Effects of the Growth Retardant CCC on Floral Initiation and Growth in Pharbitis nil [1, 2]

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Growth-retarding chemicals such as Amo-1618 (2, 12), CCC [(2-chloroethyl)-trimethyl-ammonium chloride] (2, 11), and Phosfon D (2) reduce the height of many plants, leaving, in most cases, flowering and fruiting unaffected. Thus, of 55 species studied by Cathey and Stuart (2) control of flowering by photoperiod and temperature was unaffected by application of growth retardants. Flowering was delayed by such applications only in the cases of Chrysanthemum and Cleome.

In the day-neutral plant tomato Wittwer and Tolbert (13) found that flower clusters in CCC-treated plants appeared earlier and on lower nodes. Application of both CCC and Phosfon caused prompt initiation of flower buds in Rhododendron (10).

On the other hand, floral initiation as induced by shift from long to short days in the long-short-day plant *Bryophyllum daigremontianum* was fully suppressed by application of CCC (16). The CCC-related compound, allyl trimethylammonium bromide, reduced stem elongation and flower formation in the cold-requiring rosette plant *Beta vulgaris* (6). Inhibition of flowering in both Bryophyllum and Beta could be overcome by application of gibberellin A_3.

No specific effects of growth retardants on floral initiation in short-day plants (SDP) have heretofore been reported. The present paper concerns the effects of the growth retardant CCC on flower formation and growth of the SDP *Pharbitis nil* and the reversibility of these effects by gibberellin A_3.

Materials and Methods

Seeds of *Pharbitis nil* Chois., strain Violet, were treated with concentrated sulfuric acid for 60 minutes and subsequently washed overnight with tap water. The swollen seeds were planted in vermiculite and kept at 30° for 2 days. The emerging seedlings were then transplanted to 10-ml vials containing half strength Hoagland's nutrient solution. If necessary the pH of the solution was adjusted to 5.6 with KOH. The seedlings were grown in the Earhart Plant Research Laboratory at 23 to 25° under continuous light from fluorescent and incandescent tubes yielding approximately 750 ft-c at plant level.

Unless otherwise indicated the growth retardant CCC (supplied by American Cyanamid Co., Stamford, Conn.) was applied for 24 hours from the third to the fourth day after planting of the seeds, and immediately before the inductive long night of 16 hours, which was usually given at 27°. The root tips of the plants were cut off under water with a razor blade and the seedlings then transferred to vials containing CCC of the required concentration. Deionized water was used as control. At the end of treatment the roots of all plants were rinsed in deionized water and transferred to nutrient solution.

After the end of the 16-hour night, seedlings were transplanted into 180-ml plastic beakers, one seedling per container, in a mixture of vermiculite and crushed granite, and watered daily with nutrient solution. The plants were grown under artificial light for 3 to 4 days. Then they were transferred to a greenhouse at a day temperature (0800–1700 hours) of 27° and a night temperature of 23°. This regime is referred to below as 27/23°. Natural daylength was supplemented with light from incandescent lamps to give a photoperiod of 16 hours.

Gibberellin A_3 (GA_3) in aqueous solution containing Tween 20 was applied with the aid of a microsyringe to either plumules (0.01 ml) or cotyledons (0.1 ml).

Eight or nine plants were used per treatment. All experiments were repeated at least twice.

CCC was applied either once or twice to plants grown continuously under long day (LD) conditions (photoperiod 16 hours, temperature 27/23°). The first application was made on the third day when seedlings were still growing on nutrient solution. After transplanting of these seedlings to containers (volume 950 ml) with a mixture of vermiculite and granite, a second CCC application was made via the roots. One hundred ml of a solution of 250 mg CCC/liter were applied per container. Control plants received 100 ml of deionized water. Before and after this application, nutrient solution was applied sparsely so that the containers were never drained.

The quantitative criteria for measurement of flowering were used as previously described (1, 14). Internodes were measured when fully mature.

Plants grown continuously in LD were measured at regular intervals and the number of leaves produced was counted. Young leaves were not counted until their blades had begun to unfold. For practical reasons, initiation of floral primordia was observed

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only on the lowest 30 nodes. The term, node position, is used to indicate the number of the node bearing the first flower bud, counting in acropetal direction.

