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Reduction of Chloroplast DNA Content in *Solanum nigrum* Suspension Cells by Treatment with Chloroplast DNA Synthesis Inhibitors[1]

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ABSTRACT

Suspension cell cultures of *Solanum nigrum* were grown in the presence of six different chloroplast DNA synthesis inhibitors in order to determine whether the pool size of chloroplast DNA (cpDNA) could be selectively reduced relative to the nuclear DNA content. One of the effects of the inhibitors was a reduction in cell growth and viability. Cell growth (fresh weight) was reduced 50% (in 8 day cultures) by: 100 micromolar bisbenzimide, 8 micromolar ethidium bromide, 0.3 micromolar 5-fluordeoxyuridine (Fudr), 200 micromolar nalidixic acid, 30 micromolar novobiocin, or 10 micrograms per milliliter rifampicin. At these concentrations, three of the inhibitors, ethidium bromide, Fudr, and rifampicin, also substantially reduced the viability of the cultures. Analyses of the chloroplast and nuclear DNA content per gram fresh weight by dot blot hybridizations indicated that the reduction of cpDNA content was greatest at inhibitor concentrations which reduced cell growth by more than 50% but this depended on the culture conditions. For example, the two DNA gyrase inhibitors, nalidixic acid and novobiocin, were more effective in lowering cpDNA content in cultures which were transferred (2 × 4 days) once during the eight day incubation. Because several inhibitors were toxic to cell growth, the DNA content of treated cells was also determined on the basis of cell (protoplasts) number. Analyses of nuclear and cpDNA content per cell for each treatment indicated that only the DNA gyrase inhibitors, nalidixic acid, and novobiocin reduced cpDNA content. Neither inhibitor reduced nuclear DNA content. These results suggest that DNA gyrases participate in cpDNA replication. The selective reduction of cpDNA content in regeneratable cultures may facilitate the generation and selection of cpDNA mutants or transformants from higher plants.

Previous investigators have reported that cpDNA[1] synthesis can be selectively inhibited by a variety of compounds. In *Euglena,* inhibition of cpDNA synthesis by nalidixic acid, a prokaryotic DNA gyrase inhibitor, has been shown to lower the steady state copy number of chloroplast genomes without affecting the nuclear DNA content (14, 15, 20). In the green alga *Chlamydomonas,* treatment with the thymidine synthesis inhibitor Fudr and/or the DNA binding dye ethidium bromide results in the specific reduction of cpDNA content which recovers to normal levels following removal of the inhibitor (6, 29). In addition, Fudr treatment of *Chlamydomonas* has also been shown to increase the level of transmission of chloroplast DNA for mt^- parent in crosses and recovery of chloroplast mutations (29). This feature, reduction in chloroplast genome number, has been exploited as a means to increase the frequency of chloroplast transformation in *Chlamydomonas* (2).

Unfortunately, there have been few studies on the manipulation of cpDNA content in higher plants. In one of the few studies involving higher plants, Weisbach and coworkers (9, 10) demonstrated that nalidixic acid inhibited cpDNA synthesis in higher plant suspension cultures. However, it was not determined whether nalidixic acid treatment was toxic or, in fact, lowered steady-state levels of cpDNA. To determine whether cpDNA steady-state levels could be effectively reduced in higher plants without causing cell death, we screened a number of potential cpDNA synthesis inhibitors in suspension cell cultures of *Solanum nigrum* for their effects on chloroplast and nuclear DNA content and cytotoxicity. These studies were carried out with cultures which were either grown to stationary phase or rapidly transferred so as to maintain them in an active state of cell division and cpDNA replication (30). The results of our experiments demonstrate that certain inhibitors can selectively lower the steady state level of cpDNA in actively dividing cultures with minimal effects on culture viability. It is proposed that such treatments may be exploited as means to facilitate the generation and segregation of cpDNA mutants and/or cpDNA transformants in higher plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of wild type *Solanum nigrum* L. were surface-sterilized in 10% Clorox for 20 min, rinsed three times with sterile water, and geminated on MS medium (18) at 28°C. Excised hypocotyl sections from 10 to 14 d old seedings were used to initiate fresh callus cultures by culturing on MS medium supplemented with 2 mg/L 2,4-D and 0.25 mg/L kinetin at monthly intervals. Suspension cell cultures were initiated by transfer of 2 g callus to 50 mL of liquid MS (supplemented) medium and maintained on a shaker in the dark at 28°C.

