Factors Influencing Haploid Callus Initiation and Proliferation in Megagametophyte Cultures of Sitka Spruce (Picea sitchensis)[1])

By S. BALDURSSON[2]), J. V. NØRGAARD and P. KROGSTRUP

Botanic Garden, University of Copenhagen,
Ö. Farimagsgade 2B, DK-1353 Copenhagen K, Denmark

(Received March 1992)

Abstract

Haploid and diploid cell lines were obtained from *in vitro* cultured megagametophytes of *Picea sitchensis* (BONG.) CARR. during 2 consecutive years. The best response was obtained on a modified MS medium with a combination of cytokinins (5μM 6-benzylamino purine and kinetin) and auxin (10 μM 2,4-dichlorophenoxyacetic acid). Haploid callus proliferation depended strongly upon the genotype of the mother tree. The timing of explant collection was also a critical factor, but varied between the 2 years. In both years, haploid calli were only obtained from explants collected during a 2 to 3 week period following fertilization in open pollinated gametophytes, although fertilization per se was not a prerequisite for response. In developmental terms, the period for haploid callus induction coincided with the formation of corrosion cavity in the megagametophytes. The responsive period was furthermore defined in terms of accumulated degree-days. In one megagametophyte culture, initial stages of direct embryogenesis were observed, but all attempts to induce morphogenesis in subcultured haploid calli have failed so far.

Key words: Picea sitchensis, conifers, megagametophyte culture; haploid callus.

Introduction

Although temperate conifers can be inbred to a certain degree by conventional means, their long life cycles and high genetic loads (see SEDGLEY and GRIFFIN, 1989), make this method for production of pure lines unacceptably slow. Production of doubled-haploid plants *in vitro* has therefore long been considered a potentially attractive short cut to homozygocity in these organisms. Haploids could furthermore be a valuable material for genetic studies of conifers (BONGA et al., 1988).

Experimental androgenesis in gymnosperms has been of limited success, whereas the multicellular haploid megagametophyte has proven more responsive *in vitro* (reviews: ROHR, 1987; BONGA et al., 1988). Haploid cell lines, exhibiting different degrees of morphogenesis, have been produced in megagametophyte cultures of *Pinus spp.* (BONGA, 1974, 1981), *Picea abies* (HUHTINEN et al., 1981; SIMOLA and HONKANEN, 1983), and *P. glauca* (BONGA, 1981). Plants of *Sequoia sempervirens*, regenerated from megagametophytes, grew normally in the field, but root smears from these plants gave exclusively diploid chromosome counts and an embryo-origin could not be ruled out entirely (BALL, 1987). Lately, embryogenesis has been reported from megagametophyte cell lines of *Larix spp.* (NAGMANI and BONGA, 1985; VON ADERKAS et al., 1990), resulting in haploid plantlets surviving a few weeks in soil, and SIMOLA and SANTANEN (1990) have described early stages of embryogenesis in 4 year old megagametophyte callus of *Picea abies.*

The developmental stage of the female gametophyte is important for the induction of haploid cultures, although the optimal stage may differ between species. In *Zamia integrifolia* (*Z. floridana*) (LARUE, 1984; NORSTOG, 1965), and *Ephedra foliata* (BHATNAGAR and SINGH, 1984), megagametophytes excised prior to fertilization were able to differentiate organs. In *Larix decidua*, a 4 week responsive period coincides with the onset of fertilization and the subsequent formation of corrosion cavity in the gametophyte (VON ADERKAS and BONGA, 1988). In *Picea abies*, gametophytes removed about 1 month after fertilization were successfully used for callus induction (HUHTINEN et al., 1981; SIMOLA and HONKANEN, 1983).

The objective of the present study was to produce haploid or doubled-haploid plants of sitka spruce. In this paper we describe different types of response obtained from megagametophyte tissue *in vitro*, and some important factors for the induction and proliferation of haploid calli in this species.

Materials and Methods

1. Plant material

Open-pollinated female cones were collected every 2 weeks in 1990, in the period from June 11 to August 6, from four different clones of sitka spruce (V2706, V2710, V2718, V27221) at the Arboretum in Hørsholm, Denmark. An additional collection of mature cones was made on October 10. In 1991, collections were made from clones V2710 and V2718 (the remaining 2 clones did not flower) at about 10 day intervals from May 25 to July 31. Furthermore, in 1991, 10 cones on 1 tree of clone V2710 were bagged before anthesis and other 10 cones were mentor-pollinated with *Picea abies* pollen, generally considered incompatible with sitka spruce (Professor HANS ROULUND, The Royal Veterinary and Agricultural University, personal communication).

