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Induction of microtubule damage in *Allium cepa* meristematic cells by pharmaceutical formulations of thiabendazole and griseofulvin

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ABSTRACT

Microtubules (MT) are formed by the assembly of α- and β-tubulins and MT-associated proteins. We characterized the effects of pharmaceutical formulations containing the microtubule disruptors thiabendazole (TBZ) and griseofulvin (GF) on the mitotic machinery of plant (*A. cepa*) meristematic cells. GF concentrations between 10 and 250 μg/ml were tested. GF induced mitotic index inhibition and genotoxic effects, including chromosome fragments, bridges, lagged chromosomes, C-metaphases, tripolar cell division, disorganized anaphases and nuclear abnormalities in interphase cells. Efects on the mitotic machinery were studied by direct immunofluorescence with β-tubulin labeling and by DNA counterstaining with 4′,6-diamidino-2-phenylindole (DAPI). Exposure of meristematic root cells to TBZ or GF, 100 μg/ml, caused microtubular damage which led to abnormal MT arrays. Our results suggest that GF induces abnormalities in spindle symmetry/polarity, while TBZ causes chromosome missegregation, polyploidy, and lack of cytokinesis.

1. Introduction

Inhibition of tubulin polymerization may lead to chromosome missegregation during mitosis [1]. Damage to microtubules (MT) affects major cellular functions, including intracellular transport, motility, and cell division [2,3]. MTs are formed by the assembly of α- and β-tubulins and microtubule-associated proteins (MAPs). Damage to MAPs may also result in MT disruption. Although the tubulin genes have been well conserved through evolution [4,5], there are differences in the MT arrays between the mitotic cells of plants and animals. Plant MTs assemble in three successive arrays: the preprophase band (PPB), the mitotic spindle (MS), and the phragmoplast (Phrag); these may be detected by immunofluorescence microscopy of tubulins [6]. PPB and Phrag are exclusive to plants, while MS is common to all eukaryotic cells [7]. In plants, exposure to chemical agents may affect MT organization, altering the dynamics of MT polymerization and formation of MT organizing centers (MTOCs) [8]. Abnormal chromosome segregation has been classified morphologically into two types: mitotic polarity/symmetry and segregation of individual sister chromatids [9].

The structures responsible for nucleation of the mitotic spindle are the MTOCs in vascular and non-vascular plants and the centrosomes in animals [10]. Plant MT nucleation sites are either dispersed in the cytoplasm or localized in endomembranes, such as those of the endoplasmic reticulum, nucleus, and plastid [11]. Therefore, the polarity/symmetry abnormalities in proliferating cells of plants and animals are expected to have different origins. The present study examines the effects of MT-disrupting agents on the mitotic machinery and nuclear architecture of meristematic cells of *Allium cepa*, a commonly used plant model for genotoxicity studies [12–14]. We applied direct immunofluorescence using β-tubulin labeling and DNA counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) to meristematic cells of *A. cepa* exposed to pharmaceutical formulations containing the MT disrupters thiabendazole (2-(4-thiazolyl)benzimidazole; TBZ) or griseofulvin (7-chloro-4,6,2′-trimethoxy-6′-methylgris-2′-en-3,4′-dione, GF). TBZ is used as a postharvest fungicide for fruits and vegetables and as an anthelmintic agent in veterinary and human medicine [15,16]. GF is a potent antifungal agent, extensively used to treat superficial dermatomycoses in animals and humans [17]. In vitro studies suggest that TBZ acts through inhibition of MT polymerization, by binding to β-tubulin [18]. The mechanism of MT disruption caused by GF is uncertain but several studies indicate that the drug may interact with MAPs [19]. In a previous

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genotoxicity study from our group, *A. cepa* meristematic root cells were exposed to the TBZ-containing pharmaceutical formulation Foldan®, 10–250 μg/ml; effects included mitotic index inhibition and induction of C-metaphases, lagging chromosomes, polyploidy and binucleated cells, which resulted in alteration of chromosomal segregation and cytokinesis [20]. Here, we used the pharmaceutical formulation Grisovin®, which contained GF, 10–250 μg/ml, to evaluate chromosome aberrations (CA) and, accordingly, to determine the most suitable concentration for further analysis of the mitotic machinery.