For anatomical observations first internodes were fixed in 70 % ethanol and sectioned by hand. The lengths of 20 cells in the cortical and pith parenchyma in each internode were measured with the aid of an ocular micrometer.

Results

Four-day-old Pharbitis seedlings grown in the standard vermiculite-granite mixture respond to exposure to one 16-hour dark period at 27° by the subsequent initiation of 6 to 7 flower buds, including a terminal one (1, 14). The axillary buds of the lowest 2 nodes of such seedlings remain vegetative, but the buds on the third and all higher nodes are transformed into flower buds. Seedlings grown in nutrient solution instead of in the vermiculite-granite mixture exhibit a flowering response similar to that of plants grown in the mixture.

The data of figure 1 concern an experiment in which Pharbitis seedlings were grown in different concentrations of CCC for 24 hours prior to the inductive night. Clearly such treatment resulted in a considerable decrease in the number of flower buds produced per plant. At concentrations of 30 mg/liter CCC and higher no terminal flower buds were produced. However, complete suppression of flower formation in Pharbitis by CCC was never obtained. With 250 mg CCC/liter, node 3 and 4 still had a flower bud. CCC concentrations of 500 mg/liter and higher caused considerable paling between the veins of the cotyledons and often resulted in death of the seedlings.

It is clear from figure 1 that decrease in number of flower buds with increasing CCC concentration is closely paralleled by a shortening of the first inter-

