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Morphactin Effects on Soybean Leaf Anatomy and Chlorophyll Content*

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Abstract. The effect of chlorflurenol (methyl 2-chloro-9-hydroxyfluorene-9-carboxylate) (CF) on chlorophyll (chl) content was studied in intact plants and floating leaf disks. For intact soybean (*Glycine max* (L.) Merrill) plants grown in the growth chamber, 2.5 μg/ml CF applied 10 to 20 d after planting retarded chl decline in senescing tissues such as cotyledons and unifoliate leaves and increased chl content in recently expanded tissues such as trifoliate leaves. CF did not retard chl decline in the dark unless regulator application was followed by a period of 24 h in the light prior to darkness. In floating leaf disk tests, CF retarded chl decline in dock (*Rumex obtusifolius* L.) and radish (*Raphanus sativus* L.) at concentrations of 10^{-4} M, but was ineffective at lower concentrations. Chl decline was significantly hastened by CF in tobacco (*Nicotiana tabacum* L.) and soybean, but was unchanged in barley (*Hordeum vulgare* L.).

CF treatment increased tissue weight (g fresh wt/cotyledon; g dry wt/cm^2 for unifoliate and trifoliate leaves), decreased moisture content, and

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increased leaf thickness, palisade layer thickness, and palisade and spongy mesophyll cell counts. We conclude that plants treated with morphactins show greater green coloration predominantly because of growth effects, and only in small part because of prevention of chl decline in senescing tissues.

Of the many growth regulating activities attributed to morphactins, one discovered early in their development was delay of leaf yellowing. First reports indicated that treated plants became greener (Schneider 1964). This was later interpreted as senescence delay on the basis of studies with floating leaf disks (Harada 1967) and intact plants (Schneider 1970, Uma 1972, Ram and Jaiswal 1978, Gupta and Mukherjee 1980). However, methods by which morphactins retard chlorophyll (chl) degradation are unknown.

The present work is a continuation of experiments having the objective of extending the life of crop plants through growth regulator treatments (Dybing and Lay 1981a). Morphactins, particularly chlorflurenol (methyl 2-chloro-9-hydroxyfluorene-9-carboxylate) (CF), showed promise for enhancement of seed yield of flax (Dybing and Lay 1981b) and oil content of flax, soybean, wheat, and oats (Dybing and Lay 1982). These responses presumably were mediated through prolonged leaf retention, chl retention, and blossoming, plus unknown effects on lipid metabolism. Preliminary studies (Dybing et al. 1980) indicated that the delayed leaf yellowing might be due to effects other than prevention of chl degradation. Data reported here resulted from greenhouse, growth chamber, and laboratory studies conducted to evaluate more thoroughly the effects of morphactin on chl content and leaf anatomy.

Materials and Methods

Plant Materials

Plants used were tobacco (*Nicotiana tabacum* L. var. 'Samsun NN'), barley (*Hordeum vulgare* L. var. 'Godiva hulless'), broadleaf dock (*Rumex obtusifolius* L.), soybean (*Glycine max* (L.) Merr. var. 'Swift'), and radish (*Raphanus sativus* L. var. 'Scarlet Globe'). Tobacco, barley, and dock were grown in the greenhouse in 2-l glazed crocks containing vermiculite/peat moss (1:1, v:v) watered twice weekly with nutrient solution (Dybing 1969). Greenhouse day length was extended to 14.5 h with high-pressure sodium lamps. Soybean and radish were grown in vermiculite/peat moss/sand (3:1:3, v:v:v) in growth chambers operated at 14-h photoperiods (16 h for radish), 31/22°C day/night temperature, and 450 $\mu Em^{-2}s^{-1}$ (400–700 nm) irradiance. Complete nutrient solution was applied three times per week for soybean and two for radish.