Cones were kept in plastic bags at 4° C to 5° C for a maximum of 4 days prior to *in vitro* culture. Entire cones (early collections) or immature ovules were surface sterilized in 7% calcium hypochlorite, or commercial bleach (4.5% sodium hypochlorite) with a drop of Tween 20, for 15 min, followed by 2 min treatment in 70% ethanol and rinsing 3 times in sterile, double-distilled water. Megagametophytes excised from ovules from the central part of each cone were used. All megagametophytes in 1990, and the open pollinated gametophytes collected after June 13 in 1991, were cut transversely in half, embryo fragments were removed, and the distal (chalazal) part cultured with the cut surface in contact with the medium. In 1991, open-pollinated megagametophytes collected before

[1]) Presented at the Workshop of the IUFRO Working Party S2.04-07 — Somatic Cell Genetics — on "Trends in the Biotechnology of Woody Plants", in Dehra Dun, India, 25 to 29 November, 1991.

[2]) Present address: Iceland Forest Research Station, Mógilsá, IS-270 Mosfellsbaer, Iceland

June 13 (prearchegonial) and all mentor-pollinated megagametophytes were cultured intact.

2. Culture procedure

Callus induction

In 1990, 2 basal culture media were tested: half strength MS minerals (MURASHIGE and SKOOG, 1962) and full strength MS vitamins with thiamin HCl increased to 2 μM (hereafter called 1/2 MS), and a modified MS medium (BMI-S1) (KROGSTRUP, 1986), both supplemented with a total of 1000 mg/L casein hydrolysate (Sigma, enzymatic hydrolysate), 500 mg/L glutamine, 3% sucrose, and 0.18% Gelrite (Kelco). Three combinations of growth regulators were added to both media: cytokinins only (5 μM 6-benzylamino purine, BAP, plus 5 μM 6-furfurylaminopurine, KIN), auxin only (10 μM 2,4-dichlorophenoxyacetic acid, 2,4-D), and both cytokinins and auxin (5 μM BAP, 5 μM KIN and 10 μM 2,4-D). The pH was ajusted to 5.7 with 0.1 M KOH and 0.1 N HCl before autoclaving at 121° C for 20 min. After 2 to 3 days in culture, the explants were moved to a fresh part of the medium and cultured without further subcultivation in the dark at 24° C for a minimum of 4 weeks, or until callus growth was initiated.

In 1991, the BMI-S1 media with 5 μM BAP, 5 μM KIN and 10 μM 2,4-D (hereafter called standard BMI-S1) was compared with a modified N6 medium, used successfully in *Picea abies* megagametophyte cultures (SIMOLA and HONKANEN, 1983: medium a).

Callus maintenance

After the initial induction period, calli were subcultured every 4 to 5 weeks, the first 2 to 3 times on the induction medium, but subsequently on the standard BMI-S1. The cultures were kept in a growth chamber at 24± 1° C with 16-hr photoperiod.

Experiments to induce organogenesis

Vigorously growing haploid callus lines (10 and 6 for 1990 and 1991, respectively, were selected for experiments to induce organogenesis, in which cytokinin/auxin ratios in the BMI-S1 medium were altered (0-60 μM BAP and KIN in combination with 0-2 μM 2,4-D and naphthaleneacetic acid, NAA). Different gelling agents were also tested, as well as the addition of coconut milk (10%) in combination with different concentrations of BAP and NAA.

3. Developmental stage of megagametophytes

In 1991, several morphological features of the female cone and megagametophyte development were noted: length of cone, length and width of bracts, attachment of ovuliferous scales to bracts, length of isolated gametophytes, colour and texture as well as the presence or absence of archegonia and corrosion cavity in the gametophyte. In addition, a few gametophytes (inclusive nucellar tissue for the first 3 collections) vere fixed in 3% glutaraldehyde, dehydrated and embedded in glycol methacrylate (FEDER and O'BRIEN, 1968) for microtome sectioning and histological analysis. Sections were stained in Toluidine blue.

4. Chromosome counts

Actively growing callus was fixed in 3:1 alcohol, glacial acetic acid and stored at 4 °C. Callus tissue was hydrolyzed in 1 N HCl at 60 °C for 5 to 20 min, depending on the type of callus, and then squashed in aceto-orcein. Ploidy levels were determined from at least 3 metaphase plates for every cell line classified.

5. Accumulated degree-days

Climatic records for 1990 and 1991 from the meteorological station at Sjaelsmark (within 5 km from the Arboretum) were provided by Danish Meteorological Institute. From these records, accumulated degree-days (VON ADERKAS and BONGA, 1988) from February, March, or April, until the responsive collection dates, were calculated for days with mean temperatures above 4° C, 5° C or 6° C.

