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Index of Abbreviations

Cis Platin	CisPt
Imatinib Mesylate	IM
Etoposide	Etop
5-Fluorouracil	5-FU
Maleic hydrazide	MH
Arsenic trioxide	As_2O_3
LOEL	Lowest Observed Effect Level
PEC	Predicted Environmental Concentration
Micronuclei	MN
Mitotic indices	MI
Tradescantia micronucleus	Trad MN
Allium micronucleus	*Allium* MN
Pollen fertility	PF
Plant bio assay	PBA
Root length	RL
Food and Drug Administration	FDA
Organisation for Economic Co-operation and Development	OECD

Abstract

Aim of the present study was to investigate if cisplatin (CisPt) and imatinib mesylate (IM), which are among the most used cytostatic drugs worldwide, cause genotoxic and acute toxic effects in higher plants. In order to assess if the release of these drugs into the environment may cause adverse effects, induction of DNA damage was studied in micronucleus (MN) assays with meiotic tetrad cells in *Tradescantia paludosa* and with mitotic root tip cells of *Allium cepa*. MN are formed as a consequence of numerical and structural chromosomal aberrations. Furthermore, we monitored also the inhibition of cell division and the impact of the drug on root growth in the onions and analysed the impact of the drugs on the formation of abortive pollen grains in three wild life species (*Tradescantia paludosa clone* #03, *Arabidopsis thaliana*, *Chelidonium majus*). These assays provide information on the impact of toxins on the fertility of plants. In the MN experiments two different exposure protocols were used. In *Tradescantia* the plants were treated either for 24h followed by a 24h recovery (24/24h) or continuously for 30h (30/0), in *Allium* MN assays the exposure times were also 24h followed by a 24h recovery phase (24/24h) or continuously 72h (72/0h). Both drugs induced MN formations in the plant bioassays in the meiotic and mitotic cells. The Lowest Observed Effect Level (LOEL) of CisPt was in Trad MN experiments 1.0µM (24/24h) and 0.1µM (30/0h). The corresponding in *Allium cepa* was 0.5µM (24/24h) and 0.1µM (72/0h). The mitotic indices were decreasing significantly at doses ≥0.5µM after 24/24h and ≥0.1µM after 72/0h. The LOELs of IM were in *Tradescantia* 10µM (24/24h and 30/0h exposure) and in *Allium* 1µM (24/24h) and 0.1µM (72/0h). Mitotic inhibition was seen in the roots at doses 0.1µM (24/24h) and 0.05µM (72/0). Also in the pollen abortion assays positive results were obtained; effects were seen with both drugs at levels ≥62.5µM in *Tradescantia*, ≥125.0µM in *Arabidopsis thaliana* and ≥50.0µM in *Chelidonium majus.* CisPt was in all experiments more toxic than IM. The results of the different experimental series were compared with the predicted environmental concentrations (PEC). The results show that the doses which

cause measurable effects in higher plants are several orders of magnitudes higher than the PEC values in different European countries. However, in the case of CisPt, concentrations were found in the waste waters from an oncological ward which are in the range of the levels which caused MN formations in the spiderwort.

Zusammenfassung

Ziel der vorliegenden Studie war es zu untersuchen, ob Cisplatin (CisPt) und Imatinib (IM), die zu den am häufigsten verwendeten Zytostatika weltweit zählen, in höheren Pflanzen gentoxische und akuttoxische Effekte verursachen. Um zu beurteilen, ob die Freisetzung dieser Medikamente in die Umwelt, schädliche Auswirkungen haben könnte, wurde die Induktion von DNA-Schäden in Kleinkernen (MN) in Tetradenzellen von *Tradescantia* und in mitotischen Wurzelspitzenzellen von *Allium cepa* untersucht. Kleinkerne sind die Konsequenz numerischer und struktureller Chromosomenaberrationen. Darüber hinaus, wurden auch die Hemmung der Zellteilung und des Wurzelwachstums in Zwiebeln untersucht sowie die Auswirkungen der Medikamente auf die Bildung abortiver Pollenkörner in drei Wildpflanzenarten (*Tradescantia paludosa* Klon#03, *Arabidopsis thaliana*, *Chelidonium majus*). Diese Untersuchungen liefern Informationen über die Auswirkungen von Giftstoffen auf die Fruchtbarkeit der Pflanzen. Für die MN Experimente wurden zwei verschiedene Behandlungsprotokolle verwendet. Bei *Tradescantia* war die Behandlung der Pflanzen entweder 24h mit einer folgenden 24h Erholungsphase (24/24h) oder eine kontinuierliche Behandlung für 30h (30/0h), bei *Allium* MN Versuchen waren die Behandlungszeiten ebenfalls 24h mit einer folgenden 24h Erholungsphase (24/24h) oder eine kontinuierliche Behandlung von 72h (72/0).

Beide Medikamente induzierten in meiotischen und mitotischen Zellen die Bildung von MN. Die LOEL (Lowest Observed Effect Level) für CisPt waren in den Trad MN Versuchen 1,0µM (24/24) und 0,1µM (30/0h). Die entsprechenden Dosen für *A. cepa* waren 0,5µM (24/24h) und 0,1µM (72/0h). Der mitotische Index (MI) nahm bei Dosen ≥0,5µM (24/24h) und ≥0,1µM (72/0h) signifikant ab. Die LOEL für IM in den Trad MN Versuchen waren 10,0µM (24/24h und 30/0h Exposition) und in *Allium* 1,0µM (24/24h) und 0,1µM (72/0h). Der MI wurde in den Wurzeln bei Dosen ≥0,1µM (24/24h) und ≥0,05µM (72/0) signifikant kleiner. Auch in den abortiven Pollentests wurden positive Ergebnisse erzielt. Mit beiden Zytostatika wurden mit ≥62,5µM Effekte in *Tradescantia paludosa* gefunden, bei ≥125,0µm in *Arabidopsis thaliana* und ≥50,0µm in *Chelidonium majus*. CisPt war in allen Experimenten toxischer als IM. Mit den Ergebnissen der verschiedenen

Versuchsreihen wurden Vergleiche mit den vorhergesagten Umweltkonzentrationen (PEC) durchgeführt. Die Ergebnisse zeigen, dass die Konzentrationen, die messbare Effekte in höheren Pflanzen verursachen, mehrere Größenordnungen höher sind als die PEC-Werte in verschiedenen europäischen Ländern. Allerdings wurden im Fall von CisPt Konzentrationen im Abwasser einer onkologischen Abteilung gefunden, welche sich im Bereich der MN-Bildung in *Tradescantia* befinden.

1 Introduction

The present thesis was realised in the frame of the EU project CytoThreat and concerned the question whether the release of two cytostatic drugs, cisplatin (CisPt) and imatinib mesylate (IM), into the environment may cause adverse effects in higher plants. Two plant bioassays were employed to study induction of genetic damage (micronuclei induction) and acute toxicity (retardation of root growth and inhibition of cell division); furthermore investigations were conducted with wildlife plants in order to find out if the drugs cause infertility via induction of formation of abortive pollen grains. It is well known that the release of DNA reactive substances to the environment may affect the fertility of certain species which leads to adverse effects of ecosystems. The subsequent chapters describe the principles of the different bioassays and the current use of the cytostatic drugs. The data which were obtained in the individual investigations are presented in the results section and were used to determine the LOEL. In the discussion section, predicted environmental concentrations (PEC) are compared to the doses which caused effects in the different endpoints and conclusions in regard to potential environmental hazards caused by release of the drugs.

1.1 Ecogenotoxicology

Due to the anthropogenic pollution of the environment with a broad variety of chemicals it is important to focus the impact of these compounds on biota, which are the primary targets for genotoxic agents. These “genotoxic chemicals can damage the genetic material of humans as well as that of organisms living in the environment” [1]. Important goals of egogenotoxicological research are the detection of DNA-damaging agents which are toxic in the environment, the determination of the amounts of these agents, the mode of action and the identification of the No Observed Effect Level (NOEL). For these tasks, new analysis systems and biomarkers had to be developed due to the fact, that laboratory tests which are used for the screening of pure chemicals were not sufficiently sensitive enough [2].

1.1.1 Plant bioassays

Already in the 1960's, plant bioassays were invented for the detection of environmental mutagenes. Many of these experimental models e.g. *Zea mays* L., *Hordeum vulgare* L., *Arabidopsis thaliana* L. or *Nicotiana tabacum* L. were based on the phenotypic alterations and had the disadvantage of being costly and time consuming. Therefore, they were only used in a relatively small number of studies, while micronucleus assays (MN), which are easy to perform, are still widely used. At present, most environmental studies are conducted with meiotic cells of *Tradescantia sp*. [3] and mitotic root cells of *Allium cepa* [4].

These assays were invented in the early 1970's and are based on the scoring of MN which are defined as extra-nuclear DNA containing structures. They are formed as a consequence of clastogentic effects (chromosomal breakages) aneugenic effects [5] (see figure 1). In most investigations with root cells, additional parameters are scored which provide information of the acute toxicity; namely roots lengths (RL) and mitotic indices (MI).

Pollen fertility assays were developed in the 1980's and primarily used for the detection of toxic substances in the environment in plant species which are at economic relevance. [6].

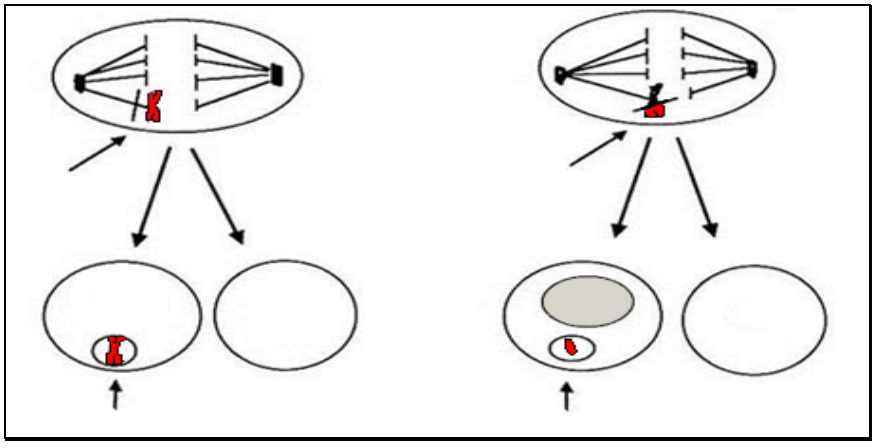

Figure 1: Induction of MN as a consequence of numerical (1A) or structural (2B) chromosomal abbreviations.