Intact-Plant Experiments

Most studies with intact plants were done with soybeans; one experiment each was done with dock and radish. Soybeans were seeded four per pot and thinned to three plants one week after planting. CF (EM Industries Inc) was applied at rates of 0, 0.25, 2.5, 12.5, or 25 μg/ml with all foliage wetted to runoff. The

surfactant Tween 80 (oxysorbic) (20 POE) (polyoxyethylene sorbitan monooleate) (ICI United States Inc.) was included in all spray solutions at the rate of 0.075% (w/v). Dock and radish were handled similarly except that number of plants per pot was higher for radish, and dock was grown in the greenhouse. Leaf samples of soybean and dock were obtained as 1.5-cm disks removed with a cork borer during and at the end of each experiment. Soybean and radish cotyledons were removed whole for weight and chl determinations. Decapitation treatments in certain soybean experiments were made by excision of the main stem just above the node bearing the leaf to be measured. All intact plant experiments were conducted in randomized complete block or factorial design with subsampling; most utilized three replications with three subsamples per treatment. Data were subjected to analysis of variance with comparison of treatment and check means by LSD.

Leaf Disk Experiments

Leaf disk experiments were conducted by standard procedures (Mitchell and Livingston 1968, Hsia and Kao 1977, Kuraishi 1976) using leaf disks (whole cotyledons in the case of radish) floated adaxial side up in the dark at 25°C in 20 ml of solution per 8.5-cm Petri dish. Leaves used were the oldest leaves still having apparently normal green color or, for growth-chamber-grown plants, leaves of known age. Disks were cut to avoid veination; diameter was 1.0 cm for tobacco and barley and 1.5 cm for soybean, dock, and radish. Solutions were prepared in water for tobacco and barley, and in 0.1 M phosphate buffer at pH 7.0 for other plants. Test duration was 3–4 d for radish, 3–5 d for tobacco and barley, 5–6 d for dock, and 6–12 d for soybean, depending on rate of chlorophyll loss in the checks. Experimental design in most cases included subsampling within replications. Chemicals tested were kinetin, GA_3, chlorflurenol, dichlorflurenol (methyl 2,7-dichloro-9-hydroxyfluorene-9-carboxylate) (DCF), and n-butyl flurenol (n-butyl-9-hydroxyfluorene-9-carboxylate) (nBF).

Chlorophyll Determination

Chloropohyll was determined by measuring absorbance of 80% (v:v) ethanol extracts at 665 nm with a Zeiss PM2DL spectrophotometer (Kao 1980). Treatment effects were determined by comparing absorbances of extracts prepared at the start of an experiment with those prepared at the end, or by comparing final absorbances of treated and control extracts. Sample size per 10 ml of solvent was 3.9 cm^2 for tobacco and barley leaves, 5.1 cm^2 for dock and soybean leaves, 1 g for soybean cotyledons, and 0.5 g for radish cotyledons.

Leaf Cell Analyses

Cell numbers were determined by microscopic sectioning and by maceration. In the former method, a centrally located 1-cm^2 segment of the terminal leaflet of the first trifoliate leaf was sampled 26 d after treatment, fixed 12–24 h in

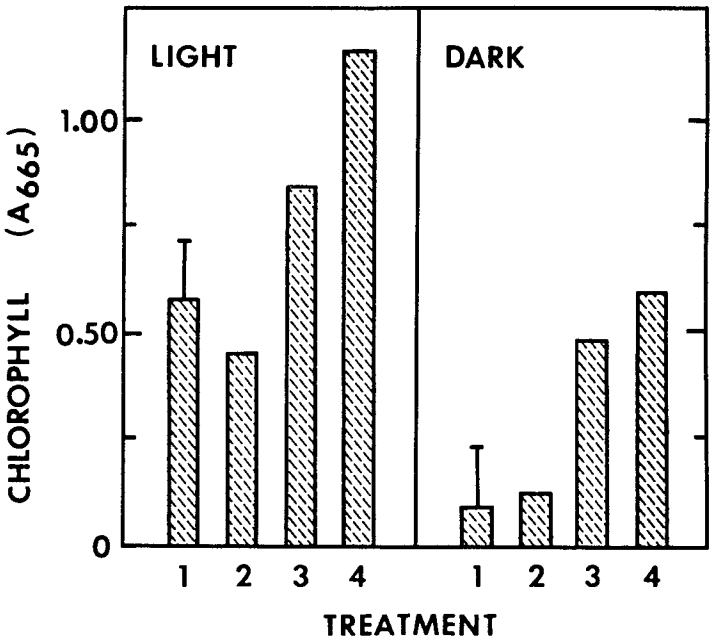

Fig. 1. Chlorophyll content of unifoliate soybean leaves 9 d after chlorflurenol treatment. Treatments 1, 2, 3, 4 = 0, 0.25, 2.5, and 25 μg/ml, respectively, applied 19 DAP. Light = 450 $\mu Em^{-2}s^{-1}$ irradiance, 31/22°C day/night temperature, 14-h photoperiod before and after CF treatment; dark = same until 4 d after CF treatment and then continuous dark at 25°C. Vertical bar = LSD (0.05).