Results

1. Callus induction and chromosome numbers of callus lines

An initial cell proliferation of gametophytic cells was observed within three weeks of culture in 21% and 14% of the explants on the average for 1990 and 1991 (*Table 1*). This we have termed induction, following VON ADERKAS *et* al. (1987). In some instances only a few cells were involved, whereas in other cases the whole gametophyte proliferated. Most often, however, these initial cell divisions stopped after an additional 1 to 2 weeks and the gametophyte tissue started to die. In 1990 and 1991 a few embryogenic cell lines (11 and 2, respectively) proliferated from the corrosion cavity of the gametophytes within 3 to 4 weeks of culture. These embryogenic lines were easily separable from the gametophyte and may have originated from embryo fragments. In one case we observed gametophytic embryogenesis, i. e. an early stage embryo with a direct connection to the gametophyte (*Fig. 1a*), but this culture was not subculturable.

True calli (i. e. non-embryogenic calli that could be isolated and subcultured at least once) proliferated from 3.3% and 2.4% of the explants on the average for 1990

Table 1. — Number of megagametophytes of four sitka spruce clones exhibiting different responses *in vitro* for 2 consecutive years (1990/1991), and ploidy levels of the cell lines obtained. Clones V2706 and V2721 did not flower in 1991.

Clone	Cultured gametophytes	Type of response			Ploidy of cell lines			
		Induced	Embryogenic cultures	Non-embryogenic calli	Haploid	Diploid	Mixoploid	Unknown
V2706	708	117	4	20	2	11	1	10
V2710	816/ 753	236/ 160	2/ 2	39/ 25	17/ 15	8/ 5	2/ 0	14/ 5
V2718	774/ 324	126/ 76	3/ 0	21/ 1	0/ 0	15/ 1	0/ 0	9/ 0
V2721	732	141	2	23	12	7	0	6

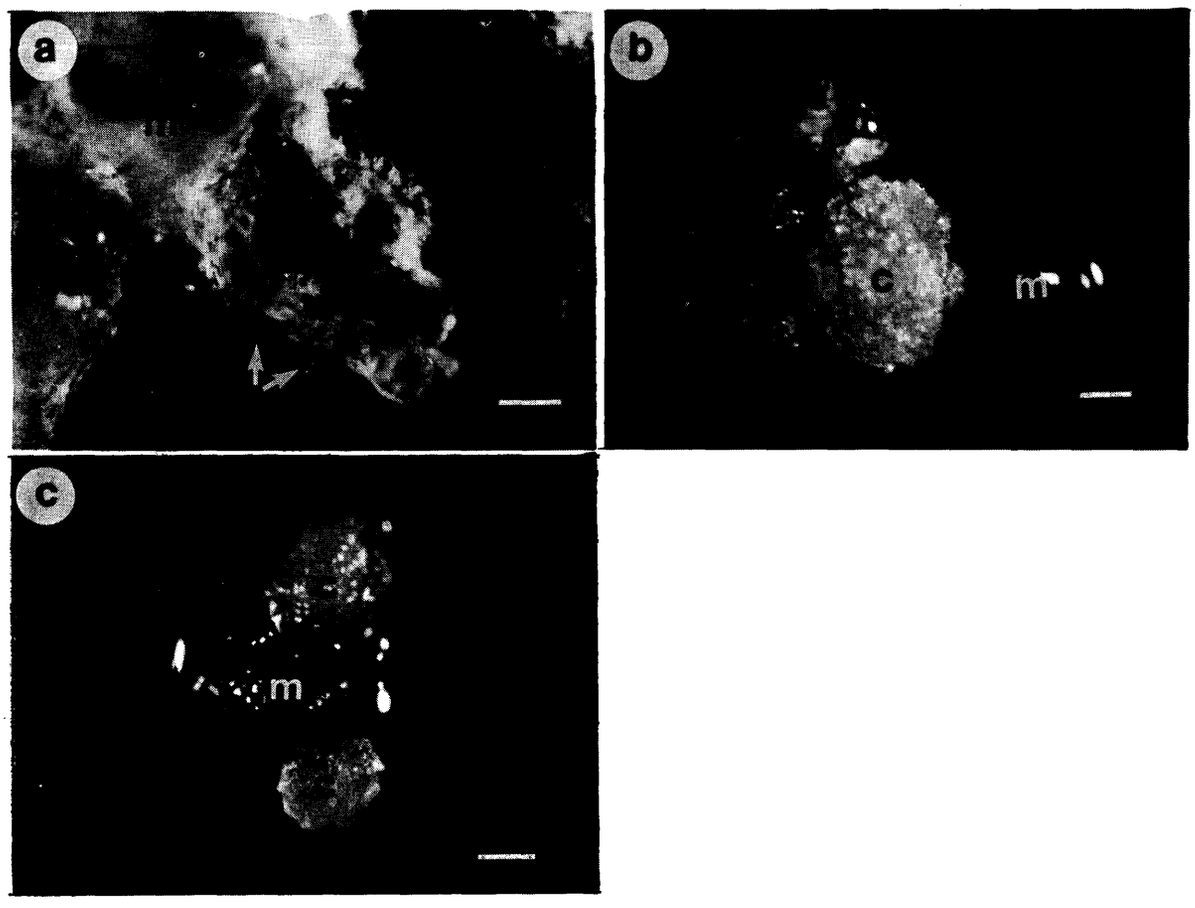

Figure 1. — Different responses in megagametophyte culture of *Picea sitchensis* (Bong.) Carr. in 1990. A) Embryogenic tissue proliferating directly from the megagametophyte (m). Arrows point to an early stage gametophytic embryo. Bar = 2 mm. B) "Fast" callus type proliferating from the mid section of a young megagametophyte (m). Bar = 5 mm. C) "Slow" callus type (c) emerging through the tip (chalazal end) of a megagametophyte (m). Bar = 5 mm.

