CHAPTER 4

Promotion of shoot formation in *Clivia miniata* (Lindley) Regel with paclobutrazol and Promalin™

4.1 Summary

Propagation of superior clones of *Clivia* occurs through division of basaly produced suckers which is relatively slow or by tissue culture which is not always readily available to small scale growers. This study investigates the use of paclobutrazol (PAC) and Promalin™ (PRO) (benzyl adenine + GA_4 + GA_7) as a foliar spray to promote shoot formation. It was found that PAC promoted bud formation in the axils of older proximal leaf bases and the mean number of shoots produced at concentrations between 250 and 25 000 ppm PAC varied from 2.3 to 7.1 without any statistically significant difference among treatments, 7 months after application. However, at concentrations of 5 000 ppm and higher, growth inhibition was unacceptable and death of the parent plant occurred in some individuals due to abortion of the apical meristem. GA_3 applied 3 times fortnightly, at 500 ppm, appeared to be useful in alleviating growth inhibition caused by PAC, but the effect could not be quantified. It was found that PRO also promoted branching, but from the axils of younger, distal leaf bases. PRO also resulted in dichotomous branching of apical meristems. When applied 10 times, at either 200 or 500 ppm active ingredient, PRO resulted in acrotonic branching of 50% of treated plants into 2 or 3 modules. However, the latter results could not be analysed statistically. The most significant benefit arising from the use of PRO was survival of the parent plant without any inhibition of growth.

4.2 Introduction

Clivia miniata can be vegetatively propagated by division of suckers. The rate at which suckers are produced varies from clone to clone and is often not satisfactory. Published tissue culture methods are still imperfect, resulting in varying degrees of success (Wang, LI & Yang, 1995, Finnie, 1998, Chapman, 1999). In addition, tissue culture facilities are not within reach of many amateur growers who are in possession of desirable clones. This investigation was undertaken to determine whether the plant growth regulators paclobutrazol and Promalin™ could be used as a foliar spray to promote shoot formation and branching in *Clivia*.

Paclobutrazol (PAC) has been used in *in vitro* propagation to stimulate shoot formation in a range of plants such as *Nerine* sp. (Ziv, Kahany & Lilien-Kipnis, 1994), *Gladiolus* sp. (Nagaraju, Parthasarathy & Bhowmik, 1997) and *Tulipa* (Kuijpers & Langens-Gerrits, 1997). PAC applied as a foliar spray resulted in production of side shoots in *Cordyline* sp. (Higiladi & Watad, 1992) and when applied to *Clivia* to reduce plant size, caused the same effect (Van Huylenbroeck, 1998). However, in the latter work, a wide range of concentrations was not tested and the yield of shoots was relatively low. PAC is a plant growth regulator which inhibits the synthesis of gibberellic acid (Grossmann, 1990). However, in *in vitro* culture of potato, growth inhibition could be reversed by using GA_3 in the medium (Simko, 1993).

Benzyl adenine (BA) can also be used to stimulate shoot formation. This was achieved in *Cordyline* sp. (Maene & Debergh, 1982) and geranium (Foley & Keever, 1992). In *Spathyphyllum* sp. grown *in vitro*, shoot induction by BA was dramatically enhanced in the presence of imidazole fungicides. As with PAC, the latter effect was obtained by inhibition of GA_3 production (Werbrouck & Debergh, 1995, Werbrouck *et al.*, 1996). PRO (GA_4 + GA_7 + BA; 19g / l active ingredient) is registered in South Africa for the promotion of branching in apples (Vermeulen, Grobler & Van Zyl, 1997).

4.3. Materials and methods

4.3.1 The effect of paclobutrazol

PAC (Cultar™; 250g / l active ingredient) was applied to flowering size plants as a foliar spray until run off, at concentrations of 1, 2, 4, 10, 20, 50 and 100ml Cultar™ / l, corresponding respectively to 250, 500, 1 000, 2 500, 5 000, 12 500 and 25 000 ppm a.i. Eight replicates per treatment were used. The number of shoots formed was then recorded and the statistical analysis comprised a regression analysis of the number of shoots formed as a function of concentration. An analysis of variance (ANOVA) using Tukey's least significant difference was done to determine whether the mean number of shoots differed significantly among the treatments ($\alpha = 0.05$).