cold 10% acrolein, dehydrated 15 min in two changes of 2,2-dimethoxy-propane (DMP), and embedded in Paraplast-plus. Sections were cut at 9 μm and stained with ferric ammonium sulfate-tannic acid-safranin 0-fast green. Width and length of palisade cells were measured with an ocular micrometer. Palisade and spongy mesophyll cells were counted per microscopic field, with any portion of a cell in the field included in the count. Cell counts by the maceration technique employed five 7-mm leaf disks from the middle leaflet of the first trifoliate leaf. These were weighed, fixed at room temperature in 2.5% (w/v) glutaraldehyde, and macerated using 2.5% (w/v) CrO_3. Counts were made with a hemacytometer. Weight of the blade region was used to determine total leaf weight and number of cells per leaflet.

Results

Effects of Chlorflurenol on Intact Plants

When analyzed a few days after treatment, leaves of soybean plants exposed to 2.5 or 25 μg/ml CF had significantly more chl than leaves from untreated check plants whether held in the light or dark following treatment (Fig. 1). At 25 μg/ml CF, treated plants were stunted and their apical meristems appeared dormant; lateral buds broke dormancy but the shoots did not elongate normally. Leaves that were fully expanded when sprayed appeared to be stiffer to the touch than untreated leaves. Leaves produced by lateral buds had tissue

Table 1. Effects of age and light on changes in chlorophyll content of soybean leaves following decapitation or treatment with chlorflurenol.

	Age[a]				Final chl (as % of initial chl)			
Leaf	1	2	3	Initial chl (A_{665})	Check	Decapitated	2.5 μg/ml Chlorflurenol	Decap. and chlorflurenol
Cotyledon	10	—	18	0.909	42	104**[b]	81**	142**
	15	—	23	0.280	88	96	57	75
Unifoliate	10	—	18	1.017	94	143**	136**	173**
	15	—	23	0.899	59	119**	93**	159**
	17	—	26	0.935	51	58	78*	—
	20	—	26	0.884	59	106**	100**	—
	17	22	26	0.935	9	36*	31	—
	20	26	33	0.884	16	21	50**	54**
First trifoliate	17	—	26	0.997	68	90*	100**	—
	20	—	26	0.921	93	115**	113**	—
	17	22	26	0.997	8	63**	49**	—
	20	26	33	0.921	24	48**	65**	59**
Second trifoliate	17	—	26	0.909	99	115	136**	—
	17	22	26	0.909	40	94**	86**	—

[a] Days after planting: 1 = start of experiment; 2 = start of dark period, if any; 3 = end.

[b] (*) Significantly different from final chl of check at $p = 0.05$; (**) significant at $p = 0.01$. For comparison with initial chl levels, LSD at $p = 0.05$ and $p = 0.01$ for cotyledons = 25% and 34%, respectively; for unifoliate = 18% and 25%; for first trifoliate = 12% and 16%; for second trifoliate = 19% and 27%.

"puckered" between veins, apparently a result of continuing division and growth in the mesophyll at a time when leaf apical meristems had ceased division. Stem internodes above unifoliate leaves became swollen. Deformations were much less pronounced at 2.5 μg/ml rates of CF treatment, and no deformation or chl effect was detected at 0.25 μg/ml. Greenhouse-grown broadleaf dock plants did not respond to CF treatment at the rate of 2.5 μg/ml, and radish appeared intermediate in responsiveness compared with dock and soybean.