and 1991 (*Table 1*). These calli could be divided into 2 categories: "fast", proliferating within 3 to 4 weeks of culture from the mid section (corrosion cavity) of the gametophyte (*Fig. 1b*), and "slow", emerging through the top (chalazal end) of the necrotized gametophyte after 6 to 16 weeks (*Fig. 1c*).

Ploidy estimates for the cell lines are summarized in *table 1*. A good separation of the chromosomes could not always be obtained for the putative diploid lines, resulting in chromosome numbers one or two fewer than 24 (the diploid chromosome number of sitka spruce). The low counts were assumed to result from overlap of chromosomes and the cells were consequently classified as diploid. The embryogenic cell lines in both years were all diploid. The ploidy levels of approximately one third of the non-embryogenic calli produced in 1990 and 1991 could not be determined due to slow growth and early browning in culture. The remaining calli were equally divided between haploid (*Fig. 2*) and diploid in 1990, whereas in 1991, 75% of the calli were haploid (*Table 1*). In general the "fast" calli tended to be diploid and the "slow" haploid. In 1990 3 mixoploid cell lines were found containing both haploid and diploid cells.

The haploid callus lines usually turned green when transferred to light. They were soft and friable or nodular, and composed mostly of unorganized masses of parenchymatous cells when examined under the light microscope. Some of the haploid lines grew slowly and died after only a few subcultures, whereas others were quite vigorous, increasing their weight several times between subcultures. Formation of adventitious buds or organs was never observed on the standard BMI-S1 medium.

Figure 2. — An aceto-orcein squash of a megagametophyte callus showing haploid number of chromosomes (n = 12). Bar = 2 μm.

Table 2. — Number of induced sitka spruce megagametophytes and different ploidy levels of cell lines by collection dates in 1990 and 1991. The numbers for 1990 represent all 4 clones and the 4 auxin supplemented media.. The numbers for 1991 represent clone V2710 and V2718 on 2 media. Percentages within brackets.

Collection date	Cultured gametophytes	Induced	Haploid calli	Diploid cell lines	Ploidy unknown	Total cell lines
1990						
June 11	444	230 (52.0)	14 (3.1)	10 (2.3)	12 (2.5)	36 (8.1)
June 25	468	97 (20.7)	15 (3.2)	4 (0.4)	8 (1.5)	27 (5.8)
July 9	354	77 (21.7)	2 (0.6)	0	5 (1.4)	7 (2.2)
July 23	336	40 (11.9)	0	7 (2.1)	5 (1.5)	12 (3.6)
August 6	354	48 (13.6)	0	15 (4.2)	7 (2.0)	22 (6.2)
October 10	300	118 (39.3)	0	5 (1.7)	5 (1.7)	10 (3.3)
1991						
May 23	18	-	-	-	-	-
June 3	92	0	0	0	0	0
June 13	224	6 (2.7)	0	0	0	0
June 25	172	21 (12.5)	3 (1.7)	0	0	3 (1.7)
July 4	224	63 (28.1)	9 (4.0)	3 (1.3)	2 (0.9)	14 (6.2)
July 15	134	35 (26.1)	3 (2.2)	1 (0.7)	3 (2.2)	7 (5.2)
July 31	112	6 (5.3)	0	1 (0.9)	0	1 (0.9)

2. Effect of genotype of mother tree

In 1990, megagametophytes from clones V2710 and V2721 produced the majority of haploid cell lines, and in 1991, all the haploid calli were induced in gametophytes from clone V2710 (*Table 1*). In terms of induction and total callus (inclusive diploid and unknown) the effect of the genotype of the mother tree was not very clear, although gametophytes from clone V2710 showed the highest response in both years (*Table 1*).

3. Effect of collection date

In 1990, gametophytes collected on June 11 (the first collection date), showed the highest overall response *in vitro* (*Table 2*). Induction frequencies declined until the first week in August but increased again in the October control collection. The date of collection was critical for haploid callus production. Over 90% of the haploid calli in 1990 were obtained from explants collected in June and no haploid calli were obtained from explants collected after July 9. Due to genotype differences, the responsive period for individual trees was further reduced to 10 to 14 days. Diploid cell lines and calli with unknown ploidy were found throughout the collection period in 1990.