2. Materials and methods

2.1. Test chemicals

Foldan® (thiabendazole, 500 mg, CAS N° 148-79-8; excipients: microcrystalline cellulose CAS N° 9004-34-6; starch CAS N° 9005-84-9; croscarmellose sodium CAS N° 74811-65-7; povidone CAS N° 9003-39; copolyvidon CAS N° 89335-67-1; glycerin CAS N° 8043-29-6; magnesium stearate CAS N° 557-04-0) was obtained from Laboratorios Andrómaco (Buenos Aires, Argentina); dimethyl sulfoxide (DMSO, CAS 67-68-5) from Backer (Buenos Aires, Argentina); and Grisovin® (griseofulvin 500 mg, CAS N° 126-07-8; excipients: sodium lauryl sulfate CAS N° 151-21-3; povidone CAS N° 9003-39; corn starch CAS N° 9005-84-9; potato starch CAS N° 9005-25-8; magnesium stearate CAS N° 557-04-0) from Glaxo-Wellcome (Buenos Aires, Argentina). Bovine serum albumin (CAS 9048-46-8), mouse fluorescein isothiocyanate (FITC)-conjugated anti β-tubulin monoclonal antibody, 4′,6-diamidino-2-phenylindole (DAPI, CAS 47165-04-8), and paraformaldehyde (CAS 68476-52-8) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol (CAS 67-56-1), EGTA (CAS 67-42-5), cellulase (CAS 9012-54-8), and pectinase (CAS 9032-75-1) were obtained from Merck KGaA (Darmstadt, Germany). PIPES (CAS 5625-37-6) was purchased from MP Biomedicals (Solon, OH, USA).

2.2. Allium cepa meristematic cells and fungicide treatment

Fresh onion bulbs (*A. cepa*, $2n = 16$) were procured from a local market. Hand-picked bulbs of uniform size were carefully scraped so that the apices of the root primordial were exposed and their dry scales peeled off. The bases of the bulbs were thoroughly washed in running tap water for 1 h and then placed in tap water to facilitate root sprouting. After 24 h, bulbs with roots 1–2 mm long were used in the experiments.

2.3. Bulb treatments with GF and cytogenetic analysis

Using the same criteria as for TBZ exposure [20], a stock solution containing 500 mg Grisovin® in 20 ml DMSO was prepared and all test concentrations were obtained by appropriate dilution of the stock solution with filtered tap water. The following test concentrations were selected based on literature: 10, 50, 100 and 250 μg/ml GF. DMSO was used as solvent control at a maximum concentration of 1%. Independent experiments were performed in triplicate. The exposure lasted for 30 h to ensure the completion of two mitotic cycles. At the end of the exposure, root tips were cut and fixed in 3:1 ethanol–glacial acetic acid for 24 h. Finally, the meristematic cells were squashed and stained with 2% acetic orcein.

2.4. Scoring of slides and data analysis

The slides were examined under a Leica DMLB light microscope (1000×). 1000 cells from the negative control and treated groups were examined, with1000 cells per concentration tested. MI was expressed as the number of dividing cells per 1000 cells scored. Chromosomal aberrations (CA) were scored in 100 anaphases or telophases. The CA were classified into the following categories: chromosome fragments, bridges, vagrant or lagging chromosomes, C-metaphases and tripolar or multipolar cell division, interphase aberrations, i.e., micronuclei (MN), mini cells (MC) and nuclear buds (NB), were scored in 100 cells.