4.3.2 Alleviation of PAC induced growth inhibition by GA_3

A small number of plants (8) were used to determine if the growth retarding effect of PAC could be alleviated by GA_3. Ten months following a PAC spray at 25 000 ppm, GA_3 (Berelex™

100g / kg a.i.), at a concentration of 500 ppm, was applied to 4 of the plants treated with PAC. Three applications were made; a single spray until runoff, every fortnight, for six weeks. The plants treated with PAC had produced a large number of stunted, basal shoot primordia.

4.3.3 Promalin™ application through the leaves

In a second set of flowering size plants, a foliar spray of PRO until runoff, was applied during April when plants were not in flower. Concentrations of 5.3, 10.4 and 26.3 ml PRO / l were used (corresponding respectively to 100, 200 & 500 ppm a.i.). Ten applications were made; one application every second day, for 20 days. Eight replicates of each treatment were used. The number of plants which reacted was then recorded.

After observation of the effects of the first PRO application, PRO was applied to a third set of plants for the purpose of an anatomical investigation (section 4.3.5). A concentration of 375ml PRO / l (7 125 ppm a.i.) was applied to small number of seedlings at the six leaf stage. The solution was painted onto the crown and leaf axils of each plant with a paint brush, wetting the areas until runoff. Two application regimes were used; a single application per plant and three applications per plant. In the case of three applications, one application was given fortnightly, for six weeks. At various intervals, plants were harvested for anatomical investigation.

4.3.4 Promalin™ application through the roots

In a forth set of plants, seedlings at the 6-8 leaf stage, an attempt was made to apply PRO via the root system. All soil was washed from the roots before plants were placed in an aerated water medium (hydroculture). The nutrient solution comprised Chemicult™ hydroponic nutrient powder at the rate of 0.5g / l. After 1 month, a PRO spray was applied to the roots at concentrations of 0, 4 750 and 7 125 ppm a.i. comparing one and three applications. In the case of three applications, roots were sprayed once every evening, for three days. After removing plants from hydroculture in the early evening and allowing them to dry for 10 minutes, PRO was sprayed onto roots until runoff. After spraying, roots were covered in a plastic bag to prevent evaporation, left overnight and returned to hydroculture in the morning. After the last spray, plants were replanted in a decomposed bark medium. Each treatment was replicated eight times.

4.3.5 Anatomical investigation

An anatomical investigation was conducted to elucidate shoot architecture and to identify the origin of shoots produced after PRO and PAC treatments. Material was prepared by fixation in formalin acetic acid alcohol followed by dehydration in sequential alcohol and alcohol xylene mixtures. After embedding in paraffin wax, microtome sections were cut and stained with toluidine blue (O'Brien & McCully, 1981).

4.4 Results and discussion

4.4.1 The effect of paclobutrazol

Ten months after the application of a 2 500 ppm PAC foliar spray, a clearly visible reduction in plant height could be seen (Figure 4.1). This was accompanied by branching in the older proximal axils of leaf bases (basitonic branching) which was especially prolific at higher concentrations. At and above concentrations of 5 000 ppm PAC, complete and near complete disintegration of the parent plant and much of the root system occurred in some individuals (Figure 4.2). However, it follows that the viability and probability of survival of shoots on plants compromised to this extent would be much reduced. It was interesting to note that PAC caused the death of the apical meristem, but stimulated bud formation in the meristematic zones in the axils of basal leaves (Figure 4.10).

Seven months after PAC application, a relationship between PAC concentration and the number of shoots formed could be found and was given by $S = 2.799 + 0.179\ k - 0.001\ k^2$ where S and k represented the number of shoots and PAC concentration, in ml Cultar / l water, respectively (Figure 4.3). All three terms were highly significant ($Pr > t$; <0.0001, <0.0007 and <0.0044 respectively). A low value for R^2 (0.22) was obtained and indicates that there were other factors which also played a role in the number of shoots formed. Since the plants used were raised from seed, genetic variation is likely to have played a role. This was true for the response of different cultivars of *Gladiolus* to PAC (Nagaraju *et al.*, 1997). The ANOVA indicated that the number of shoots formed at all PAC concentrations differed significantly from the control, but not from one another (Table 4.1).

The effect of PAC on flowering was negative because of death of the apical meristem. Furthermore, existing inflorescences disintegrated completely at concentrations of 5 000 ppm and higher and where this did not occur, peduncles were unacceptably short.