The same picture emerged in 1991 (*Table 2*). The first response was noted on June 13 with a peak of induction as well as callus production on July 4. Haploid calli were obtained from explants collected over a three week period from the last week in June until mid-July. On June 25, however, only mentor-pollinated gametophytes produced

Table 3. — Accumulated degree-days for collection dates in 1990 and 1991 when sitka spruce megagametophytes were most responsive for haploid callus induction. Degree-days are calculated from the 1st of February, March or April, for days with mean temperatures of 4° C, 5° C, or above.

	February 1			March 1			April 1		
Collection date	4°C	5 °C	6°C	4 °C	5 °C	6°C	4°C	5°C	6°C
June 11, 1990	1033	997	922	896	860	823	740	721	710
June 25, 1990	1243	1206	1132	1106	1070	1032	950	931	920
July 4, 1991	938	912	838	938	905	831	855	841	804
July 15, 1991	1163	1128	1053	1153	1121	1047	1071	1057	1020

Table 4. — Number of sitka spruce megagametophytes in 1990 exhibiting different responses on 1/2 MS and BMI-S1 media with three combinations of growth regulators. Combined data from clones V2710 and V2721 and the three most productive collections in terms of haploid callus induction (June 11, June 25, July 9). Percentages within brackets.

Growth regulators (µM)	Media	Cultured gametophytes	Induced	Haploid calli	Total cell lines
5 BAP, 5 KIN	1/2MS	162	11 (7.1)	0	0
	BMI-S1	168	9 (5.6)	0	0
10 2,4-D	1/2MS	168	82 (48.8)	5 (3.0)	8 (4.8)
	BMI-S1	168	69 (41.1)	4 (2.4)	8 (4.8)
5 BAP, 5 KIN, 10 2,4-D	1/2 MS	156	40 (25.6)	9 (5.4)	16 (10.3)
	BMI-S1	168	59 (35.2)	11 (6.5)	15 (8.9)

Table 5. — Number of sitka spruce megagametophytes (clone V2710) on 2 media both supplemented with 10 µM 2,4-D, 5µM BAP and 5 µM KIN, showing different responses *in vitro*. Data from the 3 most responsive collections in 1991 (June 25, July 4, July 15). Percentages within brackets.

Media	Cultured gametopytes	Induced	Haploid calli	Total cell lines
BMI-S1	207	85 (40.9)	12 (5.7)	21 (10.1)
N6[1)]	114	46 (35.8)	3 (2.2)	4 (3.5)

[1)] as modified by Simola and Honkanen (1983)

calli (see below). The response in 1991 was lower as well as delayed compared to 1990. Direct comparison between years is not possible because different media and clones were used, but comparing clone V2710 on the BMI-S1 standard medium revealed that haploid callus induction was also lower in 1991.

Table 3 shows accumulated degree-days, calculated in various ways, for the best collection dates for haploid callus induction in 1990 and 1991. The best fit between years was obtained when degree-days were calculated from March 1 for days with mean temperatures above 6° C. Using this criterion the megagametophytes were most responsive over a period from 800 to 1059 degree-days, corresponding approximately to 2 weeks.

4. Effect of media and growth regulators

Average induction rate of gametophytes cultured on media with cytokinins as the only growth regulator was 6.1%, but no calli were produced (*Table 4*). The highest induction rate (44.9%) was obtained on media supplemented solely with 2,4-D. Average haploid, and total callus production was 2.7% and 4.8% on these media. Media containing both cytokinins and auxins were intermediary in terms of induction (30.6%) but sustained the highest callus proliferation, 5.9% and 9.9%, averaged for haploid and total callus.

The 2 media, 1/2 MS and BMI-S1, tested in 1990 did not differ in terms of average induction rates (27.4% and 27.2%) or haploid callus production (4.3% and 4.5%) (*Table 4*). Induction frequencies were also similar on BMI-S1 and the modified N6 medium in 1991, but haploid as well as total callus production was considerably higher on the BMI-S1 standard medium (*Table 5*).

Table 6. — Number of open- versus mentor-pollinated (Norway spruce) gametophytes of sitka spruce (clone V2010), from four collection dates in 1991, exhibiting different responses *in vitro*. Percentages within brackets.