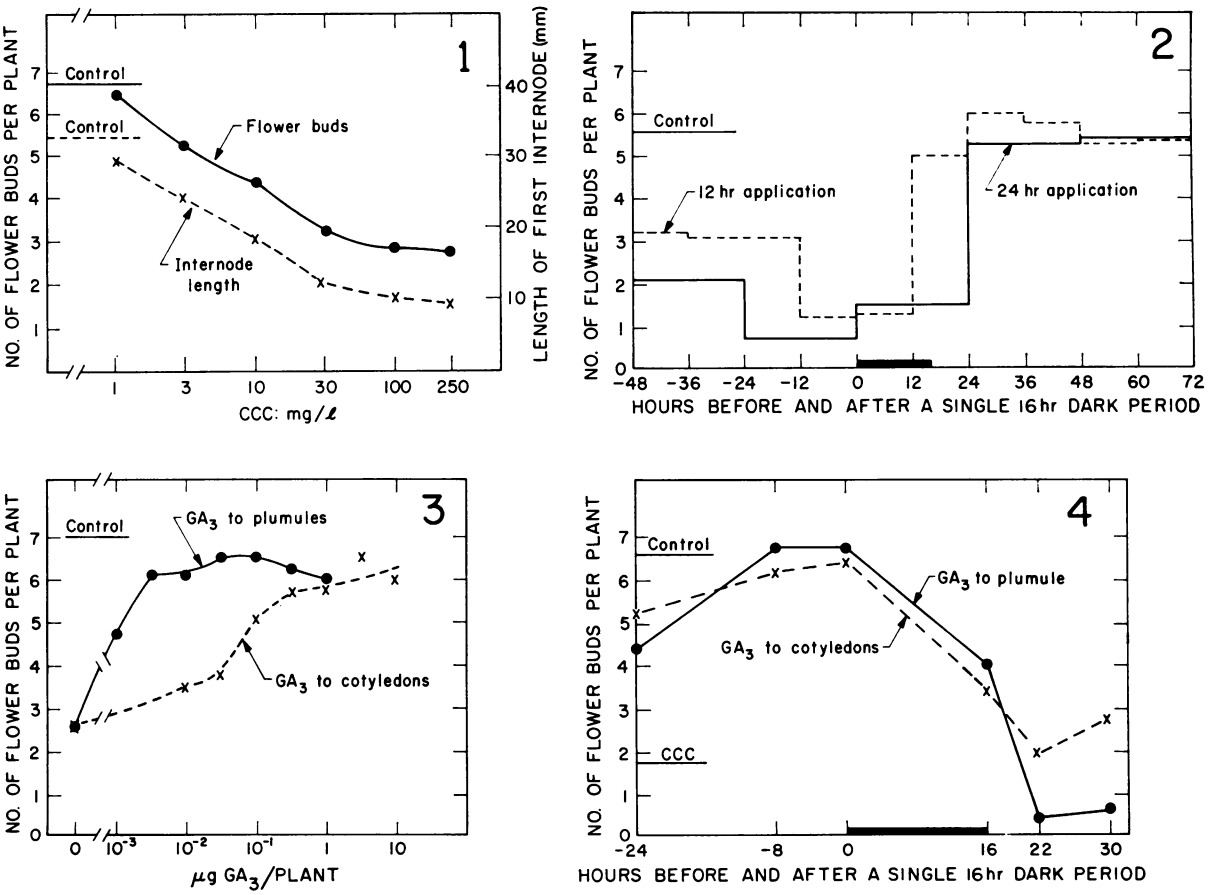


FIG. 1 (*upper left*). Inhibition of flower formation and extension of first internode in Pharbitis as a function of the concentration of growth retardant CCC applied via the roots for 24 hours prior to the 16-hour dark period at 27°.

FIG. 2 (*upper right*). Inhibition of flower formation in Pharbitis as a function of time of CCC application. CCC applications via roots either for 12 or 24 hours. Inductive night at 30°.

FIG. 3 (*lower left*). Reversion of CCC inhibition by GA_3 as a function of amount of GA_3 applied per seedling. GA_3: 0.01 ml per seedling applied via plumule, or 0.1 ml via cotyledons of different GA_3 concentrations immediately before long night.

FIG. 4 (*lower right*). Reversion of CCC inhibition by GA_3 as a function of time of GA_3 application. GA_3: 0.5 μg applied per seedling via plumules, or 5 μg via cotyledons.

node. Plants 2 weeks after application of CCC showed a dwarf habit due mainly to short internodes. After 4 to 5 weeks such seedlings would again start to grow normally. Since their terminal buds were vegetative, they would ultimately grow much taller than induced plants in which growth was arrested by the terminal flower bud.

The effect on flowering of the time of CCC application in relation to the inductive dark period is considered in figure 2. Seedlings were grown on solutions containing 250 mg CCC/liter for 12 or 24 hours before, during, or after the long night. The 2 periods of application yielded basically the same results, although the 12-hour treatment supplies a more detailed picture. Strongest suppression of flower formation was obtained when CCC was applied for 12 or 24 hours preceding the long night. No terminal flower buds were produced by plants subjected to these treatments. CCC applications started after the end of the long night caused no inhibition of flower formation. In further experiments CCC was therefore applied for a period of 24 hours prior to the beginning of the dark period in order to secure maximal inhibition of flower formation.

Reversal of CCC inhibition by GA_3. Inhibition of flowering in Pharbitis by CCC is completely reversed by the application of GA_3 (fig 3). Seedlings were grown on 250 mg CCC/liter for 24 hours prior to the long night. Immediately before the beginning of the long night GA_3 in different amounts was applied either to the plumules or to the cotyledons. Plants treated only with CCC did not form terminal flower buds. All plants supplied with at least 0.01 μg GA_3 via the plumule or 0.1 μg GA_3 via the cotyledons produced terminal flower buds. Obviously, inhibition of flowering by CCC is overcome more efficiently by GA_3 application to plumules than by such application to the cotyledons. In all similar experiments 0.01 μg GA_3 per apex fully reestablished flower formation. In some experiments even 0.003 μg GA_3 per plumule has been sufficient to cause normal flowering.

The data of figure 4 concern the reversibility of CCC inhibition as a function of time of GA_3 application. Complete suppression of the effects of CCC was obtained when GA_3 was applied shortly or immediately before the beginning of the long night. Application of GA_3 at the end of the long night did not cause complete reversal and later applications were ineffective. No differences were observed as between applications to cotyledons or plumules.

