J Plant Growth Regul (2004) 23:135–145
DOI: 10.1007/s00344-004-0050-2

JPGR
Journal of Plant Growth Regulation

Promotion of Female Strobili Flowering and Seed Production in two Japanese Pine Species by 6-Benzylaminopurine (BAP) Paste Application in a Field Seed Orchard

Satoru Wakushima

Hiroshima Prefectural Forestry Research Center, 168-1 Tokaichi, 728-0015, Miyoshi, Hiroshima, Japan

Abstract

Treatment with 6-benzylaminopurine (BAP) to promote the flowering of female strobili and to increase seed production in a seed orchard was examined at different times of conebud differentiation for Japanese red pine (*Pinus densiflora*) and Japanese black pine (*P. thunbergii*), from late August to mid-September 1998. BAP was mixed with lanolin and Vaseline to prepare a paste (BAP, 2000 mg/L), which was used for a "terminal bud treatment" and also applied in a "girdling treatment." The BAP treatment in early September transformed lateral buds on lower branches that would have normally differentiated as male strobili into female strobili. The highest percentages of female flowering branches were 21.1% (red) and 40.5% (black) produced by the terminal bud treatment, while control branch percentages were 5.0% and 7.1%, respectively. Girdling combined with BAP treatment further promoted female flowering branches to 32.3% (red) and 64.3% (black), while those of controls were 8.3% and 9.9%, respectively. Moreover, the numbers of female strobili per female flowering branch were 6 to 11-fold greater than controls. Mature seed cones were harvested in October two years later from which full seeds with a high germination ratio were recovered. Use of BAP treatment is thus highly effective for enhancing seed production in seed orchards of these two pine species.

Key words: 6-benzylaminopurine (BAP); Female strobili; Male strobili; Sex transformation; Seed cone; Seed production; Japanese red pine (*Pinus densiflora*); Japanese black pine (*Pinus thunbergii*)

Introduction

Conifer species in the *Pinaceae* family are grown worldwide for commercial plantations in forestry. A number of effective treatments for the promotion of conebud development in the *Pinaceae* have been reported. The most successful treatments have involved application of less polar gibberellins ($GA_{4/7}$) (see Pharis and Kuo 1977; Pharis and others 1987; Bonnet-Massimbert 1987; Smith 1998). More specifically, in experiments using seedlings or grafts of

Online publication: 21 October 2004
**Corresponding author; e-mail:* wakusima@urban.ne.jp

Pinus species that were over 5 years old, $GA_{4/7}$ applications enhanced the production of female strobili 2–5 fold as compared with control trees (Chalupka 1987; Fogal and others 1994; Greenwood 1982; Hare 1984; Harrison and Slee 1991; Longman 1982; Lukkanen and Johansson 1980; Ross and others 1984; Sheng and Wang 1990). Wheeler and others (1980) reported that the largest promotion of female flowering of *P. contrta* by $GA_{4/7}$ treatment was a 2–14 fold increase compared with the control treatment, although the total number of female strobili per new shoot induced by $GA_{4/7}$ treatment was lower than 1.0, and ranged from 0.2 to 0.7.

In studies with Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) and Japanese black pine (*Pinus thunbergii* Parl.), Kanekawa and Katsuta (1982) and Hashizume (1985) reported that $GA_{4/7}$ or GA_3 application could promote the production of female strobili. Their procedure enhanced the production of female strobili 2–6-fold or 2–10-fold, respectively, compared with control trees. However, the total number of female strobili was comparatively low, only 0.1 to 0.4 strobili were induced per shoot.

Herein is a report on a method for promoting female conebud development and seed fructification in two important Japanese pine species using 6-benzylaminopurine (BAP), a cytokinin. Wakushima and others (1996) reported that BAP applied to the terminal bud of red pine at the time of conebud differentiation could enhance the transformation of potential male conebuds to females. That is, lateral buds that would normally have differentiated into male strobili were transformed into female strobili in the next spring. Because the number of lateral (usually male) strobili occurring in the lower part of *Pinus* trees is 10–100-fold the number of apical strobili (Wakushima and others 1996, 1997), if numerous lateral (male) strobili can be transformed into female strobili, it would elevate the number of seed cones and thus seed productivity. Wakushima and others (1996) applying BAP in a greenhouse obtained 0.69 female, 0.37 hermaphroditic and 5.54 male strobili per new shoot at the optimum treatment. This value was equal to or higher than female strobili induced just with $GA_{4/7}$ treatments.

To develop a practical approach for use in field seed orchards, we tried several methods of BAP application: spraying (aqueous solution of BAP with 0.1% (v/v) surfactant (Tween 20) was directly applied to terminal buds, Wakushima and others 1996, 1997); a covering treatment (aqueous BAP solution was applied to a piece of cotton put on top of terminal buds then the terminal bud was covered with a plastic bag to shed the rainwater, Wakushima and Yoshioka 1997a); mousse treatment (BAP solution with 1% (v/v) surfactant was applied to the terminal bud using a special bottle to make small bubbles, Wakushima and Yoshioka 1997b); and lanolin paste treatment (BAP solution was mixed with the same volume of lanolin and then applied to terminal buds, Wakushima and Yoshioka 1998, Wakushima 1999). Only the lanolin paste treatment succeeded in promoting the development of lateral female strobili in the field seed orchard; 1.54 (red pine) and 3.31 (black pine) female strobili per treatment were obtained from the treated buds. In this study, I used a mixture of lanolin and white Vaseline as the carrier for BAP and applied it to terminal buds and branches which had their bark partly stripped. Because Wakushima and others (1996, 1997) showed that the timing of the BAP treatment greatly influenced the induction of female strobili, I treated the trees with BAP at eight timings, from late August through mid-September in 1998 to find the optimal timing. Furthermore, after the harvest of mature seed cones in October 2000, the number of seed cones and their seeds were counted and weighed for each treatment and their germinability assessed.

