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# Cytotoxic Effects of Metaphase-Arresting Methods in Barley[1]

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**Abstract**—Metaphase-arresting agents amiprophos-methyl (APM), colchicine (COL) and cell cycle-synchronization (CCS) with APM and hydroxyurea (HU) were tested for growth, metaphase index and cytogenetic abnomalities in barley (Hordeum vulgare cv. Bornova-92). Seeds were germinated for 2 days and then seedlings were treated with 8 μM (2.4 mg/L) APM for 2 h or 1.25 mM (0.5 g/L) COL or synchronized (CCS) with 1.25 mM (95 mg/L) hydroxyurea for 18 h and with 4 μM (1.2 mg/L) APM for 2 h. APM and CCS caused metaphase indices 12.57 and 38.82% respectively. COL also arrested metaphase (14.10%) but also resulted in nuclear aberrations (11.15%). After removal of APM and CCS, cells were released to grow and divide. However, COL caused irreversible effects on cell division and growth and meanwhile was shown to be effective for micronucleus formation.

*Keywords*: amiprophos-methyl, colchicine, cell cycle-synchronization, metaphase index, micronucleus

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## INTRODUCTION

For examination of chromosomes, one should have plant material containing dividing cells and arrest mitosis at metaphase step [1]. Chromosome- and microcell-mediated gene transfer requires chromosome isolation. High metaphase index is necessary for isolation of metaphase chromosomes [2]. This can be achieved by treatment of tissues with ice-cold water and colchicine (COL) [1]. Ice-water treatment arrests root tip cells in metaphase [3]. Although its effectiveness is low, ice-water treatment may improve chromosome spreading in cells treated with anti-microtubular drugs [4]. COL blocks mitosis by forming a tubulin-COL complex thus preventing the assembly of microtubules [5, 6]. COL was used for chromosome doubling in several plant species [7]. COL is highly toxic for mammals and also required in much higher concentrations for the inhibition of tubulin assembly in plants than in animals [8]. Therefore, there have been attempts to find COL alternatives [6]. Antimicrotubule herbicides *e.g.* oryzalin, amiprophos-methyl (APM) and trifluralin function as such alternatives because they have been reported to inhibit microtubule assembly in a way comparable to COL [9]. Antimicrotubule herbicides have been used in vitro for chromosome doubling in plant cells [10–12], and it was shown that these compounds are less toxic than COL [6]. Despite its relatively low phytotoxicity [11, 12]; APM is capable of metaphase arrest [13, 14]; chromosome doubling [6, 11] and micronucleus formation [2]. Moreover, effects of APM on spindle inhibition are reversible [13]. Cells can also be synchronized (cell cycle-synchronization, CCS) using hydroxyurea (HU) and APM to accumulate high number of metaphases. During CCS, tissues were incubated in HU solution to inhibit DNA replication. HU reversibly inhibits the enzyme ribonucleotide reductase and the production of all deoxyribonucleotides [15]. After this step, cells are accumulated in G1 or early S phase. Tissues are transferred to HU-free medium to release HU block and then to APM solution to accumulate cells in metaphase. However, CCS procedure with HU and APM takes longer time [4]. CCS protocol was developed for faba bean [16] and optimized for barley [17]. It was also shown that survival rates of COL-treated plants are lower than antimicrotubule herbicides [6, 18, 19]. COL has long-lasting effects upon plant growth and development independent from chromosome doubling.

In this study, cytophysiological effects of APM, COL, and CCS treatments on growth, mitotic index (MI), chromosomal abnormalities (AI), DNA and protein content were investigated in barley (*Hordeum vulgare* cv. Bornova-92). Toxicity of these treatments were compared and discussed.

## MATERIAL AND METHODS

Barley (*H. vulgare* cv. Bornova-92) seeds were provided by Aegean Agricultural Research Institute. Two-day-old barley seedlings were divided into three groups for APM, COL and CCS with HU and APM treatments. Each group had its own control (non-treated), treatment and post-treatment (recovery) subgroups.

[1] The article is published in the original.

