# TIMING OF MALE CONE INITIATION IN *PINUS RADIATA*

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## ABSTRACT

Initiation and early development of microsporangiate strobili (male cones) in *Pinus radiata* D. Don were studied during two growing seasons (1992/93 and 1996/97) at the Amberley Seed Orchard (43°10´S) in the South Island of New Zealand. Further collections were made at the Seddon Seed Orchard (41°42´S) and in a breeding archive located at Rotorua (38°10´S). Long-shoot buds from putative pollen-producing branches were collected from December to March from different clones and different-aged ramets. On one clone male cones were found on bicyclic shoots. In both years axillary buds, which would develop into male cones, had formed by mid-December. Generally, development was earlier on older ramets, but it also varied between clones, years, and locations. Development was earlier in 1992/93 than in 1996/97. In 1996/97, material collected in the Seddon Seed Orchard was about 1 month ahead of material from the Amberley Seed Orchard. Primordia developing into male cones appeared to start swelling very early. These shoots had far fewer basal cataphylls than did short shoots. Anatomically, male-cone, vegetative long-shoot, and female-cone primordia all go through a similar intermediate stage of development whence their ultimate fate might be altered by environmental factors or hormone applications.

**Keywords**: pollen induction; cone bud initiation; male strobili; phenology; morphogenesis; *Pinus radiata*.

## INTRODUCTION

With increasing demand for genetically improved breeds of trees that produce large volumes of wood with desirable intrinsic wood properties, it is important to understand the biology of cone production. Heavy cone crops in *Pinus radiata* have been associated with

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losses in wood production (Cremer 1992). For the production of genetically improved seed of pine, an adequate production of both female (megasporangiate) and male (microsporangiate) cones across genetically improved genotypes is critical (Owens & Blake1985). Promotion of male cone production offers several advantages to tree breeders. Pollen production is an effective means of transferring improved genes from one genotype across a broad range of select genotypes through controlled pollination. Clones that are recalcitrant female cone producers often produce pollen and thus ensure the contribution of their genes to a tree improvement programme (Shaf van Ballekom pers. comm.). To achieve rapid genetic gain, the breeding cycle may be shortened by the judicious application of cone-induction treatments that promote precocious cone production across both fecund and recalcitrant clones. Hormone application (Ross *et al.* 1984; Siregar & Sweet 1996; Sweet 1979) and/or exposure to physiological stress could routinely shorten the breeding cycle in Pinaceae. However, if such treatments are to succeed they need to be timed correctly, which in turn depends on a clear understanding of the time when cone-bud initiation occurs (Syamsuwida & Owens 1997).

Understanding the timing of male and female cone bud formation is also important for genetic engineering research, particularly for controlling sterility in order to prevent the escape of modified genes into wild populations and to prevent unwanted diversion of resources into reproductive effort (Selås *et al.* 2002). By understanding the "flowering trigger", timing of cone bud initiation and differentiation, and when these are influenced by cultural treatments, one may gain an insight into the action of flowering gene(s) and when and how it/they can be blocked (Mellerowicz *et al.* 1998).

There have been extensive studies on the formation of branch systems and female cones in *Pinus radiata* (Bollmann 1983; Bollmann & Sweet 1976, 1979; Sweet 1979). However, little work has been done on the development of male cones. Wang (1995) and Wang *et al.* (2000) reported a recent study on male cone development with special emphasis on the biochemistry of cone initiation and subsequent development of pollen grains.

This paper reports a study exploring the timing of male cone bud initiation in *P. radiata*. The objectives were to establish when male cone buds first become microscopically visible and to track their early development. This knowledge could assist with understanding when treatments may be applied to promote/inhibit the development of shoots that differentiate into male strobili.

## MATERIALS AND METHODS

Long-shoot terminal buds (LSTBs) were collected from branches bearing male strobili on random trees growing in the pollen archives at the Amberley Seed Orchard (43°10′S) located in Canterbury, New Zealand, on 16 December, 3 January, 1 February, and 18 February 1992/93. On 13 February 1993 material was collected from three clones (A, B, C) in the main portion of the seed orchard. In 1996/97 two other clones (D and E) growing in the Amberley Seed Orchard were selected for more detailed study. These clones consisted of a relatively good pollen source (Clone E) and a weaker pollen producer (Clone D). Ten LSTBs per clone were collected on 11 dates between 14 December 1996 and 19 March 1997, and on 6 June 1997. (*See* Dickson *et al.* (1999) for a brief description of establishment of trees in the Amberley Seed Orchard.) LSTBs were also collected from two clones (F and G) on

15 December 1996 in the Seddon Seed Orchard (41°42′S), located about 400 km north of the Amberley Seed Orchard, and from a single clone (H) on 22 November 1996 at Rotorua in the central North Island of New Zealand (Table 1).

