of embryogenic callus. Aside from nutritional, hormonal and environmental factors, the developmental state of the explant appears to play a significant role on the successful initiation of embryogenic callus. For example, in Larix decidua, fertilized ovule explants could be induced to form embryogenic callus only from late Jan. to early July [6]; In Pinus lambertiana, embryogenic callus was initiated from immature embryos 3-4 weeks after fertilization [2]; in Pinus taeda fertilized ovules obtained 4-5 weeks after fertilization were the requisite explant for the initiation of embryogenic callus [3].

The brief period during which explants are amenable to the in vitro induction of embryogenesis requires that strict attention be given to identifying the appropriate stage of development. In conifers identifying the stage of zygotic embryogenesis on the basis of the calendar or the length of time after fertilization leave much to be desired because the timing of fertilization in conifers is (1) difficult to determine and (2) subject to seasonal variation as is the subsequent development of the embryo.

In the present study an attempt was made to identify the stage of zygotic embryo development from which embryogenesis could be induced in eastern white pine, the most widely planted pine in the northeastern United States.

MATERIALS AND METHODS

PLANT MATERIAL

Seed cones of Pinus strobus L. (white pine) were collected from 5 trees; 3 trees from a plantation near Appleton, WI, and 2 clones from a seed orchard (A = 1588 and B = 1590) near Wooster, OH, on a weekly basis starting July 1, 1986. Cones were stored in kraft paper bags at 4°C for up to 5 months. Seeds were removed from the cones and surface sterilized by treatment with a commercial bleach solution (Hilex 25% v/v) for 15 min, followed by three rinses with sterile, distilled water. Embryos were aseptically removed from the surrounding gametophytic tissue and placed on culture medium.

CULTURE CONDITIONS

MSG medium [1] and DCR medium [2] with 3% sucrose were used in the following experiments. In DCR medium, the level of glutamine was increased from 50 to 250 mg/L. For callus initiation and maintenance of embryogenic callus 2,4-dichlorophenoxyacetic acid (2,4-D) was used singly or in combination with N^6-benzyladenine (BA). All media were solidified with 0.8% Difco Bacto agar and were adjusted to pH 5.8 prior to autoclaving.

In each initiation experiment, 5 embryos were cultured in each 5-cm Petri dish, and typically 30 embryos were used per treatment. After 4 weeks, cultures were transferred to fresh medium, and the initiation frequency was evaluated after an additional 4 weeks. The stage of embryo development (cotyledonary or precotyledonary) giving rise to embryogenic callus was also noted. Embryogenic callus lines were subsequently maintained by transfer to fresh medium on a monthly basis. All cultures were incubated in the dark at 23°C.

RESULTS AND DISCUSSION

Embryo development as measured by embryo length vs. time is illustrated in Figure 1 for seeds from the two clones (A & B) obtained from Ohio. Embryo length vs. time curves for the other trees (C, D, and E) were nearly identical to the curves shown for clones A and B. Fertilization in eastern white pine usually occurs in northern Ohio during the third week in June [5]. In 1986, precotyledonary embryos (collections 1 and 2) could be isolated starting in the first week of July. By the third week in July nearly all the embryos were at the cotyledonary stage (collection 3). Embryogenic callus could be initiated from precotyledonary embryos only (collections 1 and 2) in the dark on MSG and/or DCR medium containing combinations of 2,4-D and BA (see Table 1). However, embryogenic callus was not initiated under these conditions from the predominantly cotyledonary embryos contained in collection 3. In total, over 1000 cotyledonary embryos from collections 3-5 were cultured under a variety of conditions, and embryogenic callus was initiated only once (results not shown).

Figure 1 and Table 1 here

Although the frequency of initiation in collections 1 and 2 was on the order of 5%, embryogenic callus was readily recognized as the only callus proliferated from any of the precotyledonary embryos cultured. In the remaining 95% of the cultures, no callus formation of any kind was observed, and typically the embryo explant was brown and only slightly swollen. Deterioration of the embryo explant was also observed in cultures yielding embryogenic callus. However, in those cultures, the suspensor cells near the point of attachment to the embryo proper appeared to be the region where callus proliferation began. Collections 1 and 2 were characterized by white, turgid suspensors that remained firmly

attached to the embryo. Beginning with collection 3, suspensor cells began to turn rubbery, became less turgid, and in many cases were easily detachable from the embryo. The observation that the apparent loss of suspensor viability was coincidental with the corresponding loss of embryogenic potential of the explant also implies that suspensor cells were the site of initiation of embryogenic callus.