In control subgroups, distilled water was added instead of compound. In post-treatment subgroups, compound was replaced with distilled water. All experiments were performed independently. At the end of the treatment and post-treatment steps, seedlings were rinsed with distilled water; briefly-dried on filter paper and root and shoot lengths of seedlings were measured. One part of seedlings was used for estimation of fresh weight (FW) and dry weight (DW). Seedlings were dried at 80°C for 2 days for estimation of DW. One part was used for chromosome analysis. Remaining seedlings were frozen in liquid nitrogen and used for protein and/or genomic DNA (gDNA) extraction. All laboratory ware were sterilised before experiments.

**Preparation of solutions.** 25 mg COL (C3915, Sigma) was dissolved in 1 mL distilled water to achieve a 2.5% w/v (62.5 mM) stock solution and kept at −20°C. 6.08 mg APM (03992, Fluka) was dissolved in 1 mL dimethyl sulfoxide (DMSO) to give a 20 mM stock solution and kept at −20°C. 7.6 mg HU was dissolved in 1 mL DMSO to give a 100 mM stock solution and kept at −20°C.

Feulgen and acetocarmine stains were prepared in accordance with early described method [3]. To prepare Feulgen stain, 0.25 g basic fuchsin (251332, Panreac) was dissolved in 50 mL boiling water throughly. When reached to 50°C, dye was filtered through filter paper into a dark bottle, 7.5 mL 1 mol/L HCl and 0.75 g potassium metabisulfite (P2522, Sigma) were added. Bottle was tightly-capped and incubated at room temperature (RT) for 24 h. Then 0.125 g activated charcoal (Merck) was added, shaked throughly and incubated at 4°C for 24 h. Dye was filtered into a dark bottle and kept at 4°C not longer than 3 weeks. To prepare 1% w/v acetocarmine stain, 1 g carmine (C-579, Fisher) was dissolved in 45 mL boiling acetic acid for 30 min. After that 55 mL water was added and mixed for another 30 min, filtered into a dark bottle and kept at RT.

**Germination and Treatments.** Twenty-five seeds were germinated for 48 h in a Petri dish (10 cm diameter) between two sheets of filter paper moistened with 10 mL of distilled water. Then seedlings were transferred to a new Petri dish (6 cm diameter) between two sheets of filter paper and 5 mL test solution. For APM treatment, seedlings were kept in 8 μM (2.4 mg/L) APM at RT for 2 h. For COL treatment, seedlings were kept in 1.25 mM (0.5 g/L) COL at RT for 18 h. For CCS seedlings were placed on filter paper soaked with 1.25 mM (95 mg/L) HU for 18 h and after rinsing with distilled water, incubated on wet filter paper for 5 h prior to placing them on filter paper moistened with 4 μM (1.2 mg/L) APM for 2 h. Then, seeds were rinsed with distilled water and incubated in ice-cold water 24 h in a refrigerator (4°C).

**Protein and gDNA Extraction.** gDNAs were isolated from seedlings in accordance with [20]. Extractions were performed gently using wide-bore pipette tips to prevent shearing of DNA. Integrity of gDNA samples were checked with agarose gel electrophoresis. 2 μg gDNA was mixed with 1/5 volumes of 6× DNA loading buffer (100 mmol/L EDTA (pH 8.0), 1% SDS, 60% glycerol, 0.03% bromophenol blue, 0.03% xylene cyanol), incubated at 65°C and resolved on 1% agarose gel containing 0.5 ng/μL ethidium bromide (EtBR) (Sigma) in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA). Gel was run at 80 V for 30 min and visualized under UV transilluminator. Band sizes were determined by comparison with a lambda DNA Hindlll digest marker (D9780, Sigma). Purity of gDNA samples were performed by UV spectrophotometer (Nanodrop)

Total proteins were extracted by homogenising plant samples with extraction buffer (56 mM $Na_2CO_3$, 56 mM DTT, 2 % SDS, 12% sucrose, 2 mM EDTA) and incubation at 70°C for 15 min. After centrifugation at 15000× g, 4°C for 10 min, supernatants were transferred into new tubes [21]. Protein concentration was determined by Bradford method [22].