[1] Abbreviations: cpDNA, chloroplast DNA; Fudr, 5-fluordeoxyuridine; MS medium, Murashige-Skoog medium; SSC, 0.15 M NaCl, 0.017 M Na citrate (pH 7.0); SSPE, 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA (pH 7.4); DAPI, 4,6-diamido-2-phenylindole.

Suspension cultures were subcultured by transferring 10 mL of suspension to 50 mL of fresh medium at 5 d intervals.

Isolation of Protoplasts

Protoplast were isolated by digestion of 4 g of suspension cells in 15 mL of 2% (w/v) cellulase (Onozuka RS), 0.2% (w/v) macerozyme (Onozuka R-10), 0.5 M mannitol, and 0.1% $CaCl_2 \cdot 2\ H_2O$. Cells were incubated on a platform shaker at 30 rpm for 14 to 16 h. The protoplasts were isolated by filtration through 200 μm and 74 μm sieves, washed two times with 0.5 M mannitol and harvested by floatation on a 20% sucrose solution which was centrifuged at 100*g* for 10 min. Protoplasts were then collected and rinsed twice with 0.5 M mannitol.

Treatment of Suspension Cells with cpDNA Synthesis Inhibitors

Suspension cells were broken up into small clumps by vortexing in an impingement tube and then filtered through a 20 mesh (890 μm) sterile stainless steel wire cloth. The homogenous cells were incubated in fresh MS (supplemented) medium for 2 d to allow the cells to reach early log phase. One gram (fresh weight) of cells was then transferred to 50 mL fresh MS (supplemented) medium plus 1 g casein and one of the following potential cpDNA synthesis inhibitors: bisbenzymide, ethidium bromide, Fudr, nalidixic acid, novobiocin, and rifampicin. The cell cultures were incubated with inhibitors for 8 d at 27 to 28°C and cell growth (fresh weight) was determined. The cells were then pelleted, frozen in liquid nitrogen, and stored at −80°C prior to DNA extraction. In another set of culture conditions, 2 g of inoculum were grown in fresh medium plus inhibitor for 4 d (it was necessary to use 2 g of tissue instead of 1 g due to cell death at higher concentrations of the inhibitors) and then transferred (1 g) to new culture medium plus inhibitor for 4 more d growth prior to DNA extraction. The effect of inhibitors on cell viability was determined by the recovery rate of cell growth after transfer to inhibitor free medium. Treated cells were washed and then grown in fresh medium for 7 d prior to fresh weight determination. Rates of growth were compared to cultures which had not been treated with inhibitors.

Analyses of Chloroplast and Nuclear DNA Content

Three different DNA isolation procedures (4, 12, 19) were compared for yield of chloroplast and nuclear DNA and degradation of DNA (data not shown). The procedure that proved to be most effective for complete extraction of intact nuclear and cpDNA was a modification of the mini-prep procedure developed by Dellaporta *et al.* (4). Briefly, 1 g of tissue was ground in liquid nitrogen to a fine powder. The dry powder was placed in 15 mL of extraction buffer containing 0.1 M Tris-HCl (pH 8.0), 0.05 M EDTA, and 0.01 M β-mercaptoethanol. After adding 1.0 mL of 20% SDS, the solution was mixed thoroughly by vortexing and heated at 65°C in a water bath for 10 min. Five mL of 5 M potassium acetate was added, and the solution was incubated on ice for 20 min to precipitate proteins and polysaccharides. These contaminants were removed by pelleting at 2,500*g* for 20 min. The supernatant was then poured through sterile miracloth into a 30 mL vortex tube containing 10 mL of isopropanol and placed at −20°C for 30 min. Total nuclei acids were pelleted at 20,000*g* for 15 min, resuspended in 600 μL water, and treated with 1.0 mg of RNase A for 1 h at 37°C. After phenol and $CHCl_3$ extraction, the DNA was precipitated with 1 volume of isopropanol and 0.1 volume of 3 M sodium acetate at −20°C overnight. The precipitate was then pelleted in a microfuge for 10 min, washed with 70% ethanol, and resuspended in 300 μl TE buffer.