1.1.1.1 Principle of the *Tradescantia* bioassay (Trad MN assay)

The test procedure was developed in the 1980's by Ma at the Western Illinois University in Macomb, USA. More than 160 chemicals and numerous complex mixtures have been analysed and published in more than 100 studies so far. In most studies, the hybrid clone #4430 and the wild type *T. paludosa* #03 were used. Due to the high sensitivity of the plant, it is possible to test complex chemical substances [3]. For investigations of pure chemicals different laboratory protocols have been developed [7], furthermore it is also possible to expose the plants directly in the environment [8](see figure 2).
Since it is known that the formation of MN requires cell divisions, a specific tetrad stage is used since it can be easily identified and the time period which elapses between the formation of pollen mother cells and the formation of early tetrad cells is constant [9]. Two experimental protocols are commonly used:

a.) Stem absorption: Following the protocol of Ma [7], this is the optimal protocol for tests with chemicals (e.g. cytostatics). Cuttings of *Tradescantia* are exposed to the test compounds which are dissolved in water to a solution.

b.) Root absorption: According to the modified protocol of Steinkellner et al. [10], *Tradescantia* plants are directly exposed in pots with the test compounds.

In addition to the formation of MN, effects like pyknosis as well as other forms of nuclear fragmentation can be scored in the tetrads as additional endpoints [11]. Since cytostatic drugs are released primarily into wastewaters the stem absorption was used in the current study.

In the last years a number of MN studies with *Tradescantia* were conducted to analyse mutagenic effects e.g. of polluted soils, radionuclides, chemical residues in waste waters of industries (e.g. from the textile and paper industry) ([11], [12],[13], [14],).

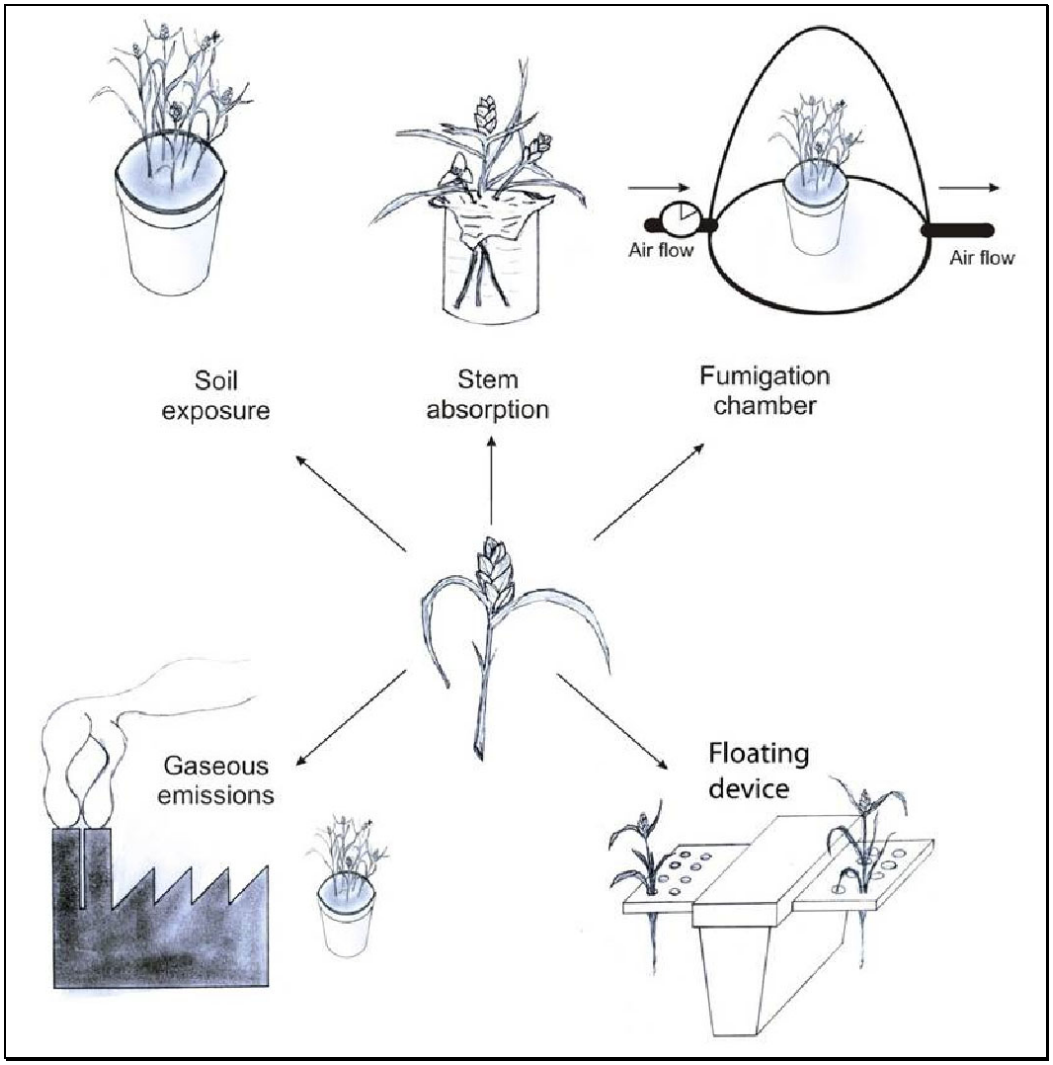

Figure 2: Various treatment protocols are used in experiments with *Tradescantia*. For the realisation of the CytoThreat project, soil exposure (for pollen abortion assays) and stem absorption (for MN assays) were used.

1.1.1.2 Experiments with *Allium cepa*

Onions have relatively large chromosomes; therefore they have been used in experiments since the early 1940's [4]. In these early studies, mainly chromosomal aberrations and mitotic disturbances, such as bridges and lagging chromosomes, were analysed. Levan was the first who used *A. cepa* to demonstrate that cholchicine causes disturbances in the mitotic spindel [15] and in a follow-up study he described for the first time chromosomal effects in meristematic root cells by treating the plant with various solutions of organic

salts [4]. However, some of the endpoints such as “bridges” and “lagging chromosome” are not indicative for persisting DNA-alterations.

The first articles on MN formation in mitotic root cells were published by Evans et.al., in the early 1970’s [16]. More than 60 publications have appeared in which *A. cepa* was used in MN-experiments [4]. In 1995 Ma et al. published a standard protocol which implied that only F1 cells should be analysed [17]. It is known that the formation of MN require cell divisions, therefore the inhibition of mitosis may have an impact on the outcome of MN. To take this possible confounding factor in consideration the mitotic indices were determined in the present study. Furthermore, also the impact of exposure to the drug on the root growth was recorded.

With the *A. cepa* assay different endpoints can be monitored (chromosomal aberrations, nuclear abnormalities, MN, MI and toxicity tests [4], [18], [19], [20]).

In a comprehensive study of Leme and Marin-Morales [4] previous studies were reviewed and overall the evaluation of the data indicated that the *Allium cepa* assay is highly sensitive to chemicals, which do not need activation. It was used successfully for the detection of heavy metals in water and soil samples, wastewaters from various industries, pesticides and disinfection products ([4], [21], [22], [23]).

1.1.1.3 Pollen abortion assay with wild plants

These experiments were included as they provide information of the impact of chemicals on the fertility of higher plants. It is known from a model study in which the air quality of different urban sites was monitored, that the induction of DNA damage, which was measured in Trad MN assays, is paralleled by induction of morphological aberrations of pollen in higher plants [24]. The experimental model was developed by Murin at the Comenius University in Bratislava and was mainly used to monitor the air pollution [25]. The experiments can be conducted with variety of wildlife plants; aquatic as well as terrestrial plants can be used in the experiments.

Certain criteria have to be fulfilled which are listed below:

a. The indicator plant has to be diploid with haploid grains, to be certain that pollen abortion at a low level whereas it is high in polyploids.

b. The background rate of abortive pollen grains should be lower than 5% and pollen should be viable and adequate established in standard climate conditions.

c. The possibility of distribution in urban and/or industrial habitats should be given.

d. Flowering of different plants should continue over longer time periods during a year to cover the whole year.

e. For better handling in common practice, it should be easy to identify the plant[26].

The frequencies of abortive pollen grains are also of agricultural interest since it is known that they have an impact on the fertility of plants. In the 1970's different assays were invented with different indicator species which were widely used for the detection of toxicological substances in the environment and for the identification of compounds which reduce the fertility of crop plants. The later tests were performed to investigate the impact of herbicides on the viability of pollen [27]. Murin and Micieta summarized available data in a review in 2000. With heavy metals (Al, Pb and Ni) contaminated soils were investigated in Slovakia and it was found, that the pollen abortion frequencies were increased. Furthermore, hydrocarbonated soils and soils of a nuclear power plant were investigated and it was shown that the pollen abortion rates was increased [26],[28],[29].

In the present study three species, namely *Tradescantia paludosa clone* #03, *Arabidopsis thaliana* and *Chelidonium majus*, were used which belong to major groups of higher plants (Commelinaceae, Brassicaceae and Papaveraceae).