TABLE 1–Sample information

| Collection/ clone | Seed orchard | Ramet age (years) | Collection dates |
|---|---|---|---|
| Archive | Amberley (43°10′S) | >7 | December 1992–March 1993 |
| A, B, C | Amberley | 2 | 13 February 1993 |
| D, E | Amberley | 7 | December 1996–June 1997 |
| F, G | Seddon (41°42′S) | 7 | 15 December 1996 |
| H | Rotorua (38°10′S) | – | 22 November 1996 |
| I | Amberley | 2, 3, 4 | 19 February and 31 March 1993 |

All material was killed and fixed in formalin-acetic acid-alcohol (FAA). Some of the buds were dissected and photographed; the rest were processed through a tertiary-butyl alcohol series, embedded in paraplast, and sectioned longitudinally at 10μm on a rotary microtome. Sections were stained in safranin-fast green (Johansen 1940) or toluidine blue (Berlyn & Miksche 1976). Photographs were taken on a Zeiss Universal Microscope on Agfa APX - 25 film.

Initial development of male cones was contrasted with development of vegetative long-shoots and female cones from Clone I, collected at Amberley on 19 February and 31 March 1993 (Dickson *et al.* 1999).

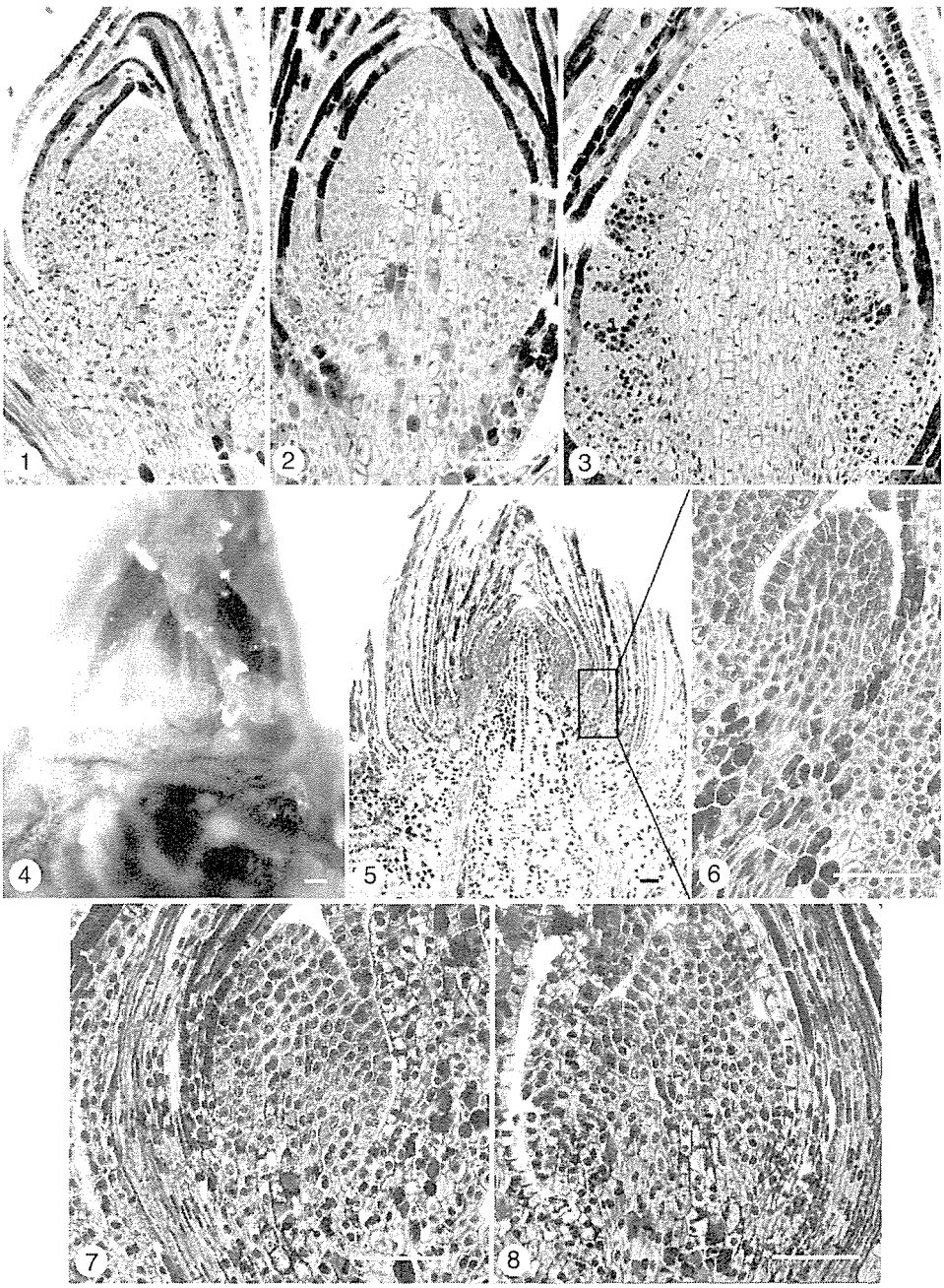
## RESULTS

### Initiation of Male Cones

Collections in different years and locations indicated that cone development is dependent upon clone, ramet age, year, and location. At Amberley on 13 February 1993, on 2-year-old ramets, LSTBs of Clone A were vegetative (Fig. 1) while those from Clones B (Fig. 2) and C (Fig. 3) were in different early stages of male strobili production. Male strobili in the older pollen archive ramets (over 7 years old) were producing sporogenous tissue on 18 February 1993.

Development of cones was earlier in the 1992/93 collections than in the 1996/97 collections from 7-year-old ramets (Clones D and E) at Amberley. In 1996/97 the material from Seddon (Clones F and G) collected on 15 December was similar to the material from Amberley on 8 February.

