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MOLECULAR BIOLOGY AND PHYSIOLOGY

Towards Improved Cell Cycle Synchronization and Chromosome Preparation Methods in Cotton

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ABSTRACT

Cotton is an economically important crop that needs more extensive genetic characterization. Highly effective methods for consistent cell cycle manipulation are needed to efficiently produce high quality cytological preparations of chromosomes, and to improve methods of chromosome doubling, both of which impact cotton genomics and breeding. This manuscript reports a procedure for cell cycle synchronization of root tips using hydroxyurea (HU), and the efficacy of several known chemicals for metaphase accumulation. Experimental treatments were administered hydroponically, followed by cytological determination of the metaphase index. An 18-hour treatment of HU at 3.5 mM gave a maximum synchronization of about 6%. Seedlings were treated with five antitubulin compounds of diverse chemistry; colchicine, amiprophos-methyl (APM), nitrous oxide gas, a benzamide designated RH-4032, and a novel phenylcyclohexene colchicine mimic RH-9472. Three of the antitubulin compounds, amiprophos-methyl, RH-9472, and RH-4032, provided average metaphase indices of 0.3 or higher on synchronized root tips and were preferable to colchicine, the standard agent for metaphase accumulation. The chromosome dispersing effects of nitrous oxide allowed the production of high quality chromosome preparations, although nitrous oxide showed little ability to increase metaphase indices or to act concertedly with the other mitotic blocking compounds, which tend to cause chromosome aggregation.

Cotton (*Gossypium hirsutum* L.) is an economically important fiber and oilseed crop. Because of its value, genetic improvements through conventional and biotechnological means are being sought throughout the world. Efforts to characterize the cotton genome are expanding domestically and internationally (International Cotton Genome Initiative, 2004) and prospectively include complete genome sequencing. Chromosome manipulation and cytogenetic analysis are essential to germplasm introgression, genetic analysis, and genomics, all of which are vital to breeding and breeding-related research. Procedures that enable reliable production of high quality mitotic chromosome preparations from cotton will help render the species more amenable to diverse protocols, e.g. cytogenetic analysis, chromosome doubling for ploidy manipulation, and harvesting of chromosomes for flow sorting and chromosome-specific BAC library development. Development of these capabilities in cotton would be facilitated by the development of suitable cell synchronization procedures.

A variety of methods have been developed to induce cell cycle synchrony in plant systems. Nutrient starvation followed by transfer to fresh medium has been used with some success in suspension-cultured cells (Arumuganathan et al., 1991). Synchrony is critically dependent on the state of the cells, so most methods employ DNA synthesis inhibitors that have nominal affects on cell viability. One of the most commonly used inhibitors is hydroxyurea (HU), which targets the nucleotide biosynthesis pathway needed for DNA synthesis by reversibly inhibiting ribonucleotide reductase and the production of deoxyribonucleotides and ribonucleotides. Treatment periods are generally longer than the length of the cell cycle, and result in the accumulation of cycling cells that have been arrested in late G1 to early S phase. Removal of the inhibitor allows the arrested cells to resume cycling in a synchronous manner.

Following synchronization, cells can be accumulated in metaphase by a treatment that is antagonistic to chromosome movement. Disruption of the spindle is a common approach, because it eliminates tension on centromeres and invokes a tension-reporting metaphase/anaphase cell cycle checkpoint. Disruption of the mitotic spindle apparatus typically involves treatment with an antitubulin chemical and/or cold

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temperature. The length of the treatment generally correlates with the percentage of cells in arrested metaphase, although longer treatments are associated with chromosome decondensation and chromosomes splitting into chromatids (Dolezel et al., 1992). Cytotoxicity of the chemicals makes extended treatment undesirable. The most popular antitubulin chemical is colchicine, despite being notoriously inconsistent for plant cell cycle synchronization (Bordes et al., 1997). Colchicine is known to be more potent toward mammalian cells than toward plant cells, which makes it a strong mammalian toxin and mutagen that must be used with extreme caution.

Alternatives to colchicine have been developed that are largely specific to plant tubulin. Antimicrotubule herbicides belong to a few chemically diverse classes, including the dinitroanilines (e.g. oryzalin and trifluralin), the phosphorothioamidates [e.g. amiprophos-methyl (APM)], the benzamides/carbamates [e.g. pronamide, chlorpropham, and isopropyl N-(3-chlorophenyl) carbamate], as well as others (reviewed in Molin and Khan, 1997 and Vaughn, 2000).