In a further experiment (fig 5) flower formation in Pharbitis was suppressed by different concentrations of CCC in order to see if these require different amounts of GA_3 to overcome the inhibition. A concentration of 400 mg/liter of CCC was the highest concentration that could be applied for 24 hours without being too toxic. As the lowest concentration capable of suppressing formation of all terminal flower buds, 100 mg/liter of CCC was chosen. Before the long night different groups of seedlings were given different amounts of GA_3 via the plumules. The results presented in figure 5 indicate that no statistically

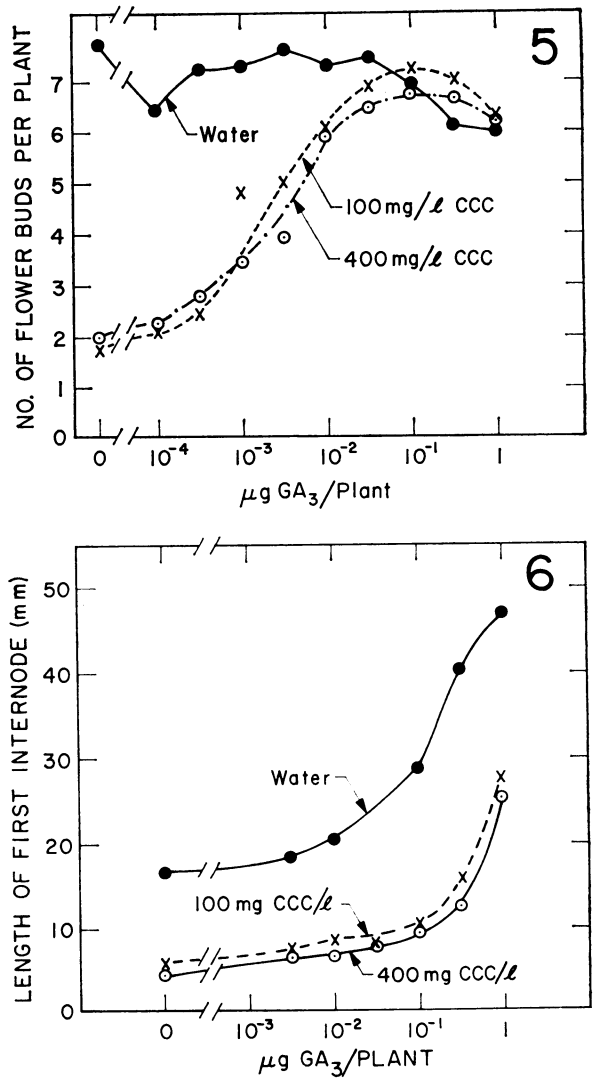
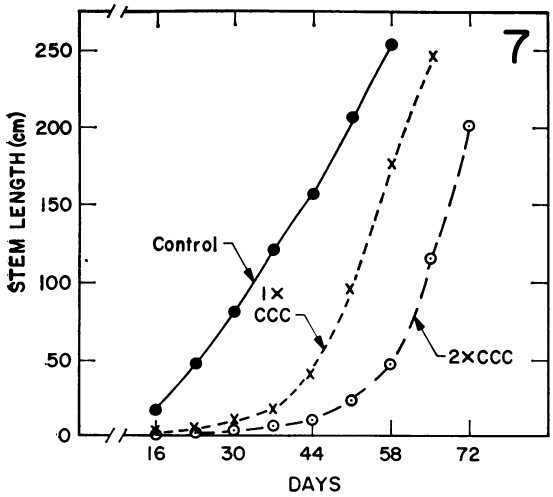
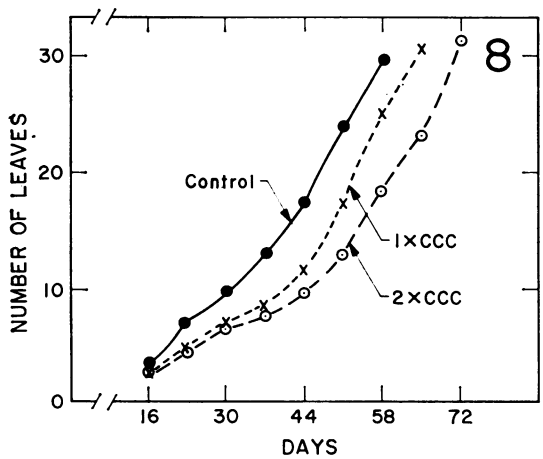


FIG. 5 (*upper*). Reversion of CCC inhibition by GA_3 as a function of amount of GA_3 applied at two different concentrations of CCC. CCC applied via roots in concentrations of 0, 100, or 400 mg/liter for 24 hours before long night at 27°. GA_3: 0.01 ml of different concentrations applied per seedling via plumules before long night.