In a separate experiment with collection 2, the effect of several levels of 2,4-D was examined along with the requirement of cytokinin (see Table 2). Collection 2 was chosen for this experiment because it represented an "optimum" stage of development. Embryos from this collection were precotyledonary but were sufficiently well developed that isolation was facilitated. From this experiment, it appears that BA is required for initiation and that a level of 2,4-D near 2-3 mg/L is adequate for initiation.

In the present study, it appears that there is a genetic component in the initiation of embryogenic callus. Immature embryos taken from trees A and C did not form embryogenic callus under any of the treatments examined. There was no readily apparent reason for this failure in initiation as embryo explants from the unresponsive genotypes were not visually different from those taken from trees B, D, and E.

Table 2 here

Upon initiation, embryogenic callus could be maintained on a variety of media supplemented with auxin/cytokinin combinations as well as with cytokinin alone. When grown under proliferative conditions, embryogenic callus was comprised of mainly pre-embryogenic masses (see Fig 2-A) in addition to somatic embryos (see Fig 2-B). The somatic embryos proliferated under these conditions

looked like zygotic cleavage embryos of Pinus spp., and often several somatic embryos shared a common suspensor (see Fig 2-C).

Figure 2 here

Transfer of embryogenic callus to DCR or MSG media containing 2.6 mg/L ABA and 6% (w/v) sucrose promoted development of embryos to nearly the cotyledonary stage. To date embryo development has not proceeded beyond this point.

The method described here for the initiation of somatic embryogenesis in white pine is very much similar to the method reported for another 5-needled pine, sugar pine [2], as well as for Norway spruce [4,7]. However, here we have documented by both embryo size and time of its development the stage of zygotic embryo development (explant) most conducive to the induction of somatic embryogensis. It appears that among pines the developmental stage of zygotic embryo explant necessary for the initiation of embryogenesis appears to be similar, as we have used the identical procedure to initiate somatic embryogensis in loblolly pine (results not shown). Along with the published reports of somatic embryogensis in loblolly pine [3] and sugar pine, this report highlights the importance of the suspensor region in the origin of somatic embryos in pines.

REFERENCES

1. Amerson HV, Frampton LJ, McKeand SE, Mott RL, and Weir RJ (1985) Loblolly pine tissue culture: laboratory, green house, and field studies. In: Henke RR, Hughes KW, Constantin MJ, and Hollaender A (eds.) Tissue Culture in Agriculture and Forestry. Plenum Press, New York, p. 271-89.

2. Gupta PK, Durzan DJ (1986) Somatic polyembryogenesis from callus of mature sugar pine embryos. Bio/Technology 4: 643-5.

3. Gupta PK, Durzan DJ (1987) Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Bio/Technology 5: 147-51.

4. Hakman I, Fowke LC, von Arnold S, and Eriksson T (1985) The development of somatic embryos in tissue cultures initiated from immature embryos of Picea abies (Norway spruce). Plant Sci. 38: 53-9.

5. Kriebel HB (1972) Embryo development and hybridity barriers in the white pines (Section Strobus). Silvae Genet. 21: 39-44.

6. Nagmani R, Bonga JM (1985) Embryogenesis in subculture callus of Larix decidua. Can. J. For. Res. 15: 1088-91.

7. von Arnold S, Hakman I (1986) Effect of sucrose on the initiation of embryogenic callus from mature zygotic embryos of Picea abies (L.) Karst. (Norway spruce). J. Plant Physiol. 122: 261-5.

Figure Captions

Figure 1. Zygotic embryo development in white pine for July, 1986. Measurements taken on 25 embryos per collection per clone.

Figure 2. Somatic embryogenesis in Pinus strobus. (A) Pre-embryogenic masses (PEM) contained in embryogenic callus. (B) Somatic embryo. Note densely stained embryonal mass supported on tiers of suspensor cells. (C) Scanning electron micrograph somatic embryos at a later stage of development (4 weeks on MSG medium containing 2.6 mg/L ABA and 6% sucrose). Note common suspensor region for the two embryos shown. Bars represent 100 µm.