Total extracted nucleic acids were bound to nitrocellulose using the procedure recommended by Schleicher & Schuell. DNA was loaded on the basis of tissue fresh weight extracted or cell number. The DNA (100 μL) was denatured by adding 15 μL of 3.0 M NaOH and 35 μL of water followed by incubation at 68°C for 1 h. After cooling to room temperature, 1.0 volume of 2 M ammonium acetate was added and mixed. One-half of each sample was applied under vacuum to a nitrocellulose membrane (0.45 μm) which had been prewashed in 1 M ammonium acetate. Known amounts of chloroplast and nuclear DNA standards corresponding to 30, 100, 300, 1000 ng of plasmid pBM5 (17) containing the psbA gene (chloroplast marker) and plasmid pSSU-160 containing the nuclear encoded small subunit of ribulose-1,5 bisphosphate carboxylase (1) were immobilized on the same membrane in order to confirm the linearity of dot blot signals. The membrane was then baked for 2 h at 80°C in a vacuum oven.

Membranes were prewashed in 200 mL of 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM EDTA, and 0.1% SDS for 1 h at room temperature prior to prehybridization in a heat seal bag containing 4 mL of 5× SSPE, 0.1% SDS, 5% dry milk, and 100 μg denatured calf thymus DNA at 40°C overnight. DNA fragments encoding the psbA and pSSU-160 genes were labeled by the random primer extension method (5). Approximately 3×10^6 cpm of denatured probe was then added to the bag and incubated for 20 h at 40°C. Filters were then washed three times in 2× SSC containing 0.1% SDS at room temperature, followed by three washes with 1× SSC containing 0.1% SDS at 65°C for 1 h. The filters were then autoradiographed and signals quantified by densitometry.

Quantification of Total Nucleic Acids by Fluorescent DNA Binding Dyes

Equal numbers of protoplasts (3×10^5/mL) from each treatment were stained with 1 μg/mL DAPI dissolved in Suzuki-Nishibayashi buffer as described in Miyamura *et al.* (16). Examination of labeled protoplasts using a fluorescence microscope indicated that virtually all fluorescence was localized in either the nucleus or chloroplasts. Relative nucleic acid content of DAPI-labeled protoplasts (3×10^5/mL) was determined spectrofluorometrically using an excitation wavelength of 372 nm and emission wavelength of 456 nm and expressed on the basis of fluorescence values from untreated cells.

RESULTS

Effects of DNA Synthesis Inhibitors on Cell Growth and Viability

The cpDNA synthesis inhibitors tested include: nalidixic acid and novobiocin, chloroplast DNA gyrase inhibitors (7–9, 11, 13, 24, 25), rifampicin, an inhibitor of chloroplast RNA polymerase (26, 28), ethidium bromide and bisbenzimide, DNA template binding dyes that inhibit cpDNA synthesis (6, 9, 10), and Fudr, which inhibits thymidine synthesis in *Chlamydomonas* chloroplasts (29). Cultures were either grown to stationary phase in continuous culture (8 d) or maintained in log phase of growth by transfer to fresh media on d 4 of the 8 d cycle (Fig. 1). The selection of inhibitor concentrations used for these studies was based on their effects on reducing culture growth and viability. The effects of various inhibitor concentrations on culture growth are shown in Figure 2. Treatment with 100 μM bisbenzimide, 8 μM ethidium bromide, 0.3 μM fluorodeoxyuridine, 200 μM nalidixic acid, 30 μM novobiocin, and/or 10 μg/mL of rifampicin inhibited cell growth by 50% in cultures maintained for 8 d in the same culture medium. Interestingly, several inhibitors were apparently more toxic in rapidly dividing cultures (transferred at 4 d intervals to new medium plus inhibitor, see Fig. 1 growth rates) than in cultures that maintained for 8 d in the same medium. Those inhibitors which were more toxic to cultures which were transferred once included bisbenzimide, ethidium bromide, and Fudr (Fig. 2). Transfer of novobiocin treated cells to new medium during the 8 d treatment had no effect on culture growth.

To determine whether exposure to the cpDNA synthesis inhibitors was lethal to the cultures, an inoculum of inhibitor treated cells was transferred to inhibitor free medium to determine whether growth would resume at normal rates. In general, treatments which substantially reduced growth rates, *i.e.* Fudr, ethidium bromide, bisbenzimide, and rifampicin also suppressed growth upon transfer to inhibitor free medium (Fig. 2). However, the toxicity of some inhibitors was greater than others. Ethidium bromide was the most toxic of the compounds tested followed by Fudr and bisbenzimide and rifampicin. In contrast, concentrations of novobiocin and nalidixic acid that reduced culture growth by 50% did not affect culture growth following transfer to new media without inhibitor (Fig. 2).