**Chromosome Preparations.** Roots were excised and fixed (1 : 3 acetic acid : ethanol) at 4°C for 24 h. Fixed roots were kept in 70 % EtOH indefinitely. Squash chromosome preparations were made by the method described in [3]. For this, fixed roots were rinsed with water; hydrolyzed in 1 M HCl at 60°C for 18 min and stained with Feulgen at RT for 1.5–2 h. Darkly-stained root tip (appr. 2–3 mm) was excised and macerated on a slide in one drop of acetocarmine and then squashed under a coverslide. Slides were examined under light microscope (Olympus).

**Data Analysis.** At least 1000 nuclei from 2 root tips of each group were scored for calculation of MI and AI. The MI was calculated as the ratio between the number of mitotic cells and the total number of cells scored and expressed as percentage [23]. AI was calculated as the ratio between number of nuclei with any aberration and the total number of nuclei scored and expressed as percentage.

Root and shoot length, FW, DW, MI, protein and DNA content data were the arithmetic mean of three independent experiments ($n = 3 \pm$ standard deviation). Data were analyzed by One-Way Analysis of Variance (ANOVA). After ANOVA, statistical significance between two subgroups was examined by Least Significant Data (LSD) test.

## RESULTS

It has been found that APM, COL and CCS-treated roots are harder than control roots. However, after 2 days (in post-treatment subgroups), roots became softer. COL-treated and recovered after COL treatment roots had a distinctive root morphology. Root tips were swelled after COL treatment just above the elongation zone (data not shown). All treatments affected ($p < 0.01$) shoot length and FW; COL ($p < 0.05$), APM alone and with HU affected ($p < 0.01$) root

**Table 1.** Root and shoot length (cm), FW (mg) and DW (mg) of subgroups

| Group | Root | Shoot | FW | DW |
|---|---|---|---|---|
| Control (APM) | 1.71 ± 0.04 | 1.24 ± 0.31$^{A}$ | 97.44 ± 8.47$^{A}$ | 38.88 ± 8.38 |
| APM-treatment | 1.41 ± 0.08$^{a}$ | 0.94 ± 0.14$^{B}$ | 103.16 ± 6.44$^{B}$ | 43.33 ± 2.88 |
| APM-recovery | 2.06 ± 0.27$^{a}$ | 4.76 ± 0.28$^{AB}$ | 150 ± 8.66$^{AB}$ | 31.66 ± 7.63 |
| Control (COL) | 2.78 ± 0.41 | 3.24 ± 0.05$^{C}$ | 145 ± 5$^{C}$ | 33.33 ± 3.33 |
| COL-treatment | 2.13 ± 0.09 | 2.46 ± 0.4$^{D}$ | 135 ± 5$^{D}$ | 40.00 ± 8.66 |
| COL-recovery | 2.15 ± 0.11 | 5.19 ± 0.47$^{CD}$ | 176 ± 6.55$^{CD}$ | 35.00 ± 5.00 |
| Control (CCS) | 3.80 ± 0.51$^{BC}$ | 4.6 ± 0.33$^{E}$ | 205.55 ± 13.47$^{Ef}$ | 40.43 ± 4.04$^{a}$ |
| CCS | 1.78 ± 0.14$^{B}$ | 2.16 ± 0.21$^{EF}$ | 113.22 ± 12.15$^{E}$ | 36.53 ± 3.13 |
| CCS-recovery | 2.29 ± 0.04$^{C}$ | 3.89 ± 0.24$^{F}$ | 136.66 ± 20.81$^{f}$ | 22.33 ± 6.65$^{a}$ |

Here and Table 2 statistically-significant (LSD) data ($p \le 0.05$) are marked with same letter. Capital letters indicate very significant ($p \le 0.01$) data.

