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In vitro Plantlet Regeneration of Loblolly Pine, Pitch Pine, and Their Hybrid[1]

—The Culture of Embryonic Tissues—

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組織培養에 의한 테다, 리기다 및 交雜種 소나무의 植物體 繁殖[1]

—胚組織의 培養—

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ABSTRACT

The embryos of *Pinus taeda*, *P. rigida*, and *P. taeda* x *rigida* were cultured for adventitious shoot regeneration in vitro. Culture media were modified from Gresshoff and Doy (MGD), Murashige and Skoog (MMS), Lloyd and McCown (MLM), and Schenk and Hildebrandt (MSH). NAA was added to initiation media at a concentration of 0.1 or 0.01 mg/l. BAP was used at the concentrations of 0.1, 0.5, 1, 2, or 5 mg/l. Each explant was induced for 3-4 weeks on solid medium. All explants were cultured up to 16 weeks. Illumination was about 1506±540 lux at the level of the tissues in the growth room with a temperature of 25±2℃. A 16-hour photoperiod per 24 hours was used. Half-strength medium was used for all the subcultures.

For shoot production by loblolly pine, MMS, MLM, or MSH is preferred with 5 mg/l BAP with either 0.1 or 0.01 mg/l NAA. For shoot production by pitch pine, MMS, MLM, or MSH is recommended with 2 or 5 mg/l BAP with 0.1 mg/l NAA. For shoot production by the hybrid pine, MMS or MLM is more effective with 1, 2 or 5 mg/l BAP with 0.1 mg/l NAA. There were no differences recognized among the species tried in the patterns of bud formation and shoot development. Different composition of media, in major and minor salts or possibly in vitamins, should be tested for the two developmental stages of adventitious shoots ; the induction of shoot buds and the elongation of them into shoots.

Key words : Pinus taeda, Pinus rigida, Pinus taeda x rigida, *embryonic tissue*, *adventitious shoot*
Abbreviations : BAP (6-benzylaminopurine), NAA(1-naphthaleneacetic acid), GD(Gresshoff and Doy medium, 1972), MS(Murashige and Skoog medium, 1962), LM(Lloyd and McCown medium, 1981), SH (Schenk and Hildebrandt medium, 1972), CK(cytokinin)

要 約

시험관내 배양에 의해 테다, 리기다 및 그 교잡종 소나무의 胚로부터 不定枝 生産을 시도하였다. 배지1

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은 Gresshoff와 Doy, 배지2는 Murashige와 Skoog, 배지3은 Lloyd와 McCown, 배지4는 Schenk와 Hildebrandt 식으로부터 각각 수정·변화되었다. 不定芽 유도 외의 배지는 무기성분(단, 철 제외)을 반으로 감소시켰다. NAA(0.1 또는 0.01 mg/l)와 BAP(0.1, 0.5, 1, 2, 또는 5 mg/l)가 10개 조합으로 첨가된 배지에서 배양체는 3-4주간 유도되었다. 광도는 1500±540 룩스이었고 光週期는 16시간이었다. 25±2℃의 배양실에서 16주(부정아 유도 기간 포함) 배양후 부정지 생산 胚의 비율과 胚當 평균 부정지의 수를 조사하여 분석하였다.

테다 소나무의 부정지 생산을 위해서는 5 mg/l BAP와 0.1 또는 0.01 mg/l의 NAA를 첨가한 배지2, 배지3 또는 배지4가 바람직하며, 리기다 소나무의 경우는 2 또는 5 mg/l BAP와 0.1 mg/l NAA의 배지2, 배지3 및 배지4가 좋은 것으로 나타났다. 교잡종의 경우는 배지2와 배지3이 1, 2, 또는 5 mg/l BAP와 0.1 mg/l NAA를 첨가했을 때 효과적이었다. 不定芽 형성 및 不定枝 발달 양식에 있어 수종간 차이는 관찰되지 않았다. 부정아 유도와 유도된 부정아의 성장을 위한 기본배지는 각기 다른 것을 사용하는 것이 바람직하다.