Concentrations necessary for significant effects in vivo are typically much lower than concentrations needed to inhibit taxol-induced polymerization of purified plant tubulin (Morejohn and Fosket, 1991). This has been attributed to the lipophilic nature of these chemicals, which facilitates their uptake and allows for high effective concentrations at sites of microtubule nucleation, i.e. the nuclear envelope (Stoppin et al., 1994) and cell cortex (Chan et al., 2003). The observation that lipid levels correlate with tolerance to these chemicals, which may explain why dicots are less susceptible than monocots (Molin and Khan, 1997), is perplexing. Particularly relevant to applications in cotton is herbicide partitioning into lipid-rich gossypol glands (Stegink and Vaughn, 1988).

Many reports have compared potencies of these agents and their relative utility for botanical studies. Hess and Bayer (1974) specifically studied the effect of trifluralin on cotton microtubules. Some polyploidy and polymorphonucleate cells resulted from formation of restitution nuclei, but treatments were prone to yield an undesirable range of mitotic disruption in single samples attributed to the exceedingly low water solubility of trifluralin. The phosphorothioamidate herbicides are symptomatically indistinguishable from the dinitroanilines and bind to the same site(s) on α- and β-tubulin (Blume et al., 2003) but have greater than 100-fold higher solubility in aqueous solutions than dinitroanilines (Morejohn and Fosket, 1991). Carbamate herbicides interact with plant tubulin in a poorly understood manner, but the effect of these herbicides on plant microtubules is generally much less than APM and the dinitroanilines (Morejohn and Fosket, 1991). APM was more effective than oryzalin, trifluralin, and pronamide for chromosome doubling in *Beta vulgaris* L. ovule culture (Hansen et al., 1998).

Two novel chemicals, 3,5-dichloro-N-(3-chloro-1-ethyl-1-methyl-2-oxopropyl)-benzamide, designated RH-4032 (Young et al., 2000), and *trans*-4-nitro-5-(2,3,4-trimethoxyphenyl)cyclohexene, a colchicine mimic designated RH-9472 (Young et al., 2001), have been developed with significantly increased affinity for plant tubulin. In assays for inhibition of tobacco root growth, both RH-4032 and RH-9472 strongly inhibited elongation and produced swollen root tips, a characteristic feature of antitubulin herbicides (Vaughn and Lehnen, 1991), and each chemical was more potent than all other herbicides that were compared (trifluralin, pronamide, zariliamide, APM, and chlorpropham for RH-4032, and trifluralin and pronamide for RH-9472). Another method for mitotic disruption is the use of nitrous oxide, a nontoxic gas that in maize has proven more effective than colchicine, trifluralin, APM, and temperature pretreatments for metaphase accumulation and chromosome counting (Kato, 1999).

Several reports have linked the use of microtubule disruptors with chromosome clumping. Chromosomes treated at metaphase tend to aggregate tightly into clumps, making them impossible to distinguish by microscopy or separate by flow cytometry (Lysak et al., 1999). Lee et al. (1996) noted that chromosomes began to clump with trifluralin treatments over five hours, and recommended that treatments be kept as short as possible to minimize the problem. Lysak et al. (1999) and Dolezel et al. (1999) reported that overnight incubation of APM-treated roots in ice water improves chromosome spreading for several cereal species.

In contrast, Kato (1999) indicated that nitrous oxide does not cause the chromosome-clumping side-effect. In fact, it disperses maize metaphase chromosomes so that the distances among chromosomes increase with higher treatment pressures. This makes nitrous oxide a potentially valuable pretreatment agent when used in combination with other mitotic blocking compounds, if the dispersal effect can overcome chromosome aggregation.

This manuscript describes a cell cycle synchronization procedure for *G. hirsutum* using HU, compares a variety of anti-microtubule agents of diverse chemistry (APM, RH-9472, RH-4032, nitrous oxide, and colchicine) for their ability to arrest metaphases, and describes approaches that enhance somatic chromosome preparations using nitrous oxide.

MATERIALS AND METHODS

Germination. All experiments used line 96WD22 of *G. hirsutum* (courtesy of Peggy Thaxton), subsequently released as cultivar Tamcot 22 (Thaxton et al., 2005). Seeds were germinated in "ragdolls" by evenly spreading them on moist germination papers and rolling the papers loosely into bundles. The ragdolls were soaked for at least 10 min in an antifungal solution of etridiazol plus thiophanate methyl (Banrot; Scotts Co.; Marysville, OH) to prevent seed rot and to allow the seeds to imbibe water, and then were placed vertically with the secured end up in 1 cm of Banrot solution in the dark at 30 °C for 2 d.