In field observations on 6 June 1997, it was found that the male cones on Clone D tended to be on bicyclic branches rather than monocyclic branches. As the collections from Clones




All sections are longitudinal sections cut at 10 μm and stained with either safranin-fast green or toluidine blue. The bars are 100 μm. Abbreviations: C = cambium; P = phloem; PX = primary xylem; X = differentiating xylem; SX = secondary xylem.

FIG. 1–Near-median section of a short shoot from the base of a long-shoot terminal bud (LSTB) from Clone A collected in Amberley on 13 February 1993.

FIG. 2–Near-median section of a microsporangiate strobilus from the base of a LSTB from Clone B collected in Amberley on 13 February 1993. Sporophylls are just beginning to form on the flanks of the primordia.

FIG. 3–Near-median section of a microsporangiate strobilus from the base of a LSTB from Clone C collected in Amberley on 13 February 1993. Sporophylls are being initiated up the flanks of the primordia.

FIG. 4–Dissection of a LSTB from Clone H collected in Rotorua on 22 November 1996, showing the presence of axillary primordia.

FIG. 5–Near-median section of a LSTB from Clone H collected in Rotorua on 22 November 1996. Although the bud is still expanding, axillary primordia are evident in the axils of some basal bracts.

FIG. 6–Enlargement of axillary bud from Fig. 5. Definitely off-median; however, it shows that cataphylls are forming on the axillary bud and that vascular tissue to the subtending bract is becoming lignified (white arrow).

FIG. 7–Near-median section of axillary primordium from Clone E collected in Amberley on 15 December 1996. Provascular tissue to the subtending bract is evident to the left of the bud. This bud has initiated some cataphylls and is beginning to broaden and is most likely a developing microsporangiate strobilus.