Figure 4.1 Reduction in plant height 11 months after application of 2 500 ppm paclobutrazol (front) compared to the untreated control (back).

Figure 4.2 Disintegration of the parent plant and root system 13 months after application of 25 000 ppm paclobutrazol.

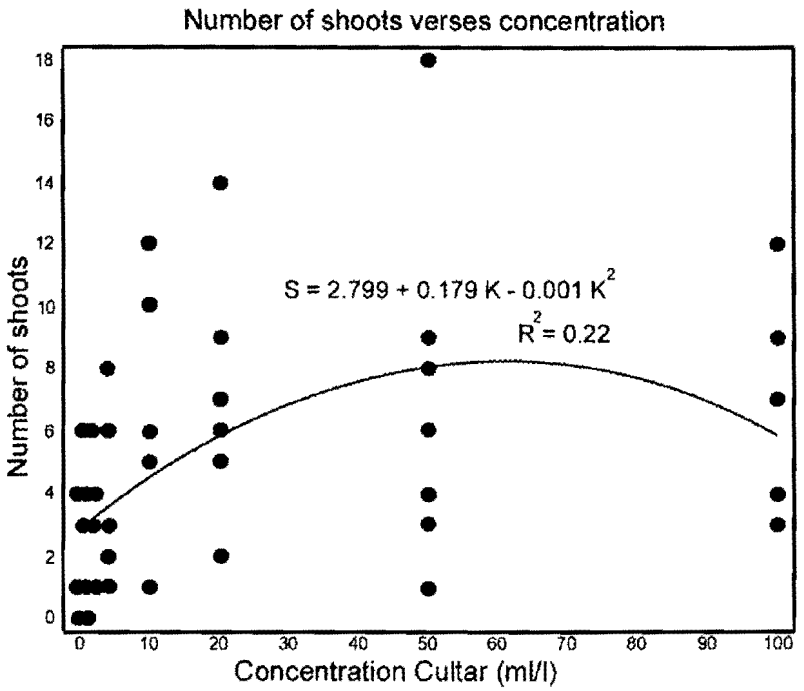

Figure 4.3 Regression analysis of number of shoots as a function of paclobutrazol concentration (ml Cultar™ / l water). $R^2 = 0.22$.

Table 4.1 Mean number of shoots as a function of paclobutrazol (PAC) concentration (ml Cultar™ / l), seven months after application. Means with the same letter do not differ significantly. $\alpha = 0.05$, Tukey, n = 64.

ml Cultar™ / l	0	1	2	4	10	20	50	100
Mean number of shoots	1	2.3[a]	3.3[a]	3.5[a]	7.1[a]	6.5[a]	6.6[a]	6.1[a]

4.4.2 Alleviation of PAC induced growth inhibition by GA_3

It appeared that the 3 applications of GA_3 at 500 ppm alleviated the inhibition caused by PAC to some extent (Figure 4.4). It was felt that the use of GA_3 could have practical benefits but its effect could not be quantified or statistically analysed.