Synchronization. To establish the synchronizing effects of HU, actively growing seedlings were selected and suspended with a pipette tip holder in 1X Hoagland's solution (Sigma-Aldrich Chemical Co., St. Louis, MO) containing various concentrations of HU (Sigma-Aldrich) (Fig. 1). The seedlings were then placed back in the dark at 30 °C with vigorous aeration for 18 h. A fish tank aerator and plastic tubing were used for aeration. At the end of the 18-h treatment, seedlings were rinsed vigorously with 30 °C water prior to placement in fresh 1X Hoagland's solution. Primary root tips were harvested at various time periods afterwards and fixed overnight in "3:1" fixative, 3 parts ethanol : 1 part glacial acetic acid.

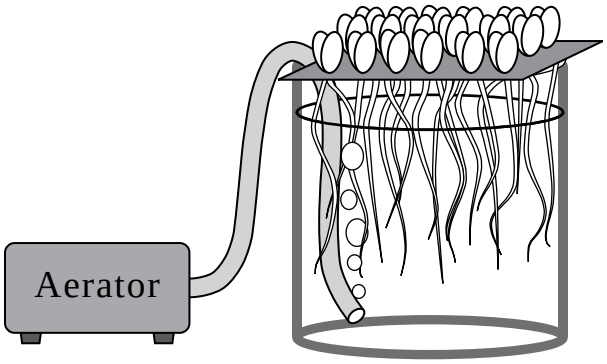

Fig. 1. Hydroponic treatment apparatus used for synchronization.

Metaphase accumulation. The effects of antimicrotubule chemicals were assessed on both synchronized and nonsynchronized seedlings. For synchronized seedlings, aliquots of APM (courtesy of Bayer Agriculture Division, Kansas City, MO), RH-9472, RH-4032, or colchicine in DMSO were added to the recovery solution 2 h after removal from HU. RH-4032 and RH-9472 were obtained courtesy of Dow Agrosciences, Indianapolis, IN. For nonsynchronized seedlings, HU was left out of the 18-h pretreatment solution. Seedlings were administered nitrous oxide in a 20-cm-dia. air-tight iron chamber (Fig. 2) that was pressurized over a 10-min period. Root tips were then harvested and fixed as above at various time periods.

Fig. 2. Pressure chamber for nitrous oxide treatment of seedlings flanked by the chamber gas valve (black arrow) and pressure gauge (white arrow). The chamber was accessed by unscrewing the circular top (ca. 24-cm diameter).

Slide preparation, staining, and microscopy. Chromosome preparations were made according to the methods of Kim et al. (2002), except for the pretreatments and slight modifications. Root tips were removed from fixative and placed for an hour in two changes of water. One- to 2-mm segments behind the root cap were excised with a razor blade and digested in a cell wall hydrolytic enzyme solution of 5% cellulase (Onozuka R-10; Yakult Honsha Co. Ltd., Tokyo), and

2.5% pectolyase Y-23 (Seishin Corporation, Tokyo) in 0.1 M citrate buffer at 37 °C for at least 1 h, after which the root tips were suspended in water. Groups of 10 tips were placed on slides; the excess water removed using a fine-tip pulled Pasteur pipette, and immediately spread to homogeneity in a drop of 3:1 fixative to a nickel-sized region. To facilitate cell separation by briefly elevating the relative humidity at the slide surface, a gentle breath was applied to the glass slide immediately after spreading and again after 30 s.

Dried slides were then stained with azure B (Sigma-Aldrich), 25-200 µg/ mL of 0.1 M phosphate buffer at pH 6.4-7.0 (Crane et al., 1993) and analyzed by light microscopy. For consistency and to allow comparison between data sets, both synchronization and metaphase accumulation were assessed by the metaphase index. In samples blocked for normal microtubule function, this is taken as the fraction of cells with condensed chromosomes. In non-blocked samples (control or HU only), this fraction equals prometaphase, metaphase, and anaphase cells. No less than three slides were evaluated per sample, with a total cell count per slide of 300 to 1200. All data reported combine three independent trials. Data were analyzed with SPSS (ver. 11.0.1; SPSS Science; Chicago, IL).