INTRODUCTION

A lot of research has been done and is still in progress in the area of mass-propagation of forest trees using tissue culture, since the first report of organogenesis was made in *Ulmus campestris*[12]. 'Tissue culture' was initially applied to the in vitro culture of callus. However, currently the term has been extended in use to cover culture of organs, tissues, free cells, and protoplasts. The tissue culture of forest trees draws upon information and techniques developed for fruit or ornamental trees and nonwoody plants. However, two major differences are associated with forest trees : 1) a high variability in culture responses, and 2) morphogenetic unresponsiveness due to maturity of some explant material.[37] For a few tree species the commercial needs are met in terms of mass-propagation research.[30]

The main area of concentration of research in pines is on micropagation by organogenesis or embryogenesis. Most of the research has been done with embryos (mature of immature), cotyledons, primary needles, dormant buds, or calli. Some preliminary studies on conifer protoplasts have been reported.[8,9,16,19] So far successful regeneration has not been routinely achieved from gymnosperm protoplast culture.[4,15] Embryonic cotyledons have been successfully used to produce adventitious shoots (as parts of intact embryos or alone) : in *P. palustris*[36], *P. radiata*[32], *P. taeda*[24], *P. rigida*[27], *P. rigida* x *taeda*[18], and *P. elliottii*[29].

It is widely accepted that 6-benzylaminopurine (BAP) is more efficient than any other cytokinin (CK), with or without auxin, for adventitious bud formation and shoot elongation in many coniferous species in vitro.[7] The basal medium has also been reported to affect morphogenesis. The basal media modified from Gresshoff and Doy (GD)[14], Murashige and Skoog (MS)[25], Lloyd and McCown (LM)[22], and Schenk and Hildebrandt (SH)[34] are those most widely used in pine tissue cultures. The basic differences among them are : 1) NH^{4+}/NO_3 ratio, 2) concentration and source of K, N, and P, and 3) concentration of minor salts. However, in reality the choice of medium and growth regulator is rather arbitrary, primarily because the physiological and biochemical interactions between explants and nutrients are still far from being understood.

The goals of this article are to develop micropropagation methods for loblolly pine, pitch pine, and their hybrid by attempting to culture embryonic tissues, and to investigate the effects of medium composition and phytohormones on in vitro morphogenesis of those tissues.

MATERIALS AND METHODS

Explants used

Two pine species and their hybrid were adopted for this experiment : *Pinus taeda* L., *P. rigida*

Mill., and *P. taeda* x *rigida*. Loblolly pine seeds originated from 5 seed sources of southeastern regions of the United States; pitch pine from South Carolina, USA. For hybrids, 12 combinations of hybrid seeds were obtained from Westvāco Co., Summervile, South Carolina, USA.

Intact mature embryos with their embryonic cotyledons still attached were used. Seeds were imbibed for 48-72 hours at room temperature prior to removal of the embryo. The seeds were surface-sterilized before embryo removal as follows. Seeds were dipped in 70%(v/v) ethanol for 3-5 minutes and then rinsed with sterile distilled water for 5 minutes. Seeds were then submerged with frequent stirring in about 2.5% sodium hypochlorite solution (1 : 1 dilution of Clorox, a commercial laundry bleach, with sterile-distilled water) for 15 minutes, followed by rinsing with sterile-distilled water for 5 minutes and by dipping in sterile 0.01-N HCl for 5 minutes, and then rinsed 3 times with sterile-distilled water for 5 minutes per rinse.

Medium

Table 1 showes the composition of four basal media; MGD, MMS, MLM, and MSH. Adventitious bud initiation was stimulated by growth regulators. After this stimulation no phytohormones were added to the medium. Naphthaleneacetic acid(NAA) was added to initiation media at a concentration of 0.1 or 0.01 mg/l. BAP was used over a wider range at the concentrations of 0.1, 0.5, 1, 2, or 5 mg/l. Casein hydrolysate (enzymatic) was included in every initiation medium and activated charcoal (neutralized) (Sigma Chemical Co., Lot 86C-0101) was added to the first subculture medium only. Basal medium was diluted to one half normal concentration in major and minor elements except iron for all the subcultures (half-strength medium). All the media were adjusted with either HCl (0.1 or 1-N) or NaOH (0.1 or 1-N) to pH 5.6-5.7, after all the components except agar had been added, prior to autoclaving. The media were sterilized by autoclaving for 15 minutes at 121 ℃ and 18 psi.