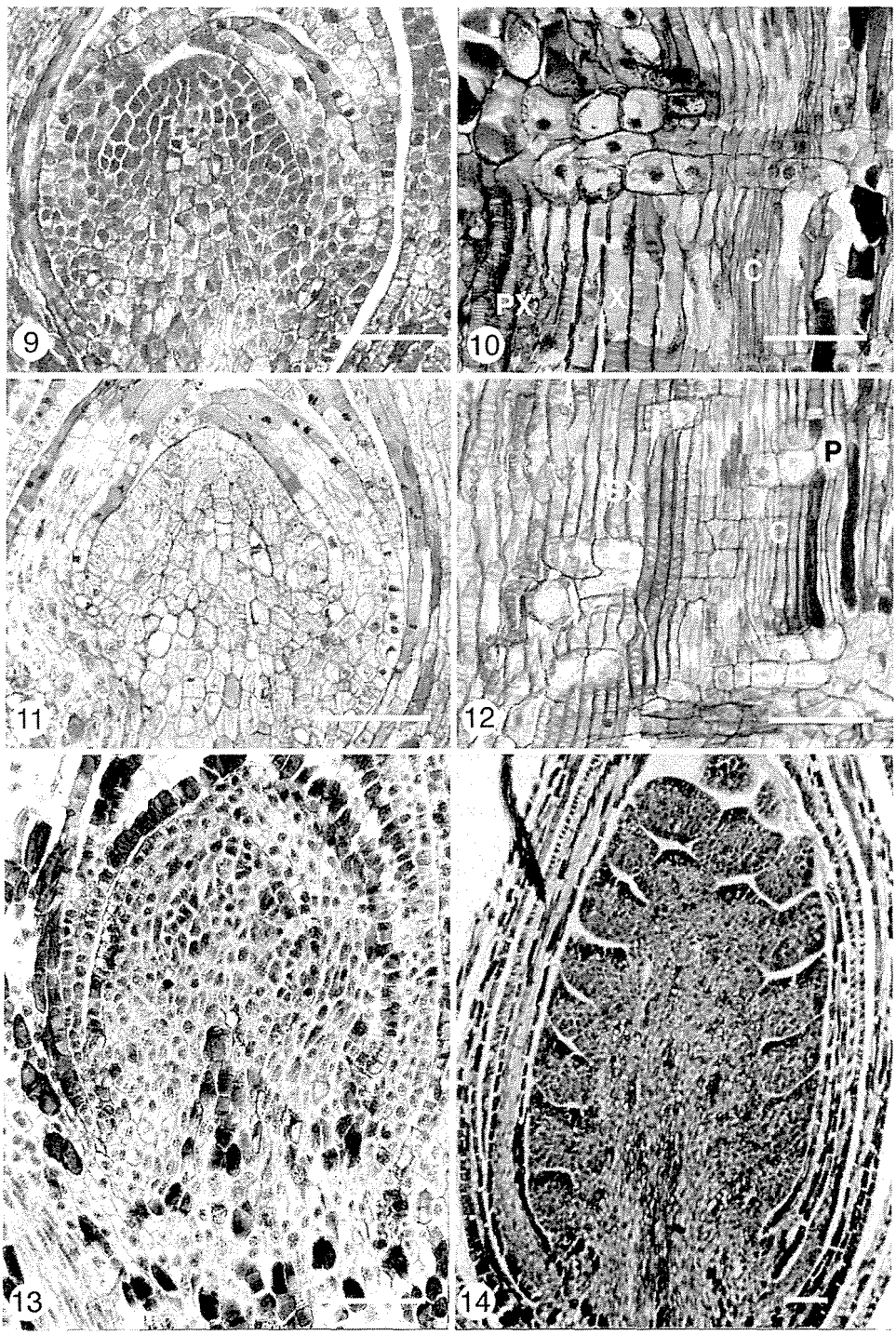
FIG. 8–Near-median section of an axillary bud from Clone D collected in Amberley on 15 December 1996. Provascular tissue is evident going to the subtending bract on the right. This bud is fairly narrow and is most likely a developing short shoot.

D and E happened to have been made only on monocyclic branches, most of the buds from D were vegetative.

Axillary primordia were evident in all the buds collected in Rotorua (Clone H) on 22 November 1996 (Fig. 4, 5, 6). The buds were in the axils of bracts that had provascular tissue going into them (Fig. 5 and 6). There was cataphyll formation on axillary primordia in some buds. Where this occurred there was secondary wall formation and lignification on some of the tracheary elements leading to the subtending bracts (Fig. 6). The apical meristems of the LSTBs were still very elongated, indicating they were in a period of bud extension and rapid bract formation (Fig. 5). All material collected in December 1996 had axillary buds with cataphylls in the axils of the lower bracts above the region of sterile cataphylls. The number of cataphylls varied between the axillary buds. Within a LSTB the basal axillary buds were more developed. Generally, the axillary buds from Clone E, putatively developing as microsporangiate strobili, had a more zonate appearance than those from D, putatively developing as short shoots (Fig. 7, 8).

Many axillary primordia in buds collected at Seddon on 15 December 1996 (Clones F and G) had three or more cataphylls present in median section and had started to swell (Fig. 9) approaching the "bullet" stage (Wang 1995). In 1997, however, male strobili from Clone E in Amberley did not reach this stage of development until February. Yet in 1993, male-cone primordia in the Amberley open-pollinated seed orchard were at a similar stage of development on 3 January (cf. Fig. 9, 11, 13). Development of male-cone primordia at Amberley in 1997 was very rapid during February, going from the bullet stage to cone primordia with incipient sporogenous tissue in 4 weeks (Fig. 13, 14). Development from the bullet stage to cones was equally rapid in 1993; the main difference was that in 1993 the cone primordia on 18 February were similar to those of Clone E on 19 March 1997.

The rapid differentiation of the male cones occurred after the completion of elongation of the portion of the shoot subtending the LSTB. Evidence that elongation was completed




Captions on facing page

FIG. 9–Near-median section of a developing axillary primordium from the base of a LSTB from Clone F, collected in Seddon on 15 December 1996. Based on its shape (relatively broad sub-apical zone) and position in the LSTB, this is a developing microsporangiate strobilus.