RESULTS AND DISCUSSION

Synchronization with HU. Timing and concentration effects HU on the metaphase index are shown in Fig. 3 (A) and (B), respectively. The collective results from three experiments are depicted in Fig. 3 (A), where seedlings were treated with 3 to 4 mM HU, and then samples were taken at 3 to 5 h post-treatment. The graphs reveal a peak of mitotic activity around 4.5 h following removal from HU. Figure 3 (B) represents combined data from three experiments aimed at determining useful concentrations, where bars are mean metaphase indices between 3.5 and 5 h (samples taken at 30 min intervals). These results demonstrate that HU alone at 3-5 mM increases the metaphase index at least 3-fold over untreated root tips with resulting slide preparations similar to Fig. 4, which shows a high percentage of metaphase and anaphase cells at 5 h after removal from 3.5 mM HU.

While a 3-fold increase in mitotic activity is significant, it is not consistent with the potent synchronization capacity of HU suggested by Dolezel et al. (1999), who used it alone in root tips of *Vicia faba* L. to attain mitotic indices exceeding 50%. While a direct comparison cannot be made to our figures, which exclude the prophase fraction, it is clear that *V. faba* was more responsive to HU. Species-specific differences were noted, but optimum HU concentrations never exceeded 2.5 mM for a range of both dicot and monocot species, and it was suggested that higher concentrations lead to delayed recovery from the block and poor synchrony. The relatively high HU concentrations required for cotton synchrony indicate that cotton is only weakly susceptible to HU-induced synchronization, and requires correspondingly high concentrations that also lead to unfavorable recovery. This could reflect the tetraploid nature of *G. hirsutum* in that paralogues of HU's target enzyme, ribonucleotide reductase, could exist and have variable susceptibility. These results warrant analysis of other synchronizing agents, such as aphidicolin and mimosine, and combinations of synchronizing agents, which may be more effective.

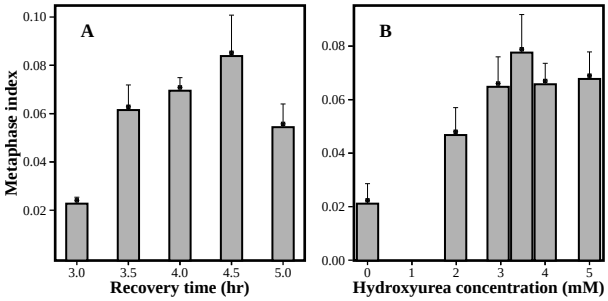

Fig. 3. Analysis of length of recovery from 3-4 mM HU (A) and HU concentration (B) for peak synchrony (between 3.5 and 5 h). Bars show mean metaphase indices, and error bars show +/- 1.0 SE.

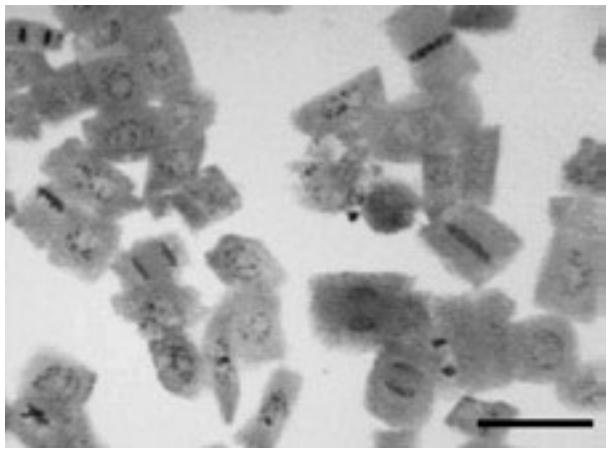

Fig. 4. Mitotic synchrony at 5 h following removal from 3.5 mM HU. Bar represents 50 µm.