Materials and Methods

Plant Materials

All experiments were done in the Shobara Resistant Pine Seed Orchard located in Kawanishi, Shobara, Hiroshima Prefecture (lat. 34.65°N and long 133.04°E.; 330–360 m above sea level). The trees used were 48 individual red and 48 individual black pines. These trees had been transplanted into the seed orchard in March 1988. In 1996, each main stem was decapitated at a height of 3 meters. Hence, tree heights were 4 to 5 m at the time of the experiments. Branches located in the lower crown of these trees at 2 m or lower were randomly selected for treatment. Branches located at the same positions were selected for a control lot on each tree.

Preparation of BAP Paste

One gram of BAP powder (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in 15 ml aqueous 1*N* KOH solution, and the concentration of BAP solution was adjusted to 4000 mg/L with distilled water. Purified lanolin (Yamazen Chemicals, Tokyo, Japan) and white Vaseline (Wako Pure Chemical Industries, Ltd.) were dissolved in a hot water bath at 60°C and gradually mixed with the BAP solution to prepare a white

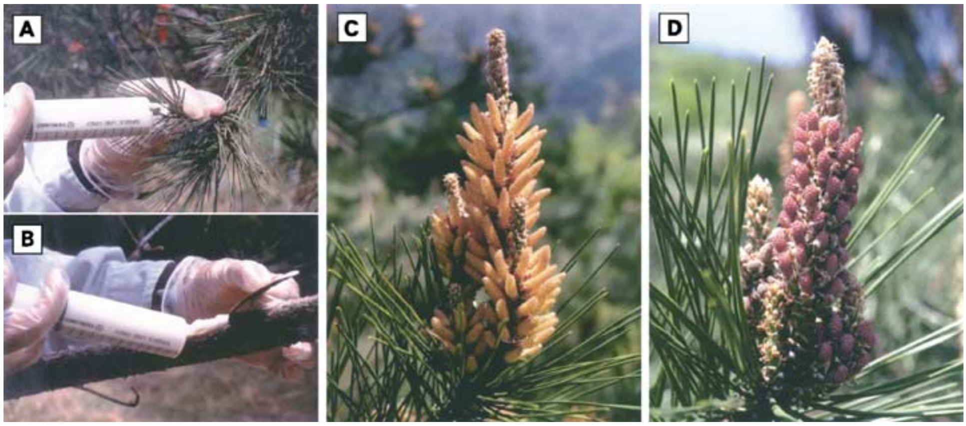

Figure 1. (**A**) Terminal bud treatment. Terminal buds were covered with 2 ml volume of BAP paste. (**B**) Girdling treatment. Selected branches (4-year or 5-year-old portions) were girdled with a craft knife. About 1–2 cm (width) × 5 cm (length) of bark was stripped off. Five ml volume of BAP paste from a syringe was applied to the stripped part. (**C**) Normal male strobili developed at the lower part of new shoots (Japanese black pine). Photographed in early May 1999. (**D**) Numerous lateral female strobili developed on the lower part of new shoots treated with BAP (Japanese black pine). Photographed in early May 1999.

paste. The mixing ratio of BAP solution, lanolin and white Vaseline was 2:1:1 (v/v/v) with a final BAP concentration of 2000 mg/L. The paste was freshly prepared just before the treatments and dispensed with 50-ml plastic syringes.

Terminal Bud Treatment on Lateral Branches

The trees were treated at 8 timings on August 25, 27, 31, September 4, 8, 11, 14 or 18 in 1998 for red pine and at 7 timings on the same dates except for August 25 for black pine. The terminal buds were covered with the BAP paste (Figure 1A). This treatment was done only once, and the volume of paste for the treatment was about 2 ml per one branch (the treatment was designated as terminal bud treatment). The control lot was not treated with any chemicals because KOH was shown earlier to have no effect on the transformation into female strobili (Wakushima and others 1997; Wakushima and Yoshioka 1997a).

Girdling Treatment

A branch girdling with BAP applied to the girdled area was done on the same dates as for the terminal bud treatment. Four-year or 5-year-old portions (diameters of about 1–4 cm) of lateral branches were subjected to the girdling plus BAP treatment. Bark was stripped off at a width of about 1–2 cm and a length of about 5 cm with a craft knife. The paste in the syringe was applied on the stripped part (Figure 1B). The treatment was done only once, and the volume for the treatment was about 5 ml per site (the girdling and BAP treatment was designated as girdling treatment). There was no ''girdling only'' treatment.

Counting of Strobili

In May the following year, female strobili and number of branches bearing female strobili were counted. The numbers of branches with or without female strobili were evaluated by the Fisher's exact probability test between control and BAP-treated branches. In the counts, female strobili were separated into apical and lateral, that is, on the lower parts of the new shoots. The number of female strobili induced for each treatment time was evaluated relative to controls by the Bartlett test for homogeneity of variance. At a 1% or less probability, the variance among the individual groups was not homogenous. Thus, the non-parametric Kruskal-Wallis test was carried out. A significant difference was observed among the multiple groups ($p < 0.001$), which was then evaluated by the Mann-Whitney U test to determine the level of significance between the control and the BAP-treated groups (Nagata 1993; Lehmann 1975).