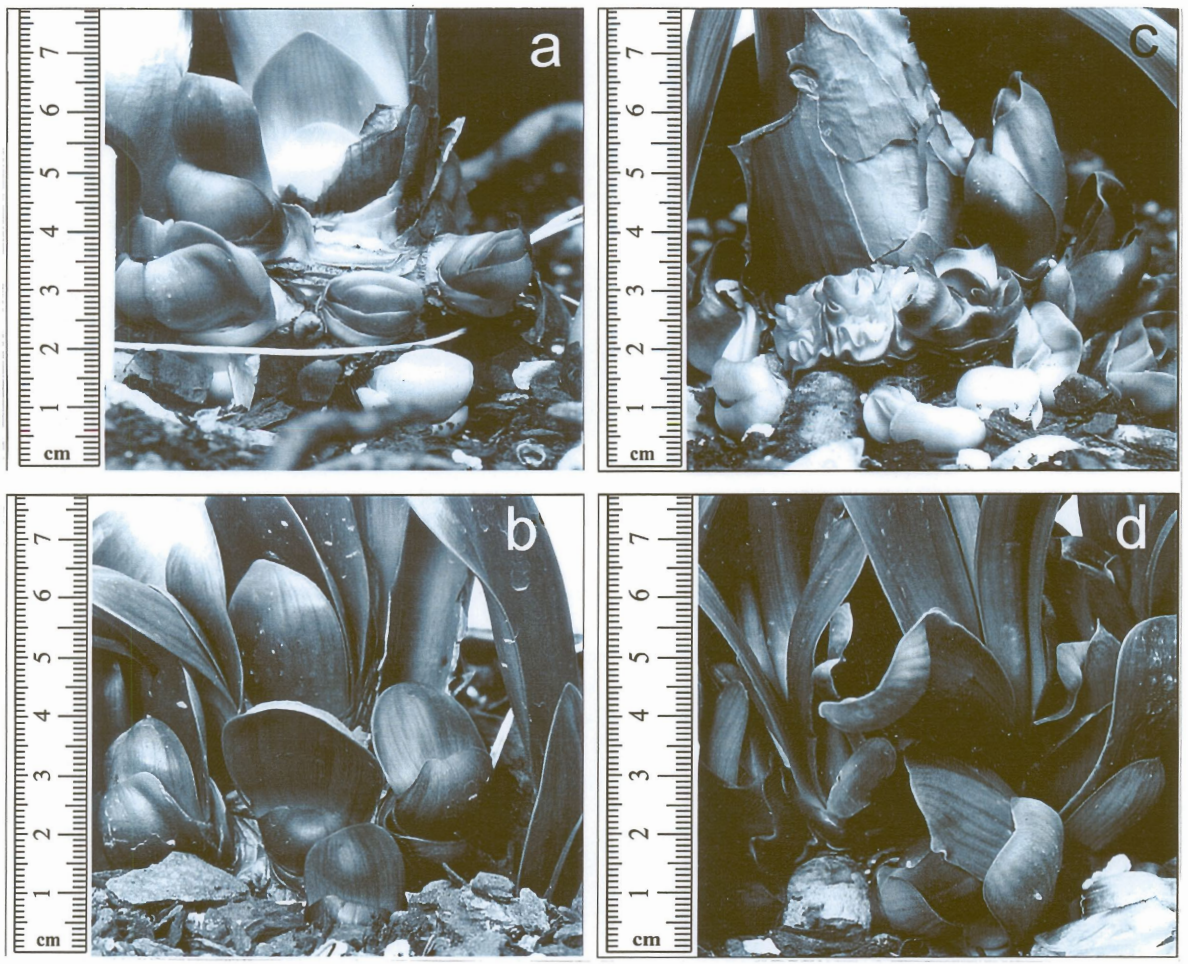

Figure 4.4 Alleviation of growth inhibition after application of 25 000 ppm paclobutrazol. Ten months after application of PAC, following formation of shoot primordia, 3 fortnightly applications of GA_3 at 500 ppm were made. Photo taken 2 months after the last application of GA_3.

a: Plant no.1 before, without GA_3. **c:** Plant no. 2 before, with GA_3.

b: Pant no.1 after, without GA_3. **d:** Plant no. 2 after, with GA_3.

4.4.3 Promalin™ application through the leaves

The result of PRO application was branching of treated plants in the region around the apical meristem. Shoots formed in this way were different in morphology to those formed by PAC in the sense that leaf size and shape were normal (Figure 4.5). One year following the 10 applications of PRO at 0, 100, 200 and 500 ppm, the percentage of plants exhibiting formation of additional shoots was 0%, 25%, 50% and 50% respectively. However, these results could not be statistically analysed. The most significant benefit which arose from the use of PRO was that there was no inhibition of vegetative growth or destruction of the parent plant. However, flowering was negatively affected and deformed flowers could be observed when using 100, 200, 300 or 7 125 ppm PRO (Figure 4.6). The excessive formation of green tissue in the perianth probably occurred because cytokinins promote chloroplast development and chlorophyl synthesis (Salisbury & Ross, 1992) while the abnormal thickness of the tissue could be due to cell proliferation and expansion caused respectively by BA and gibberellins in PRO. A degree of green colouration sometimes occurs normally in certain individuals of *Clivia*.

4.4.4 Promalin™ application through the roots

PRO at 7 125 ppm a.i. proved to be phytotoxic when applied the roots. When applied at 7 125 ppm a.i., 100 % of individuals exhibited necrosis of the root system within two weeks, followed by gradual death of the entire plant. Phytotoxicity could also be observed, to a lesser extent, at 4 750 ppm a.i. Table 4.2 shows the number of plants surviving at the various application rates, 4 months after the last PRO application. From the first experiment in which PRO was applied through the leaves (section 4.3.3), it was apparent that visible branching could only be detected approximately 1 year after application. At the date of publication it was not possible to determine whether PRO application through the roots had achieved this.