1.1.2 Advantages and disadvantages of plant bioassays

The experiments, which were performed in the present study, are time and cost effective and it is known from earlier investigations that plants are in general highly sensitive towards certain groups of environmentally relevant genotoxic carcinogens such as heavy metals, herbicides, pesticides and radionuclides [30]. The sensitivity of plant cells towards these toxins is sufficiently high to conduct experiments without concentration procedures, which are required in tests with mammalian cells and bacterial indicators which are in general less sensitive [10]. As mentioned above, it is possible to conduct *in situ* monitoring studies in which the indicator plants are directly exposed in the environment. However, one of the disadvantages of plant bioassays (PBA) is that indirectly acting compounds, which are activated by drug metabolizing enzymes, give in general negative or at best marginally positive results [31]. Typical examples are polycyclic aromatic hydrocarbons, mycotoxins and heterocyclic aromatic amines. On the basis of the evaluation of a relatively small database, Ennever and Rosenkranz concluded in 1988 that PBA have a relative high specificy but a relative low sensitivity for the detection of environmental carcinogens [32].

PBA appear to be suitable for the detection of cytostatics since most of these drugs do not require enzymatic activation.

1.2 The CytoThreat project

While the toxic effects of cytostatics in humans are well known, hardly any data exist which concern environmental organisms. As these substances are released to the environment and were detected in waste waters and surface waters of rivers ([33], [34], [35], [36]) investigations of these highly toxic drugs are an important issue in order to draw conclusions in regard to long term effects in ecosystems. The abovementioned CytoThreat project concerns the investigation of the fate of the most frequently used cytostatics (cisplatin, imatinib mesylate, 5-fluorouracil, etoposid), their distribution in aquatic systems, their chronic and acute toxicity in higher plants, aquatic organisms and studies concerning their impact on the stability of ecosystems. The project will provide new knowledge how these compounds influence human health and the environment. The

ambition is to present a new risk assessment of the drugs on objective arguments for instructions, recommendations and regulations.
It is supported by the 7th Frame Work Program of the European Commission and seven European states (five EU and 2 associated) are involved in realization of this project which consists of nine work packages.

1.3 Cytostatic drugs

Cytostatic drugs cause inhibition of the division of cancer cells via different molecular mechanisms. Some of them are listed in Table 1. At present more than 126 anticancer drugs are used in human cancer therapy. Many of them (85) e.g. Vincristine, Vinblastine or Etoposid have been isolated from plants [37]. Due to differences in their molecular mode of action, it is necessary to investigate the impact if each individual drug on environmental stability.

Three factors are of particular interest: the amount of the substances which are released, the possibility of their elimination and their toxicity.
Cytostatics of the first generation do not only affect cancer cells by inhibition of cell division, but normal cells as well. The release of these drugs occurs not only in hospitals but also takes place via excretion by cancer patients in households. Since cytostatics are among the most potent toxic agents which are produced, it is at high relevance to study their impact on the environment [38].

Table 1: Different modes of actions of cytostatic drugs[1)]

Type of cytotoxic agent	Selected active substances	Mechanism of action	Main effect phase in the cell cycle
Alkylating agents	Ifosfamide Cyclophosphamide Treosulfane Carboplatin Cisplatin	Alkylating of DNA ▼ Cross-linking ▼ Prevention of DNA-replication	Non-specific in all cycle phases
Antimetabolites	Cytarabine 5-Fluorouracil Gemcitabine Mercaptopurine Methotrexate*	Incorporation into DNA as fraudulent nucleotide ▼ Inhibition of enzymes in DNA-synthesis	S-phase
Mitotic inhibitors	Paclitaxel Vinorelbine Docetaxel Vincristine Vinblastine Vindesine	Disturbance of spindle foundation ▼ Arresting of mitosis during metaphase	M-phase
Cytotoxic antibiotics	Daunorubicin Doxorubicin Epirubicin Mitozantrone	Intercalation between DNA-bases ▼ Inhibition of DNA-biosynthesis	S-phase G_2-phase
Topoisomerase I- and II-inhibitors	Etoposide Teniposide Topotecane	Inhibition of Topoisomerase I, which removes the torsion of the DNA Inhibition of Topoisomerase-II, which catalyzes the torsion of the DNA and others	S-phase G_2-phase M-phase S-phase

[1)] (from Eitel et al. [39])

1.3.1 CisPlatin

The effects of CisPt were discovered in 1965 by Rosenberg et al., who studied the impact of electric currents on the growth of bacteria. In subsequent experiments it was found that these compounds affect also the division of cancer cells [40].

CisPt is an inorganic heavy metal complex (platinum is the central atom and has two chloride ligands and two ammine ligands) (Figure 3).

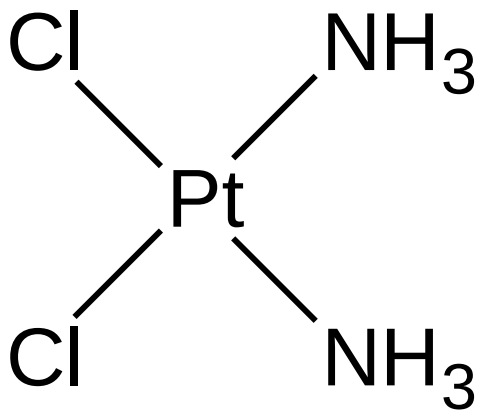

Figure 3: Chemical structure of (SP-4-2)-diamminedichloridoplatinum (CisPt); a planar platinum complex with two cis-chlorine ligands and two amine ligands.

Most cancer patients are treated with the drug intravenously but other application forms are also used. After medication of 20.0-120.0mg/m² body surface area, the highest platinum concentrations are found in liver, prostate and kidney, followed by bladder, muscles, gonads, pancreas and spleen. Platinum can be detected in the body tissues up to 180 days after intake, more than 90.0% are bound to the proteins within two hours after application ([38])

The high chloride amount in the serum is responsible for the persistence of CisPt - in the beginning - in its dichloride form. After it passes the cell wall, it exchanges its ligands in the cytoplasm. There are three different possibilities how CisPt can pass the cell membrane. One is diffusion, another is active transport via specific receptors and the third is the penetration through the cell membrane via copper transporting proteins. (Figure 4)

The central platinum core binds to DNA-bases (5-10%), the rest to nucleophilic sites of intracellular constituents. Platinum binds to DNA preferentily to guanine (65% intrastrand crosslinks) and adenine (25% intrastrand crosslinks) the rest are interstrand crosslinks and monofunctional adducts [41] (Figure 5).

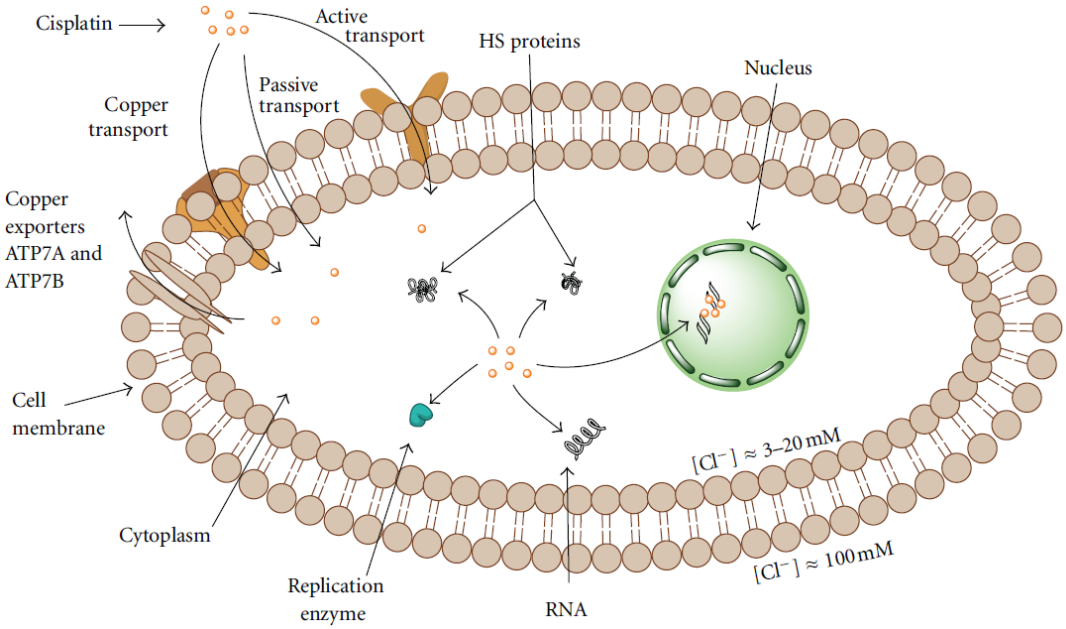

Figure 4: CisPt passes cell membrane via three different pathways: via active (specific receptors), passive (diffusion) or copper transport system; 5-10% of the drug binds to DNA and 75-85% to proteins, replication enzymes, thiol containing peptides and RNA (from Gomez-Ruiz et al. [41]).