Collection date	Pollination method	Cultured gametophytes	Induced	Haploid calli	Total cell lines
June 13	Open	65	12 (18.4)	0	0
	Mentor	50	5 (10.0)	0	0
June 25	Open	60	14 (23.3)	0	0
	Mentor	52	22 (42.3)	2 (3.8)	3 (5.7)
July 4	Open	50	20 (40.0)	4 (8.0)	7 (14.0)
	Mentor	50	28 (55.6)	5 (10.0)	6 (12.0)
July 15	Open	65	33 (50.8)	4 (6.1)	7 (10.8)
	Mentor	-	-	-	

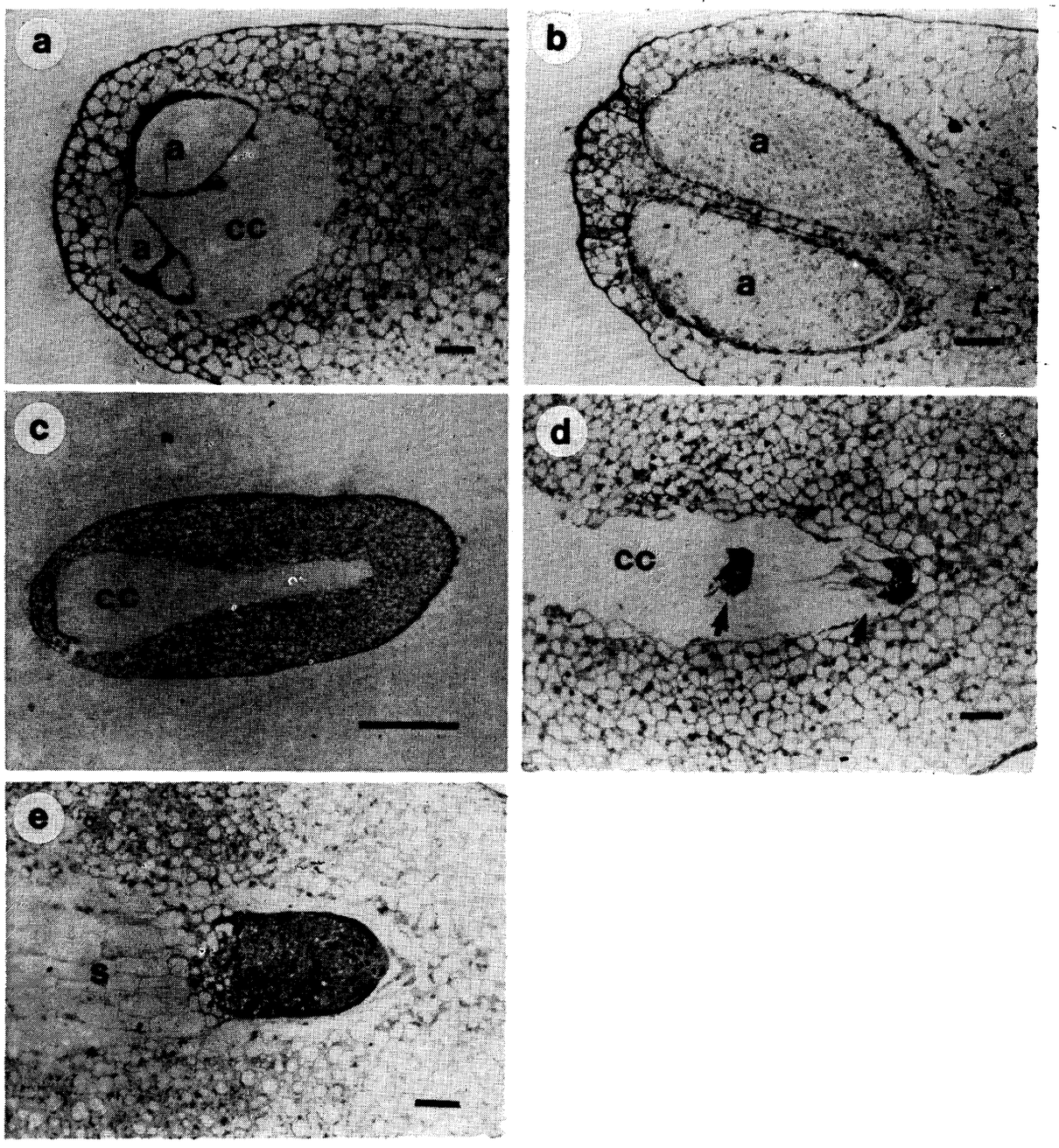

Figure 3. — Histology of *Picea sitchensis* (Bong.) Carr. megagametophytes from the responsive collection dates in 1991. a) Section through a mentor (*Picea abies*) pollinated megagametophyte from June 25 showing degenerating archegonia (a) and the beginning of corrosion cavity (cc) formation. Bar = 0.1 mm. b) Section through open pollinated megagametophyte from June 25 showing well developed agchegonia (a). Bar = 0.1 mm. C) Section through a mentor (*Picea abies*) pollinated megagametophyte from July 4 showing fully formed corrosion cavity cc). Bar = 0.5 mm. C) Section through an open pollinated gametophyte from July 4 showing corrosion cavity (cc) containing two small zygotic embryos (arrows). Bar = 0.1 mm. E) Section through an open pollinated gamethophyte from July 15 showing a zygotic embryo with distinct embryo head and suspensor (s). Bar = 0.1 mm.