Harvesting of Seed Cones and Counting of Seeds

In October 2000, about 2 years after BAP treatment, all seed cones were harvested, counted and weighed. Seed cones were then dried in the sun and seeds were collected. Membranous wings were removed by hand. According to the general procedure

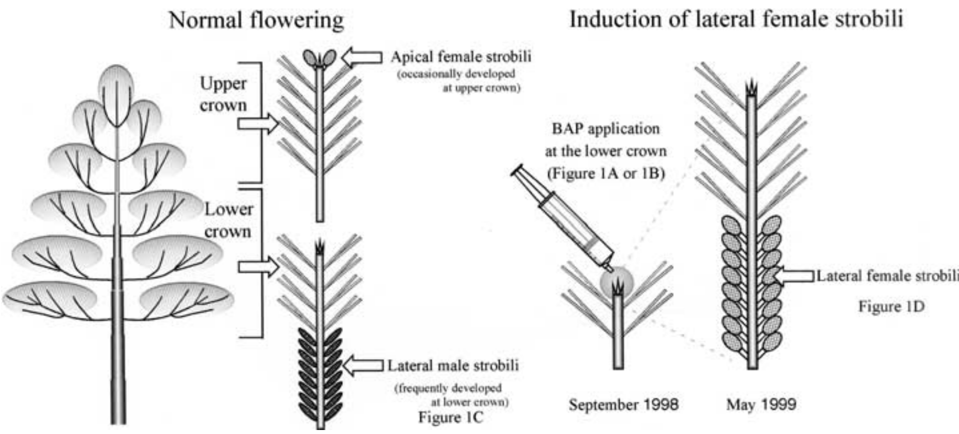

Figure 2. Zone of female and male strobili in a shoot and a crown of Japanese red pine and black pine with or without BAP treatment. Normal flowering of female and male strobili without BAP application is shown at left. Zone of female strobili induced by BAP application in 1998 is shown at right.

(Japanese Govt. For. Expt. Sta. 1969), the seeds were visually classified into two groups: full, heavy and black seeds and empty, white and light ones. After grouping, the seeds were counted and the dry weight of full seeds was measured.

For comparison, seed cones were also harvested from all the non-treated trees and non-treated (comparable) branches from the same clones as those used for the experiments. Additionally, seed cones were harvested from the upper crown of 36 non-treated red pine trees to compare cone numbers on the upper crown parts of the 48 red pines of the BAP-treated trees. Similarly, seed cones were harvested from all the 18 non-treated black pines and non-treated branches of the 48 BAP-treated black pine trees. Seeds were collected from these seed cones and assessed as above.

Germination was examined by placing full seeds in an agar culture medium (0.9%, w/v) under total darkness at 25°C (736 seeds for red pine and 1203 for black pine of the BAP-treated trees and 800 for red and 600 for black pine of the non-treated trees). After 21 days, the number of germinating seeds was counted. Germination was evaluated between treatments by the chi-square test.

Results

The BAP treatment successfully transformed lateral buds that would normally have produced male strobili into lateral female strobili. However, all of the treated buds and the branches did not always succeed in flowering of the lateral female strobili. The cases in which new shoots elongating from the BAP-treated buds developed only apical female strobili were rare. Both no lateral and no apical female strobili occurred frequently. Figure 2 schematically shows the normal zone of lateral male and apical female flowering in pine trees and the successful case of transformation of lateral strobili to female after BAP treatment without apical female strobili. Figure 1C shows a non-treated branch with the normal pattern of male strobili distribution without apical and lateral female, and Figure 1D shows a comparable BAP-treated branch with the increased numbers of lateral female strobili but without apical female strobili.

Terminal Bud Treatment

Table 1 shows the number and percentage of treated and non-treated branches with induced female strobili for all treatment times. For red pine, 5% of the control branches developed female strobili. Terminal bud treatment did not significantly increase the percentage of branches bearing female strobili, except for the September 14 treatment. In total, 159 of 2631 branches (6%) with BAP-treated terminal buds emerged with female strobili. This total percentage (6%) was not significantly higher than that of controls (5%) at the 5% level. The numbers of apical female strobili per treated bud on six treatment dates and the total were significantly smaller than the number of controls at the 5% level, except for the treatment on September 14 and 18. However, the numbers of lateral female strobili on six treatment dates and the total were significantly greater than that of the control at the 5% level, except for the August 27 and September 18 treatment for red pine.

For black pine, the percentage of female flowering BAP-treated branches (28.8–40.5%) on six dates (from August 31 to September 18) was significantly higher than that of controls (7.1%), significant at the 1% level. In total, 717 of 2134 branches (33.6%) with BAP-treated terminal buds emerged with female strobili (significantly different from the control at the 1% level). The numbers of apical female strobili per treated bud in the treatment dates

Table 1. The Date of Terminal Bud Treatment with BAP, the Number of Branches Treated, and Female Strobili Produced

		No. of branches			No. of female strobili	
Species	Date	Treated	With female strobili	Percentage	Apical	Lateral
Red pine	Aug. 25	300	11	(3.7%)	4 a	46 b
	27	343	5	(1.5%)*	2 a	26
	31	468	27	(5.8%)	0 a	122 b
	Sept. 4	402	10	(2.5%)	0 a	22 b
	8	355	24	(6.8%)	2 a	156 b
	11	256	20	(7.8%)	2 a	86 b
	14	218	46	(21.1%)**	9	198 b
	18	289	16	(5.5%)	22	9
	Treated total	2,631	159	(6.0%)	41 a	665 b
	Control	179	9	(5.0%)	9	0
Black pine	Aug. 27	68	5	(7.4%)	4	7 b
	31	311	99	(31.8%)**	12	712 b
	Sept. 4	309	89	(28.8%)**	1 a	417 b
	8	393	133	(33.8%)**	28	678 b
	11	392	138	(35.2%)**	17 a	801 b
	14	330	119	(36.1%)**	8 a	556 b
	18	331	134	(40.5%)**	32	683 b
	Treated total	2,134	717	(33.6%)**	102 a	3,854 b
	Control	156	11	(7.1%)	12	0