Metaphase accumulation with APM. Metaphase accumulation of cells enhanced the level of synchrony achievable by HU treatment. Antitubulin chemicals disrupt polymerization of the mitotic spindle, causing failure of the chromosomes to align at the metaphase

plate and a delay at the metaphase/anaphase checkpoint of the cell cycle. APM was used to demonstrate the additional synchrony gained by temporary metaphase accumulation of synchronized cells (Fig. 5). Cells depicted by graphs (A) and (B) were synchronized with 3.5 mM HU, while cells depicted by (C) were maintained in HU-free medium. At 2 h after removal from HU, 10 µM APM was added to (B) and (C). Bars for each graph represent mean metaphase indices of three trials. Not all time points were collected for each experiment. Graphs (A) and (C) show the synchronization induced by HU alone and APM alone, respectively, while graph B shows the results of trials with both HU synchronization and APM metaphase accumulation. Peak mitotic activity occurred between 4 and 5 h after removal from HU, corresponding to 2 to 3 h of exposure to APM. Included in the 9-h metaphase indices of both APM-treated groups was a substantial proportion of decondensing metaphases, characterized by slightly elongated chromosomes. Metaphase accumulation by APM results in an additional 3- to 4-fold increase in synchrony over HU treatment alone, and APM treatment by itself produced metaphase indices intermediate to those of HU alone and the combined (HU/APM) treatment. Figure 6 shows accumulated metaphase cells resulting from the HU/APM treatment. Note the abnormal metaphase appearance because of chromosome aggregation.

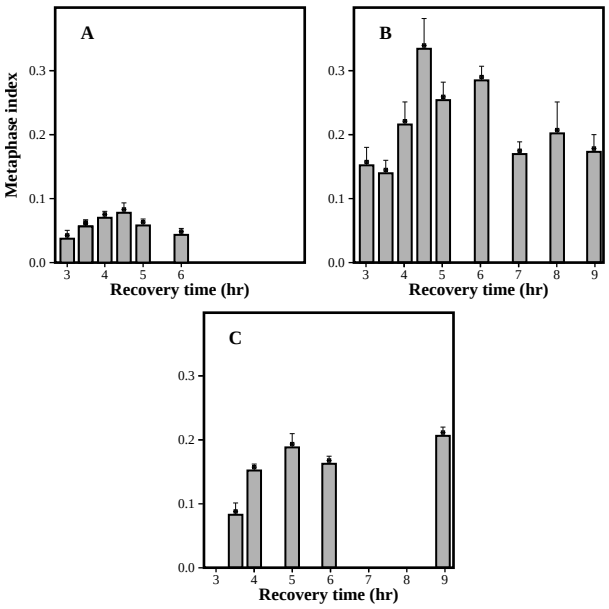

Fig. 5. Metaphase indices from (A) 3.5 mM HU alone, (B) APM-induced metaphase accumulation of HU-synchronized cells, and (C) nonsynchronized cells, where APM was added to 10 µM at hour 2 of "recovery time". Bars show mean metaphase indices, and error bars show +/- 1.0 SE.

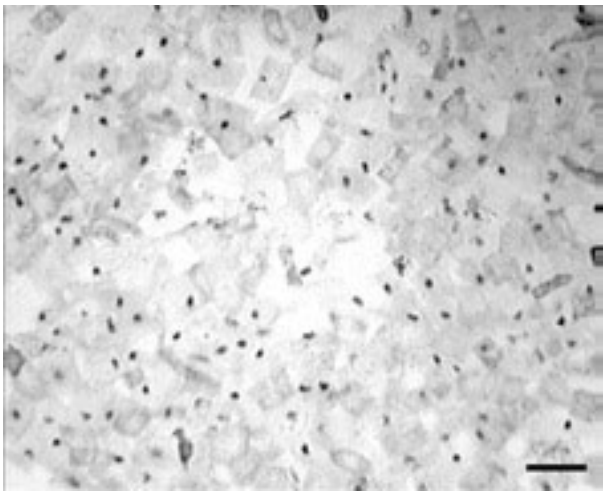

Fig. 6. APM-induced accumulation of metaphases. Bar represents 50 µm.

The graphs indicate that accumulation of arrested metaphases does not increase linearly with duration of APM treatment, and that the metaphase block is only temporary. The peak metaphase index occurs at 4.5 h of recovery in both (A) and (B), followed by a gradual decline, while that of nonsynchronous cells in (C) does not peak but rather plateaus at 0.2. Comparison of these three graphs indicates a transient APM-induced block that lengthens metaphase only minutes more than non-inhibited metaphase. The greater than 3-fold increase that APM contributes to HU-induced synchrony demonstrates the significance of the APM block, regardless of its short duration. In effect, APM integrates a small expanse of the HU-induced synchrony curve. Samples taken after 24-h treatments (not shown) had reduced metaphase indices comparable to untreated control roots and indicative of reduced cell cycling, possibly because of toxic effects.