Table 1. Effect of medium and stage of explant development on initiation of embryogenic callus in white pine.

Collection (Date)	Tree	Initiation Frequency, %	
		MSG 2/1[a]	DCR 3/0.5
1 (7/2)	C	--	0
	D	--	8
	E	--	0
2 (7/9)	A	0	0
	B	3	0
	C	0	0
	D	3	6
	E	3	5
3 (7/16)	A-E	0	0

[a]2/1 etc. refers to auxin/cytokinin levels (mg/L).

Table 2. Effect of auxin level and cytokinin on initiation of embryogenic callus in white pine.

Clone	Initiation Frequency on DCR Medium, %			
	3/0[a]	0.1/0.5	3/0.5	10/0.5
A	0	--	0	0
C	0	0	0	0
D	0	0	6	0
E	0	0	5	0

[a]etc., refer to auxin/cytokinin levels (mg/L).

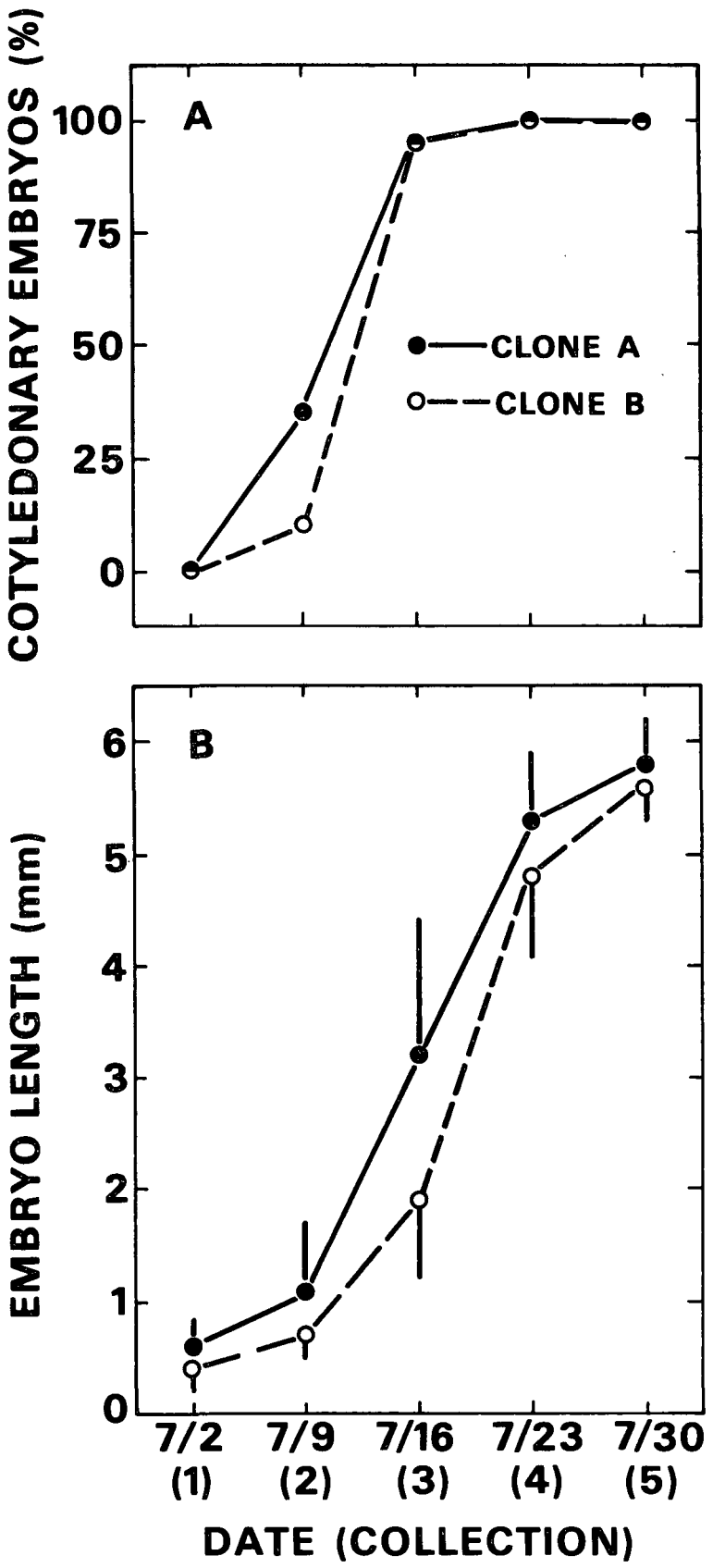

COTYLEDONARY EMBRYOS (%)
100
75
50
25
0
A
CLONE A
CLONE B
EMBRYO LENGTH (mm)
6
5
4
3
2
1
0
B
7/2
(1)
7/9
(2)
7/16
(3)
7/23
(4)
7/30
(5)
DATE (COLLECTION)

Figure 1.