*The numbers of branches with or without female flowering were evaluated by Fisher's exact probability test between control and BAP-treated branches. * and ** are significantly different from the control at 5% and 1% level, respectively. The numbers of apical and lateral female strobili per branch were evaluated by Mann-Whitney U test between the control and the BAP-treated groups. The numbers of female strobili appended ''a'' were significantly smaller and ''b'' were significantly greater than the control at 5% level, respectively*

on September 4, 11, 14 and the total were significantly smaller than the control at the 5% level, whereas the numbers of lateral female strobili from all treatment dates and the total were significantly greater than the control lot.

Generally, more apical female strobili occur on vigorously growing branches in the upper part of a crown than in the lower part (Marquard and Hanover 1984; Ross 1985; Smith 1998; Young and Young 1992). Because the positional height of the treated branches was set at 2 m or lower in the lower part of a crown for both control and BAP treatment, the number of growing apical female strobili on the control lower branches was smaller than that on the higher branches.

The total number of apical and lateral female strobili per female flowering branch was examined at each treatment stage (Figure 3). All BAP-treated branches produced significantly more ($p < 0.05$) female strobili than the control branches. For red pine, the number was the largest, 6.58 ± 0.99 strobili/female flowering branch (mean ± SE) in the September 8 treatment, whereas that of the control lot produced only 1.00 ± 0.00 strobili/female flowering branch. For black pine, the number was the largest, 7.31 ± 0.69 strobili/female flowering branch for the August 31 treatment although the control lot gave only 1.09 ± 0.09 strobili/female flowering branch.

Girdling Treatment with BAP

For red pine, 0.0%–32.3% of the girdled branches developed female strobili across all treatment days whereas 8.3% of the control branches developed female strobili (Table 2). Percentages of female flowering branches for girdling treatments on September 8 and 14 are significantly higher than their controls at the 1% level. In total, 83 of 572 branches (14.5%) had at least one female strobilus (significantly different from the control at the 5% level). The numbers of apical female strobili per branch in the treatment dates of August 27, 31, September 4, 8 and 18 and the total were significantly smaller than that of the control at the 5% level; the numbers of lateral female strobili per branch were significantly greater than the control lot in the seven treatment dates and the total, except for August 25.

For black pine, 21.1%–64.3% of the girdling treated branches developed female strobili, whereas only 9.9% of the control branches developed female

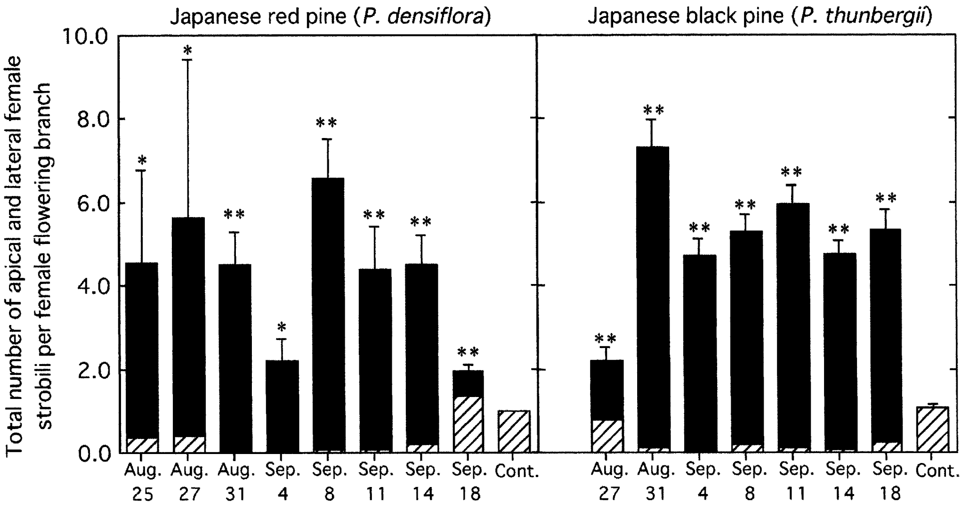

Figure 3. Total number of apical and lateral female strobili per female flowering branch by terminal bud treatment in Japanese red pine and black pine. Means of apical and lateral female strobili are shown by hatched and solid columns, respectively. Vertical bars indicate standard error. Significant differences were evaluated by the Mann-Whitney *U* test; * and ** are significantly different from the control at the 5% and 1% level, respectively.

strobili. Percentages of female flowering branches were significantly higher on any treatment date compared to controls at the 1% and 5% level. In total, 204 of 467 branches (43.7%) had at least one female strobilus (significantly different from the control at 1% level). Only the numbers of apical female strobili per branch in the treatment on August 27 were significantly smaller and on September 11 were significantly greater than that of the control, respectively, while the numbers of lateral female strobili from all treatment dates and the total were significantly greater than the control.