Table 4.2 Percentage of plants surviving 4 months after application of PRO to the roots once (1x) and 3 times (3x) at concentrations of 4 750 and 7 125 ppm a.i.

PRO (ppm a.i.)	0	4 750; 1x	4 750; 3x	7 125; 1x	7 125; 3x
% survival	100	100	62.5	87.5	0

Figure 4.5 New (1 & 3) and original (2) modules, 1 year following10 applications of 500 ppm a.i. Promalin™.

Figure 4.6 Abnormal flower development (bottom) compared to control (top), after a single foliar application of 7 125 ppm a.i. Promalin™, before emergence of the inflorescence.

4.4.5 Anatomical investigation

In an untreated mature plant, Figure 4.7 depicts the development of a new module adjacent to the old one which has terminated in an inflorescence. This suggests a modular, sympodial plant architecture (Chapter 6). Potentially meristematic zones are located in leaf axils, on the abaxial side of leaf bases, but no differentiated axillary buds are present (Figure 4.8). These meristematic zones are believed to be the source of new modules produced when a PAC treatment is applied. This would be consistent with the findings that PAC enhanced meristem formation on stem explants of *Tulipa* (Kuijpers & Langens-Gerrits, 1997). Figure 4.9 indicates these zones accentuated in a seedling, 5 months after treatment with 5 000 ppm PAC while Figure 4.10 indicates a new bud forming in the most proximal axil of another seedling following the same treatment. It is evident that the bud is orientated downwards and this is consistent with the "U" shape which can be observed in suckers attached to the parent plant. At high PAC concentrations, it is believed that bud formation from proximal axillary meristems occurs continuously in subsequent modules and that this is responsible for the prolific regeneration of shoots not characterised by any distinguishable pattern (Figure 4.3).

It is believed that the action of PRO is different to that of PAC. Modules formed in response to PRO also appear to arise from axillary meristems but from those directly adjacent to the apical meristem. This is illustrated in a seedling, 3 months after application of PRO at 7 125 ppm a.i., where axillary meristems have given rise to a new module on either side of the original apical meristem (Figure 4.11). In addition, it appears that following the application of PRO to seedlings, dichotomous branching of the apical meristem may occur (Figure 4.12). The occurrence of both dichotomous and axillary branching in the same individual has been observed in a seedling (Figure 4.13).

In mature plants, axillary branching would explain the formation of the shoots in Figure 4.5. It is thought that dichotomy also occurs in mature plants because leaf pairs, fused along the abaxial surface, were seen emerging from the apical meristem after treatment with PRO. These leaves were followed by the emergence of two new modules of similar size; one next to each adaxial surface of the fused leaf pair. Furthermore, it is proposed that in mature plants, pseudo-dichotomous branching may be the result of an early switch or reversion of the terminal bud from a reproductive to a vegetative stage. (In an untreated plant the reproductive bud would have given rise to the inflorescence.) Following reversion, the new vegetative bud and the axillary bud which was to form the new module after flowering, may then develop further, in

the form of 2 vegetative buds of more or less equal size.

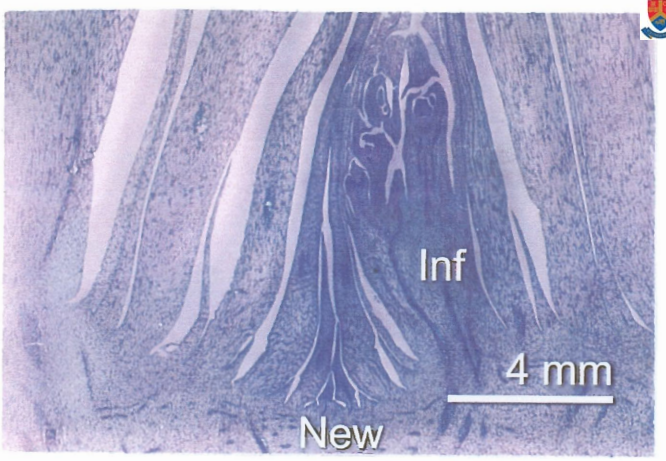

Figure 4.7 Longitudinal section of an untreated mature shoot showing termination of the old module in an inflorescence (Inf) and formation of the new module (New).