The effect of CF on soybean chl content was observed whether the tissues were at early stages of yellowing or at peak chl content, and it was quantitatively similar to the chl-preserving effect of decapitation (Table 1). From 10–18 days after planting (DAP), when cotyledon chl content was declining rapidly, decapitation or CF treatment reduced cotyledon chl loss, and a treatment combining CF and decapitation increased chl content to a level greater than the starting level at 10 DAP. Cotyledons from plants treated 15 DAP, when considerable chl loss had already occurred, showed little response to either CF or decapitation. Unifoliate leaves responded to both CF and decapitation from 10 DAP, when little chl loss was occurring, to 20 DAP, when they were rapidly senescing. As with cotyledons, CF treatment combined with decapitation increased chl in unifoliate leaves significantly above starting chl content. Unifoliate leaves of plants transferred to the dark 5–6 d after decapitation or CF

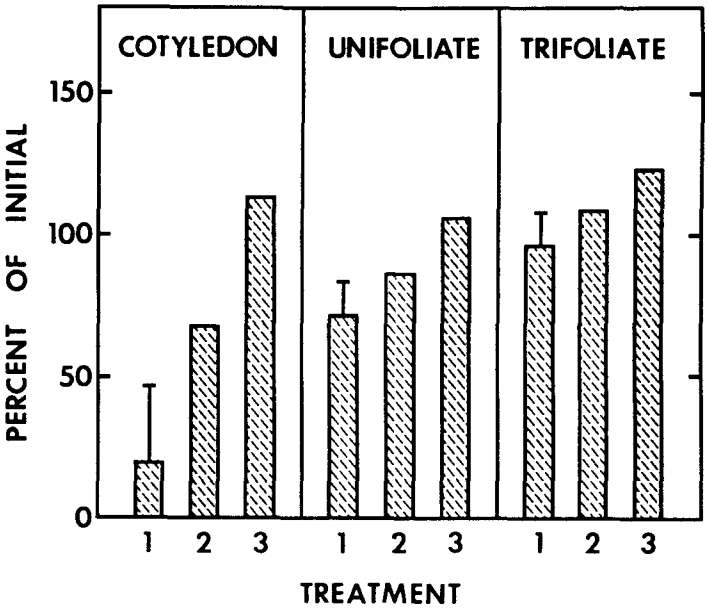

Fig. 2. Changes in weight of soybean cotyledons and leaves following decapitation or treatment with CF. Treatments 1, 2, and 3 = untreated check, decapitated, and 2.5 μg/ml CF, respectively. For cotyledons, experiment length = 8 d (treated 10 DAP); for leaves, experiment length = 9d (treated 17 DAP). Data expressed as percent of weight at treatment: 154 mg fresh weight/cotyledon, 2.5 mg dry weight/cm^2 unifoliate leaf, or 2.3 mg dry weight/cm^2 trifoliate leaf. Vertical bar = LSD (0.05).

treatment had higher chl contents than unifoliate leaves from check plants darkened at the same time, but in this case decapitation did not enhance the effect of CF in combined treatments. First and second trifoliate leaves showed chl changes after CF or decapitation similar to those in unifoliate leaves.

Changes in chl following CF treatment were accompanied by parallel changes in tissue weight (Fig. 2). Soybean cotyledons lost 79% of their fresh weight between 10 and 18 DAP, but the loss was significantly reduced by decapitation and prevented entirely by CF. Unifoliate leaves lost 28% of their specific leaf weight between 17 and 26 DAP; this loss was also diminished by decapitation or CF. Specific leaf weights of trifoliate leaves increased with decapitation or CF in the same period, even though untreated leaves were not declining in weight. Moisture contents of unifoliate and trifoliate leaves were significantly reduced by CF but were not affected by decapitation (data not shown).