The total number of apical and lateral female strobili per female flowering branch with BAP and girdling is examined in Figure 4. Except for treatment on August 25, BAP-treated branches produced significantly more ($p < 0.05$) female strobili than the control branches. For red pine, the female conebud response was greatest, 10.87 ± 2.66 strobili/female flowering branch (mean ± SE), for the September 4 treatment. In contrast, red pine control branches showed only 1.30 ± 0.15 strobilus/female flowering branch. For black pine, the best treatment date was also 4 September, with 19.05 ± 4.64 strobili/female flowering branch. Controls for black pine yielded 1.67 ± 0.23 strobili/female flowering branch.

Harvested Seed Cones and Seeds

Table 3 gives the numbers and weights of seed cones and seeds, and their germination ratio for each treatment. Total numbers of female strobili occurring on the BAP-treated branches were 1480 for red and 7002 for black pine by early May 1999. In October 2000, about two years after the BAP treatments, 888 red pine and 2722 black pine seed cones were harvested. Because lower branches (less than 2 m height) did not generally produce cones in the controls, I included cones harvested on the upper part of trees (higher than 2 m) for control branches in Table 3 (that is, in the BAP column). For black pine, seed cones were frequently attacked by the larvae of pine shoot moths, *Dioryctria sylvestrella* Ratzeburg, so many of them had aborted before harvest. Thus, the ratio of female conebuds to total seed cones was lower in black pine than in red pine. The fresh weight of an average cone resulting from BAP treatment was 4.2 g for red pine and 7.8 g for black pine. Hence, they were very much smaller than a corresponding (upper crown) control seed cone (10.4 g for red and 19.7 g for black pine).

Harvested seeds from branches treated with BAP were 5207 for red pine and 27,243 for black pine. From the control (upper crown), 9737 seeds from 84 red pines and 5287 seeds from 66 black pines were harvested. Seed numbers per single cone for BAP-treated branches were smaller in number compared to those for control cones, that is, 6.2 (red pine) and 11.2 (black pine) seeds per cone, whereas control cones were 28.2 (red) and 26.7 (black) seeds per cone. Seed weight per 100 seeds with or without BAP treatment were almost equal in red pine, 1.18 g (with BAP) and 1.19 g (control), but for black pine, seeds from branches treated with BAP weighed less (1.48 g) than control seeds (2.50 g).

Table 2. The Date of Girdling + BAP Treatment, the Number of Branches Treated, and Female Strobili Produced

		Number of branches			Number of strobili	
Species	Date	Treated	With female strobili	Percentage	Apical	Lateral
Red pine	Aug. 25	8	0	(0.0%)	0	0
	27	106	7	(6.6%)	0 a	74 b
	31	134	17	(12.7%)	2 a	142 b
	Sept. 4	103	15	(14.6%)	3 a	160 b
	8	65	21	(32.3%)**	0 a	227 b
	11	61	6	(9.8%)	2	60 b
	14	49	13	(26.5%)**	3	99 b
	18	46	4	(8.7%)	0 a	19 b
	Treated total	572	83	(14.5%)*	10 a	781 b
	Control	120	10	(8.3%)	13	0
Black pine	Aug. 27	71	15	(21.1%)*	2 a	76 b
	31	74	24	(32.4%)**	6	333 b
	Sept. 4	54	22	(40.7%)**	28	391 b
	8	72	31	(43.1%)**	52	530 b
	11	77	48	(62.3%)**	76 b	659 b
	14	56	36	(64.3%)**	60	497 b
	18	63	28	(44.4%)**	35	301 b
	Treated total	467	204	(43.7%)**	259	2,787 b
	Control	151	15	(9.9%)	25	0

*The number of branches with or without female flowering were evaluated by Fisher's exact probability test between control and BAP-treated branches. * and ** are significantly different from the control at 5% and 1% level, respectively. The numbers of apical and lateral female strobili per branch were evaluated by the Mann-Whitney U test between the control and the BAP-treated groups. The numbers of female strobili appended "a" were significantly smaller and "b" were significantly greater than the control at the 5% level, respectively*

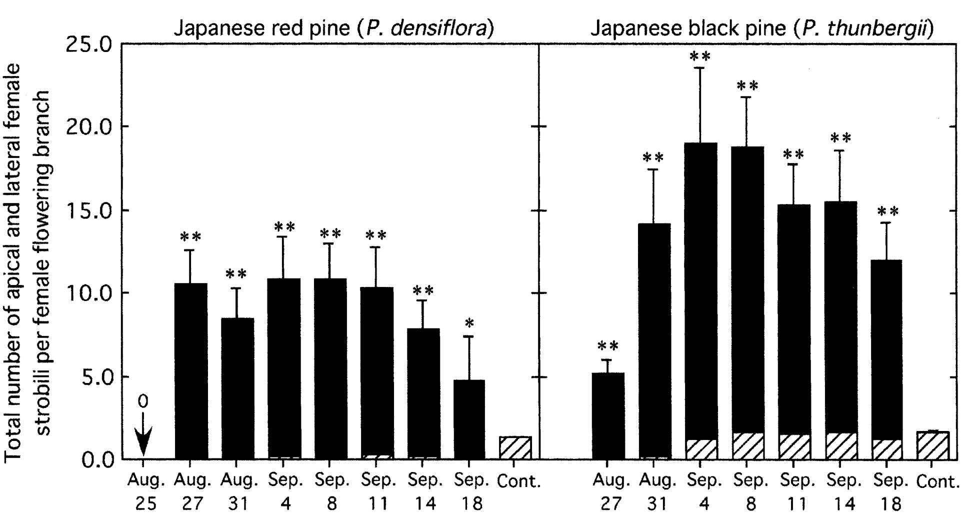

Figure 4. Total number of apical and lateral female strobili per female flowering branch of girdling treatment in Japanese red and black pine. Means of apical and lateral female strobili are shown by hatched and solid columns, respectively. Significant differences were evaluated by the Mann-Whitney *U* test; *and **are significantly different from the control at the 5% and 1% level, respectively.