2.5. Bulb treatments and direct immunofluorescence using β-tubulin labeling

Based on the genotoxic and cytotoxic damage induced by both agents, we chose 100 μg/ml as the most appropriate concentration for assessing deleterious effects on the mitotic machinery. Onion bulbs were treated and slides were processed for immunodetection as previously described in detail [21]. Briefly, two groups of roots, one exposed to 100 μg GF/ml and the other to 100 μg TBZ/ml, were kept at 23 °C, under constant aeration and light protection, for 30 h (two cell division cycles). Negative controls (untreated bulbs) were processed concurrently with treated bulbs.

2.6. Scoring of slides and direct immunofluorescence of the mitotic machinery

Two slides per concentration and the controls were incubated with mouse fluorescein isothiocyanate (FITC)-conjugated anti-β-tubulin monoclonal antibody. The antibody was diluted 1:500 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, rinsed with PBS and air-dried. Finally, the slides were stained with DAPI. The extent of damage to microtubules was determined by scoring slides using an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination and Cario FISH 1.2 software. MT arrays (PPB, MS, and Phrag) were scored according to criteria described elsewhere [21]; their frequency was determined from at least 1000 cells per experimental point. Aberrant or missing arrays were classified as abnormal. Data were expressed as frequency of MT abnormal arrays per 100 MT arrays analyzed.

2.7. Statistical analysis

Statistical analysis of data was performed using Statistica software (Stat Soft 1999). Student's *t*-test was used to compare between treatment groups.

3. Results

3.1. Mitotic abnormalities by exposure to GF

Treatment with GF (10, 50, 100 or 250 μg/ml) induced a statistically significant decrease in MI. At the highest GF concentration, the MI decreased to more than 50% of the control value, indicating critical cytotoxicity. This is in agreement with the result obtained with TBZ. The genotoxic effect was evidenced by abnormalities at anaphase and telophase, such as fragments (Phg), bridges (Bg), lagging chromosomes (Lag), C-metaphases, tripolar cell divisions (Tri) and disorganized anaphases (DA). These abnormalities were recorded at concentrations up to 250 μg/ml, above which they were less frequent, possibly due to cytotoxicity. At interphase, mini cells (MC), micronuclei (MN) and nuclear buds (NB) (Fig. 1) were statistically significant for all concentrations tested; MC was the only end point showing a threshold <50 μg/ml (Table 1).

Table 1
Mitotic index (MI), micronucleus (MN), mini cells (MC), nuclear buds (NB) and chromosomal aberrations (CA) in anaphase–telophase of *A. cepa* meristematic cells exposed to GF formulation.

GF (μg/ml)	MI ± SD (%)	MC ± SD (%)	MN ± SD (%)	NB ± SD (%)	N° Aberrations in anaphases–telophases						
					Fr	Bg	Lag	C-met	Multipol	Des An	CA ± SD (%)
0	8.50 ± 0.66	0	1.64 ± 0.32	0	0	0	0	0	0	2	1.98 ± 0.87
DMSO (1%)	8.02 ± 0.84	0	1.17 ± 0.57	0	0	0	0	0	0	1	1.32 ± 0.52
10	5.67 ± 0.61**	1.17 ± 1.01	7.27 ± 3.35*	1.20 ± 0.37**	1	1	1	2	1	1	6.10 ± 0.91***
50	5.37 ± 0.14**	12.84 ± 0.31*	9.62 ± 2.47**	6.07 ± 1.95*	2	3	4	4	4	8	25.62 ± 2.43***
100	4.52 ± 0.24***	12.47 ± 6.82**	16.46 ± 3.77**	12.53 ± 3.64**	2	5	2	7	8	9	39.03 ± 7.62***
250	3.50 ± 0.62***	15.56 ± 3.17**	36.87 ± 14.66**	20.54 ± 8.20*	5	0	0	0	5	0	5.48 ± 5.07**