CF retardation of chl decline in the dark was not apparent unless the plants were exposed to light after application as shown in two experiments. In the first (Fig. 3a), unifoliate leaves from untreated soybeans moved to the dark at the start of the experiment lost 84% of their chl content in 9 d. Leaves from treated plants held in the light 2, 8, or 24 h after CF treatment and then moved to the dark did not differ significantly in chl from untreated checks at the end of the test, whereas leaves from plants given 72 h light after CF treatment and

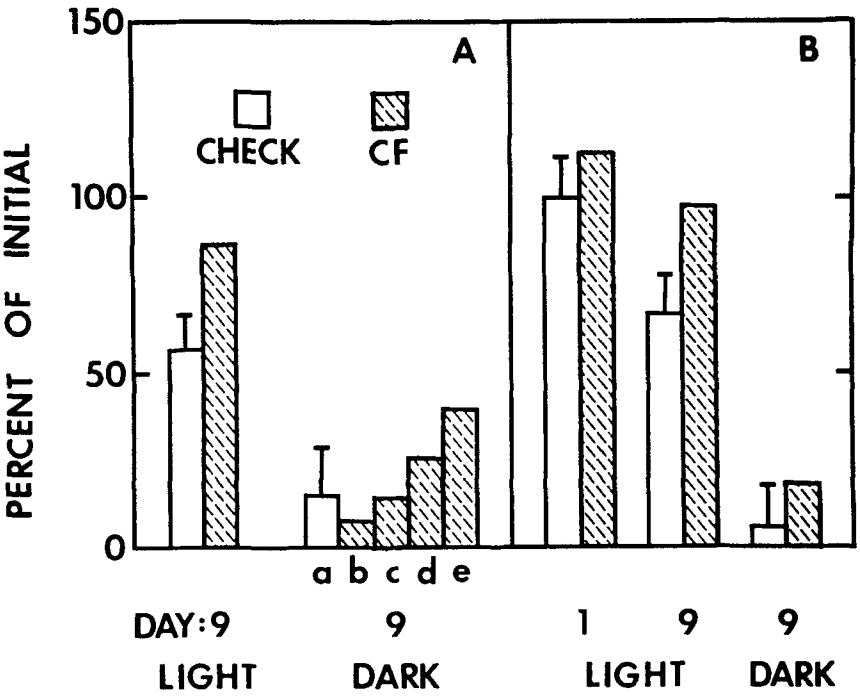

Fig. 3. Effect of CF on chlorophyll content of unifoliate soybean leaves in the dark. 3a. Chl content of untreated checks and plants treated 21 DAP and measured 30 DAP. Left = plants in light following treatment. Right = untreated checks held in the dark from 21 to 30 DAP (a) and plants transferred to the dark 2, 8, 24, or 72 h (b, c, d, or e, respectively) after CF treatment. Starting chl level 21 DAP: A_{665} = 0.940. 3b. Plants in light or dark analyzed 1 and 9 d after CF treatment. Plants in dark transferred to dark chamber 24 h after CF treatment (end of day 1). Plant age = 23 d at treatment. Starting chl: A_{665} = 0.756. Vertical bar in A and B = LSD (0.05).

then moved to the dark were higher in chl. Untreated plants held in light throughout the experiment had a much smaller decline in chl content than plants in the dark, and CF further reduced the loss. In the second experiment (Fig. 3b), CF increased chl in unifoliate leaves after 24 h for plants held in light, and chl was higher in treated leaves at the end of the experiment whether the plants were held in the light or moved to the dark 24 h after treatment. Extent of the chl decline in the dark from day 2 through day 9 was the same (0.72 absorbance units per three leaf disks) for both the check and CF-treated unifoliate leaves. Thus, the primary CF effect was enhancement of chl content during the first 24 h when the plants were in light, and not prevention of chl loss in the dark.

Leaf Disk Experiments

In floating leaf disk tests, morphactin effects on chl content differed according to concentration, molecular structure, and plant type (Table 2). Moreover, morphactins were generally less effective than kinetin and GA_3. CF at 10^{-4} M

Table 2. Effects of kinetin, GA_3, and morphactins on loss of chlorophyll by floating leaf disks in the dark.