FIG. 10–Longitudinal section through a vascular trace just below the LSTB from Clone F, collected in Seddon on 15 December 1996, showing pitted tracheids and activity of the vascular cambium indicating that extension of the subtending shoot was completed at the time of harvest.

FIG. 11–Near-median section of a developing microsporangiate strobilus from a LSTB collected from the pollen archives at Amberley on 3 January 1993.

FIG. 12–Longitudinal section of a vascular trace just below the LSTB containing the primordium pictured in Fig. 11. Secondary xylem is very evident in this shoot. Expansion of the basal internodes in the LSTB was just beginning.

FIG. 13–Near-median section of a developing microsporangiate strobilus from the base of a LSTB of Clone E, collected in Amberley on 8 February 1997. This bud is almost at the same stage as Fig. 9, a little younger than the one in Fig. 11.

FIG. 14–Off-median section of a microsporangiate strobilus collected from the base of a LSTB of Clone E, collected in Amberley on 4 March 1997, showing sporophylls with developing sporangia.

included the presence of pitted tracheids and the beginning of cambial activity immediately below the bud (cf. Fig. 10, 12). This was accompanied by expansion at the base of the LSTB which included internode expansion of the main axis and basal axillary primordia (Fig. 15, 18). Maturation of microsporangiate strobili occurred in acropetal sequence (Fig. 16, 17) and was more rapid than development of short shoots on vegetative branches (Fig. 19).

Needle elongation on the main axis below the LSTB and expansion of the LSTB continued to take place throughout this developmental period, although the rate of bud extension declined. On 19 March 1997 the shoot apex in LSTBs was still producing needle/cataphyll/bract primordia; however, no axillary buds were forming, indicating that bud scales for the next year were being initiated. It would appear that the shift from male-cone determination to short-shoot determination occurred at the end of January in 1997. Sampling in 1992/93 was not frequent enough to infer a date for the switch.

## Determination of Long-shoot Primordia

It was observed that axillary primordia in *P. radiata* followed a definite developmental sequence. The initial primordia could develop as any of the four lateral structures: male cones, short shoots, female cones, or vegetative long-shoots. On male branches the first-formed primordia immediately started to swell, leading to the formation of an intermediate meristem (Fig. 9, 11, 13).

For vegetative branches and female branches, the initial primordia remained in a fairly early stage of development, forming cataphylls but not swelling; these shoots developed into short shoots. After a period of short-shoot production, newly formed primordia underwent lateral expansion or swelling as soon as they formed, giving rise to a cluster of lateral vegetative long-shoots (Fig. 21, 23) and/or female-cone primordia (Fig. 20, 22). The vegetative long-shoots, developing from the primordia closest to the shoot tip, appeared to undergo precocious development. They proceeded through an intermediate stage of expansion and elongation (Fig. 21) leading to the formation of needles/bracts and short shoots.

The female-cone primordia, arising from the basal primordia in a cluster, developed to an intermediate stage (Fig. 20) and then development slowed for a period. This was followed