Effects of DNA Synthesis Inhibitors on Nuclear and Chloroplast DNA Content

The nuclear and chloroplast DNA content per g fresh weight of treated cultures was determined by dot blot hybridization using chloroplast (psbA) and nuclear (pSSU) gene-specific probes. Chloroplast and nuclear DNA was analyzed from cultures grown at inhibitor concentrations which effectively reduced growth by approximately 10, 50, and 80%. The results from these measurements from 8 d and 2 × 4 d treatments are shown in Tables I and II, respectively. With the exceptions of bisbenzimide and novobiocin, the chloroplast and/or nuclear DNA content of 8 d treated cultures was reduced 50% or more by each of the inhibitors tested (Table I). However, the concentrations of inhibitors which reduced the content of nucleic acids by 50% or more also reduced culture growth. Interestingly, the effectiveness the inhibitors varied with different culture conditions. The two DNA gyrase inhibitors, nalidixic acid and novobiocin, were more effective in reducing cpDNA content in cultures which were transferred during the 8 d growth period, whereas rifampicin and ethidium bromide preferentially reduced cpDNA content in 8 d continuous cultures and not in transferred cultures. In contrast, Fudr reduced cpDNA content in both 8 d continuous and 2 × 4 d cultures. Only one of the inhibitors tested, bisbenzimide, did not reduce cpDNA content under any conditions tested. The differences in effectiveness of inhibitors in reducing cpDNA content in 8 d *versus* 2 × 4 d cultures may be due to selective inhibition of cpDNA replication following transfer of cultures to new media (30). As shown by Yasuda *et al.* (30), there is a burst in cpDNA synthesis during the first 2 d after transfer of suspension cells to new media, whereas nuclear DNA synthesis rates do not peak until several days later.

At inhibitor concentrations which reduced culture growth by 80% or more, each inhibitor tested substantially reduced the content of nuclear DNA (ranging from 30–65%) as determined by dot blot hybridizations expressed on the basis of fresh weight (Tables I and II). The loss of nuclear DNA can not be readily accounted for by a reduction in the copy number of genomes similar to the reduction in cpDNA content. However, as demonstrated in Figure 2, high concentrations of inhibitors can be lethal and may result in the generation of heterogeneous populations of viable and nonviable cells. Since it was not apparent whether determination of DNA content on the basis of fresh weight was biased due to the presence of dead cells, alternate methods of DNA quantification were used to determine independently the total DNA content of viable cells only. Protoplasts were prepared from 2 × 4 d inhibitor treated suspension cells and the relative amounts of total nucleic acids from the different treatments was determined spectrofluorometrically using the fluorescent DNA binding dye, DAPI. As shown in Table III, bisbenzim-

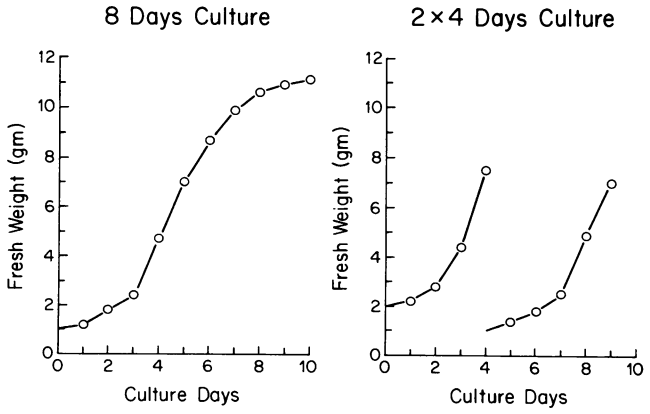

Figure 1. Growth of *S. nigrum* cultures in culture medium for 8 continuous d or following transfer on d 4 to new medium for 4 more d (2 × 4 d); see "Materials and Methods."