Table 1. Composition of media (unit ; mg/l)

Media	MGD	MMS	MLM	MSH
Compound				
$NH_4H_2PO_4$				300
NH_4NO_3			400	
$(NH_4)_2SO_4$	200			
$CaCl_2 \cdot 2H_2O$	150	440	96	200
$Ca(NO_3)_2 \cdot 4H_2O$			556	
$MgSO_4 \cdot 7H_2O$	250	370	370	400
KCl	300			
KNO_3	1000	1900		2500
KH_2PO_4		170	170	
K_2SO_4			990	
Na_2HPO_4	30			
$NaH_2PO_4 \cdot H_2O$	90			
H_3BO_3	3.0	6.2	6.2	5.0
$CoCl_2 \cdot 6H_2O$	0.25	0.25	0.025	0.025
$CuSO_4 \cdot 5H_2O$	0.25	0.25	0.25	0.25
$MnSO_4 \cdot H_2O$	20.0	22.3	22.3	20.0
KI	0.75	0.75	0.075	0.075
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.25
$ZnSO_4 \cdot 7H_2O$	3.0	8.6	8.6	1.0
$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.8	27.8
Na_2-EDTA $2H_2O$	37.3	37.3	37.3	37.3
myo-inositol	100	100	100	1000
nicotinic acid	1.0	1.0	1.0	5.0
pyridoxine HCl	0.1	0.1	0.1	0.5
thiamine HCl	1.0	1.0	1.0	5.0
agar	6000	6000	6000	6000
casein hydrolysate*	1000	1000	1000	1000
sucrose	20000	20000	20000	20000
activated charcoal**	5000	5000	5000	5000

*Only added to the induction media

**Only added to the first subculture media

Culture methods

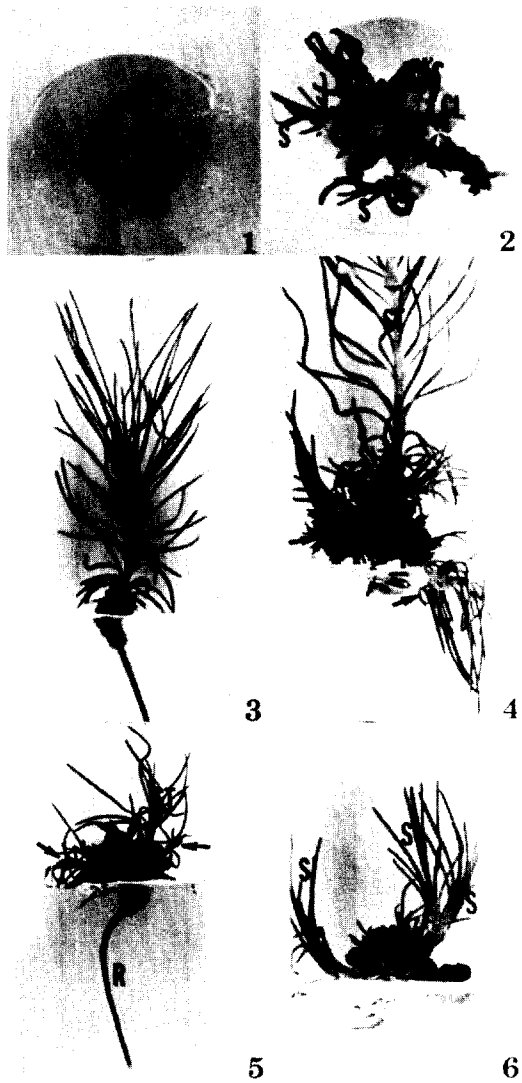
Each group of cultures was maintained for as long as 16 weeks consisting of an 3-4 week induction and 3 subcultures every 4-5 week. Illumination was given by cool white fluorescent lamps with approximately 1506±540 lux at the level of the tissues in the room under 25±2℃. A 16-hour photoperiod per 24 hours was used. The embryos were cultured in 25 x 150 mm test-tubes containing 15 ml of medium. For each species, a split-split-plot design with two replications was