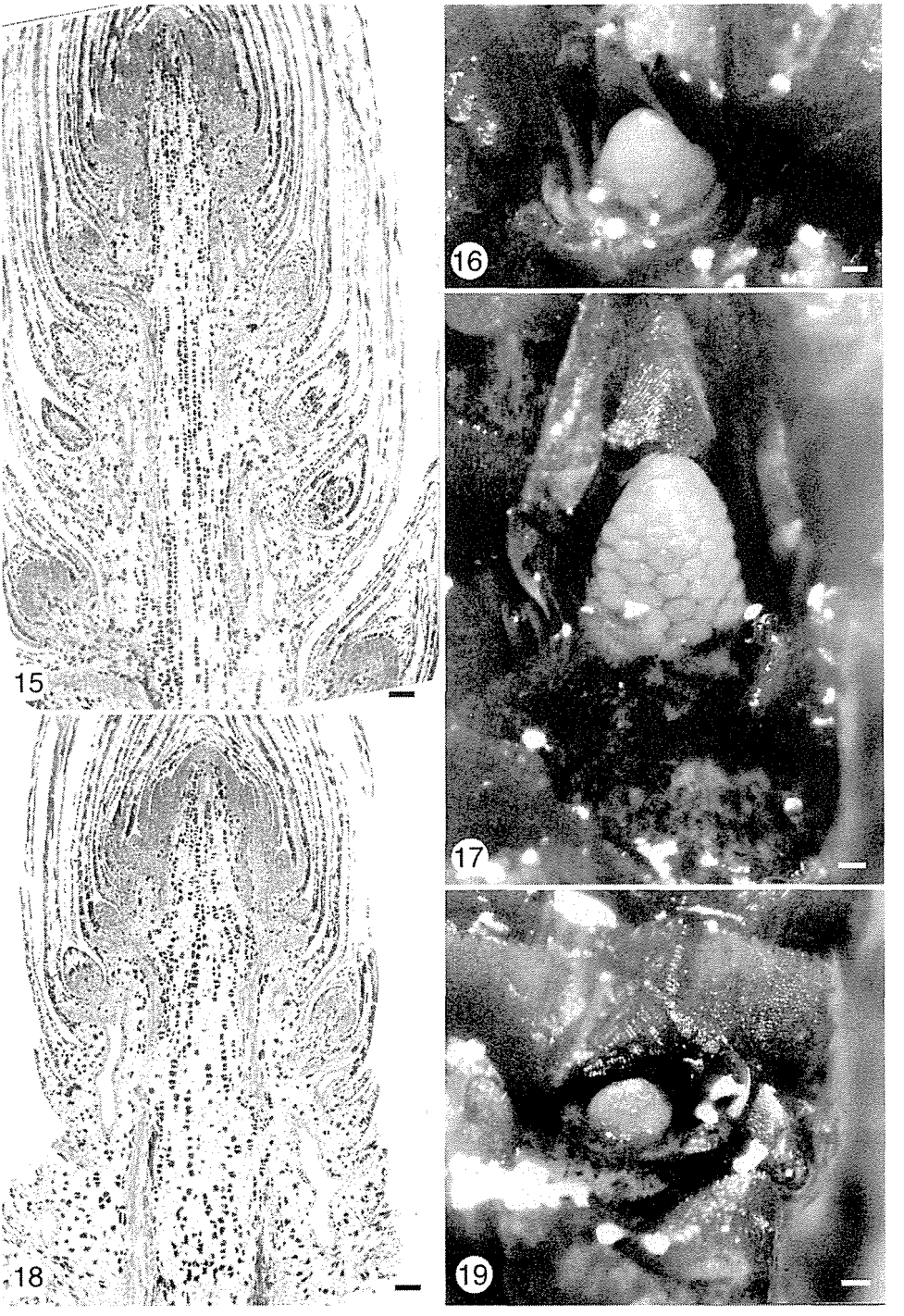



FIG. 15–Near-median section of a LSTB from Clone E, collected at Amberley on 18 February 1997. This section includes only the top of the bud to the region of microsporangiate strobili. Near-

median sections of developing strobili are present at the bottom of the figure. Note that the meristem is still quite pointed and is producing bract primordia.

FIG. 16–Dissection of a microsporangiate strobilus from the top of the zone of microsporangiate strobili from a LSTB similar to that pictured in Fig. 15. The cone would be similar to the ones at the bottom of that picture.

FIG. 17–Dissection of a microsporangiate strobilus from the base of the zone of microsporangiate strobili from a LSTB similar to that pictured in Fig. 15. Note that this cone is more developed than Fig. 16, illustrating the acropetal development of primordia along the shoot axis.

FIG. 18–Near-median section of a LSTB from Clone D collected at Amberley on 18 February 1997. This section includes the whole bud. The meristem is still quite pointed and producing bract primordia.

FIG. 19–Dissection of a short shoot from the base of LSTB similar to that pictured in Fig. 18. Note that the needles have been initiated (small protuberances on the sides of the primordium) but have not extended beyond the tip of the meristem.

by a very rapid development of a megasporangiate strobilus (Fig. 22). The major difference between the vegetative branch and megasporangiate strobilus in early differentiation was the broadening of primordia developing as female cones and an expansion of the height of the apical dome.

From sections of buds it appeared that the determination of reproductive or vegetative long-shoots compared to short shoots occurred very close to the time of initiation of the primordia and was reflected by the swelling of primordia immediately following initiation (cf. Fig. 11, 20, 21). The further development of primordia into a particular type of long shoot could be determined after the formation of an intermediate stage.

In vegetative branches this intermediate stage was followed by a period of rapid primordia formation and the differentiation of pro-vascular tissue (Fig. 23). In the reproductive axes differentiation of vascular tissue was delayed.