5. Effect of pollination and fertilization

No gametophytes developed in cones bagged before anthesis. Mentor-pollinated gametophytes developed normally, though they were unfertilized. Mentor-pollinated gametophytes grew more rapidly than open-pollinated ones and produced the first calli on June 25, whereas open-pollinated gametophytes did not respond until July 4 (*Table 6*). Unfortunately, we did not have enough mentor-pollinated cones for the whole season, and consequently do not have comparisons for all collection dates. On July 4, however, when both types were responsive, they appeared to be equally so (*Table 6*).

6. Morphological markers for callus induction

The responsive period began approximately when individual cones had reached their full length. Absolute cone and bract size could not be used as markers for response capacity due to large variations within and between trees. A more useful macroscopic marker was the detachment of the ovuliferous scales from the under-

lying bracts at the time when gametophytes became responsive.

The megagametophytes developed as described by OWENS and MOLDER (1980). Histological study of sections revealed a considerable variation in internal gametophyte development within cones, but the general picture was the following. On June 25, when the first mentor-pollinated gametophytes produced calli, they had attained full size (2 mm to 2.3 mm). Their texture was firm and their colour white. Archegonia were rapidly degenerating and formation of corrosion cavities had begun (*Fig. 3a*). The open-pollinated gametophytes were still soft and translucent on June 25, but with well developed archegonia (*Fig. 3b*). On July 4, the archegonia were fully degenerated and the formation of corrosion cavities complete in mentor-pollinated gametophytes (*Fig. 3c*). At this time degeneration of archegonia and the formation of corrosion cavity was apparent in most open-pollinated gametophytes and in one of them small twin embryos were seen (*Fig. 3d*). On July 15, embryos with distinct heads and suspensors had developed in the open-pollinated gametophytes (*Fig. 3e*).

7. Regeneration experiments

Macroscopic examination of haploid calli, cultured on media with raised cytokinin levels (BAP, KIN) in relation to auxin (2,4-D or NAA), did not reveal any signs of morphogenesis. Most of the cell lines appeared to require exogenously applied auxins for continuous growth, whereas a few calli grew quite vigorously on all media. Enrichment of the medium with coconut milk in combination with different levels of BAP and NAA, and exchanging gelrite with agar was also ineffective in inducing organogenesis in the haploid cell lines.

Discussion

Although a high percentage of sitka spruce gametophytes cultured *in vitro* could be induced to undergo initial cell divisions (*Tables 2* and *3*), only a small fraction of these had the ability for sustained proliferation. Whether this developmental block was due to the expression of lethal recessive genes, as suggested by VON ADERKAS *et al.* (1987), or some limiting physical or chemical factors in the medium is unknown at present.

The fact that many of the diploid cell lines were of the fast growing type and obtainable throughout the collection period, contrary to haploid calli, leads us to suspect that they originated from fragments of diploid tissue. However, a spontaneous diploidization of initially haploid lines cannot be excluded. Three cell lines contained a mixture of haploid and diploid cells. Furthermore, BORCHERT (1968) has found that callus from mature gametophytes of *Pinus lambertiana* diploidizes spontaneously. In *Taxus*, ROHR (1987), has shown that the mature endosperm contains many multinucleate cells, and approximately 70% of determined cell lines, derived from relatively mature endosperm of *Picea abies*, were diploid or mixoploid (HUHTINEN et al., 1981; SIMOLA and HONKANEN, 1983).

Of the different responses observed, the genotype of the mother tree was only critical for haploid callus production (*Table 1*). Genotype effect on callus growth from megagametophytes was also demonstrated in *Picea abies* (HUHTINEN et al., 1981; SIMOLA and HONKANEN, 1983). On the other hand, haploid embryogenesis was induced at a low rate from megagametophytes of all *Larix leptolepis*, *L. decidua* and hybrids tested (VON ADERKAS et al., 1990), and VON ADERKAS and BONGA (1988) observed no difference in the induction of megagametophytes among 3 trees of *Picea glauca*.

A low nutrient status of the gametophytes in 1991 as a result of large seed crop in the previous year (see OWENS and BLAKE, 1985), combined with the cold spring, may explain the generally lower response this year compared to 1990. The approximately 3 week delay in response in 1991 compared to the previous year is directly attributable to the cold spring of 1991, whereas the early spring of 1990 was exceptionally warm. Climatic fluctuations between years and the fact that the most responsive period for haploid induction is only about two weeks for each mother tree, indicate the need for a more reliable marker than calendar date for initiating collections. In developmental and morphological terms the best time to start collections in sitka spruce, seems to coincide with the formation of corrosion cavity and archegonial degeneration in the gametophyte. In fertilized gametophytes this corresponds to the period when the embryo is elongating but before the development of cotyledons (OWENS and MOLDER, 1980). Our results are similar to those obtained for *Larix decidua* by VON ADERKAS and BONGA (1988), except that the period of inducibility in *Picea sitchensis* appears to be considerably shorter, and support their suggestion that the above mentioned morphological markers may also be used for other Pinaceae. Definition of the active period in terms of degree-days is not a universally applicable marker due to evolutionary and adaptional differences among species and provenances, but may be used to direct collections toward the time of maximum inducibility in local areas.