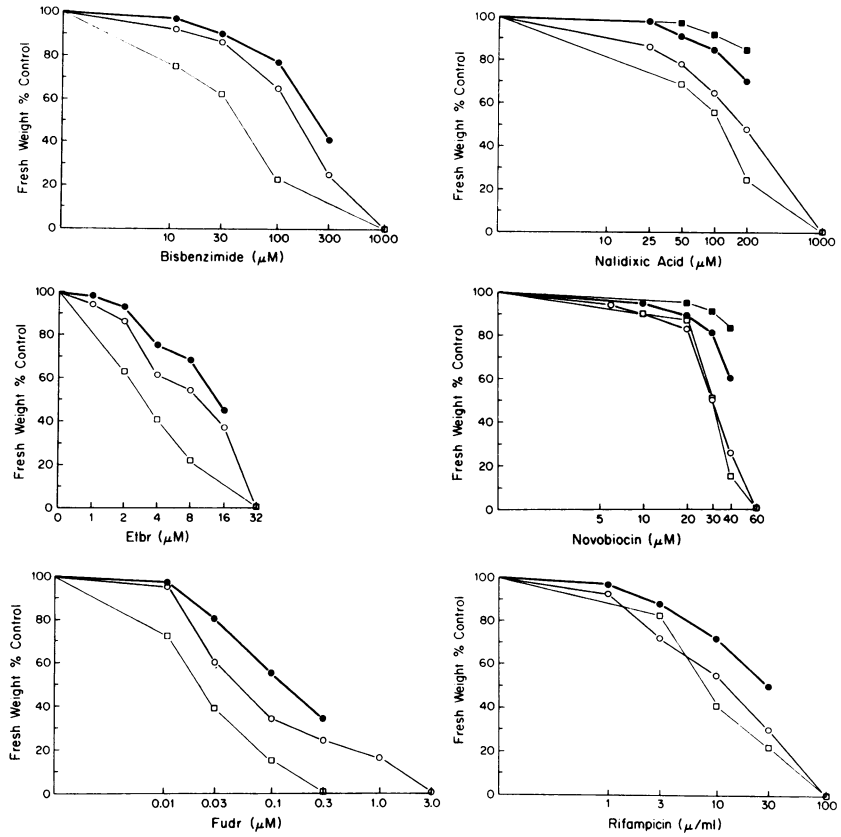

Figure 2. Effects of inhibitors on growth of cell suspensions of *S. nigrum*. (○), 8 d; (□), 2 × 4 d; (●), recovery after transfer to inhibitor free media (8 d); (■), recovery after transfer to inhibitor free media (2 × 4 d).

ide, Fudr, and rifampicin treatments reduced the total nucleic acid content of protoplasts by less than 10% although they were shown to reduce chloroplast and nuclear DNA content/g fresh weight by 25 to 60% as determined by dot blot hybridizations. In contrast, ethidium bromide, nalidixic acid, and novobiocin reduced total nucleic acid content per cell by 20% or more as determined by DAPI fluorescence values (protoplasts).

Since DAPI measurements do not discriminate between cpDNA and nuclear DNA content, nuclear and cpDNA per unit cell (protoplast) was determined by dot blot analyses using DNA extracted from protoplasts. As shown in Table IV and Figure 3, the nuclear DNA content per cell of inhibitor-treated cultures was nearly identical to that of untreated cultures on a per cell basis. These results were in contrast to results expressed on the basis of fresh weight (Tables II and III) indicating that measurements of DNA content on the basis of fresh weight were biased probably due to the presence of nonviable cells. Chloroplast DNA content expressed on the basis of cell number was not affected (≤15%) by Fudr, ethidium bromide, or rifampicin treatment. However, the two DNA gyrase inhibitors, nalidixic acid and novobiocin, reduced cpDNA/cell by 35 and 45%, respectively. Significantly, novobiocin and nalidixic acid treatment had little effect on nuclear DNA content/cell. Based on these results, and those obtained from growth and recovery studies, it appears that nalidixic acid and novobiocin treatment were the most effective in selectively reducing cpDNA content/cell without reducing cell viability.