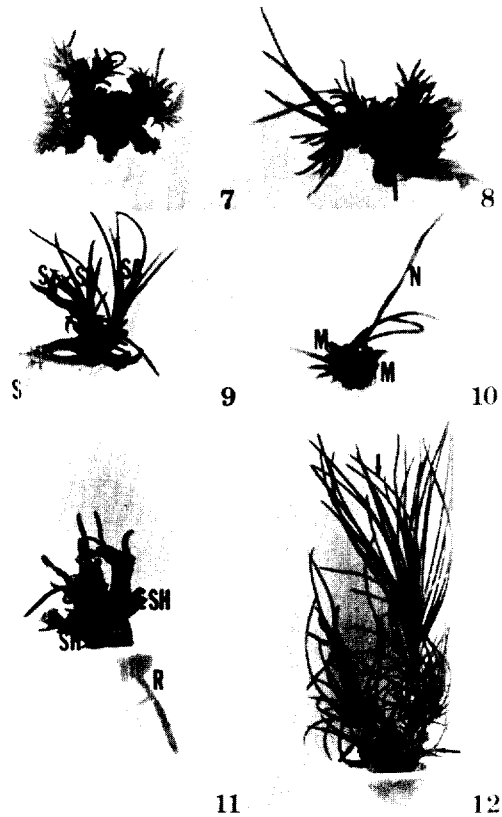
used. Each provenance source provided 2 seeds for each replication in loblolly pine. Two to three seeds per replication were used for pitch pine from each mother tree. Two to three seeds per replication were used for hybrid pine from each female parent source. Each replication was made using 10 explants. Data, which were collected as a ratio, were transfomed by the equation, y=arc sin (square root (x)).

RESULTS

No characteristics specific to each species were observed in the pattern of bud formation and shoot development. Within 1-2 weeks after transferred from induction media to non-hormonal media, many embryos were observed to form small swellings mainly at the tips of cotyledons and/or along the cotyledons. Some had already shown swollen regions on induction media. Some embryos developed callus-like structures mostly at the hypocotyl and radicle, but rarely at the cotyledons in contact with medium. If adventitious buds were not formed, these callus-like structures grew and covered the whole embryo (Fig. 1). This overgrowth by callus-like tissues always happened during bud initation or the first subculture period. Even though shoots developed, some calli overgrew the growing shoots (Fig. 2). This occurred mainly during the first subculture period. As soon as this was observed, shoots were excised and transferred to the fresh subculture medium. Some embryos developed into normal plantlets, without the formation of adventitious shoot (Fig. 3). These plantlets completed their seedling growh during the first subculture period. Some embryos developed a plantlet stem without development of a root (Fig. 4). Some embryos were observed that showed the normal development of plantlets in addition to the formation of adventitious shoots (Fig. 5). A cluster of buds, usually covering the whole embryo, appeared on other embryos (Fig. 6). Many buds in such a cluster grew into shoots, but other buds showed no further growth. Many adventitious shoots were produced at the tips and/or along the cotyledons (Figs. 7 to 9). Axillary shoots between the cotyledons were frequently observed (Fig. 9).

Few embryos differentiated shoots on the hypocotyl or radicle (Fig. 11). Some buds produced elongated needles but had poorly developed apical meristematic growth (Fig. 10). These buds did not yield harvestable shoots until the end of the experiment. Unsynchronized growth of buds was common as well as a high degree of variability in the ability of buds to grow into shoots (Fig. 12).





Legend for Figs. 1 to 12 : Description in parentheses is the species and the medium on which plant(s) in photograph was (were) cultured during induction period. Almost all the photos were taken at the time of the second and third subculture.

Fig. 1. Embryo covered with callus (CL) except one cotyledon (C). (Pitch pine ; MLM medium with 1 mg/l BAP and 0.1 mg/l NAA).

Fig. 2. Embryo showing adventitious shoots (S). Calli (C) are developing from the base of cotyledons. (Pitch pine ; MLM medium with 2 mg/l BAP and 0.01 mg/l NAA).

Fig. 3. Seedling-like plantlet developed from embryo without the formation of adventitious shoots. (Loblolly pine ; MMS medium with 0.5 mg/l BAP and 0.01 mg/l NAA).