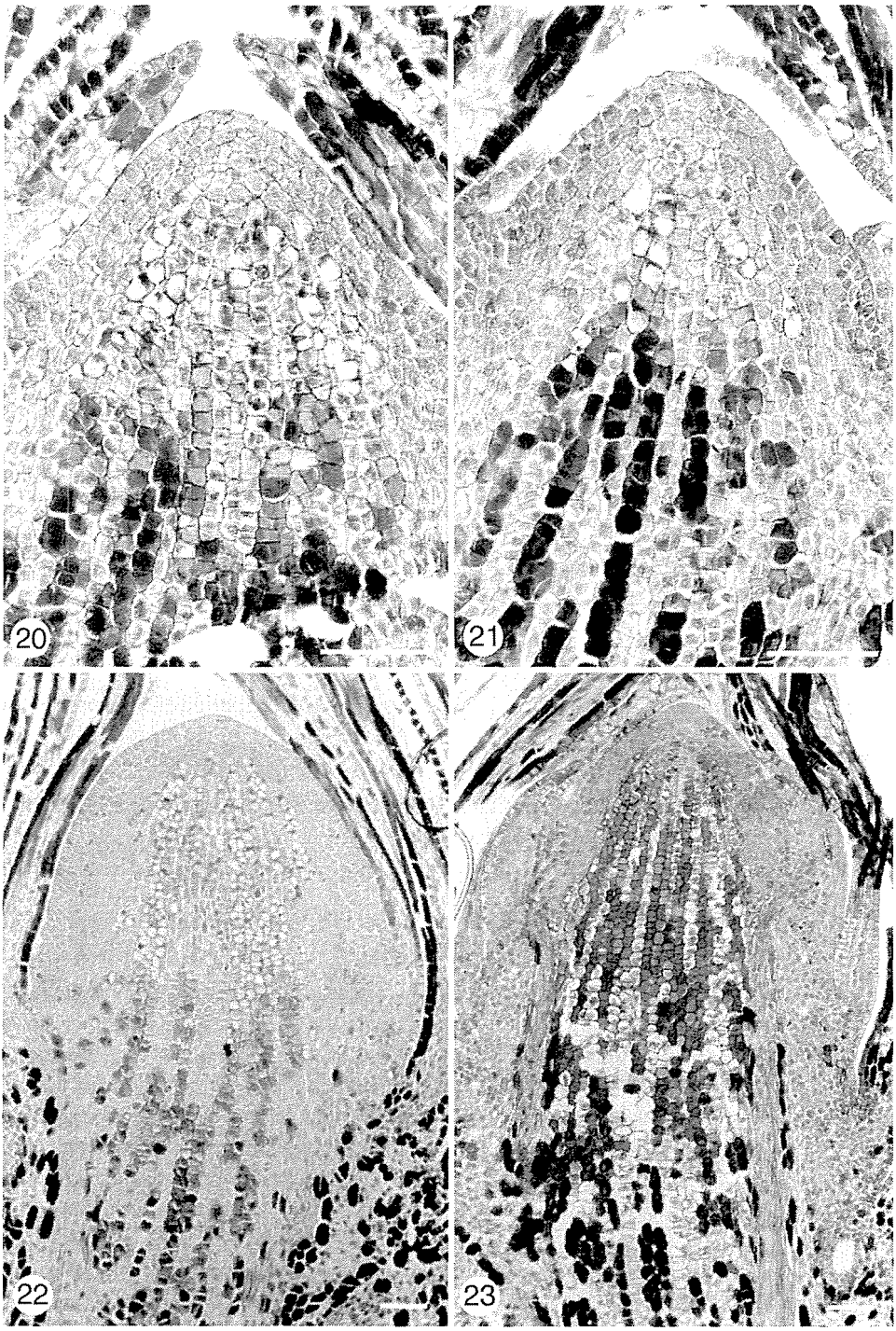
In female-cone primordia more cell divisions occurred, increasing the diameter of the primordia before elongation of the axis (Fig. 22).

In male cones the intermediate stage was followed immediately by a period of sporophyll formation (Fig. 13, 14).

## DISCUSSION

At early stages of lateral-shoot development male cones, vegetative long-shoots (branches), and female cones all go through an initial phase of broadening (swelling) of the axillary meristem which yields an intermediate condition, referred to as the bullet stage by Wang (1995) and Wang *et al.* (2000). Bollmann (1983) made a similar observation for female strobilus initiation in *P. radiata*. In vegetative branch initials the intermediate stage was followed by a period of rapid primordia (bracts and short shoots) formation and differentiation of pro-vascular tissue. In the female cone primordia cell divisions continued, increasing primordium diameter and height, before initiation of bracts and ovuliferous scales.

In the male cones sporophyll formation was pronounced after the intermediate stage. Generally, the pattern of differentiation of short shoots and male strobili followed that described for *P. contorta* Loudon and *P. monticola* D. Don (Owens & Molder 1975, 1977). However, there appeared to be differences between short shoots and male strobili very shortly after initiation of the axillary primordia. Those developing into male cones expanded




Figures 20 to 23 from Clone I (cf. Dickson *et al.*1999)

FIG. 20–Median section of an axillary shoot from the first cycle of long-shoot primordia from a 1990 ramet of Clone I collected in Amberley on 18 February 1993. This is most likely a megasporangiate strobilus primordium. Note the apical portion of the primordium is beginning to broaden as compared to the bud in Fig. 21.