DISCUSSION

Ideally, a specific inhibitor of cpDNA synthesis should not have any secondary or cytotoxic effects on cell growth unassociated with the reduction in cpDNA content. Although it was difficult to identify secondary effects of the various inhibitors screened, we demonstrated that certain inhibitors, while reducing DNA content, also substantially reduced cell viability. The most cytotoxic of the inhibitors tested were ethidium bromide and Fudr. In contrast, cell suspensions treated with the DNA gyrase inhibitors, novobiocin and nalidixic acid, effectively recovered cell growth following transfer to media lacking these inhibitors. Furthermore, we observed that among those inhibitors tested only novobiocin treated suspension cultures were capable of regenerating plants from calli. Thus, among the inhibitors tested, the DNA gyrase inhibitors appeared to have the least number of side effects on the cultures.

Both cpDNA and nuclear DNA content were determined on the basis of fresh weight as well as cell number. However, as previously mentioned, several of the inhibitors had adverse effects on culture viability. Since several of the inhibitors appeared to be cytotoxic, determinations of nucleic acid content from dot blot signals standardized on the basis of fresh weight may give an inaccurate estimate of the biologically active DNA content. As a result, we also quantified DNA isolated from protoplasts by dot blot hybridization and expressed DNA content on the basis of cell number. Two of the inhibitors, Fudr and rifampicin, which were previously shown

Table I. *Effects of DNA Synthesis Inhibitors on the DNA Content/g fresh weight of* S. nigrum *Suspension Cells (8 d Cultures)*

Cells were cultured for 8 d with various concentration of inhibitor. DNA extracted from these cells was applied to nitrocellulose and probed with chloroplast (psbA) and nuclear (ssRubisco) probes. Abundance of chloroplast and nuclear DNA was determined by densitometry of autoradiographs. The experiments were done in triplicate.

Inhibitor	Concentration	Nuclear DNA	cpDNA
	μM	% of control	
None		100 ± 3.3	100 ± 3.6
Bisbenzimide	10	67 ± 4.3	105 ± 5.3
	30	69 ± 3.2	63 ± 4.9
	100	71 ± 3.7	83 ± 6.6
Ethidium bromide	2	51 ± 3.9	33 ± 3.4
	4	61 ± 4.4	45 ± 3.6
	8	49 ± 3.3	30 ± 3.5
Fluorodeoxyuridine	0.1	78 ± 4.9	90 ± 3.1
	0.3	57 ± 4.6	28 ± 2.9
	1.0	57 ± 3.5	35 ± 4.3
Nalidixic acid	50	84 ± 6.2	100 ± 4.1
	100	49 ± 5.0	53 ± 5.7
	200	53 ± 2.9	63 ± 6.1
Novobiocin	20	84 ± 4.9	80 ± 5.6
	30	82 ± 3.8	80 ± 4.1
	40	73 ± 5.0	73 ± 2.4
Rifampicin	3 μg/mL	69 ± 4.8	55 ± 4.0
	10	61 ± 4.3	30 ± 4.2
	30	45 ± 4.9	25 ± 2.8

to reduce substantially cpDNA and nuclear DNA content on a fresh weight basis, had no effect on either chloroplast or nuclear DNA content expressed on the basis of viable cell numbers. Since it is difficult to rationalize 50% losses of nuclear DNA content without lethality we suggest that DNA determinations based on cell number are more accurate than those based on fresh weight. Based on measurements of DNA content per cell number only two of the inhibitors substantially reduced cpDNA content. These were the DNA gyrase inhibitors, nalidixic acid and novobiocin, which reduced cpDNA content per cell by 35 to 45%. Inhibition of cpDNA synthesis by the DNA gyrase inhibitor, nalidixic acid, has previously been demonstrated in *Euglena* (15, 20). Nalidixic acid treatment led to irreversible bleaching of *Euglena* cells accompanied by a substantial decrease in cpDNA (as determined by density gradient fractionation of total nucleic acids). Interestingly, the chloroplast DNA content of dark-grown *Euglena* or mutant strains unable to carry out photosynthetic electron transport was not reduced by nalidixic acid treatment (14, 15). Thus, nalidixic acid was only effective in reducing *Euglena* cpDNA content when cells were photosynthetically competent. In contrast, it does not appear that light is required for nalidixic acid dependent cpDNA content reduction in *S. nigrum* suspension cells (nongreen, dark grown). However, nalidixic acid and/or novobiocin treatment of *S. nigrum* suspension cultures did reduce culture growth by 50%. The fact that both nalidixic acid and novobiocin reduced culture growth suggests that reductions in cpDNA content in suspension cells leads to reduced culture vigor. One possible outcome of cpDNA reductions could be a reduction in plastid numbers per cell (21). While we have not determined whether plastid numbers are reduced by these treatments it is conceivable that reductions in plastid numbers could lead to reductions in the nonphotosynthetic metabolic activities compartmentalized in plastids (*e.g.* lipid, amino acid, and terpenoid synthesis) and, therefore, a reduction in culture growth.