Comparison of chemicals for metaphase accumulation. Comparisons were made with a variety of antitubulin chemicals in an effort to find the most effective agents and respective concentrations for metaphase accumulation. All chemicals demonstrated a time-dependent synchrony curve similar to that of APM in Fig. 5. Treatments were made on HU-synchronized root tips, and metaphase indices at 4 to 6 h of recovery were used for comparison of metaphase accumulation between chemicals (Fig. 7).

Colchicine had the lowest performance in the concentration range tested, with RH-9472, APM, and RH-4032 inducing higher metaphase indices at 10-fold lower concentrations. RH-9472 was espe-

cially potent, yielding average metaphase indices above 0.3 at just 1 µM. Even at 0.5 µM, the lowest concentration shown, RH-9472 shows a significant effect ($P = 0.021$ by *t*-test) compared with the untreated control. Note that the graphs do not include values for 0 µM treatments. Occasional instances of metaphase indices exceeding 0.7 were observed with both RH-9472 and APM, although significant variability may have been due to inconsistency of HU synchronization in these trials, as well as imprecise harvesting of root tip meristems.

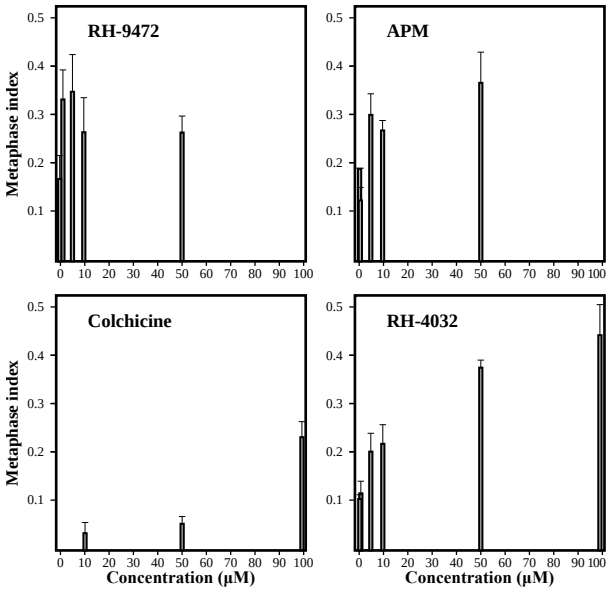

Fig. 7. Analysis of effects of antimicrotubule chemicals and concentrations on metaphase indices. Bars show mean metaphase indices from 4- to 6-h recovery, and error bars show +/- 1.0 SE.

Preliminary evaluations made with nitrous oxide gas indicated that pressures exceeding 600 kPa would be necessary to prevent cotton cells from progressing through metaphase (A. Kato, personal communication, 2005). Treatments of 800 and 1000 kPa (upper limit of suggested pressures) on nonsynchronized root tips were evaluated for metaphase-accumulating ability with the peak metaphase indices occurring with 1000 kPa for 4 h (Fig. 8). The results for nitrous oxide were well below those of the other chemicals tested, suggesting that it is inferior for metaphase accumulation in cotton.

Attempts to reduce chromosome clumping. As seen in the aforementioned figure of accumulated metaphase cells (Fig. 6), APM-treated cotton metaphase chromosomes demonstrated extreme susceptibility to the clumping effect of APM. Colchicine, RH-4032, and RH-9472 also exhibited this unwanted side effect. Existing methods to improve chromosome spreading were evaluated, including decreased treatment durations and ice water pretreatments, but had no significant effect on clumping.

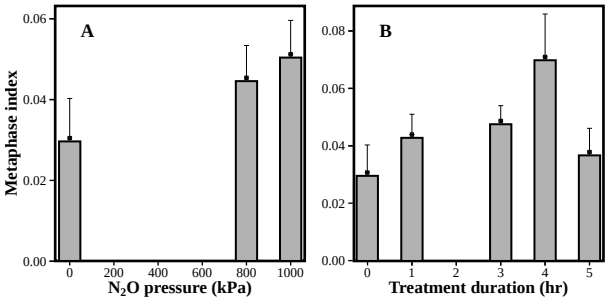

Fig. 8. Analysis of pressure of nitrous oxide treatments for (A) and analysis of nitrous oxide treatment duration at 1000 kPa (B). Bars show mean metaphase indices, and error bars show +/- 1.0 SE.