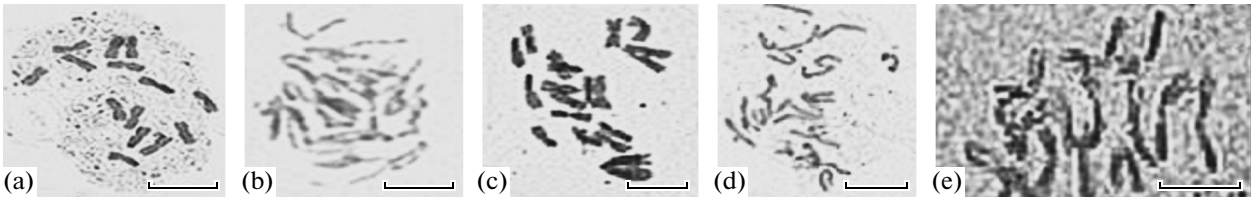
**Table 2.** Total protein (mg/g FW) and DNA content (μg/g FW), MI (%) and AI (%) of subgroups

| Group | Protein | DNA | MI | AI |
|---|---|---|---|---|
| Control (APM) | 39.42 ± 6.65 | 170.09 ± 61 | 6.92 ± 1.86$^{a}$ | 0.53 ± 0.05 |
| APM-treatment | 44.21 ± 2.35 | 149.06 ± 60 | 12.57 ± 0.87$^{ab}$ | 1.98 ± 1.55 |
| APM-recovery | 29.69 ± 8.91 | 198.53 ± 21 | 6.37 ± 1.86$^{b}$ | 2.62 ± 0.63 |
| Control (COL) | 46.35 ± 5.28 | 192.53 ± 91 | 4.79 ± 1.78$^{C}$ | 0.37 ± 0.13$^{A}$ |
| COL-treatment | 45.47 ± 15.04 | 203.74 ± 68 | 14.10 ± 2.88$^{CD}$ | 11.15 ± 3.21$^{B}$ |
| COL-recovery | 42.34 ± 6.25 | 173.60 ± 60 | 0.40 ± 0.19$^{D}$ | 28.39 ± 5.29$^{AB}$ |
| Control (CCS) | 40.39 ± 5.78 | 122.05 ± 5 | 1.48 ± 0.18$^{E}$ | 1.21 ± 0.78 |
| CCS | 47.87 ± 3.30 | 238.47 ± 33 | 38.82 ± 7.92$^{EF}$ | 2.19 ± 0.81 |
| CCS-recovery | 45.06 ± 11.08 | 188.55 ± 81 | 2.17 ± 0.76$^{F}$ | 1.35 ± 0.40 |

length (Table 1). APM alone increased FW compared to control. CCS decreased ($p < 0.01$) DW while APM alone or COL did not affect DW ($p > 0.05$). There are positive correlations between FW and root length ($r(7) = 0.84$, $p < 0.01$) and FW and shoot length ($r(7) = 0.86$, $p < 0.01$). After removal of compound, root and shoot length and FW increased.

APM- and COL-treatments and CCS did not have significant ($p > 0.05$) effect on protein and gDNA content (Table 2). All methods increased ($p < 0.01$) MI compared to control. In treatment subgroups, all dividing cells were in metaphase. In control and recovery subgroups, all stages of cell cycle were present. Only COL-treatment affected ($p < 0.01$) AI. Nuclear aberrations also occurred after removal of COL. Nuclear aberrations in treatment and post-treatment subgroups mainly included grouping of chromosomes, polyploid cells, binucle-ated cells, micronuclei formation and separation of chromatids at metaphase (figure). Micronucleus formation was the most prevalent aberration in recovery subgroup of COL.

According to values above (Tables 1 and 2), all methods were positively correlated (Table 3).




Nucleus morphology of root tip cells of seedlings treated with APM, COL and CCS. Letters on the figure indicate subgroups: (a) normal metaphase after APM-treatment; (b) aneuploid cell after APM-recovery; (c) grouping of chromosomes after COL-treatment; (d) aneuploid cell after CCS-recovery (APM + HU); (e) normal metaphase after CCS. Scale bars 25 μm.