The mechanism by which cpDNA content/cell is reduced by DNA gyrase inhibitors is most likely due to inhibition of cpDNA synthesis rather than by acceleration of cpDNA degradation. In support of this hypothesis, we note that Weisbach and coworkers (10) have shown that cpDNA synthesis is inhibited in nalidixic acid treated suspension cultures (tobacco and soybean) as determined by the level of [^{3}H]thymidine incorporation into cpDNA. We found that, in addition to nalidixic acid, the DNA gyrase inhibitor novobiocin also preferentially reduced cpDNA content/cell. Both of these inhibitors have been shown to reduce DNA synthesis levels in bacteria (11, 22, 27). Recently, a novobiocin sensitive chloroplast DNA gyrase has been partially purified from peas which can alter the superhelical density of cloned cpDNA sequences (11). Alterations in the superhelical density of cloned chloroplast genes has been shown to affect their transcription rates *in vitro* (11, 23). However, it is unlikely that inhibition of cpDNA transcriptional activity by DNA gyrase

Table II. *Effects of DNA Synthesis Inhibitors on the DNA Content/g fresh weight of* S. nigrum *Suspension Cells (2 × 4 d Cultures)*

Cells were cultured for 2 × 4 d (one transfer to fresh medium) with various concentrations of a inhibitor DNA extracted from these cells was applied to nitrocellulose and probed with chloroplast (psbA) and nuclear (ssRubisco) probes. Abundance of chloroplast and nuclear DNA was determined by densitometry of autoradiographs. The experiments were done in triplicate.

Inhibitor	Concentration	Nuclear DNA	cpDNA
	μM	% of control	
None		100 ± 4.1	100 ± 3.7
Bisbenzimide	10	81 ± 3.5	81 ± 5.7
	30	76 ± 5.6	81 ± 4.3
	100	64 ± 5.2	61 ± 3.7
Ethidium bromide	2	81 ± 5.1	106 ± 4.7
	4	79 ± 4.5	84 ± 4.3
	8	69 ± 4.7	65 ± 6.2
Fluorodeoxyuridine	0.1	93 ± 5.3	103 ± 3.7
	0.3	50 ± 5.2	39 ± 5.1
	1.0	40 ± 4.1	26 ± 5.2
Nalidixic acid	50	55 ± 5.4	42 ± 4.3
	100	38 ± 3.8	32 ± 3.9
	200	36 ± 2.2	19 ± 4.3
Novobiocin	20	98 ± 6.1	94 ± 3.3
	30	67 ± 3.3	65 ± 4.5
	40	71 ± 5.2	48 ± 3.0
Rifampicin	3 μg/mL	74 ± 4.0	65 ± 3.7
	10	43 ± 5.4	45 ± 4.3
	30	48 ± 3.9	35 ± 2.1

Table III. *Quantification of Total DNA in Protoplasts Labeled with the Fluorescent DNA Binding Dye, DAPI*

Cultures were transferred once during the 8 d treatment (2 × 4 d). Inhibitor concentrations were: 30 μM bisbenzimide, 4 μM ethidium bromide, 0.3 μM Fudr, 100 μM nalidixic acid, 30 μM novobiocin, and 10 μg/mL rifampicin.

Treatment	DAPI Fluorescence Values			Control
	Experiment 1	Experiment 2	Average	
				%
Control	121	130	126	100
Bisbenzimide	127	135	131	104
Ethidium bromide	97	101	99	79
Fudr	115	119	117	93
Nalidixic acid	96	99	98	78
Novobiocin	102	104	103	82
Rifampicin	111	116	113	90

inhibitors causes reductions in cpDNA content/cell, since rifampicin, an inhibitor of chloroplast RNA polymerase, had no effect on cpDNA content. We suspect that, similar to gyrase catalyzed replication processes in bacteria, the chloroplast DNA gyrase may be required for removal of DNA supercoils introduced during DNA replication or for decantenation of replicated cpDNA circles (11, 22, 27).