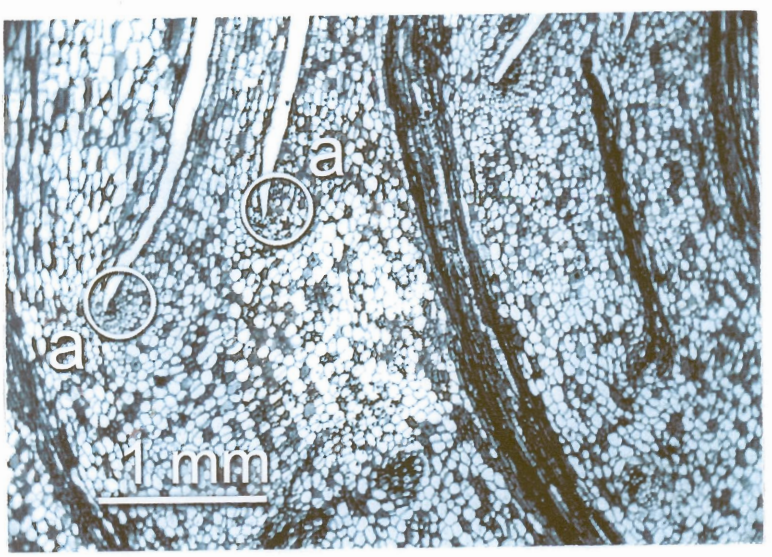

Figure 4.8 Longitudinal section of an untreated seedling at the 6 leaf stage, showing potentially meristematic zones (a) located on the abaxial side of leaf bases.

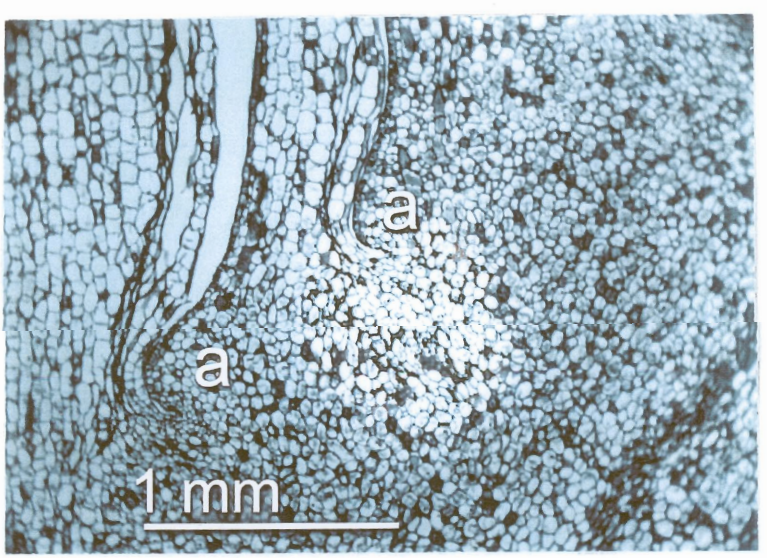

Figure 4.9 Longitudinal section of a seedling, 5 months after treatment at the six leaf stage, with 5 000 ppm paclobutrazol, indicating accentuated meristematic zones (a).