FIG. 6 (*lower*). Lengths of first internodes as affected by different concentrations of CCC and GA_3. CCC applied via roots in concentrations of 0, 100, or 400 mg/liter for 24 hours before long night. GA_3: applied via plumules before long night. Measurements: 26 days after end of long night.

significant differences in number of flower buds per seedling resulted from application of a given amount of GA_3 to plants supplied with CCC at the 2 different concentrations. Further detailed experiments with GA_3, in the range 10^{-4} to 10^{-2} μg of GA_3 per plant, applied to plumules of plants inhibited by different CCC concentrations, indicated that essentially the same amount of GA_3 is required to reestablish flower formation at all levels of inhibitor.





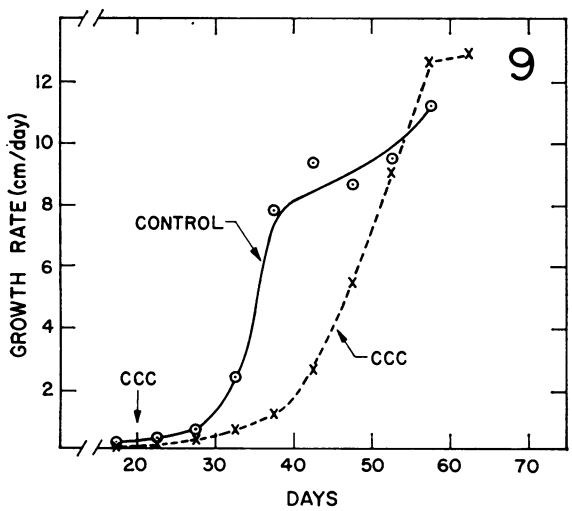


FIG. 7 (*upper*). Stem growth of Pharbitis in long days as affected by CCC. Treatments as described in table I.

FIG. 8 (*middle*). Effect of different CCC treatments on leaf formation in Pharbitis. Treatments as described in table I.

In addition to suppressing flower formation, CCC treatment of Pharbitis also reduces the length of the first and higher internodes (fig 1). This dwarfing effect is also reversed by GA_3 as is shown in figure 6. In this experiment different quantities of GA_3 were applied to control plants, and to plants treated with 100 or 400 mg/liter of CCC. Complete reversion of flower inhibition was obtained with 0.003 μg GA_3, but 0.3 to 1 μg GA_3 was required to cause the first internodes to reach the same legnth as untreated ones. Thus, under the present conditions in order to overcome suppression of internode growth by CCC with a single GA_3 application, at least 100 times more GA_3 was necessary than was required for overcoming inhibition of flower initiation.

First internodes of plants inhibited with 400 mg CCC/liter were always slightly shorter than those of plants treated with 100 mg/liter.

Effects of CCC on Pharbitis in LD. Three-day old seedlings were grown on a solution containing 250 mg CCC/liter for 24 hours, or as a control on deionized water. The seedlings were planted in containers (volume 950 ml) when 6 days old and grown under continuous light at 23° until 16 days old. They were then moved to a 16-hour photoperiod at 27/23°. To one group of plants 100 ml of a 250 mg/liter CCC solution was applied 9 days after the first application, resulting in the 3 different treatments listed in table I.

Results of weekly measurements of stem length and number of leaves are plotted in figures 7 and 8. During the first weeks CCC-treated plants exhibited a dwarfed habit as compared with untreated plants. Stem growth of untreated plants was essentially linear with time after the 16th day (fig 7). CCC-treated plants reached a similar growth rate but much later. The delay was approximately 3 weeks for 1, and 5 weeks for 2 CCC applications. However, once treated plants started to grow rapidly, their growth rate even surpassed that of untreated plants as is illustrated in figure 9. In the experiment of figure 9 CCC-treated plants did not attain the same growth rate as control plants until 30 days after the last CCC application. It was also established that application of 10 μg GA_3 to the 2nd and 3rd leaf at the same time as the last CCC application yielded a growth rate essentially the same as that of untreated plants.