Fig. 4. Dense cluster of adventitious shoots (arrows). Stem (ST) is grown from the apical meristem of embryo. No root development from radicle. (Pitch pine ; MMS medium with 2 mg/l BAP and 0.01 mg/l NAA).

Fig. 5. Seedling-like plantlet development and adventitious shoots (arrows). ST ; stem ; R ; root. (Loblolly pine ; MMS medium with 1 mg/l BAP and 0.01 mg/l NAA).

Fig. 6. Embryo showing a rosette of buds and several shoots (S). Counting of buds is unreliable in this case. (Pitch pine ; MLM medium with 5 mg/l BAP and 0.1 mg/l NAA).

Fig. 7. Adventitious shoots developing from cotyledons. No growth of apical meristem. (Loblolly pine ; MLM medium with 1 mg/l BAP and 0.01 mg/l NAA).

Fig. 8. A cluster of adventitious shoots from a cotyledon. No further growth of apical apex of embryo (arrow). (Hybrid pine ; MLM medium with 5 mg/l BAP and 0.1 mg/l NAA).

Fig. 9. Shoots (SA) at cotyledon axils and shoots (S) at cotyledon tips. (Pitch pine ; MLM medium with 0.1 mg/l BAP and 0.1 mg/l NAA).

Fig. 10. Elongated needles (N) from a shoot bud. This bud never developed into shoot. Small buds (M) are also shown. (Hybrid pine ; MLM medium 0.1 mg/l BAP and 0.01 mg/l NAA).

Fig. 11. Adventitious shoots(SH) formed on hypocotyl. Root (R) growth is shown without developing stem. (Pitch pine ; MMS medium with 2 mg/l BAP and 0.01 mg/l NAA).

Fig. 12. Unsynchoronized development of adventitious shoots. Note the difference in height of shoots (arrows). (Pitch pine ; MMS medium with 2 mg/l BAP and 0.01 mg/l NAA).

At the time of the second and the third subcultures shoots greater than 5mm were dissected from the embryo and put on half-strength meida for elongation. These shoots had primary needles longer than the shoots themselves. Isolated, separable buds were also dissected from the embryo and transferred to half-strength medium. Many transferred buds and shoots had developed into normal vigorous shoots at the end of the culture period. Shoots less than 10 mm were discarded at the time of the fourth subculture. Small buds that remained on the embryos after the shoots were removed grew into shoots or shoot-like structures during the subculture period.

They were dissectd and counted as shoots when fully recognizable (≧ 10 mm).

An attempt was made to count the number of buds. However, the counts were unreliable primarily because some embryos produced a cluster of buds rather than individual, separable buds. Embryos forming buds were counted and the ratio of bud-forming embryos to total embryos were calculated for each replication by the formula, ratio=No. of buds forming embryos/(No. of planted embryos − No. of contaminated embryos).

The number of shoots longer than 10 mm and usable for root initiation were counted at the fourth subculture, i.e., 12 to 13 weeks after induction. The ratio of shoot-producing embryos to total embryos was calculated per replication by the formula, ratio=No. of shoots producing embryos/(No. of planted embryos − No. of contaminated embryos).

Average number of shoots per responding embryo was calculated and compared among treatments.

Loblolly pine

Comparison of means is shown in Table 2. MGD medium showed the lowest performance in all three traits investigated ($\alpha=0.05$). Other media, i.e., MMS, MLM, and MSH, did not appear to be different from one another in effect for all traits, i.e., bud-formation ratio, shoot-formation ratio, and number of shoots per responding embryo. NAA at 0.1 mg/l showed a higher bud-forming ratio than 0.01 mg/l. However, no difference was found in shoot production traits between NAA concentrations ($\alpha=0.05$). The ratio of bud-forming and shoot-producing embryos was not affected by BAP concentration. The highest concentration (5 mg/l) of BAP, however, produced significantly more shoots per embryo than the lower three (0.1, 0.5, and 1 mg/l) ($\alpha=0.05$).

Table 2. Comparison of means for bud-forming ability, shoot-producing ability, and number of shoots per embryo for embryonic tissues of *Pinus taeda*.