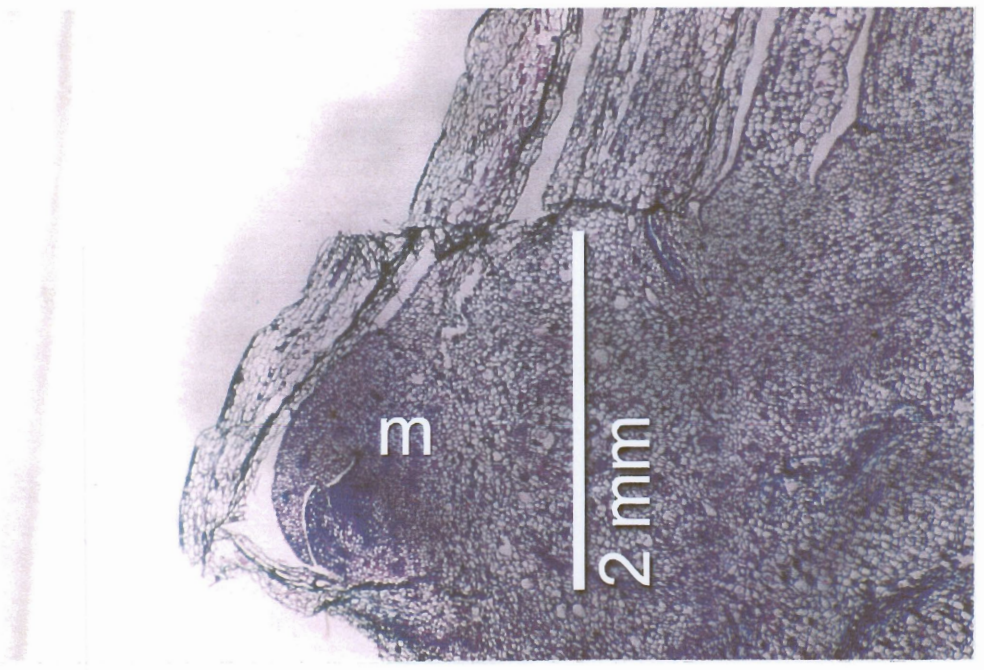

Figure 4.10 Longitudinal section of the most proximal axil of a seedling, 5 months after treatment at the six leaf stage, with 5 000 ppm paclobutrazol, indicating bud formation (m).

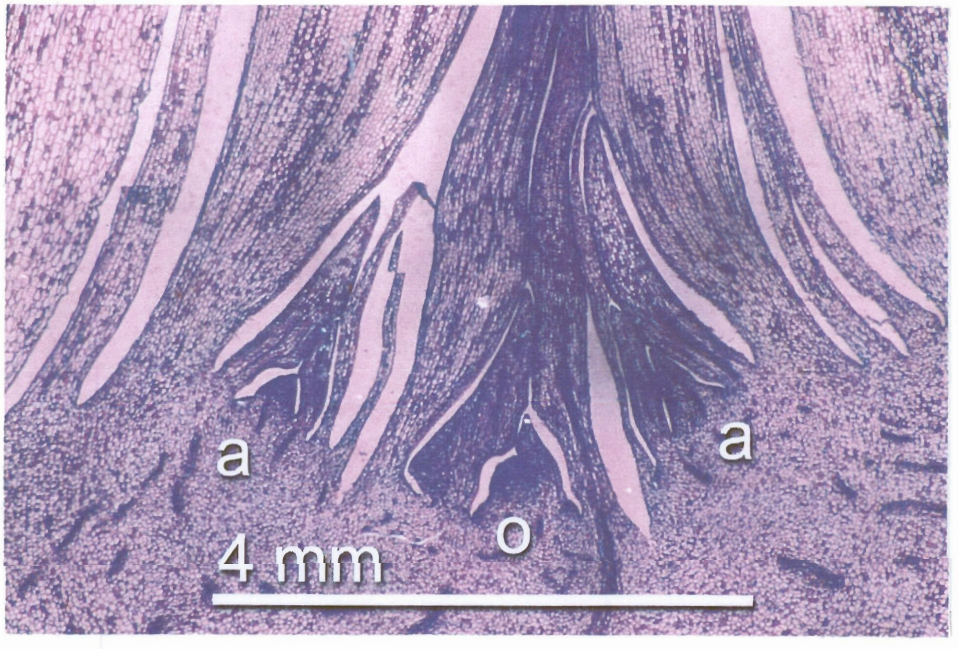

Figure 4.11 Longitudinal section of the apical meristem of a seedling, 3 months after treatment at the six leaf stage, with 7 125 ppm a.i. Promalin™, indicating new modules (a) adjacent to the original apical meristem (o). Note adnation of the buds (a) to the abaxial, basal part of the leaves.