FIG. 21–Median section of an axillary shoot from the first cycle of long-shoot primordia from a 1990 ramet of Clone I collected in Amberley on 18 February 1993. This is putatively a developing vegetative shoot, based on shape and location within the LSTB.

FIG. 22–Median section of a megasporangiate cone primordium from the first cycle of long-shoot primordia from a 1989 ramet of Clone I collected in Amberley on 18 February 1993. Bracts are beginning to form up the flanks of this primordium. The primordium is wide and there is little evidence of provascular tissue.

FIG. 23–Median section of a vegetative shoot primordium from the first cycle of long-shoot primordia from a 1989 ramet of Clone I collected in Amberley on 18 February 1993. Bracts have formed up the flanks of the meristem and axillary buds are being initiated in the axils of some of the basal scales. Provascular tissue is evident in the main axis and extending to some of the scales.

laterally early and produced very few cataphylls compared with short-shoot primordia. The initial steps in development were not rapid and appeared to be coupled with development of the main axis and the formation of scale primordia.

Development of the male cones appeared to be associated with internode development of the main axis as the axillary primordia expand. This follows completion of elongation of the subtending shoot, i.e., the axis below the LSTB. There was no distinct break in development from the time of initiation until the cones formed sporophylls. Within a LSTB there was an acropetal gradient in development of male cones, as reported by Wang (1995). This being the case, it is possible that the fate of a primordium is determined before there are visible signs of axillary bud initiation. This is partially supported by the observation that a LEAFY gene homologue (PRFLL) is expressed in buds of *P. radiata* in December before there is sure microscopic identification of male cone primordia (Mellerowicz *et al.* 1998). That male cones may be determined even before they are initiated is also supported by the observation that the production of male cones is enhanced in pine, spruce, and larch by application of gibberellin before there is any evidence of differentiation (Cecich 1983; Eysteinsson & Greenwood 1995; Smith & Greenwood 1995).

Considering the observation that determination of the fate of axillary shoots occurs very close to the time of initiation, it is proposed that for optimal male-cone development in *P. radiata* strobilus-promoting treatments should be applied in late spring before the cessation of shoot elongation and the start of initiation of axillary buds. After this time, additional treatments or stimuli to enhance the number of axillary primordia that continue to develop as male cones might be applied later in the season. For example, late summer (end of February through the beginning of March) gibberellin applications have been found to substantially promote female strobilus production across selected clones in *P. radiata* meadow tree seed orchards in New Zealand (Ross *et al.* 1984).

It is reasonable to assume that small physiological changes during an intermediate phase could regulate the production of the different cones or long shoots. Indeed, Doak (1935) reported environmental induction of female cones in the basal region of pine shoots where male cones normally develop. Further, development of primordia into particular structures may be altered after the intermediate stage. Dickson (1995) found that for *P. radiata* the numbers of strobili and other types of long shoots were not "fixed" in the early pattern of

differentiation. During the months of May to July inclusive, site and climate substantially modified the April pattern of differentiation; and warm temperatures may have induced death of cone primordia (terminal hypertrophy). Differentiating strobili modified their course of development to become branches or latent buds. It was found that the transfer of potted trees in the summer from coastal sites, where they had initiated seed cone buds, to cooler semi-continental sites caused a loss of seed-cone buds, with most of them ending up as latent buds. Transferring to a heated glasshouse caused the greatest loss of strobili, with all the differentiating buds switching to either branches or latent buds. Burdon (1977) found that production of pollen cones could be altered by elevated temperatures in a glasshouse, with cones forming throughout the growth period independent of photoperiod.

It was apparent from the present study that there were significant differences in the timing of cone initiation between years and sites. The timing of male-cone initiation in 1992/93 was found to be a month ahead of that in 1996/97. With regard to site it was found that bud development in the warmer northern sites of New Zealand was 1 month ahead of that in the cooler South Island seed orchard. The age and size of ramets have also been reported to affect the timing of seed cone bud initiation (Dickson *et al.* 1999). This effect was also reflected in the time of emergence of the strobili in the spring. Thus, it is suggested that the timing of application of strobilus-promoting treatments should be based on a morphological calendar rather than the Gregorian calendar.

Further research is proposed to equate the microscopic observations to timing of strobilus-promoting treatment application. It is also suggested that changes in hormonal content and expression of LEAFY gene homologues should be monitored across clones of *P. radiata* in order to establish which treatments are likely to stimulate cone development or delay the transition from male-cone determination to short-shoot determination.

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