Regulator	Plant	Concentration (M)			
		10^{-4}	10^{-5}	10^{-6}	10^{-7}
		Chl (treated as % of check)			
Kinetin	Soybean	135**[a]	114*	104	100
	Dock	86	86	76	82
	Radish	254**	225**	146*	100
	Barley	139**	138**	111	—
	Tobacco	—	131**	124**	—
GA_3	Soybean	164**	97	107	95
	Dock	278**	283**	289**	223**
	Radish	123*	92	111	92
	Barley	120*	119*	111	—
	Tobacco	91	97	99	89
Chlorflurenol	Soybean	72**	107	93	95
	Dock	300**	93	75	120
	Radish	222**	117	95	98
	Barley	108	108	92	—
	Tobacco	54**	82*	93	91
Dichlorflurenol	Soybean	86*	82**	88	90
	Dock	148*	98	101	95
	Radish	129*	92	100	95
	Barley	—	104	99	95
	Tobacco	26**	58**	77**	83*
n-Butyl Flurenol	Soybean	94	97	89	95
	Dock	202**	92	101	94
	Radish	—	—	—	—
	Barley	98	105	102	—
	Tobacco	78**	107	105	94

[a] (*) Significantly different from the check at $p=0.05$; (**) significant at $p=0.01$.

significantly increased chl loss in soybean and tobacco disks, but decreased loss in dock and radish. Results at 10^{-4} M for DCF and nBF were similar to those for CF. At lower concentrations, only tobacco responded to morphactins. Barley did not respond to morphactins at any concentration. Kinetin significantly retarded chl loss at 10^{-5} M for most species and at 10^{-6} M for two. Dock was particularly responsive to GA_3. Techniques such as dark aging of detached leaves before assay and increasing the duration of the dark period or age of the disks failed to increase responsiveness of soybean disks to CF (data not shown). Disks cut from unifoliate soybean leaves 24 h after plants received 2.5 μg/ml CF as a foliar spray lost more chl while floating in buffer in the dark than did disks from unsprayed plants.

Leaf Cell Analyses

Data collected from sectioned and macerated leaves are presented in Table 3. Decapitation increased specific leaf weight, leaf thickness, and palisade layer

Table 3. Effects of chlorflurenol on cell characteristics in first trifoliate leaf of decapitated and intact soybean plants.

Plant	Characteristic measured[a]	Chlorflurenol (μg/ml) 0	2.5	25
Intact	Leaf fresh wt. (mg/cm^2)	15.0	20.6**[b]	16.3*
	Leaf thickness (μm)	214.0	246.7**	265.4**
	Palisade layer thickness (μm)	61.9	65.1	77.7**
	Leaf cell count (No. × 10^6/g)	5.2	9.4**	11.5**
	Palisade cell count (No./field)	14.5	24.3**	25.9**
	Spongy cell count (No./field)	12.3	16.6**	18.5**
Decapitated	Leaf fresh wt. (mg/cm^2)	16.3	17.2	17.8
	Leaf thickness (μm)	255.0	243.0*	324.5**
	Palisade layer thickness (μm)	78.0	72.8	104.0**
	Leaf cell count (No. × 10^6/g)	6.8	9.9*	10.1*
	Palisade cell count (No./field)	18.5	23.3**	23.2**
	Spongy cell count (No./field)	12.2	14.9**	21.0**

[a] Samples collected 45 DAP (26 d after treatment).
[b] * = significantly different from 0 CF treatment at $p = 0.05$; ** = significant at $p = 0.01$.

thickness, but had little effect on cell count. CF (2.5–25 μg/ml) significantly increased most characteristics in both intact and decapitated plants. Compared to decapitation, CF was particularly effective in increasing cell numbers. Ratio of leaf thickness to length of palisade layer remained relatively unchanged at all rates of CF (3.5, 3.8, and 3.4 for 0, 2.5, and 25 μg/ml applied to intact plants, respectively), indicating uniform increase in thickness for all cell layers in the leaf. Palisade cell count did not increase beyond that attained at 2.5 μg/ml, whereas mesophyll cell count increased progressively with each increase in CF. As a result of these changes, CF-treated leaves were thicker and more compact with fewer intercellular spaces than untreated leaves (Fig. 4). Although cell counts showed that divisions had taken place in these tissues, no mitotic spindles were found, and observation of two cells in one sample that had recently divided provided the only visual evidence of cell division.

Cells of vascular cambia, which normally cease division and differentiate into phloem and xylem after the leaflet becomes fully expanded, continued to divide in midveins of leaves treated with CF. The new cells differentiated primarily into phloem (Fig. 5). Xylem cell diameter was increased in treated plants. Prolonged activity of the vascular cambium produced so many more cells that some primary and secondary phloem cells were crushed. Midvein fibers in treated plants appeared thicker and more lignified than in untreated plants.