## DISCUSSION

All three methods especially synchronization resulted in metaphase accumulation. Much higher concentrations of APM and COL and also longer duration of treatment gave lower percentages of metaphase in previous reports [2, 24]. However, those studies were performed in vitro in suspension cells. CCS was the most effective method in this study although metaphase accumulation efficiency was much lower than in previous reports [4, 17, 25, 26]. MI of 70 and 76.5% was achieved in maize and barley by synchronization with trifluralin, respectively [25, 27]. Trifluralin may be more effective than APM. Antimicrotubule effects of APM at micromolar concentration were already compared with COL at milimolar concentration. Then, it was stated that APM is a rapid, reversible antimicrotubule agent for plant cell cultures [28]. However, MI data were not calculated or presented in that study. Trifluralin and APM are regarded as potential antimalarial agents; neither trifluralin nor APM showed any inhibitory effect on mammalian cells at concentrations up to 64 μM [29]. Dinitroaniline-derived herbicides *e.g.* trifluralin inhibits formation of mitotic spindle [30] and dinitroaniline derivatives were shown to affect MI; cause cytogenetic disorders and also have a phytotoxic effects [31].

In this study, COL-treatment induced nuclear aberrations while APM and CCS did not. Grouping of chromosomes is common in root tips of spindle toxin-treated plants [2]. Micronucleus frequency increased (data not shown) even after removal of COL. Thus, it may be postulated that although metaphase-arresting capacity is lower than CCS, COL is very effective for micronucleus formation in barley while APM is not. Metaphase-arresting and micronucleus-inducing ability of APM was shown in cell suspension cultures [2, 32]. Interestingly, it was indicated that APM was more effective at micronucleus formation than COL [2]. However, according to an another report, oryzalin caused higher micronucleation indices than APM [33].

Antimitotic drugs are generally used for chromosome doubling in vitro and their toxicity is evaluated by embryo survival, necrosis and vitrification [34, 35]. In the present study, 2-day-old seedlings were used and toxic effects (APM, COL and HU+APM) were evaluated firstly on seedling growth. Germination percent, FW, root and shoot length, protein content and antioxidant enzyme acitivities are investigated for assessment of toxicity of several substances [36–39]. Toxic substances and ions, metals tend to decrease FW [36], root and shoot length [40], protein content [37, 39]. In the present study, all treatments inhibited root and shoot growth yet plants treated with APM or APM + HU were able to grow after removal of substances. However, roots could not grow after removal of COL. Protein and DNA contents were not affected. Salinity at certain levels decrease protein, DNA and RNA contents [41]. In another study, cells were able to show normal mitosis and growth after APM-treatment [32]. Very low MI in recovery subgroup of COL-treated plants showed that COL-treatment affected cell division irreversibly. This may explain the inhibition of root growth in COL-treated plants which were not able to recover. COL was known to decrease survival in treated plants and to cause sterility, chromosome rearrangements and abnormal growth [42–45]. However, tolerance to antimitotic substances varies with species [46]. High survival rates of banana explants treated with 5 mM COL for 24 h were reported [19]. COL is generally applied in a concentration range of 1.25–2.5 mM, while other antimitotic agents as oryzalin, trifluralin or APM have a final concentration of 1–50 μM [9]. Antimitotic agents are generally dissolved in DMSO which increases cell permeability yet decreases survival rates [44]. In the present study, COL was prepared in distilled water while HU and APM were dissolved in DMSO. Yet, COL-treatment was more toxic than APM and APM + HU. Plants in both treatment and recovery subgroups of COL had distinctive root tips. Plants with increased ploidy levels are sometimes apparent by their distinct morphology [45, 47, 48]. Enlarged cell size is often associated with polyploids, which can result in anatomical imbalances. These visual characteristics are sometimes helpful for identifying putative polyploids [47].

**Table 3.** Correlation coefficients between treatments ($r(22)$, $p < 0.01$)

| Group | AMP | COL | CCS |
|---|---|---|---|
| APM | – | – | – |
| COL | 0.972 | – | – |
| Synchronization | 0.904 | 0.942 | – |

In conclusion, COL-treatment and CCS was the best choice for micronucleus formation and metaphase arrest, respectively. Plants were able to overcome the toxic effects of CCS. Therefore, it may be hypothesized that root tip cells can be synchronized using HU and APM together to obtain high metaphase index without causing irreversible side-effects.

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