Rate of leaf formation (fig 8) was also reduced by one CCC treatment and even more by two applications. The plants at final harvest exhibited the following general characteristics: control plants contained more anthocyanin in the stems than CCC-treated ones. Leaf color of untreated plants was light green, but the leaves of CCC-treated plants were dark green and very large. Moreover, leaves of treated

FIG. 9 (*lower*). Effect of CCC on growth rate in Pharbitis as determined from measurements at 5-day intervals. CCC treatments: 250 mg/liter via roots when seedlings were 4 days old; second application 100 ml of 250 mg CCC/liter per container when 20 days old. Temperature 27/23°. Photoperiod 16 hours.

Table I

Effect of CCC on Flower Formation in Pharbitis under 16-hour Photoperiods

CCC was applied in the following ways: first treatment, seedlings on solution containing 250 mg CCC/liter for 24 hours, 3 days after planting; second treatment, 100 ml of solution containing 250 mg CCC/liter added per container 9 days after first treatment. The growth conditions were as follows: for first 16 days, continuous light from fluorescent tubes at an intensity of 750 ft-c, 23°; afterwards, photoperiod of 16 hours, temperature 27/23°.

Treatment	Flowering quotient*	Average node position**	Average number of flower buds***
Control	7/8	26.7 ± 0.86	3.0 ± 0.69
CCC, 1x	7/8	14.3 ± 1.12	1.4 ± 0.29
CCC, 2x	8/8	13.4 ± 0.38	16.4 ± 0.53

* Number of plants with flower buds divided by number of plants per treatment.
** Position of the node bearing the first flower primordium (3).
*** Data for first 30 nodes.

plants abcised later than those of controls. Stem diameter of the lowest 10 internodes was 2 to 3 times that of the control plants. Data concerning flower bud initiation in these plants are summarized in table I. Under these conditions (daylength 16 hours, temperature 27/23°) control plants produced a few flower buds, but always above the 25th node. This response seems to be temperature dependent. Thus, under the regime 31/27° no flower buds were observed below the 30th node, under the 23/19° regime the lowest nodes with flower buds were numbers 17 or 18. One CCC treatment caused a significant lowering of the node with the first flower bud to around number 14 (table I), although only 1 to 3 flower buds per plant were produced. Two CCC applications caused practically all nodes from the 13th and higher to produce flower buds. Thus, it is clear that under LD conditions the growth retardant CCC promotes flowering in that it causes formation of flower buds at lower nodes than in untreated plants.

Experiments were also carried out under continuous light (daylight with supplementary light from fluorescent tubes at an intensity of approximately 300 ft-c) in order to determine if under these conditions CCC could cause formation of flower buds. A few flower buds were indeed initiated between the 25th and 30th node on plants treated twice with CCC. No flower buds were produced by control plants, or by plants treated once with CCC.

Anatomical observations. Anatomical observations were made only on the first internodes as these exhibit the most conspicuous responses to CCC. First internodes were measured and fixed 39 days after CCC application. CCC caused a considerable shortening and thickening of the first internode (table II). Application of 1 μg GA_3 per seedling treated with CCC resulted in internodes considerably longer than those of untreated plants.

The results of cell measurements given in table II show that cell length in cortical and pith parenchyma in the three treatments was roughly the same. The big differences in internode lengths must therefore be due to differences in cell numbers and these are calculated in table II from the average internode and cell length. From these calculations it follows that CCC treatment reduced the number of cells to approximately one-third that of the control. GA_3 treatment on the other hand caused a considerable increase in cell number.

In addition to changes in number of cells caused by CCC and GA_3 further noticeable anatomical changes were caused by these two chemicals. In plants treated with CCC cortex and pith constituted a larger portion of the stem cross section than in control plants, or in plants that received GA_3. Pith parenchyma cells in seedlings treated with CCC also exhibited a larger diameter than those of control plants.