Last of all, we note that treatments which reduce or inhibit DNA synthesis in bacterial cells such as novobiocin and nalidixic acid treatment have been shown to enhance DNA recombinational and mutational processes (3, 22, 27). In fact, recA protein synthesis is promoted by nalidixic acid treatment of *Escherichia coli* (27). These observations suggest that nalidixic acid and novobiocin treatment of plant cells may also affect cpDNA recombinational processes. In support of this hypothesis it is noted that Boynton *et al.* (2) found that treatment of *Chlamydomonas* cells with 0.5 mM Fudr increased the frequency of cpDNA transformation (by homologous recombination) by 20- to 280-fold while also selectively lowering the cpDNA content (2). Preliminary results obtained from our laboratory indicate that novobiocin treatment of *Chlamydomonas* cells also reduces cpDNA content as well as enhances cpDNA transformation frequencies by homologous recombination. These results suggest that transformation of higher plant cpDNA by recombination mechanisms may be facilitated by treatment of cells with DNA gyrase inhibitors.

Table IV. *Effects of cpDNA Synthesis Inhibitors on DNA Content of Protoplasts Isolated from 2 × 4 d Suspension Cultures of* S. nigrum *as Determined by Dot Blot Hybridizations*

Protoplasts were isolated from 2 × 4 d cultures and treated with various inhibitors. DNA extracted from these protoplasts was applied to nitrocellulose and probed with chloroplast (psbA) and nuclear (ssRubisco) probes. Abundance of chloroplast and nuclear DNA was determined by scanning densitometry of autoradiographs.

Inhibitor	Concentration	Nuclear DNA	cpDNA
	μM	% of control	
None		100	100
Ethidium bromide	4.0	102	85
Fudr	0.3	92	85
Nalidixic acid	100.0	90	65
Novobiocin	40.0	97	55
Rifampicin	10 μg/mL	89	90

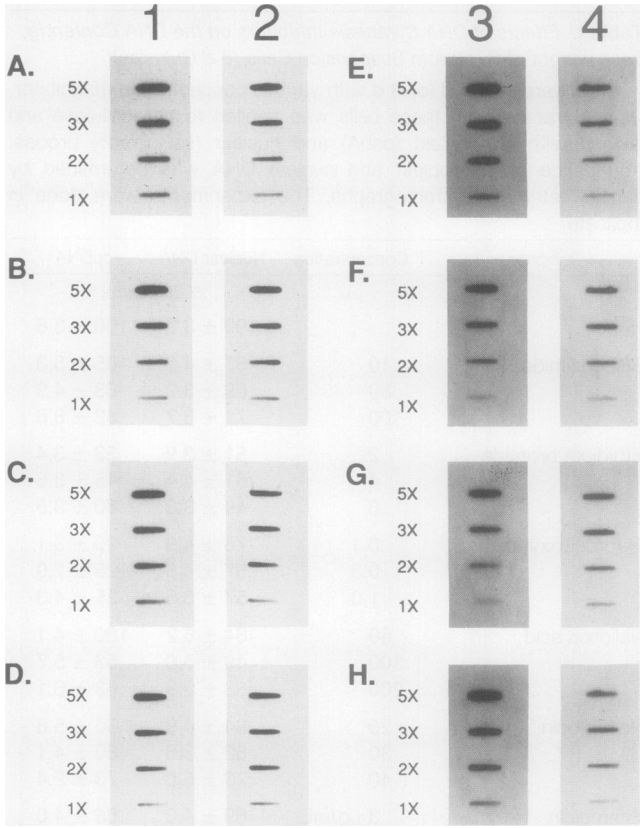

Figure 3. Dot blot of a dilution series of DNA extracted from *S. nigrum* protoplasts cultured for 2 × 4 d with various inhibitors and probed with the chloroplast encoded psbA gene, lane 1 and lane 3 or the nuclear encoded pSSU gene, lane 2 and lane 4. The treatments are: A, control; B, 30 μM bisbenzimide; C, 4.0 μM ethidium bromide; D, 0.3 μM Fudr; E, control; F, 100 μM nalidixic acid; G, 40 μM novobiocin; H, 10 μg/mL rifampicin. Approximately 18.0, 10.8, 7.2, and 3.6 μg of total DNA were blotted in a dilution series labeled 5×, 3×, 2×, and 1×, respectively.

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