The germination ratio was 61.4% for seeds from BAP-treated red pine compared to a control ratio of 65.3%. The germination ratio of seeds from BAP-treated black pine was significantly (chi-square test, $p < 0.01$) lower (74.3%) than the germination ratio of control seeds (94.2%).

Discussion

According to Hashizume (1973), the differentiation stage of conebud primordia for these two Japanese pine species continues from early July to early September; then conebud differentiation continues

Table 3. The Number and Weight of Harvested Seed Cones and Full Seeds Extracted From Cones

	Red pine		Black pine	
Harvested zone of seed cones in a tree crown	+BAP Lower	−BAP Upper	+BAP Lower	−BAP Upper
Total number of female strobili	1,480	Not counted	7,002	Not counted
Total number of harvested seed cones	888	345	2,722	198
Percentage of seed cone development from strobili	60%	–	39%	–
Total number of seed cones for weighed and dried	840	345	2,426	198
Total fresh weight of seed cones (kg)	3.5	3.6	18.9	3.9
Fresh weight of single cone (g)	4.2	10.4	7.8	19.7
Total number of full seed	5,207	9,737	27,243	5,287
Seed number/cone	6.2	28.2	11.2	26.7
Total dry weight of full seed (g)	61.5	115.8	402.7	132.1
Weight of 100 seeds (g)	1.18	1.19	1.48	2.50
Number of sown seed for germination test	736	800	1,203	600
Germinated number	452	523	894	565
Germination (%)	61.4	65.3	74.3*	94.2

**The germinated number of black pine seeds with BAP treatment is significantly smaller than that of seeds without BAP treatment at the 1% level (chi-square test for independence). Germination was assessed 21 days after sowing.*

from the middle of September until mid-October. In earlier studies, to increase the number of conebud primordia, many experiments applied $GA_{4/7}$ to *Pinaceae* trees during their formation beginning in March to July or, at the latest, in September (Cecich 1983; Chalupka 1984, 1987; Fogal and others 1993, 1995; Greenwood 1982; Harrison and Slee 1991; Hare 1984; Hashizume 1985; Kanekawa and Katsuta 1982; Longman 1982; Luukkanen and Johansson 1980; Ross and Greenwood 1979; Ross and others 1984; Sheng and Wang 1990; Wheeler and others 1980). In this work, when BAP treatment was applied in late August (at the conebud differentiation stage), it transformed the lateral (usually male) conebud primordia into females. The optimal stage for red pine was confined to a short period around September 14, whereas black pine responded to a broader range of application times in early to mid-September.

Figure 5 shows a schematic view of the timing of BAP treatments relative to results in the following spring with regard to conebud sex. Our previous research revealed that BAP treatment in August at the stage of conebud primordia differentiation and prior to the floral differentiation stage of red pine induced vegetative shoot primordia, which greatly suppressed the number of both female and male strobili produced the next spring (Wakushima and others 1996, 1997). Sheng and Wang (1990) also reported the same outcome for BAP treatment of *Pinus taburaeformis* in June. Finally, Smith and Greenwood (1995) reported a similar finding for *Picea mariana* treated with BAP, zeatin or zeatin riboside in June or July. Thus, BAP should not be used prior to mid-August in pine seed orchards to promote female conebud formation. Also, when BAP is used in October or thereafter, no sex transformation occurs, presumably because all lateral potential conebud primordia have already differentiated into male conebuds (Wakushima and others 1997; Wakushima and Yoshioka 1998). However, it is important to note that the conebud differentiation stage varies widely, depending on the species, temperature, moisture conditions and branch location within the crown. Furthermore, the individual treatment methods differ in terms of BAP absorption into either terminal buds or girdled (scraped) branches. Thus, more detailed relations among environmental factors such as weather conditions, floral differentiation stage, and optimal timing for BAP treatment need to be examined.

The paste (BAP solution: lanoline: white Vaseline = 2:1:1) used in the present experiments had a lower viscosity than that of the single lanoline paste (BAP solution: lanoline = 1:1) previously used (Wakushima 1999). Thus, the lower viscosity paste is recommended for syringe applications because its greater spreadability aids coating of both terminal buds and barked-stripped branches. The low viscosity paste neither dried nor solidified during the two-year experiment and it shed rainwater and avoided reagent run-off. It is thus useful in field seed orchards.

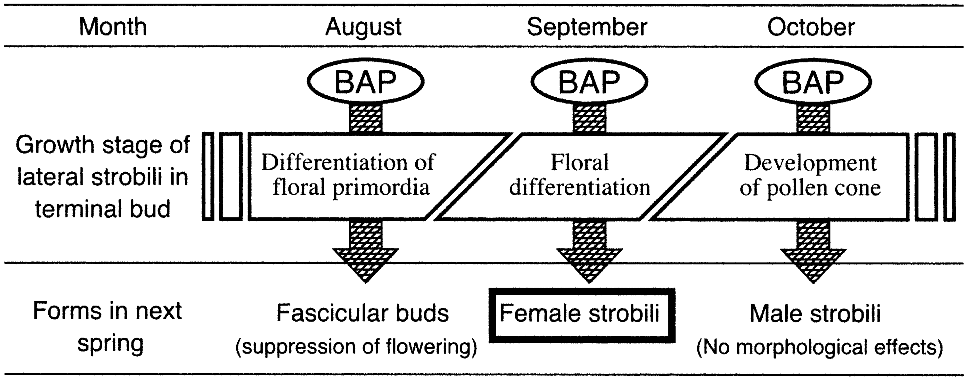

Figure 5. Relationships between the growth stage of lateral strobili and the morphological effects of BAP application. BAP treatment at the stage of floral primordia prior to the floral differentiation stage of two Japanese pine species transforms the lateral floral primordia into fascicular bud primordia. When the BAP treatment is done in October or thereafter, no floral sex transformation occurs because all lateral floral primordia are already differentiated into male strobili. Only BAP treatment during the floral differentiation stage induces sex transformation of lateral floral primordia into female strobili in the next spring.