Treatment with GA_3 caused the formation of thin, but woody internodes. In the pericycle a layer of cells with very thick walls was formed, but these thick-walled cells were never observed in seedlings

Table II

Length and Number of Cells in First Internodes of Pharbitis Seedlings Treated with CCC and GA_3

CCC was applied (300 mg/liter) via the roots for 24 hours before long night. GA_3 was applied (1 μg per seedling) via the plumule before a long night. Internodes were harvested 39 days after CCC application

	Tissue	Treatments		
		Control	CCC	CCC + GA_3
Length × diameter 1st internode (mm)		31 × 3.2	11 × 4.4	53 × 2.6
Cell length (μ)	Cortex	122	145	124
	Pith	131	115	127
Calculated number of cells in longitudinal direction	Cortex	254	76	427
	Pith	237	96	417
Relative cell number	Cortex	1.0	0.30	1.68
	Pith	1.0	0.41	1.76

treated with CCC only. Application of GA_3 also resulted in the differentiation of more secondary xylem, so that in these plants cortex and pith took up only a relatively small portion of the cross section of the stem.

Discussion

The results described here clearly demonstrate that the growth-retarding chemical CCC strongly inhibits photoperiodic induction in Pharbitis if applied immediately before the long night. Flower formation remains unaffected if CCC is applied at the end of the long night when production of the floral stimulus supposedly has been completed (15), but stem length is still greatly reduced. This brings up the question as to which partial reaction of the flowering process is affected by CCC. With compounds such as 5-fluorouracil (14) and SK&F 7997 (1) this problem has been solved by applying the chemicals at different times and to either cotyledons or plumules. CCC, however, can be applied effectively only via the roots. No inhibition of flowering was obtained by application of CCC via plumules or cotyledons. With very high concentrations (6 g/liter) the plumules were killed.

No definite conclusion can be drawn as to whether CCC inhibits flower hormone production in the cotyledons or differentiation of flower primordia in the plumules, although the following indirect evidence supports the latter view:

A) Inhibition of flowering is overcome by applying GA_3 to the plumule only. GA_3 application to cotyledons also reverses inhibition of flowering, but the material is probably rapidly translocated to the apex. B) The growth retardant Amo-1618 completely inhibits subapical meristematic activity in Chrysanthemum (9). Anatomical observations in Pharbitis showed that CCC strongly reduces cell number in the first internode which is just beginning to expand at the time of induction. Consequently, meristematic activity in the plumule must be low when the floral stimulus arrives in the apex, so that it cannot be expressed in the formation of flower buds. As concluded previously (14) DNA multiplication and cell division are a prerequisite for expression of floral stimulus in Pharbitis. It remains to be seen whether CCC, like Amo-1618 in Chrysanthemum, suppresses cell division in the subapical region only, or also in the apex, as the latter is the probable site of hormone action.

C) Unpublished results with the growth retardant B-995 (*N*-dimethylaminosuccinamic acid) (8) which can be applied via roots, plumules and cotyledons, show that this chemical exerts its flower inhibiting effect in Pharbitis in the plumule only.

We conclude tentatively that CCC inhibits flower formation in Pharbitis by reducing cell division in the shoot apex, thus preventing the complete expression of the floral stimulus.

The mode of action of growth retardants suggests the following possibilities: A) Based on kinetic studies Lockhart (5) has concluded that growth retardants function as competitive inhibitors of gibberellins and are thus antigibberellins in the strict sense of the word. B) Kende et al. (4) have shown that cultures of *Gibberella fujikuroi* grown in the presence of CCC or Amo-1618 do not produce GA and suggest that effects of growth retardants in higher plants may be explained in terms of inhibition of biosynthesis of native gibberellins.

The results of figure 5 show that with different concentrations of CCC the same minimal amounts of GA_3 are required to prevent flower inhibition. Although it has to be borne in mind that flowering is not a very suitable system for kinetic studies, this result does not support the idea that growth retardants are competitive inhibitors of gibberellins.