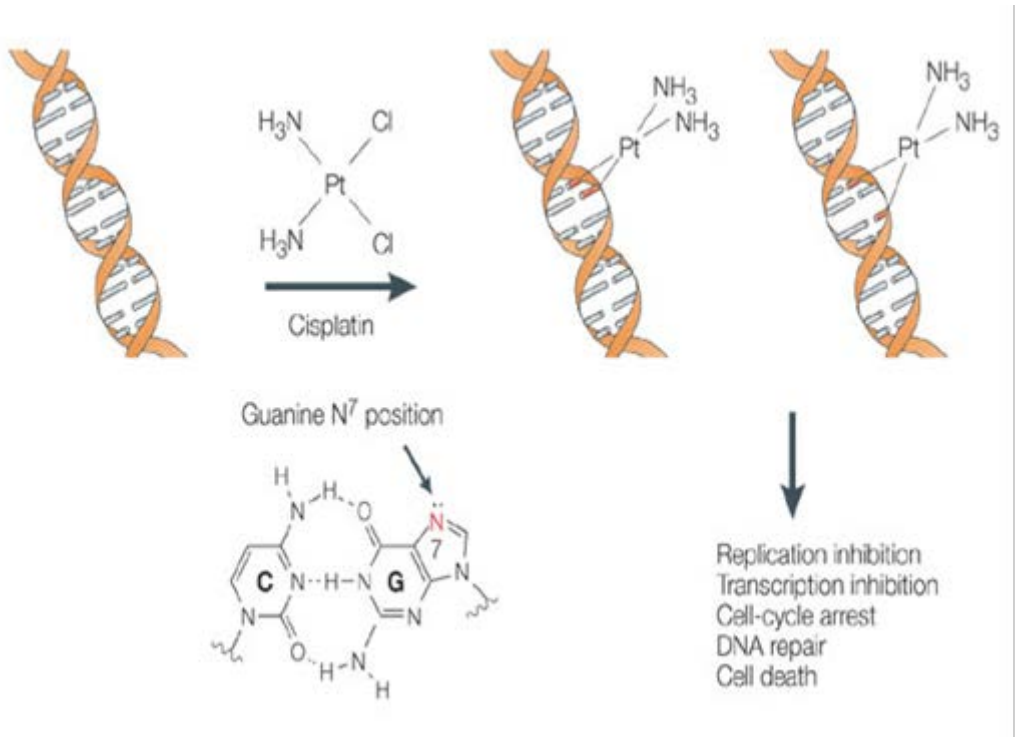

Figure 5: Formation and effects of cisplatin adducts. Once inside the cell the platinum atom of cisplatin binds covalently to the N^7 position of purines to form 1,2- or 1,3 intrastrand crosslinks, and interstrand crosslinks. (from Wang and Lippard [42])

About 75% of the CisPt is excreted and transferred via waste water to sewage plants as pharmacologically activated monoaqua-CisPt. Most likely the drug wont be removed or transformend to platin ions by sewage plants [43]. Eco-toxicological tests with CisPt show yielded the results which are listed in table 2.

Table 2: Toxicity doses from various species.[1)]

Test system	Test species	(mg/l)
Acute toxicity (EC50)	*Pseudomonas putida*	1.20
Acute toxicity (EC50)	*Pseudokirchneriella subcapitata*	2.30
Acute toxicity (EC50)	*Daphnia magna*	0.64
DNA-damage SOS-Chromotest with activation	*Escherichia coli*	0.17
DNA-damage SOS-Chromotest without activation	*Escherichia coli*	0.09
GreenScreen Assay	*Sacchormyces cervisiae*	0.44

[1)]From Kummerer et al. [38]

1.3.2 Imatinib Mesylate

Imatinib mesylate (IM) was developed in the late 1990s at the Oregon Health and Science University, U.S.A. The compound (Figure 6) was developed by rational drug design for curing patients who suffer from chronic myelogenous leukemia (CML) [44].

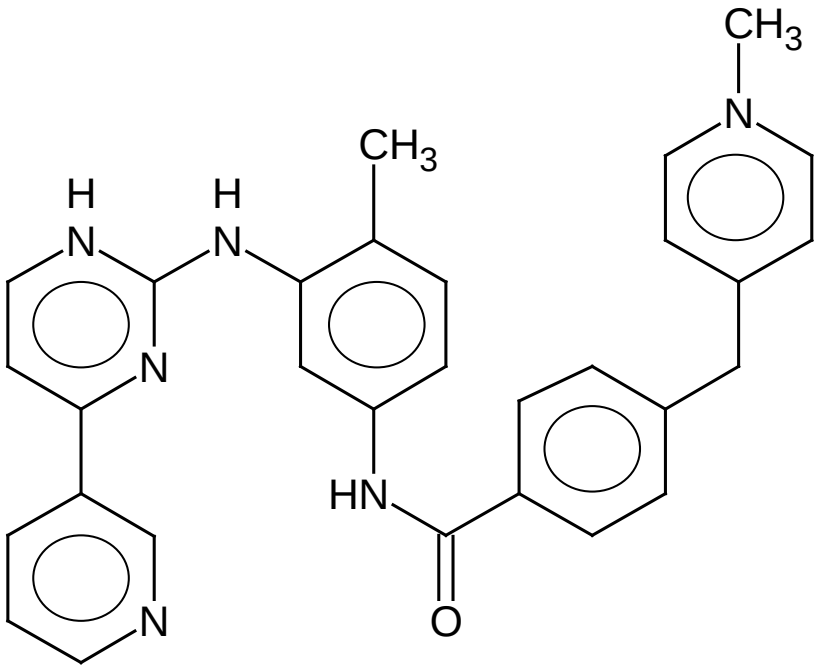

Figure 6: Chemical structure of IM

The life time risk to develop CML is 0.51 (1 of 192 persons) in the USA [45]. The disease is characterized by the “Philadelphia chromosome” (Figure 7), which is a translocation, leading to formation a fusion gene (BCR-ABL) [46]. ABL is transferred to chromosome 22 where the BCR is located. The function of the individual gene products are not known, but it is known that they are expressed in many tissues. The fusion gene produces a protein which has enzymatic activities. It functions as a tyrosine kinase which transfers the terminal phosphate from ATP to tyrosine residues. This process is known as thyrosine phosphorylation [47].

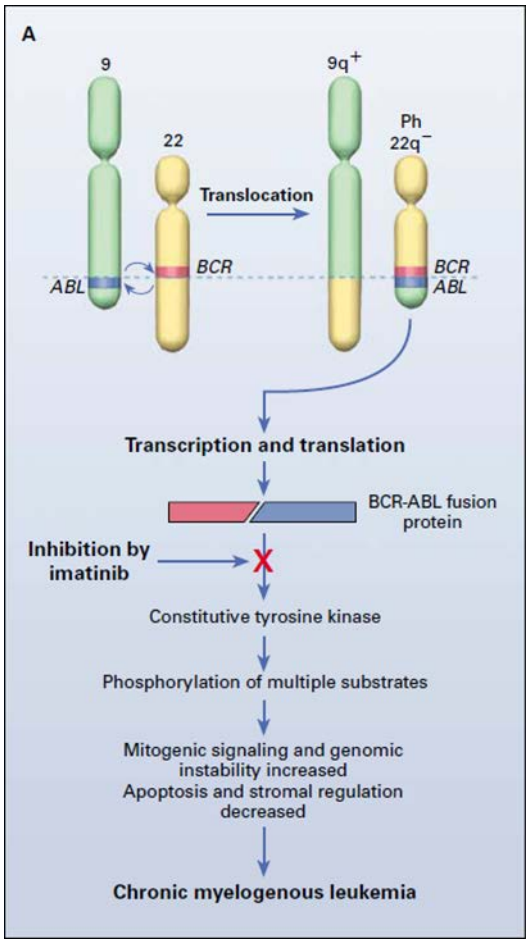

Figure 7: Origin of CML via the fusion gene BCR-ABL. The translocation (reciprocal) produces an enlarged chromosome $9q^+$ and the Philadelphia chromosome Ph $22q^-$ (From Savage and Antman [46]).

The consequence of the consecutive expression of this gene is malignant transformation. Among the substances which are substrates of the enzyme, is the RAS-protein which plays a key role in cancer (signal transduction). Consequences of the activation of BCR-ABL are also decrease of the apoptosis-rates, which leads to a survival advantage of leukemic clones [48].

The main focus of the development of the drug was the inhibition of protein kinase C which lead to the synthesis of 2-phenylaminopyrimidine. However, this was too unspecific, therefore several modifications were conducted. The most important modification was adding N-methylpiperazine which increased the bioavailability of the drug. Finally IM was developed which has improved binding properties [48, 49].

In 2001 the FDA (Food and Drug Administration, USA) accepted the new anticancer drug and it is nowadays one of the most widely used cytostatic.

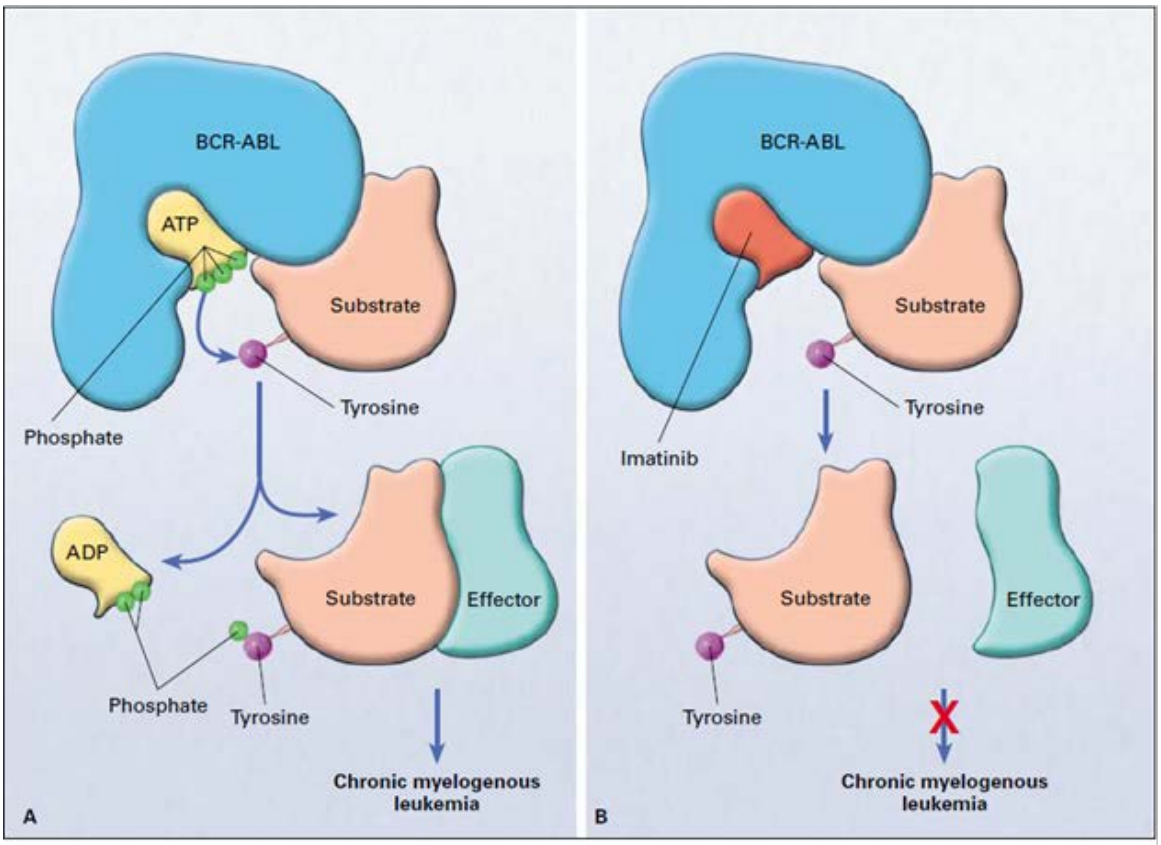

Figure 8: Panel A shows the BCR-ABL oncoprotein with a molecule of adenosine triphosphate (ATP) in the kinase pocket. The substrate is activated by the phosphorylation of one of its tyrosine residues. It can then activate other downstream effector molecules. When imatinib occupies the kinase pocket (Panel B), the action of BCR-ABL is inhibited, preventing phosphorylation of its substrate. ADP denotes adenosine diphosphate. From Savage et al. [46].