The girdling treatment was superior to the terminal bud treatment with respect to efficacy of female flowering, especially in black pine at the optimum timing (September 4 to September 14).

The numbers of apical female strobili per BAP-treated bud or branch in the terminal bud and girdling treatment showed no significant increase at the 5% level in either pine species, except for the September 11 girdling treatment for black pine (Tables 1 and 2). The morphological roles of BAP treatment in late August to mid-September seemed to be restricted to the transformation of primordia sex to female. Therefore, the noted increase of the apical female strobili by BAP treatment was not expected in these treatment timings because apical primordia would always develop into apical female strobili irrespective of the BAP treatment.

Treatment with $GA_{4/7}$ enhanced the production of female strobili per shoot 2–14-fold as compared with controls (see Introduction). However these values cannot be compared directly with the present BAP treatment for a variety of reasons. Nonetheless, because the BAP treatments increased the number of female strobili per treatment in the two species by 28–60-fold compared with controls it appears that the use of BAP on lower branches can increase the number of female strobili much more than $GA_{4/7}$ treatments.

For both red pine and black pine, the average weight of cones harvested from BAP-treated lower branches was about 40% of the weight of a control cone harvested from the upper part of the crown (higher than 3 m). The reason for the small seed cone size produced by BAP treatment may be due to the fact that these lower branches are less vigorous than upper crown branches and thus the cones have access to less photoassimilate. Additionally, photoassimilation products must be distributed to more seed cones developing on these less vigorous branches.

The numbers of full seeds per cone with BAP treatment of red and black pine were reduced to about 20% and 40%, respectively. One of the reasons may be ascribed to more self-pollination, that is, pollen shedding from male strobili on the next untreated branch occurs at the same time that the female strobilus is receptive. Thus, there is no separation in terms of timing as usually occurs for upper crown female strobili relative to their lower crown male strobili (Hashizume 1973). Finally, the germination ratio of black pine seeds with BAP treatment was significantly lower than that of the control seeds. Other than poor visual screening of seeds we cannot explain this difference. However, the germination ratio of black pine seeds with BAP treatment (74.3%) was high enough for the production of seedlings in nurseries in Japan (Ozawa 1962).

Thus, even though there are problems, BAP treatment can induce very large numbers of female strobili at a position (lower crown part) where females normally do not develop. Therefore, BAP is a practical method for seed orchards to increase seed production. Seeds recovered from BAP-treated trees were sown in a nursery to examine germination and growth. No morphological differences between the seedlings of BAP-treated and control individuals were observed in the nursery.

Currently, in Japan, the death of pine forests due to a pinewood nematode (*Bursaphelenchus xylophi-*

lus) is so serious that wood volumes of 720,000 m^3 were lost in 1999 (Forestry Agency, Ministry of Agriculture, Forestry and Fisheries, Japan 2001). As a preventative strategy, nematode-resistant pine trees have been selected (Fujimoto and others 1989) and seed and seedling production of these resistant genotypes is now under way. In the future, BAP treatment will be applied to seed orchards for the efficient improvement and mass-scale production of nematode-resistant lines of pine trees.

ACKNOWLEDGMENTS

The author is grateful to Prof. N. Sakurai (Hiroshima University) for his critical and helpful comments on this manuscript and expresses many thanks to Mr. H. Yoshioka (Hiroshima Prefectural Forestry Research Center) for his assistance with the field work.

REFERENCES

Bonnet-Masimbert M. 1987. Floral induction in conifers: a review of available techniques. For Ecol and Management 19:135–146.

Cecich RA. 1983. Flowering in a Jack pine seedling seed orchard increased by spraying with gibberellin $A_{4/7}$. Can J For Res 13:1056–1062.

Chalupka W. 1984. Time of $GA_{4/7}$ application may affect the sex of Scots pine flowers initiated. Silvae Genetica 33:4–5.

Chalupka W. 1987. Stimulation of flowering in Scots pine (*Pinus sylvestris*) grafts by gibberellin injection. For Ecol and Management 19:177–181.

Fogal, WH, Coleman, SJ, Wolynetz, MS, Schooley, HO, Lopushanski, SM, Anderson, ML (1994) Modifying sexual expression of containerized jack pine by tpping, altering soil nitrogen and water, and applying gibberellins. Can J For Res 24:869–877.

Forestry Agency, Ministry of Agriculture, Forestry, Fisheries, Japan. 2001. Outline of forestry statistics 2001. Rinyakousaikai, Tokyo, Japan.

Fujimoto Y, Toda T, Nishimura K, Yamate H, Fuyuno S. 1989. Breeding project on resistance to the pine-wood nematode-an outline of the research and the achievement of the project for ten years. Bull For Tree Breeding Inst Japan 7:1–84(in Japanese with English summary).

Greenwood MS. 1982. Rate, timing, and mode of gibberellin application for female strobilus production by grafted loblolly pine. Can J For Res 17 12:998–1002.