	Ratio of bud-forming embryos(%)	Ratio of shoot-producing embryos(%)	Number of shoots per embryo
Medium			
MGD	44.45a*	23.39a	2.22a
MMS	66.50b	62.00b	4.72b
MLM	74.95b	60.67b	4.00b
MSH	65.00b	52.78b	5.08b
NAA(mg/l)			
0.1	66.42a	51.89a	4.02a
0.01	59.03b	47.53a	3.98a
BAP(mg/l)			
0.1	57.85a	49.45a	2.35a
0.5	60.56a	47.29a	3.75ab
1	68.20a	50.42a	4.60ab
2	64.65a	48.61a	4.60bc
5	62.36a	52.78a	5.57c

* The same letters are not different at 5% significance level by the analysis of Tukey's test.

Pitch pine

Means were compared for each factor, i.e., medium, NAA level, and BAP level by Tukey's method (Table 3). MLM and MSH medium were significantly higher than MGD medium in the ratios of bud-forming and shoot-producing embryos. However, no difference was found among three media in the number of shoots per responding embryo ($\alpha=0.05$). The difference

Table 3. Comparison of means for bud-forming ability, shoot-producing ability, and number of shoots per embryo for embryonic tissues of *Pinus rigida*.

	Ratio of bud-forming embryos(%)	Ratio of shoot-producing embryos(%)	Number of shoots per embryo
Medium			
MGD	57.06a*	38.61a	3.05a
MMS	68.47ab	62.02b	5.66b
MLM	78.29bc	53.38b	4.25ab
MSH	81.38c	63.38b	3.44a
NAA(mg/l)			
0.1	75.37a	59.40a	4.29a
0.01	67.23b	49.30b	3.91a
BAP(mg/l)			
0.1	68.06a	48.28a	3.01a
0.5	70.96a	54.68a	3.05a
1	74.99a	59.89a	3.19a
2	70.84a	61.11a	5.55b
5	71.65a	55.27a	5.71b

* The same letters are not different at 5% significance level by the analysis of Tukey's test.

between MMS and MLM was not significant for any trait. A concentration of 0.1 mg/l of NAA increased the ratios of bud-forming and shoot-producing embryos significantly compared to 0.01 mg/l. A concentration of 0.1 mg/l was as effective as 0.01 mg/l in the number of shoots per embryo. All concentrations of BAP were equally effective in the ratio of bud formation, but more shoots could be producted at the two higher concentrations than at the three lower concentrations.

Hybrid pine

The means were compared for each factor using Tukey's method (Table 4). While no difference was detected among media in the number of shoots per responding embryo ($\alpha=0.05$), MMS and MLM media had significantly higher ratios of bud-forming and shoot producing embryos than MSH medium. MSH medium showed the lowest ratio for shoot-producing embryos. NAA showed the significantly higher ratio in bud-forming embryos and shoot-producing embryos at 0.1 mg/l. A concentration of 0.1 mg/l BAP appeared to be less effective for all traits than any other BAP concentration ($\alpha=0.05$). No differences were found among the three higher BAP concentrations in bud formation and shoot production ratios ($\alpha=0.05$).

Table 4. Comparison of means for bud forming ability, shoot-producing ability, and number of shoots per embryo for embryonic tissues of *Pinus taeda* x *rigida*.

	Ratio of bud-forming embryos(%)	Ratio of shoot producing embryos(%)	Number of shoots per embryo
Medium			
MGD	57.44a*	55.99a	2.77a
MMS	78.50b	66.00a	3.13a
MLM	86.50c	83.89a	3.74a
MSH	55.39a	27.22b	3.07a
NAA(mg/l)			
0.1	75.47a	59.19a	3.30a
0.01	63.44b	47.36b	3.05a
BAP(mg/l)			
0.1	53.19a	38.13a	1.89a
0.5	69.38ab	48.75ab	3.15b
1	75.63b	55.06b	3.36b
2	72.78b	62.63b	3.65b
5	76.32b	61.81b	3.83b

* The same letters are not different at 5% significance level by the analysis of Tukey's test.

DISCUSSION

Adventitious shoots were produced mainly on the cotyledons and a very low number of adventitious shoots could be obtained in the regions of hypocotyl. Axillary shoots were frequently seen between the cotyledons. The ratio of bud-forming and shoot-producing embryos varied from species to species (Table 2, 3, and 4). The hybrid pines were found to show tendency towards neither of their parents in the three traits investigated.