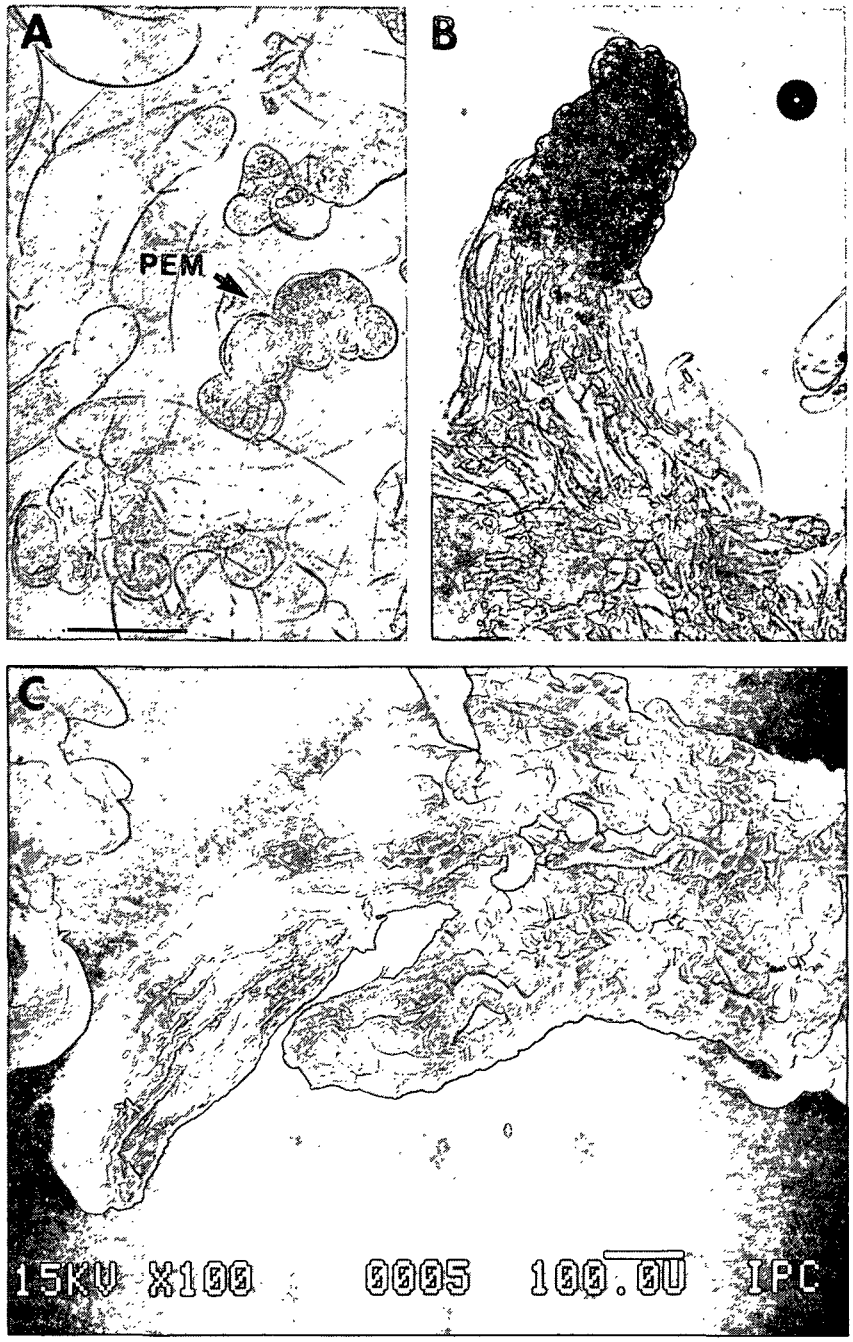


Figure 2.