Phg, fragment; Bg, anaphase bridges; Lag, lagging chromosome; C-met, C-metaphase; Multipol, multipolar division; Des. An, disorganized anaphases.
DMSO dimethyl sulfoxide; GF, griseofulvin; Student *t*-test.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

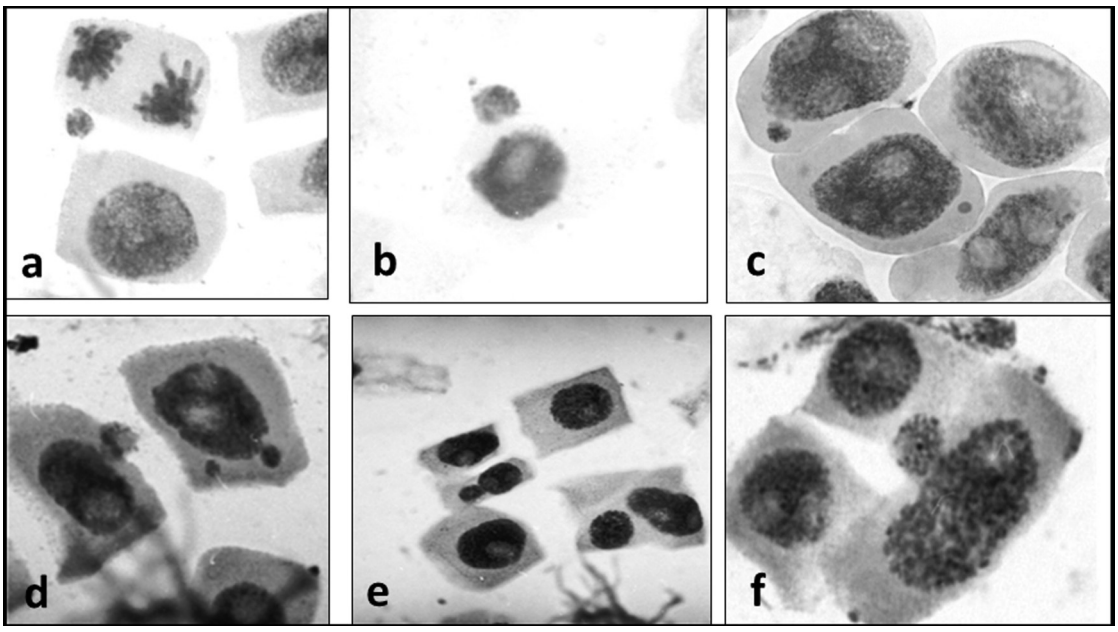

Fig. 1. Aberrations in *Allium cepa* in cells induced by the fungicide griseofulvin. (a) Mini cell located adjacent to main cells. (b)–(d) Mini cell and micronucleus, (c) micronucleus, (d) nuclear bud, (e) nuclear bud, (f) mini cells.

3.2. Induction of aberrant mitotic machinery by GF and TBZ

A GF concentration of 100 μg/ml was determined to be the optimum for analyzing the effects on MT arrays, in agreement with our previous results using TBZ [20]. For both GF and TBZ, the frequency of MT arrays was significantly lower in the group exposed to this concentration than in the control group, ($p < 0.01$–0.001) (Table 2). This is consistent with the cytotoxicity results. The frequency of abnormal microtubule arrays was significantly increased at 100 μg/ml for both GF and TBZ ($p < 0.01$–0.001) (Tables 2 and 3).

Analysis of tubulin immunolabeling and counterstaining with DAPI (Fig. 2) allowed us to characterize the different MT arrays and the damage induced by exposure to TBZ (Fig. 2a–e and a'–e'), including the absence of Phrag or PPB. As with TBZ, exposure to GF induced a significant increase in the frequency of abnormal PPB, MS and Phrag ($p < 0.01$–0.001). However, only cells exposed to GF showed other abnormalities, such as tripolar spindles (Fig. 3a–d and a'–d').