Derivatives of the vascular cambium in swollen internodes of CF-treated plants did not differentiate into vessel elements but instead remained parenchymatous. The cambium of treated plants also became broader and more active in these internodes. Cells of pith and cortex differentiated into such active meristematic zones that in some internodes the pith resembled callus.

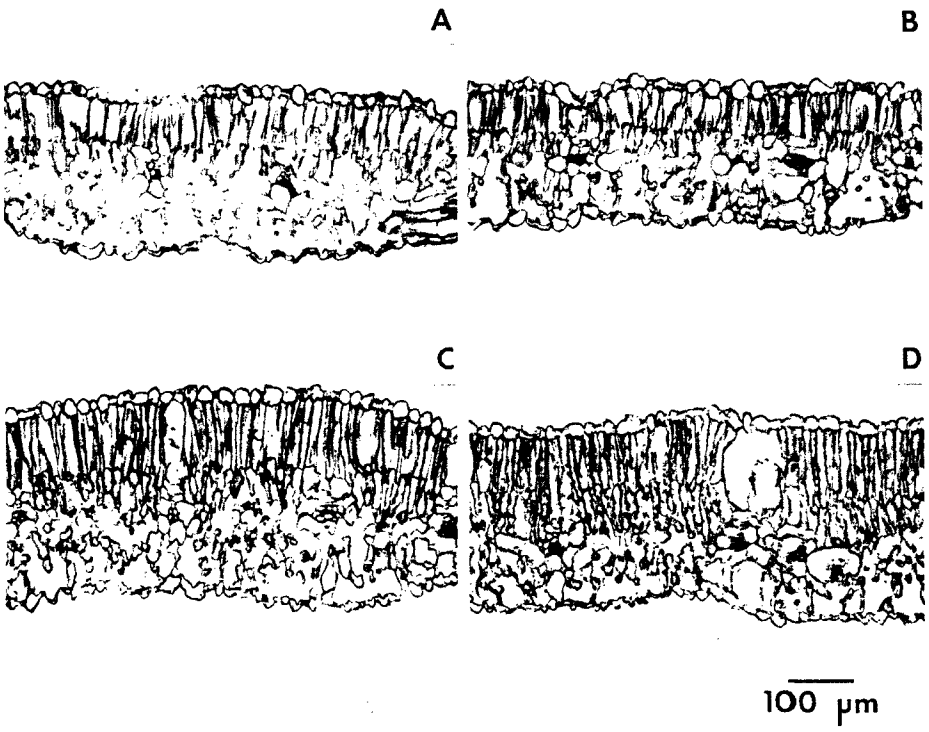

Fig. 4. Cross sections of terminal leaflet, first trifoliate leaf of soybean plants treated with CF at 19 DAP and sectioned 26 d later. A, B, C, D = 0, 2.5, 12.5, and 25 µg/ml, respectively.

Discussion

The dark green coloration that follows morphactin treatment has been ascribed to tighter "packing" of chloroplasts in new, incompletely expanded leaves (Schneider 1970), plus retarded chl breakdown in aging leaves (Harada 1967, Schneider 1970). To an extent, our results confirm both effects: dark green, deformed leaves were produced on new shoots stimulated from lateral buds of soybean, and dock and radish leaves floating in the dark on CF solutions retained more chl than the checks.

However, our results also show that these are not the only causes of increased chl in soybeans. Increased chl content occurred not only in new or senescing leaves but also in young, fully expanded leaves. Moreover, antisenescence activity of the morphactins appeared relatively unimportant in soybeans, since soybean was nonresponsive in floating leaf disk assays. Reduced chl loss in intact plants in the dark appeared to involve more than delayed chl degradation, because a 24 h or longer postapplication light period was required for positive CF response, a time that should be more than that needed for penetration of the chemical into the leaves.