Loblolly pine embryos appeared to be less capable of the formation of adventitious buds than either pitch or hybrid pine embryos. Hybrid pine embryos were more capable of adventitious shoot production than loblolly or pitch pine. However, each responding embryo of hybrid pine produced lower numbers of shoots than either loblolly or pitch pine embryos. As only shoots as tall as 10 mm were counted for calculating the frequency of shoot-producing embryos and the number of shoots per embryo, results may differ if shoots less than 10 mm were included. 10 mm is a reasonable height considering the results of rooting of adventitious shoots via hormonal stimulation in vitro or direct rooting trials on a perlite soil in a moist box as reported.[1,3,18,26]

Media

While NAA and BAP showed a reliable, predictable trend in bud formation and shoot production dependent upon their concentrations, the basal medium effect was not as predictable. Bud formation or shoot production observed for one species on a given basal medium did not always predict the response of another species. It is an obvious conclusion that the medium showing the highest adventitious bud formation frequency in a given species is not necessarily of the same

rank in a test of other species. MGD medium showed the lowest adventitious bud formation and shoot production ratio in loblolly pine embryo cultures (Table 2). In hybrid pine MSH medium was the lowest in both traits (Table 4). Even in the same species, the capacity for bud formation on one medium does not always reflect the capacity for shoot production. In the culture of pitch pine embryos, MMS medium showed much lower bud formation frequency (68%) than MLM (78%) and MSH medium (81%) (Table 3). However, for the ratio of shoot producing embryos MMS medium (62%) is similar to MSH medium (63%) and higher than MLM medium (53%). In the number of shoots per responding embryo MMS medium showed a higher value (5.7) than MLM (4.3) or MSH medium (3.4). In MLM and MSH medium 14% and 12% of the embryos, even though they formed buds, could not develop shoots, respectively (Table 2). In MMS medium only 5% of the embryos could not form shoots after they established buds. In contrast, in the hybrid pine 13% of embryos on MMS medium lost adventitious shoot producing ability even after bud formaition, and half of the bud forming embryos on MSH did (Table 4). Only 3% of the embryos on MLM medium lost shoot producing capacity after developing adventitious buds.

From these results, it is inferred that, although a certain medium caused adventitious bud formation more easily from embryonic tissues than any other medium, it could not differentiate all these buds into viable shoots. For a more successful mass-propagation, different media need to be considered for the induction of adventitious buds and for their development into shoots.

Following analyses of data for the three traits examined, we may consider MMS, MLM, and MSH media but not MGD medium as potential mass-propagation media for embryonic tissue cultures of loblolly and pitch pine. For hybrid pine embryonic tissues MMS or MLM media may be preferred to MGD and MSH media. It is thus not possible to draw a generality from these phonomena for embryo cultures for application to other pine species. MMS and MLM media generally showed favorable results in all traits investigated, but MGD and MSH media showed low capacity to support one of the traits with at least one of the species tested.

Flinn et al.[11] reported that high concentration of NH_4^+ in MS medium might inhibit shoot formation in eastern white pine embryo cultures. Pérez-Bermúdez and Sommer[23] also reported the high concentration of nitrogen (due to NH_4^+) in MS medium reduced the caulogenic capacity of *P. elliottii* embryos compared to the medium of Risser and White (RW)[33]. However, MMS medium used in this study did not contain NH_4NO_3[6], and thus was free of NH_4^+ as a nitrogen source. Contrary to the results of *P. elliottii*[29], MS medium could not promote adventitious bud initiation on *P. pinaster* embryos compared to RW medium.[31] SH medium showed a higher ratio of adventitious bud-formation in *P. brutia* embryos than GD and MS medium.[2] It was suggested that the high concentration of K^+ and Ca^+ in SH medium caused this result. However, in *P. taeda* x *rigida* MSH medium was not as effective as MMS and MGD medium. It is, possibly, not reasonable to compare the results of MMS medium with those of MS medium of those workers, because MMS medium composition is different from that of MS medium in *P. brutia*. However, MGD is basically the same as GD medium of those workers[2]. Therefore, this discrepancy may be attributable to the genetic and/or physiological differences among species. One half strength MS medium promoted more adventitious bud formation on *P. nigra* embryos than GD, MS, and SH media.[17,20] There is another report that there was no difference among SH, 1/2x SH, and 1/2x MS media in adventitious shoot forming ability of *P. strobus* embryos, but that MS medium was not as effective as any of the media mentioned.[11]