Hare RC. 1984. Application method and timing of gibberellin $A_{4/7}$ treatments for increasing pollen conebud production in southern pines. Can J For Res 14:128–131.

Harrison DLS, Slee MU. 1991. Gibberellin $A_{4/7}$ enhanced flowering in *Pinus caribaea* var. *hondurensis*. Can J For Res 21:788–793.

Hashizume H. 1973. Studies on flower bud formation, flower sex differentiation and their control in conifers. Bull Tottori Univ Forests 7:1–139(in Japanese with English summary).

Hashizume H. 1985. Effect of gibberellins on the promotion of flowering in *Pinaceae* species. Bull Fac Agric Tottori Univ 37:80–87(in Japanese with English summary).

Japanese Government Forest Experiment Station1969. Regulation for inspection method of tree seeds for forestry Tokyo, Japan: Jap Govt For Expt Sta.(in Japanese).

Kanekawa T, Katsuta M. 1982. Promotion of strobilus production in *Pinus thunbergii* Parl. and *P. densiflora* Sieb. et Zucc. by gibberellin. J Jpn For Soc 64:101–106.

Lehmann EL. 1975. Nonparametrics, Statistical methods based on ranks (translated to Japanese by Nabeya S, Kariya T, Miura R). Holden-Day, Inc., California, USA.

Longman KA. 1982. Effects of gibberellin, clone and environment on cone initiation, shoot growth and branching in *Pinus contorta*. Ann Bot 50:247–257.

Luukkanen O, Johansson S. 1980. Effect of exogenous gibberellin on flowering in *Pinus sylvestris* grafts. Physiol Plant 50:365–370.

Marquard RD, Hanover JW. 1984. Sexual zonation in the crown of *Picea glauca* and the flowering response to exogenous $GA_{4/7}$. Can J For Res 14:27–30.

Nagata O. 1993. Medical statistic manual for Macintosh Tokyo, Japan: Shinko koeki.(in Japanese).

Ozawa J. 1962. Seed of conifers, production and management. Chikyu-syuppan Co. Ltd., Tokyo, Japan (in Japanese).

Pharis RP, Kuo CG. 1977. Physiology of gibberellins in conifers. Can J For Res 7:299–325.

Pharis RP, Webber JE, Ross SD. 1987. The promotion of flowering in forest trees by Gibberellin $A_{4/7}$ and cultural treatments: a review of the possible mechanisms. For Ecol and Management 19:65–84.

Ross SD, Greenwood MS. 1979. Promotion of flowering in the *Pinaceae* by gibberellins. II. Grafts of sexually mature and immature *Pinus taeda* L. Physiol Plant 45:207–210.

Ross SD, Bollman MP, Pharis RP, Sweet GB. 1984. Gibberellin $A_{4/7}$ and the promotion of flowering in *Pinus radiata*: effects of partitioning of photoassimilate within the bud during primordia differentiation. Plant Physiol 76:326–330.

Ross SD. 1985. Promotion of flowering in potted *Picea engelmannii* (Perry) grafts: effect of heat, drought, gibberellin A4/7, and their timing. Can J For Res 15:618–624.

Sheng C, Wang S. 1990. Effect of applied growth regulators and cultural treatments on flowering and shoot growth of *Pinus tabulaeformis*. Can J For Res 20:679–685.

Smith R, Greenwood M. 1995. Effects of gibberellin $A_{4/7}$, root pruning and cytokinins on seed and pollen cone production in black spruce (*Picea mariana*). Tree Physiol 15:457–465.

Smith R. 1998. Effects of stem injections of gibberellin A4/7 and paclobutrazol on sex expression and the within-crown distribution of seed and pollen cones in black spruce (*Picea mariana*). Can J For Res 28:641–651.

Wakushima S, Yoshioka H, Sakurai N. 1996. Lateral female strobili production in a Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) clone by exogenous cytokinin application. J For Res 1:143–148.

Wakushima S, Yoshioka H, Sakurai N. 1997. Promotion of lateral female strobili production in *Pinus densiflora* by cytokinin application at a specific stage. J For Res 2:51–57.

Wakushima S, Yoshioka H. 1997a. Promotion of female flowering in Japanese red pine clone by benzylaminopurine IV: practical trial of BAP treatment by plastic bag covering on terminal bud. Forest Tree Breeding (Rinbokunoikusyu) 1–4 (in Japanese).

Wakushima S, Yoshioka H. 1997b. Lateral female strobili production by BAP application III (a preliminary report). Fertility of seeds obtained from BAP-induced strobili. Ap For Sci Kansai 6:103–106(in Japanese with English summary).

Wakushima S, Yoshioka H. 1998. Promotion of female flowering by spray, mousse and lanolin paste application of benzylaminopurine (BAP) to three red pine clones at five differential stages. Bull Hiroshima Pref For Res Center 30:1–12(in Japanese with English summary).

Wakushima S. 1999. Promotion of female flowering in Japanese red pine (*Pinus densiflora*) and black pine (*Pinus thunbergii*) by 6-benzylaminopurine paste treatment to terminal bud in seed orchard. Bull Hiroshima Pref For Res Center 31:1–8(in Japanese with English summary).

Wheeler NC, Wample RL, Pharis RP. 1980. Promotion of flowering in the *Pinaceae* by gibberellins. IV. Seedlings and sexually mature grafts of lodgepole pine. Physiol Plant 50:340–346.

Young JA, Young CG. 1992. Seeds of woody plants in North America-revised and enlarged edition Oregon, USA: Dioscorides Press..