While the metaphase-accumulating ability of nitrous oxide was much less than the other agents, it was the only chemical treatment used in this experiment that did not induce chromosome aggregation. Consequently, it seems possible that the optimized use of nitrous oxide in combination with the other agents could circumvent the problem of chromosome clumping altogether. Trials were conducted combining nitrous oxide with other antitubulin treatments in an attempt to improve the quality of metaphases while preserving the high blockage attainable with these chemicals.

The physical appearances of metaphases from the different treatments are markedly different (Fig. 9). The results of these trials indicate that the inclusion of nitrous oxide with APM and other antimicrotubule treatments results in metaphases that are well spread similar to those attainable with nitrous oxide alone. The drawback, however, is a significant decrease in metaphase index. As seen in the graphs in Fig. 10, exposure to nitrous oxide significantly reduced the gains attainable by RH-9472. The reduction was enough that the addition of RH-9472 did not yield a relevant ($P > 0.1$) increase in metaphase index with nitrous oxide treatment. Similar results were obtained with nitrous oxide in combination with the other chemicals. These data combined with the results of extended-duration treatments (not shown) indicate that nitrous oxide exposure is toxic to cotton cells, resulting in a rapid decline of cell cycling rates that arrests cells prior to reaching the metaphase block. Because of this, further evaluation may be necessary in an alternative, less susceptible species, such as maize, to determine if nitrous oxide can indeed overcome chromosome aggregation.

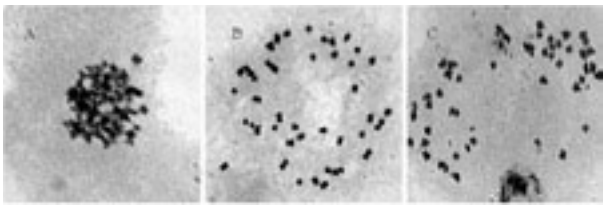

Fig. 9. Comparison of 3-hr antimicrotubule treatments with (A) APM 10 µM, (B) nitrous oxide 1000 kPa, (C) and simultaneous APM 10 µM and nitrous oxide 1000 kPa on metaphase appearance.

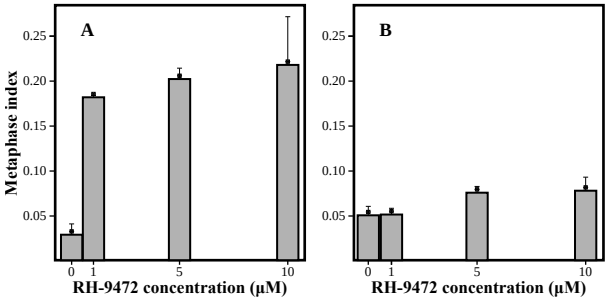

Fig. 10. Analysis of metaphase index for 3-hr treatments with (A) RH-9472 without nitrous oxide and (B) RH-9472 with 1000 kPa nitrous oxide. Bars show mean metaphase indices, and error bars show +/- 1.0 SE.

It seems that cotton represents a particularly troublesome species in regard to chromosome aggregation, and perhaps it is a problem that cannot be avoided in procedures using antitubulin chemicals. Induced chromosome aggregation represents an interesting biological phenomenon worthy of further investigation.

This research established a cell cycle synchronization procedure for cotton root tips that can be used reliably to attain metaphase indices of at least 0.3. Higher levels of synchrony are desirable, and other agents, such as mimosine, aphidicolin, and 5-aminouracil, deserve exploration. The level of synchrony depends intimately on the careful control of physical parameters during the procedure. Consistent temperature and the absence of light are essential. The roots should be rinsed thoroughly after removal from HU, as any carryover of HU into the recovery solution can lead to poor recovery. Additionally, the temperature of the rinse water should be consistent with that of the treatment solution.

APM, RH-9472, and RH-4032, are effective for metaphase accumulation in cotton, but the desire for higher indices warrants further exploration into the large array of antimitotic chemicals available with emphasis against lipophilicity. Additionally, trials could be conducted with glandless lines. The highest performing chemicals in the present study are more efficient and presumably safer to use than colchicine, and may also lend themselves to improved chromosome doubling methods in the near future. Preliminary flow cytometric data indicate that roots recovering from extended treatments are ploidy chimeras. Methods to prevent and/or disrupt chromosome clumping are in further development with particular emphasis on the use of nitrous oxide. The use of antitubulin chemicals for obtaining cytogenetics-quality metaphase cotton chromosomes will be greatly facilitated by a procedure to prevent and/or disrupt clumping.

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