The high variation of MGD and MSH media in adventitious shoot production among species may be caused by the presence of Na^+ or high

concentration of vitamins, respectively. MGD is the only one that contains Na^+. MSH medium included 5 times more vitamins than all the other media. In microelements, concentrations of Zn^+ and BO_3^- are lower in both media than in MMS and MLM media. Zinc has a close relationship with auxin content of plants[35] and is a component of an enzyme for the synthesis of tryptophan (IAA precursor)[38]. Boron deficiency results in low cytokinin synthesis[21], proliferation of cells with abnormal cell organelles in *P. contorta* suspension cultures[39], and low sucrose uptake[13]. However, in *P. strobus* embryos microelements and vitamins were not critical in stimulating or limiting adventitious bud formation.[11]

NAA

NAA might be thought to be more capable of bud formation and shoot production at 0.1 than 0.01 mg/l in all three species. Media with 0.1 mg/l NAA produced higher number of shoots per embryo than those with 0.01 mg/l, although the difference was not significant. In comparison, 0.1 mg/l NAA concentration always showed significantly higher frequency than 0.01 mg/l in one or both of the ratios of bud forming and shoot producing embryos.

Patel *et al.*[27] induced adventitious shoots from pitch pine embryos on media containing BAP, kiretin(KN), or 2-isopentyl adenine (2iP) without auxin. Kim *et al.*[18] also used no auxin for adventitious shoot bud induction or *P. rigida* x *taeda* embryos. In loblolly pine embryo cultures, 0.01mg/l NAA with 10 mg/l BAP was used for adventitious shoot bud induction.[24] Excess proliferation of callus from Calabrian pine embryos was promoted by auxin.[2] Reduction of adventitious bud formation was reported from longleaf pine embryos by NAA.[29] NAA could promote bud elongation at lower BAP concentrations, but did not at the highest BAP concentration.

BAP

In loblolly and pitch pine embryos, BAP concentration, ranging from 0.1 to 5 mg/l, did not appear to be critical in obtaing adventitius buds and shoots. However, the number of shoots per responding embryo was significantly higher at 5 mg/l BAP than at 1, 0.5, and 0.1 mg/l. There was no difference in this trait between 2 and 5 mg/l. In hybrid pine embryos, all levels of BAP concentration except 0.1 mg/l seemed to be equally effective in all three responses investigated.

As CK's other than BAP were not tested in this experiment, it is not possible to compare directly the capacity for adventitious shoot production among CK's. However, others have found that in loblolly pine BAP and zeatin could induce more adventitious buds from embryonic cotyledons than KN, although no difference was recognized in the results of two former CK's.[23] For pitch pine embryos more adventitious buds were induced by KN and BAP than 2iP.[27] In *P. ponderosa* BAP was more effective in bud formation frequency than 2iP.[10]

BAP concentration has ranged from 0.02 to 12 mg/l in much mass-propagation research on pine embryonic tissues. However, the concentration must be considered together with the induction period length. For lodgepole and pitch pine embryos 3 weeks was optimal for advantitious shoot production at 10^{-5} M (ca 2.25mg/l).[27,28] A similar period was optimal for radiata pine embryos at the BAP concentrations of 2.5×10^{-5} M (ca 5.63 mg/l).[5] In slash pine embryos it was reported that 30 day induction on liquid medium was too long and 5 day was too short for bud formation at the BAP concentrations of 2.2 x to 22.2×10^{-6} M(ca 0.5 to 5.0 mg/l).[29] If we consider BAP concentrations and induction period, the period, 23 to 25 days, is efficient enough to produce a higher numbers of adventitious shoots from the embryos of loblolly pine, pitch pine, and their hybrid at the concentrations of 0.5 to 5 mg/l. However, the higher the concentration, the higher the number of shoots that can be obtained.

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