4. Discussion

Our results indicate that the formulation containing TBZ, 100 μg/ml, affects the formation of MT structures during the mitosis of *A. cepa* meristematic cells without altering MTOCs, as

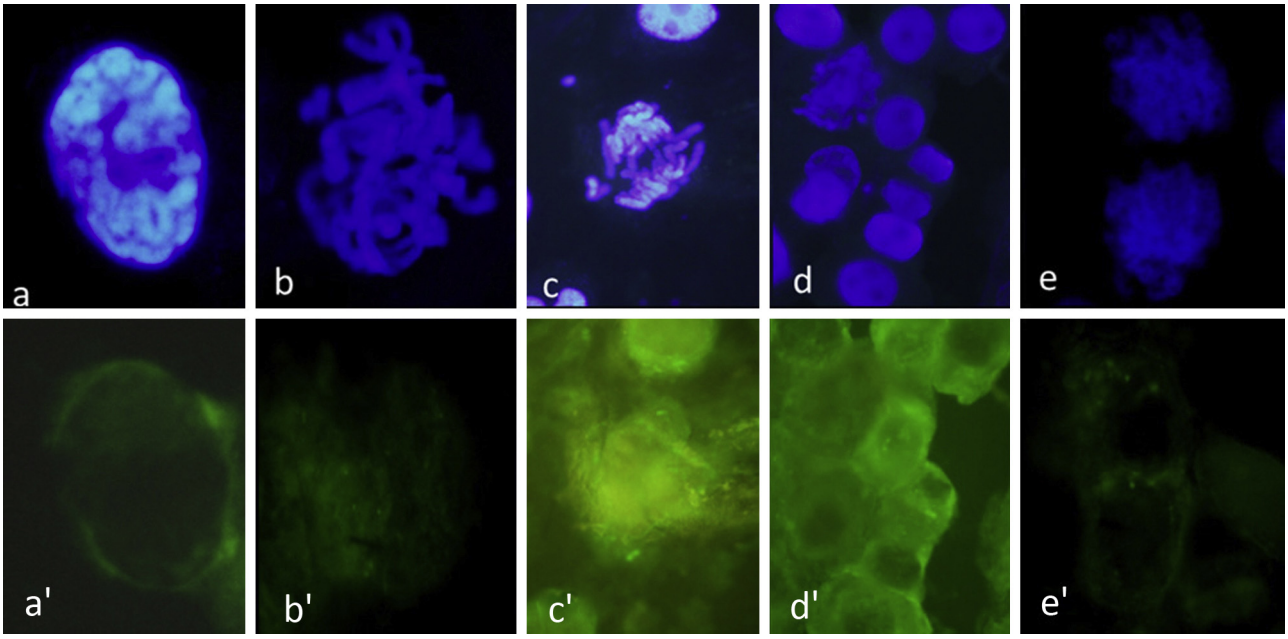

Fig. 2. Immunostaining of microtubule structures from *A. cepa* meristematic root cells(a-e) Cells stained with DAPI; (a'- e') cells labeled with FITC-conjugated anti-β-tubulin (a) prophase; (a') note the absence of preprophasic band (PPB); (b) prometaphase; (b') depolymerized microtubules; (c) metaphase; (c') depolymerized microtubules; (d) disorganized anaphase; (d') disorganized microtubules; (e) telophase; (e') aberrant phragmoplast (Phrag) (1000×).