The half-life of IM (bioavailability 98.0%) after oral application is 18h. 81.0% of C^{14} marked IM are excreted within seven days (68.0% in faeces and 13.0% in urine). 25.0% of the drug are unmodified (5.0% can be found in the urine and 20% in faeces) and the rest are metabolites [38].

In tests which were conducted with dogs (13 weeks), rats (6 month) and monkeys (9 month) similar toxic effects were found as with the clinical doses which were used in humans [50].

1.4 Realisation of the experimental work

The present study consists of three main parts. The first concernes the investigation of the genotoxic effects of the two drugs in Trad MN and *A. cepa* MN assays. In the experiments with onions, also acute toxicity parameters (growth of roots and division activities) were monitored, which are summarized in the second chapter. The third section concerns the investigation of the effect of the drugs on pollen fertility in three wildlife plants (*Tradescantia paludosa* clone #03, *Chelidonium majus* and *Arabidopsis thaliana* (Columbia, WT-02-41-02, Lehle seeds, USA).

2 Materials and methods

2.1 Micronucleus assay *Tradescantia*

The *Tradescantia* micronucleus assays (Trad MN) were performed according to the protocol of Ma et al. (1994). Clone #4430, a hybrid of *T. subacaulis* and *T. hirsutifolia*, was used in all experiments (Figure 9). In the following chapters the cultivation, the treatment and storage, the preparation, the evaluation and the statistical analyses are described.

2.1.1 Cultivation of the plants

The plants were cultivated in pots with a diameter of 15 cm in SERAMIS®, which served as substrate for hydroponic cultivation. Once per week, liquid fertilizer (NPK – nitrogen, phosphor and potassium, label: Cererit Hobby, producer: Agro) was added to the water basins, with the pots. Every three months, the plants were divided and replanted to restart their growth. After three weeks, the plants began to develop inflorescences. The cultivation was performed in a room with artificial light (day/night cycle 16/8h). The relative humidity was between 60.0-80.0% and the temperature was 22-25°C.

Figure 9: *Tradescantia* clone #4430 in glass beaker. For assay closed flower buds were used.

2.1.2 Treatment and storage

For performing an investigation with *Tradescantia* at least 150 plants are required. The experiments were conducted with young inflorescences which had a minimum of nine buds and no open flowers. Every experiment included at least four doses of a test substance. Tap water was used as a negative control, maleic hydrazide (20mg/l) served as a positive control. For each dose, 15 cuttings were used with stem lengths between 10.0 and 15.0cm. The duration of the exposure period was either 24 hours with a recovery period of 24 hours or with an extended exposure time of 30 hours without recovery. The test solutions were filled in glass beakers which were covered with aluminium foils for the separation the cuttings. As it is well known that CisPt is a light sensitive substance the solutions were protected by covering the beakers as well with aluminium foil.
At the end of the treatment periods, the inflorescences were transferred in a mix of ethanol/acetic acid (3:1) for 24 hours to fix the buds, afterwards they were stored in 70.0% ethanol (storing possibility for several months).

2.1.3 Evaluation of the Trad MN frequencies

Per experiment, five flower buds were analysed for each dose. MN formation was analysed in buds which contained early tetrads. To identify this stage the petals were removed with preparation needles and pollen was placed on glass slides. For staining, 2.0% acetocarmine solution was used. Subsequently, the pollen was squeezed gently with a preparation needle to scatter the tetrads. After this procedure, the slides were covered with a coverslip. Figure 10 shows schematically the isolation and staining of the tetrads

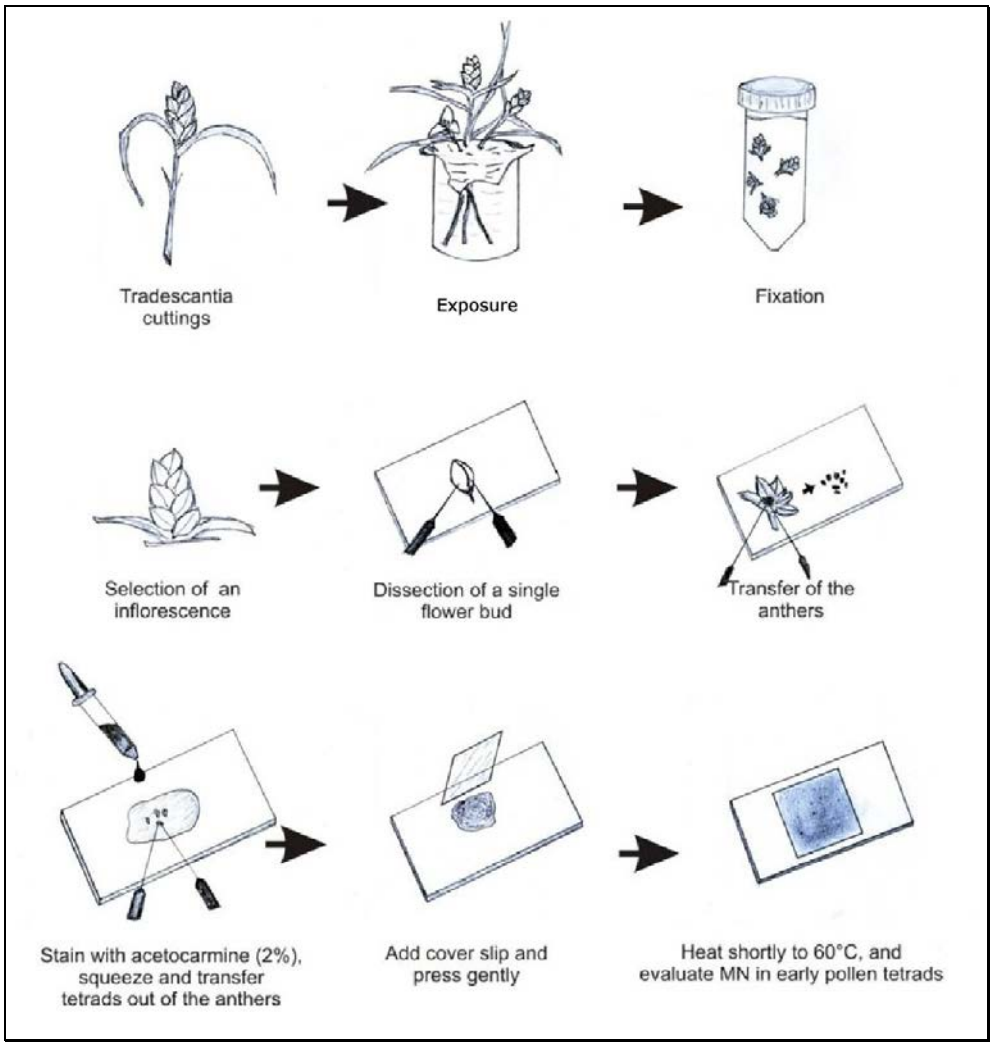

Figure 10: Scheme of the treatment of plant material and of the preparation of slides.

2.1.4 Determination of the MN-rates and statistical analyses

The evaluation was done under a light microscope (Nikon Eclipse E200, Nikon Instruments Inc. NY, USA) with 400-fold magnification. Per concentration five bulbs of five different inflorescences were taken and 300 early phase tetrads were screened for MN formation (Figure 11).

The experimental results were analysed with the software Prism 5 (Graphpad Inc., CA, USA) by using one-way ANOVA followed by Dunnett's multiple comparison test. P-values ≤0.05 were considered as significant.