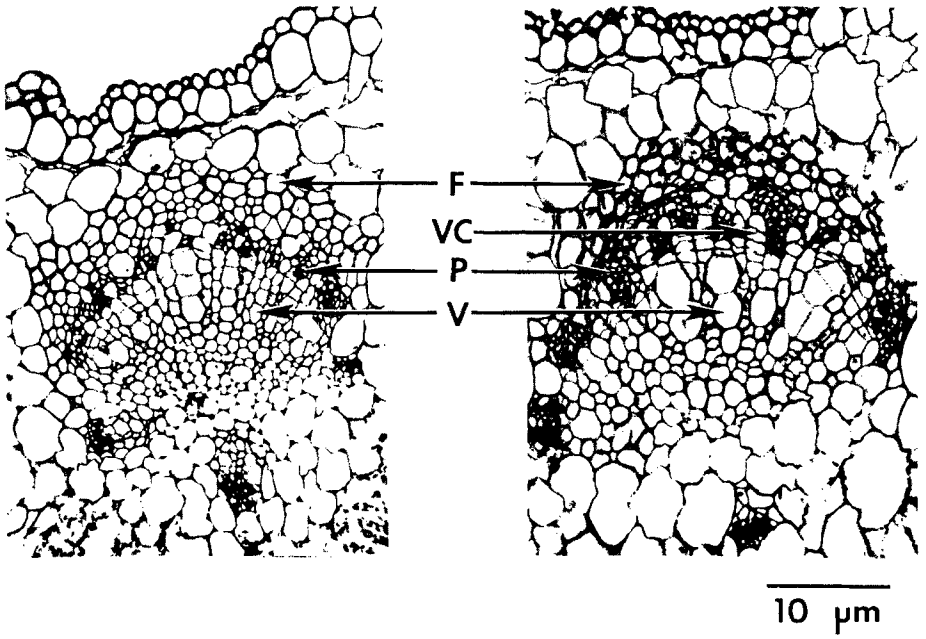

Fig. 5. Cross sections of midvein of middle leaflet, first trifoliate leaf of soybean. Left untreated; right 25 μg/ml CF applied 19 DAP. Sectioned 26 d after treatment. Arrows identify xylem vessels (V), vascular cambium (VC), phloem (P), and phloem fibers (F).

Response of intact soybean plants to CF appeared to involve close association between effects on chl content and promotion of growth in the affected leaf as well as in the whole plant. Chl responses were rapid (1–4 d), but at the same time, affected tissues changed in density. This appeared in 8 to 9 d as increased fresh and dry weight. By 26 d after treatment, extensive cell division and elongation had taken place in leaf mesophyll layers, along with renewed cambial activity in leaf vascular tissues. Stunting, lateral bud release, and other whole-plant deformities were severe at treatment rates of 25 μg/ml, and this rate gave maximum increase in chl content. Reducing CF concentration to 2.5 μg/ml markedly reduced deformation but still gave significant chl change; reduction to 0.25 μg/ml eliminated both growth and chl effects. Effects of CF on physiological changes and nutrient redistributions that customarily accompany leaf senescence (Noodén and Leopold 1978) are unknown.

Thus, the principal effect of CF treatment appears to be like the "regreening" effect observed in decapitated plants (Thimann 1980, Krul 1974) in which senescing lower leaves tend to recover and begin synthesis of chl, protein, and RNA. In fact, CF treatment and decapitation gave effects in soybeans that were not only similar but in some cases additive. Both treatments reduced chl decline in senescing tissues, increased tissue weight, increased leaf thickness, and stimulated palisade cell elongation. Yet, correspondence between

decapitation and CF treatment was not perfect, since CF increased cotyledon fresh weight and leaf dry weight/cm^2 significantly more than decapitation. Moreover, decapitation showed little tendency to stimulate division of leaf cells (as also shown for *Phaseolus* by Katagiri and Tsuji 1980), but CF markedly increased mesophyll cell count.

We conclude that effects of CF on mature and senescing soybean leaves appear more related to "regreening" (including increased leaf density resulting from elongation and renewed cell division in mesophyll layers) than to retarded chl breakdown of the type evidenced for leaf disks in the dark. In the field, where special effort is made to minimize deformation, i.e., low concentration, postbloom application (Dybing and Lay 1981a), retention of canopy greenness would appear to result from a combination of effects including increased foliage area through stimulated growth of normal shoots and retention of senescing foliage through "regreening."

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