Although the present results (fig 3, 4, 5, 6) and the appearance of seedlings treated with CCC seem to indicate that such plants are deficient in gibberellin, preliminary extraction experiments of seedlings, harvested at the end of the inductive night, failed to show any decrease in gibberellin-like activity as tested by the dwarf corn bioassay. As CCC-treated seedlings respond readily to an exogenous supply of GA_3, these observations suggest that the bulk of gibberellin-like substances extractable from Pharbitis seedlings is physiologically inactive. But a minor, active fraction absent in CCC-treated seedlings may have escaped detection in the dwarf corn bioassay. Thus, the mode of action of the growth retardant CCC in Pharbitis remains open to further speculation and experimentation.

Seedlings grown on a CCC solution for 24 hours took up 2 to 3 ml of the solution. Assuming passive uptake through the cut roots, this amounts to approximately 600 μg CCC per seedling, whereas 0.01 μg GA_3 was already sufficient to overcome flower inhibition. Thus, as in Bryophyllum (16) the ratio of growth retardant to GA_3 is high (approximately 10^5 on a molar basis).

The measurements of heights of plants grown in LD show that CCC has a long residual effect in Pharbitis. No obvious explanation can be offered for earlier flower formation under LD with CCC, except the general statement that there is an antagonism between vegetative growth and reproductive development, so that suppression of the former results in promotion of the latter. The same seems to hold true when growth of Pharbitis is reduced by lowering the temperature.

Thus, the growth retardant CCC has a dual effect on flower formation in Pharbitis: it suppresses flowering when applied to seedlings before photoperiodic induction, but promotes flower formation in plants grown under LD.

The shorter internodes which result from CCC treatment can be attributed to inhibition of cell division (table II). In the dwarf strain Kidachi of Pharbitis it has been found that shorter internodes are also due to fewer cells and that GA_3 application caused formation of longer internodes with more cells (7). Thus, whether dwarfing is controlled geneti-

cally or is induced by the growth-retarding chemical CCC, the response to GA_3 seems to be quite similar.

Okuda (7) has observed, too, that GA_3 causes thickening of cell walls in Pharbitis. On the other hand, stems of Lilium (2) treated with Phosfon were enlarged in diameter and the cell walls in and adjacent to the cortex were less thickened. From these and the present observations it can be concluded that growth retardants tend to prevent cell wall thickening whereas GA_3 has the opposite effect.

Summary

Flower formation in the short-day plant, *Pharbitis nil* Chois, strain Violet, induced by a long night of 16 hours at 27°, is strongly suppressed by application of (2-chloroethyl)-trimethylammonium chloride (CCC) via the roots for a period of 24 hours prior to induction. Formation of terminal flower buds is already suppressed with a CCC concentration of 30 mg/liter, but even with 400 mg/liter no complete suppression of formation of axillary flower buds on the lower nodes is obtained. Application of CCC after the long night does not inhibit flowering, but reduces stem growth considerably.

Inhibition of flowering by CCC can be completely overcome by application of gibberellin A_3 either to plumules or cotyledons before the inductive night. An amount of 0.01 μg gibberellin per seedling applied via the plumule is sufficient to overcome inhibition of flowering whereas 50 to 100 times more gibberellin is required in order to overcome growth suppression of the first internode. CCC does not act as a competitive inhibitor of gibberellin.

Pharbitis plants grown continuously in long days show a dwarfed habit until approximately four weeks after the last CCC application. The CCC treatment results in short, thick internodes and dark green leaves. Growth rate in plants treated with CCC remains low for a considerabe period after the last application, but ultimately reaches the same level as in untreated plants.

CCC treatment results in a promotion of flower formation in Pharbitis grown in long days.

Anatomical observations reveal that differences in the lengths of first internodes of control plants and those treated with CCC are due only to differences in cell number. CCC reduces mitotic activity, at least in the first internode. Application of gibberellin A_3 to CCC-treated plants increases the length of the first internode by increasing the number of cells per internode. Gibberellin also causes remarkable thickening of cell walls in the pericycle and xylem.

Available evidence suggests that the growth-retarding chemical CCC inhibits flower formation in Pharbitis by inhibiting cell division in the plumule during the period when the floral stimulus is present, thus preventing the expression of the stimulus in flower primordia.

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