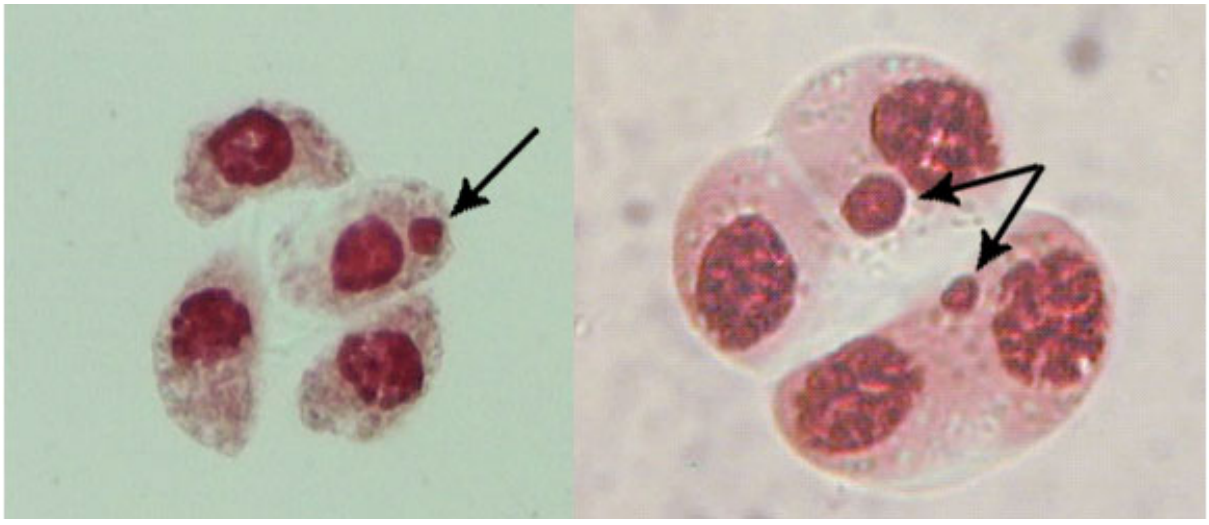

Figure 11: Pollen tetrad of *Tradescantia clone* #4430 with MN. Arrows mark MN formation (400-fold magnification, staining 2% acetocarmine).

2.2 Micronucleus and acute toxicity assays with *Allium cepa*

The method was developed by Ma et al. (1995) [17]. In addition, acute toxicity tests were conducted simultaneously according to the protocol of Fiskesjo (1995) [51]. The cultivation, treatment, storage, preparation, evaluation and statistical analyses are described in the following chapters.

2.2.1 Plant material and cultivation

In all experiments, *Allium cepa* bulbs (Austrosaat, Wien-Inzersdorf, Austria) were used. The onions were stored in the dark, under cold (4-8°C) and dry conditions. For the treatment, young onion bulbs which had a diameter between 12.0 and 21.0mm were peeled and placed on top of 13ml glass tubes which were filled with tap water. The tubes were placed in an incubator at a temperature of 26°C to induce the growth of primary roots for 24h.

2.2.2 Treatment and storage

After 24h, onions which had root lengths between 0.5 – 1cm were selected for further use. For each experimental point (at least five) ten plants were treated. The test substances, tap water and the positive control solutions were filled in the glass tubes, subsequently the bulbs were placed on top (see Figure 12). Afterwards, the glass tubes were put back into the incubator. The exposure period was either 24h followed by a 24h recovery phase with tap water or 72h with no recovery. Every day the solutions were changed and the root lengths of each onion were measured to determine acute toxic effects.

After the experiment, the onions were cut and the roots were fixed in ethanol and acetic acid (3:1) for 24h and stored in 70.0% ethanol.

Figure 12: Treatment of the onions with the cytostatic drugs.

2.2.3 Evaluation of the *Allium* MN frequencies and determination of the root growth

MN are evaluated in the meristematic and F1 zone of the root tips which contain dividing cells (Figure 13). Due to the thick cell walls, the roots have to be macerated in 5.0 N HCl solutions for four minutes, afterwards they are washed in distilled water. The root lengths are measured as an indicator for growth inhibition and acute toxicity. The material is stained for 20 minutes with 2.0% acetocarmine. After the staining, a cover slip is added and the cells are spread on the slide by use of a pencil.

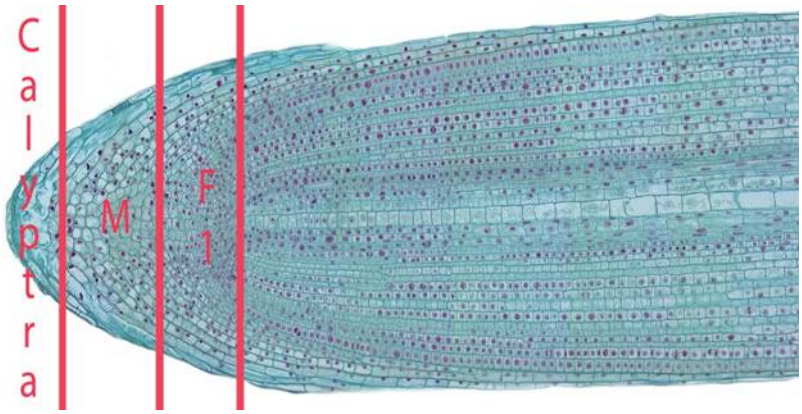

Figure 13: Root of *Allium cepa*. Only the meristem and F1 daughter cells were used for the evaluation of the MN frequencies and for the determination of mitotic index (bio.rutgers.edu [52]).

2.2.4 Determination of the MN-rates and the mitotic indices

The cells were analysed under a light microscope (Nikon Eclipse E200, Nikon Instruments Inc. NY, USA) with 400-fold magnification. For each concentration of the test substance, five onions were used and two root tips per onion were evaluated. From each root, 500 cells were scored for MN formation. Overall, 5000 cells were evaluated per dose. In addition, the mitotic indices (MI) were determined in 100 cells per root (in total 1000 per dose). The number of dividing cells is a parameter for the inhibition of the cell division caused by the test substances. Untreated onions had division rates between 12.0 – 16.0% whereas high concentrations supressed the cell division. It is known that a decrease of the mitotic activity may lead to a decline of the MN frequencies.

The results were analysed statistically with Prism 5 (Graphpad Inc., CA, USA) with one-way ANOVA followed by Dunnett's multiple comparison test. P-values ≤0.05 were considered as significant.

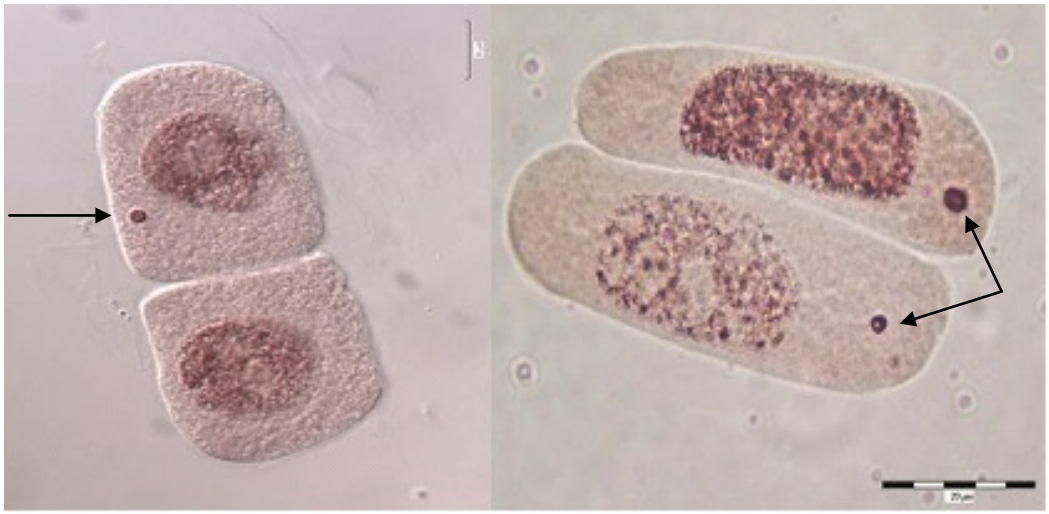

Figure 14: Cells of *Allium cepa*; arrows show micronuclei formation (400-fold magnification, staining 2% acetocarmine).

2.3 Pollen fertility assays with wildlife species

The tests were performed with different wildlife plant species according to the protocol of Murin and Micieta (2000) [26]. The following plants were used: *Tradescantia paludosa* (clone #03), *Chelidonium majus* and *Arabidopsis thaliana* (Columbia, WT-02-41-02, Lehle seeds, USA); photographs of the indicator plants are shown in Figure 15A-C. In the following chapters the cultivation, the treatment and storage, the preparation, the evaluation and the statistical analyses are described.

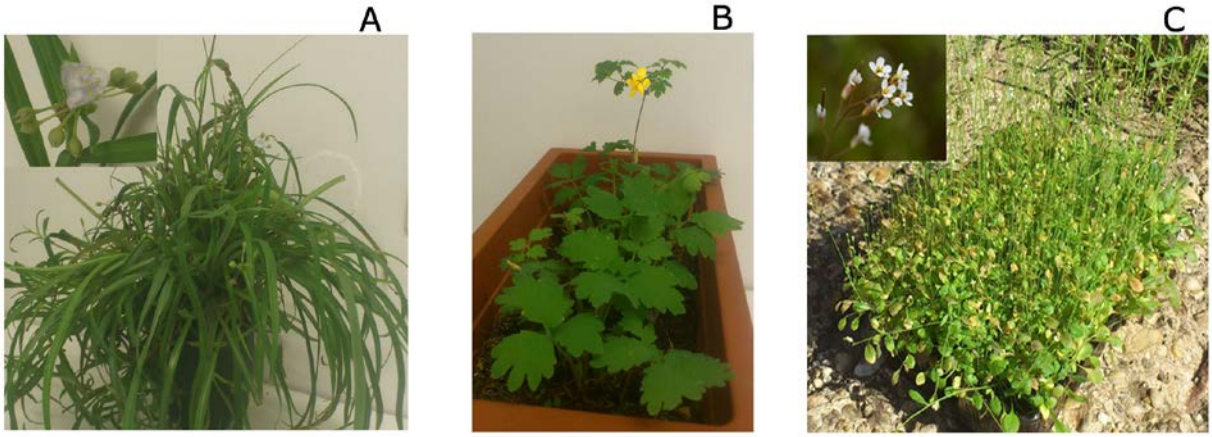

Figure 15: Photographs of the three indicator plants. *Tradescantia paludosa* (A), *Chelidonium majus* (B) and *Arabidopsis thaliana* (C).

2.3.1 Cultivation

The plants were treated under similar experimental conditions as *Tradescantia* clone #4430 in MN experiments (see chapter 2.1). The temperature was between 22 and 25°C, the relative humidity between 60-80% and the day/night cycle 16/8h. The soil was a 1:1 mix of peat and organic soil (Composana, Wien, Austria).