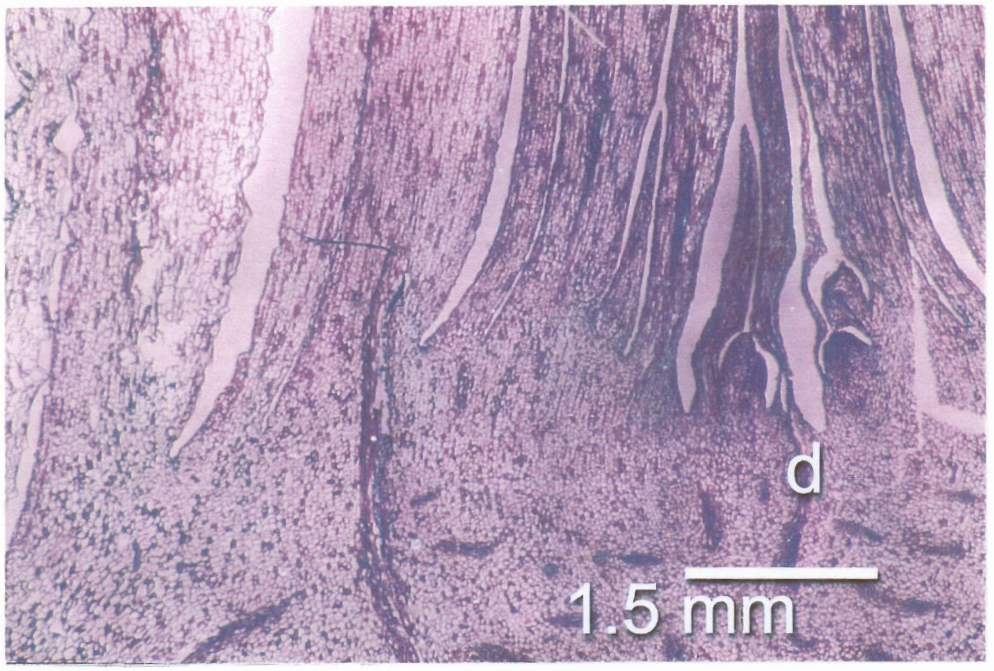

Figure 4.12 Longitudinal section of the apical meristem of a seedling, 2 months after treatment at the six leaf stage, with the first of 3 fortnightly applications of 7 125 ppm a.i. Promalin™. Dichotomous branching (d) of an apical meristem is shown.

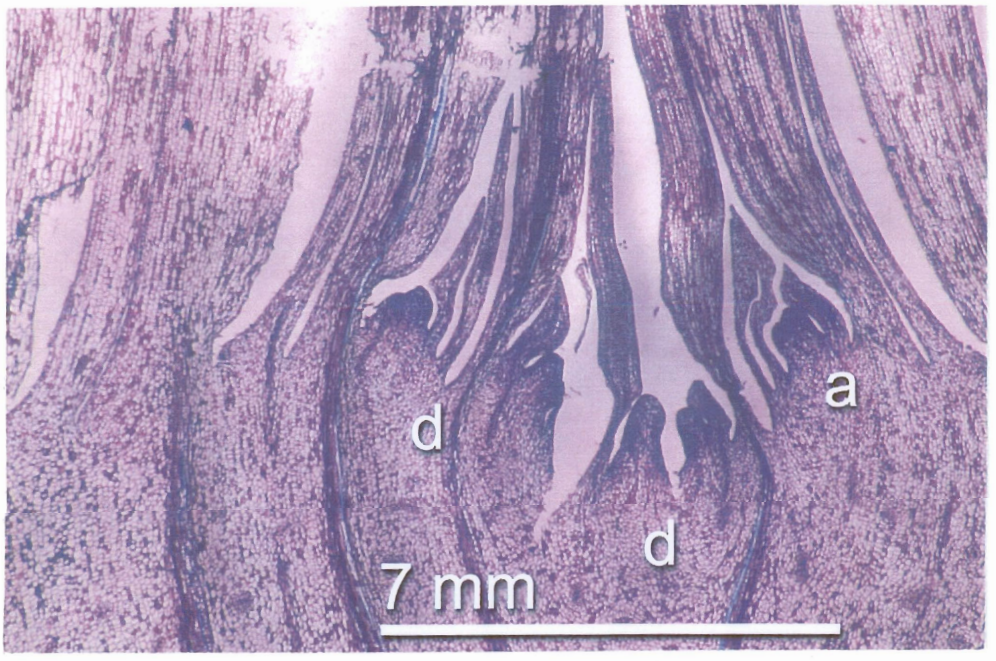

Figure 4.13 Longitudinal section of the apical meristem of a seedling, 3 months after treatment at the six leaf stage, with 7 125 ppm a.i. Promalin™, indicating dichotomous (d) and axillary (a) branching.