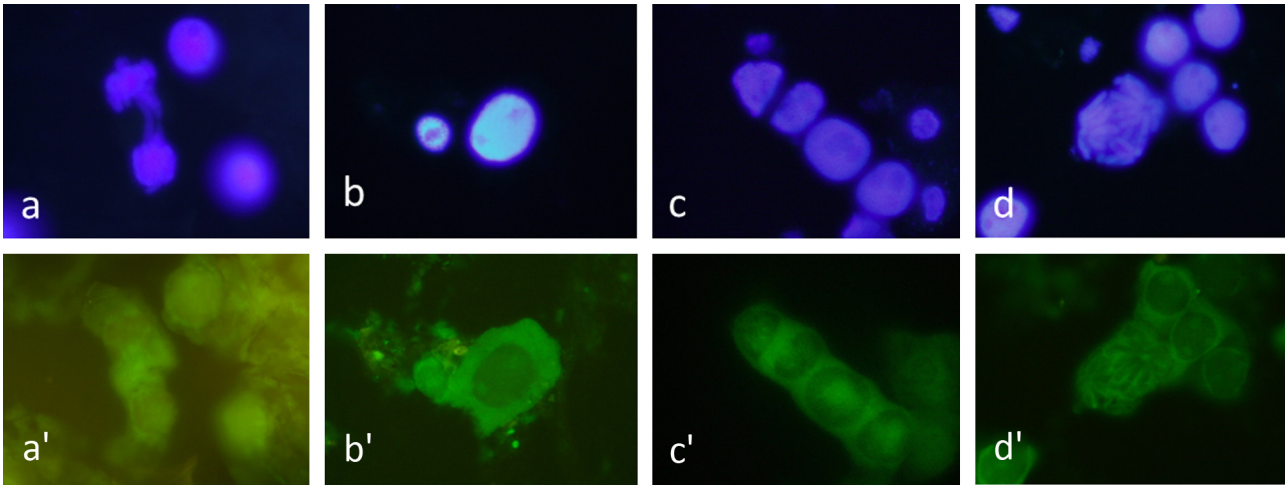

Fig. 3. Inmunostaining of microtubule structures from *A. cepa* meristematic root cells. (a-d) Cells stained with DAPI; (a'-d') cells labeled with FITC-conjugated anti-β-tubulin (a) telophase with bridges; (a') disorganized spindle (b); mini cell; (b') microtubules inside mini cell; (c) micronucleus in telophase cell, mini cell; (c') phragmoplast indicating telophase stages, note the absence of microtubules in the vicinity of the micronucleus, microtubules inside mini cell; (d) tripolar anaphase; (d') tripolar spindle (1000×).

evidenced by the absence of multipolar spindles. This is in agreement with other studies reporting that thiabendazole binds to specific sites on the β-tubulin [22]. Such interaction between thiabendazole and tubulin monomers prevents the normal formation of MT arrays and the occurrence of mitotic processes mediated by MT, such as chromatid segregation and cytokinesis. Instead, the presence of multipolar spindles has been reported in animal cells exposed to thiabendazole, such as CHO cells [23,24]. Their formation by direct interference of thiabendazole with tubulin would be associated with the centrosomes. Proteins involved in the regulation of DNA replication during the cell cycle and the metaphase–anaphase transition also regulate centrosome duplication. Aurora A plays a key role in these processes; its abundance is regulated by ubiquitination of the APC/C complex, which is involved in the control of cell cycle progression in interphase and metaphase [25]. During genotoxic stress, the cascade of reactions leading to cell cycle arrest and mitotic arrest promotes the dysregulation of the centrosome cycle; this results in supernumerary centrosomes and multipolar spindles [26]. Higher plants also have proteins belonging to the Aurora family, which are located at the centromere, in the middle spindle and the phragmoplast. However, they are implicated in the control of the mitotic cycle and play no role in regulating the nucleation of the mitotic spindle [27]. Therefore, genotoxic stress, in terms of interference of chemical agents with plant tubulins, is not expected to result in the formation of multipolar spindles. On the other hand, MT-disrupting agents may interfere directly with tubulins or MAPs [19]. The mechanism by which GF interferes with MT organization is unclear and results concerning its ability to interfere with tubulin and MAPs remain controversial [28–31].