2.3.2 Treatment and storage

Plants were exposed in flower pots, containing 700ml of the soil mix, to 200ml of aqueous solutions of the drugs. Tap water was used as a negative control; MH (10.0mg/l) was used as a positive in experiments with *Tradescantia* #03; As_2O_3 (1.0mM) was used in tests with *Arabidopsis thaliana* and in *Chelidonium majus*. The solutions were poured directly on the soils in the pots. After different time intervals, young flowers and closed flower buds were collected from at least 10 individual plants per experimental point. The material was either evaluated immediately under a light microscope (400-fold magnification) or fixed in ethanol and acetic acid (3:1) for 24h and stored in 70.0% ethanol.

2.3.3 Preparation

The petals were opened with slight pressure and the anthers were transferred to glas slides. Subsequently, the debris (petals and sepals) was removed from the slides. Then the anthers were stained for 3-5 minutes with 2.0% aceto-carmine and squeezed gently with a needle to release the pollen. After the staining phase (5-7 minutes9 a coverslip was added. The preparation procedure is depicted in Figure 16.

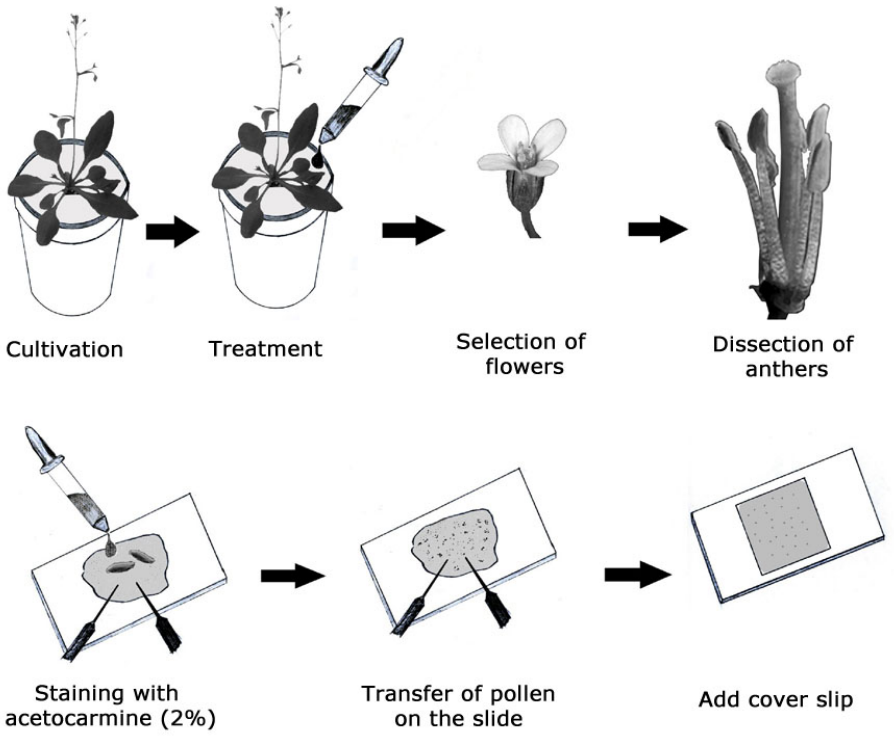

Figure 16: Cultivation and treatment of pollen abortion experiments with *Tradescantia*.

2.3.4 Evaluation and statistical analysis

The frequencies of abortive pollen grains were determined under a light microscope (Nikon Eclipse E200, Nikon Instruments Inc. NY, USA) with 400-fold magnification. Per concentration, 10 slides were evaluated and from each slide 300 pollen grains were scored and evaluated in regard to size, form, and staining ability. The experimental results were analysed statistically with Prism 5 (Graphpad Inc., CA, USA) with Kruskal Wallis followed by Dunnett's multiple comparison test. P-values ≤0.05 were considered as significant.

2.4 Chemicals and Material

Chemicals which were used for the experiments are summarized in table 3.

Table 3: Chemicals which were used for the experiments.

Name	CAS number	Company
cis-Diammineplatinum(II) dichloride (CisPt)	15663-27-1	Sandoz (Vienna Austria)
Imatinib mesylate (IM)	220127-57-1	Cayman Chemical Company (MI, USA)
Maleic hydrazide (MH)	123-33-1	Sigma-Aldrich(Munich, Germany)
Acetic acid	64-19-7	Roth (Vienna, Austria)
Arsenic trioxide	1327-53-3	Sigma-Aldrich(Munich, Germany)
Ethanol	64-17-5	Roth (Vienna, Austria)
Hydrochloric acid	7647-01-0	Sigma-Aldrich(Munich, Germany)
Carmine	1390-65-4	Sigma-Aldrich(Munich, Germany)

For hydroponic cultivation, pots with a diameter of 15 cm, water basins, SERAMIS®, soil (Composana®,Obi, Wien, Austria), glass beakers , eprouvettes and plastic tubes as well as waste boxes and pipettes were used. Preparation needles, slides (Thermo SCIENTIFIC, ISO 8037/1), cover slips and a light microscope (Nikon Eclipse E200, Nikon Instruments Inc., NY, USA) were used for the evaluation of the experiments.

3 Results

The results section consists of three chapters. The first concerns the findings which were obtained in the Trad MN assay, the second describes the findings of experiments with *Allium* (i.e. MN induction, alterations of the MI and acute toxic effects) and the last part summarizes data which were obtained in the pollen infertility assays.

3.1 Micronucleus assays with *Tradescantia*

3.1.1 Micronucleus assay with CisPt

It can be seen in Figure 17, that the drug induced significant effects after 24h with 24h recovery and also after a 30h treatment period without recovery.The extension of the treatment phase (from 24h to 30h) resulted in a strong increase of the effects; i.e. a clear induction of MN was seen with the longer treatment phase already at doses > 0.1 µM. It can be also seen in the figure that pronounced induction of the MN frequencies was found with the positive control (MH).

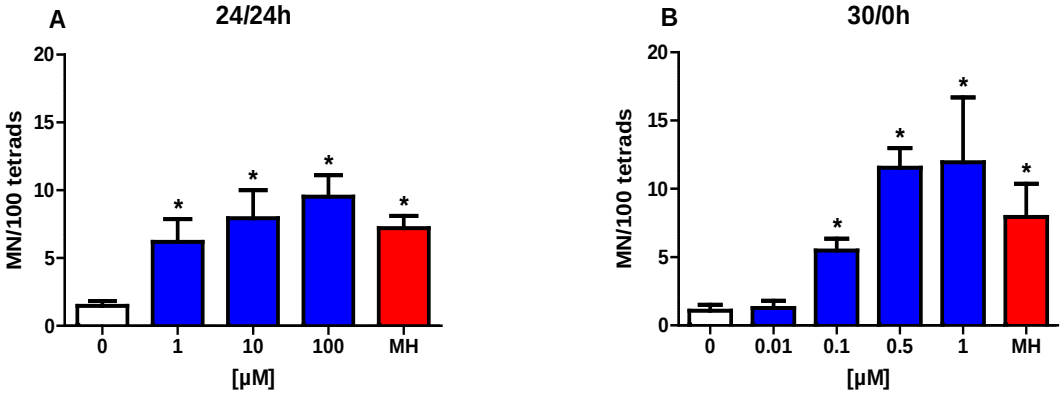

Figure 17: Induction of MN in early tetrads of *Tradescantia* #4430 by CisPt. The plants were treated in stem absorption experiments with aqueous solutions of the drug either for 24h followed by a 24h recovery phase (Figure 17A) or continuously for 30h (Figure17B). The MN rates were evaluated in early tetrads after fixation and staining. For each experimental point, five buds were analysed for MN formation (300 tetrads/bud). Tap water was used as negative control; MH (10.0mg/l) was used as a positive control. Bars indicate the means ±SD values (5 plants) stars indicate statistical significance (ANOVA, with Dunnett's multiple comparison test, p≤0.05).

3.1.2 Micronucleus assay with IM

IM caused a clear cut effect when the cuttings were exposed to 10.0µM for one day (24h). Longer treatment resulted in a substantial increase of the effect; i.e. after 30h exposure the MN rates were 3-5 folds higher than the background frequencies. It can be seen in figures 19A and 19B that the effects increased as a function of the dose.

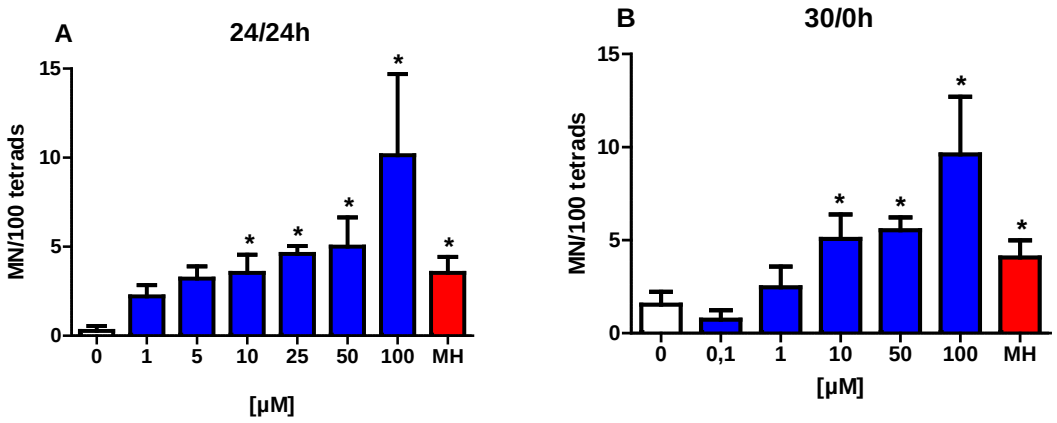

Figure 18: Induction of MN in early tetrads of *Tradescantia* #4430 by IM; figure 18A shows data obtained with 24/24h treatment; figure 18B shows the results which were obtained with a 30/0h treatment. Further details can be found in the legend of Figure 17.