The high initial induction rates of cell divisions in the cultured megagametophytes on media with 2,4-D as the sole growth regulator (*Table 4*), suggest that only 2,4-D is needed in the early phase of culture, and that the cytokinins used may even be inhibitory during this stage. However, it appears that cytokinin is generally needed to sustain growth of the induced cell lines.

The rapid development of mentor-pollinated cones compared to open-pollinated ones may be explained by favorable microclima in the pollination bags during the cold spring. Earlier, VON ADERKAS and BONGA (1988) have documented that mentor-pollinated gametophytes of *Picea glauca* were easily inducible. Our study showed that fertilization was not a prerequisite for haploid callus induction in sitka spruce. We were furthermore unable to demonstrate any differences between mentor-pollinated and open-pollinated gametophytes in this regard, but our data are limited. Due to low natural pollen shed in 1991, only a fraction of the open-pollinated gametophytes cultured were in fact fertilized. To further determine the potential effect of fertilization it is necessary to compare mentor-pollinated and control-pollinated gametophytes.

So far we have been unable to demonstrate any organogenic capacity in our haploid callus lines. In *Picea glauca*, haploid callus also had a poor capacity for organogenesis (BONGA, 1981). A more promising method for regeneration of haploid plants in conifers may be through gametophytic embryogenesis (*Larix decidua*: NAGMANI and BONGA, 1985; *L. leptolepis:* VON ADERKAS et al., 1990; *Picea abies:* SIMOLA and SANTANEN, 1990). The single case of gametophytic embryogenesis observed in this study (*Fig. 1a*), although transient, allows us to hope that this strategy may succeed in sitka spruce.

Acknowledgements

The authors wish to thank Dr. SVEN B. ANDERSEN, The Royal Veterinary and Agricultural University, for critical review of the manuscript. CHERRY NIELSEN for linguistic revision, DORTHE CHRISTIANSEN and CHERRY NIELSEN for excellent technical assistance. Financial support for this study was granted by The Agricultural and Veterinary Research Council (Contract no. 13—4301).

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Transformation of Populus Hybrids to Study and Improve Pest Resistance[1])

By N. B. KLOPFENSTEIN[2]), H. S. MCNABB JR.[3])[4]), E. R. HART[4]),[5]), R. B. HALL[4]), R. D. HANNA[4]), S. A. HEUCHELIN[3]),[4]), K. K. ALLEN[4]),[5]), NIAN-QING SHI[3]) and R. W. THORNBURG[3]),[6])

USDA Forest Service, Rocky Mountain Forest and Range Experiment Station, Center for Semiarid Agroforestry, East Campus, University of Nebraska, Lincoln, NE 68583—0822, USA[2]). Departments of Plant Pathology[3]), Forestry[4]), Entomology[5]), and Biochemistry and Biophysics[6]), Iowa State University, Ames, IA 50011, USA

(Received March 1992)

Summary

Plant defense mechanisms that have activity against a specified pest contribute to resistance of trees to that pest, provided that the defense mechanism is expressed at adequate levels in the appropriate tissues. Additionally, the stability and (or) physiological efficiency of the resistance may be increased by expressing the defense mechanism only when the tree is threatened by pest attack or challenge. In studies aimed at understanding and improving pest resistance of trees, 2 *Populus* hybrids, *Populus alba* L. X *P. grandidentata* MICHX. and *P.* X *euramericana* (DODE) GUINIER, were transformed with chimeric plant defense gene constructs based on the potato proteinase inhibitor II (*pin2*) gene. An *Agrobacterium* binary vector system was used to transform these hybrids with one of the following chimeric genes: 1) a wound-inducible *pin2* promoter linked to a chloramphenicol acetyltransferase (*CAT*) reporter gene; 2) a bacterial nopaline synthase (*nos*) promoter linked to a *PIN2* structural gene; or 3) a cauliflower mosaic virus *35s* promoter linked to a *PIN2* structural gene. All of the transgenic poplars also were transformed with a selectable marker gene consisting of a *nos* promoter linked to a neomycin phosphotransferase II (*NPT II*) structural gene which confers kanamycin resistance.

Tissue-specific expression of the *nos-NPT II* gene construct is being evaluated with enzyme-linked immunosorbent assays (ELISAs). Transgenic poplar lines from separate transformation events demonstrate variable

[1]) Presented at the Workshop of the IUFRO Working Party S2.04-07 — Somatic Cell Genetics — on "Trends in the Biotechnology of Woody Plants", in Dehra Dun, India, 25 to 29 November, 1991.