Table 2
Total of microtubule arrays (MT) each 1000 observed cells and aberrant MT each 100 observed cells in meristem root of *Allium cepa* exposed to thiabendazole and griseofulvin formulations.

Treatment (μg/ml)	MT/1000	Aberrant MT arrays (%)
0	81.33 ± 8.0	1.61 ± 1.41
DMSO (1%)	75.67 ± 5.13	2.17 ± 0.64
TBZ 100	42.33 ± 1.53**	33.04 ± 6.90**
GF 100	46.00 ± 14.93**	35.00 ± 4.16***

DMSO, dimetil sulfoxide; GF, griseofulvin; TBZ, thiabendazole.
Student *t*-test.
** $p < 0.01$.
*** $p < 0.001$.

Table 3
Frequency of abnormal microtubular arrays in meristem root cells of *Allium cepa* exposed to thiabendazole and griseofulvin formulations.

Treatment (μg/ml)	PPB	MS	Phrag
TBZ 0	1.55 ± 2.69	1.23 ± 2.14	1.85 ± 3.21
DMSO (1%)	3.08 ± 2.68	–	3.03 ± 5.25
TBZ 100	26.59 ± 13.76***	33.93 ± 2.74***	43.98 ± 6.26***
GSF 100	33.73 ± 8.94***	35.14 ± 1.60***	37.74 ± 1.98***

PPB, preprophase bands; MS, mitotic spindle; Phrag, phragmoplast; DMSO, dimethyl sulfoxide; GF, griseofulvin; TBZ; thiabendazole.
Student *t*-test.
*** $p < 0.001$.

Our results indicate that exposure of *A. cepa* meristematic cells to GF caused damage to the MTOCs and induced abnormal MT arrays and chromosome missegregation. Moreover, it altered cell division polarity and symmetry, resulting in tripolar spindles and MC, as has also been observed for some herbicides [7,32]. These evidences suggest that, unlike TBZ, GF may not only interfere with tubulin, as previously proposed but also with other molecular targets involved in MTOC formation.

The chromosomal aberrations found in interphase cells exposed to GF were MN, MC, and NB. The MN are formed by the entrapment of a chromosome fragment or a whole chromosome [33], while the mechanism of MC formation remains unknown. In the present study, MC were frequently located adjacent to the main cells (Fig. 1a). Interestingly, we detected MTs and nucleolus in the MC, suggesting the presence of a cytoplasmic portion with nuclear content (Fig. 3b and b'). In contrast, MN do not have MTs (Fig. 3 c and c'). We observed that some MC had small MN (Fig. 1b), suggesting that they resulted from a recent asymmetric cell division.

Our results indicate that GF had effects on the meristematic cells of *A. cepa* similar to those of the herbicide trifuralin [32]. The formation of NB has been attributed to trifuralin-induced polyploidy and DNA excess [21]. In the present study, the presence of NB (Fig. 1d and e), together with the absence of polyploid cells, suggests that a different mechanism is responsible for the NB formation, e.g., the preformation of the MN. Although both GF and TBZ affected MT arrays in meristematic cells of *A. cepa*, the former may act through a different and less direct mechanism. The result obtained with TBZ is probably due to a direct interaction with tubulin molecules,

affecting chromosome segregation and cytokinesis. In contrast, the interaction of GF with MAPs may interfere with tubulin polymerization. This may lead to alterations in chromosome segregation and other processes, such as MTOC organization and symmetry/polarity of cell division. Moreover, it may impair repair mechanisms, causing chromosome breaks.

We conclude that exposure of *A. cepa* meristematic root cells to TBZ and GF induces MT damage, resulting in abnormal microtubule arrays. The fact that only GF caused abnormalities in spindle symmetry/polarity may be attributed to the different targets being affected by each agent. This work attempts to contribute to the interpretation of results obtained from genotoxicity assays with plant models, in the context of exposure to MT disrupters.

Conflict of interest

The authors declare that they have no competing interests.

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