3.2 Micronucleus assays with *Allium cepa*

3.2.1 Results with CisPt

CisPt caused in the root tip experiments clear cut positive results. It can be seen in Figure 19, that the MN rates were 20-fold higher as the background levels when the plants were exposed to 0.5 and 1.0µM of the drug. With 5.0µM, no significant effect was detectable. The lack of a positive response at this concentration can be explained as a consequence of the inhibition of mitotic inhibition, which increased gradually as a function of the exposure concentrations. Figure 19B shows the number of dividing cells declined at the highest dose (5.0µM) below 95.0% in comparison to the control value. Extensions of the treatment period to 72h lead to a marginal increase of the genotoxic effects. However, it is notable that an increase of the mitotic activities was observed under these conditions; i.e. a strong mitogenic effect was seen with the lowest dose (0.05µM) which caused a 2-fold increase of the mitotic index (Figure 21).

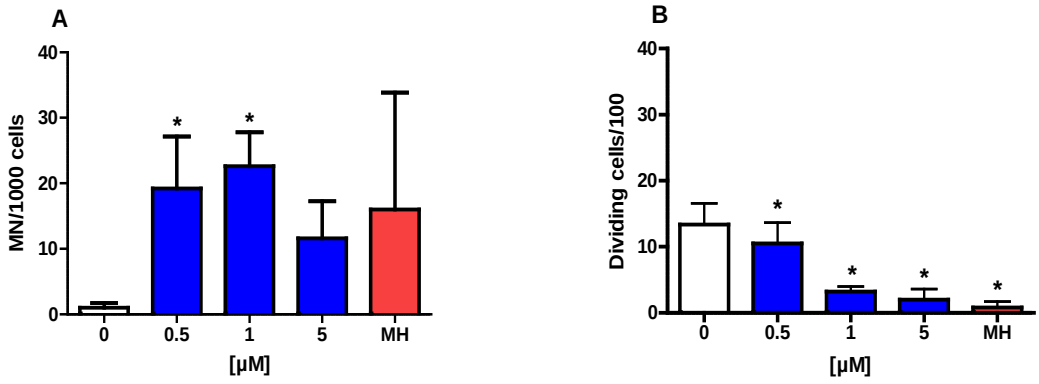

Figure 19: Induction of micronuclei (MN) (Figure 19A) in root tip cells of *Allium cepa* by CisPt. The onions were treated as described in the material and methods chapter to different aqueous solutions of the drug for 24h followed by a 24h recovery phase. The MN rates were evaluated in meristematic and F1 daughter cells after fixation and staining. For each experimental point five onions were evaluated. Two root tips per onion were used to monitor MN formation (500 cells/root; in total 1000 cells/onion). For the evaluation of mitotic indices (MI) (Figure 19B) additional 100 cells per root were analysed for mitosis (500/5 onions). MH (10.0mg/l) was used as a positive control. Bars indicate the means ±SD values (5 onions) stars indicate statistical significance (ANOVA, with Dunnett's multiple comparison test, p≤0.05).

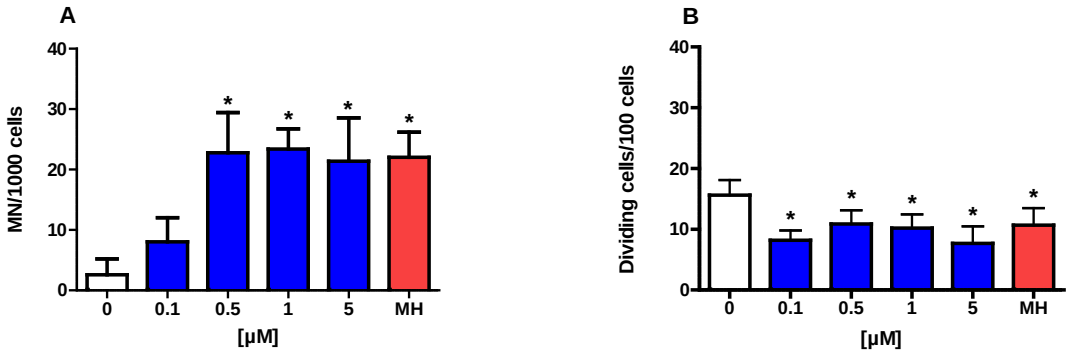

Figure 20: Induction of MN (Figure 20A) and MI (Figure 20B) in root tip cells of *Allium cepa* by CisPt 72/0h. Further details are given in the legend of Figure 19.

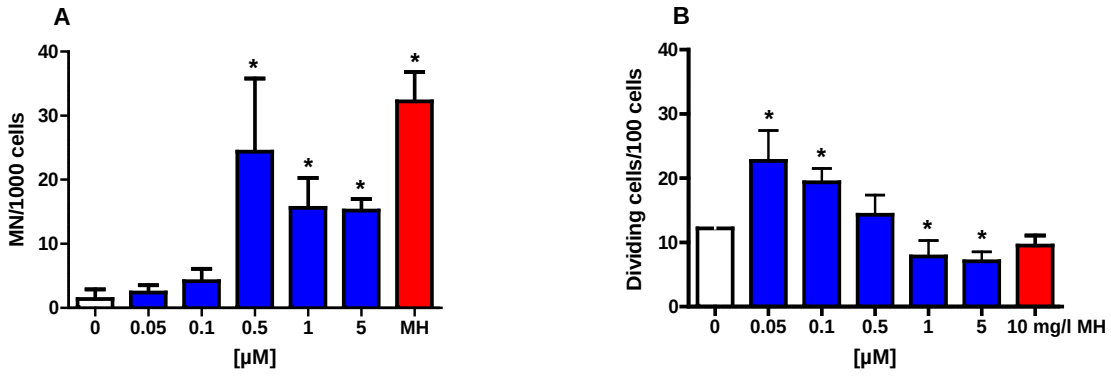

Figure 21: Induction of MN (Figure 21A) and MI (Figure 21B) in root tip cells of *Allium cepa* by CisPt 72/0h. Further details are given in the legend of Figure 19.

3.2.2 Results with IM

Also IM caused induction of MN in the roots. The lowest effective level was in the short term experiment 0.1µM; higher doses did not cause significant effects. The lack of a response is probably due to inhibition of cell division. Already with low doses (≥ 0.05µM), a reduction of the MI was observed after 24h (Figure 24B). Treatment of the plants over three consecutive days (Figures 22A and 23A) lead to an increase of the MN rates in the range between 1.0 and 10.0µM. The later dose caused a significant decline of the MI (50.0%). In order to determine effects, which are caused by low doses more precisely, a follow up experiment

was conducted in which lower concentrations were tested. The findings are shown in Figure 24. It can be seen that no genotoxic effects were detectable in the range between 0.05 and 0.5µM.

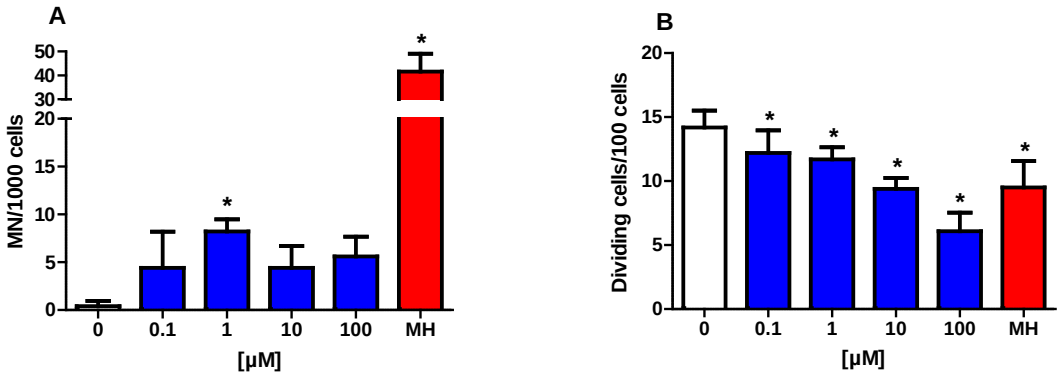

Figure 22: Induction of MN (Figure 22A) and MI (Figure 22B) in root tip cells of *Allium cepa* by IM 24/24h. Further details can be found in the legend of Figure 19.

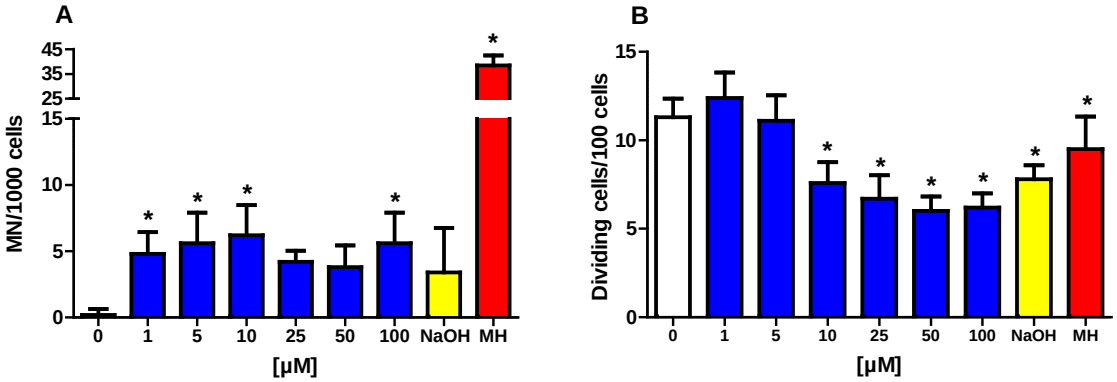

Figure 23: Induction of MN (Figure 23A) and MI (Figure 23B) in root tip cells of *Allium cepa* by IM 72/0h. Further details can be found in the legend of Figure 19.

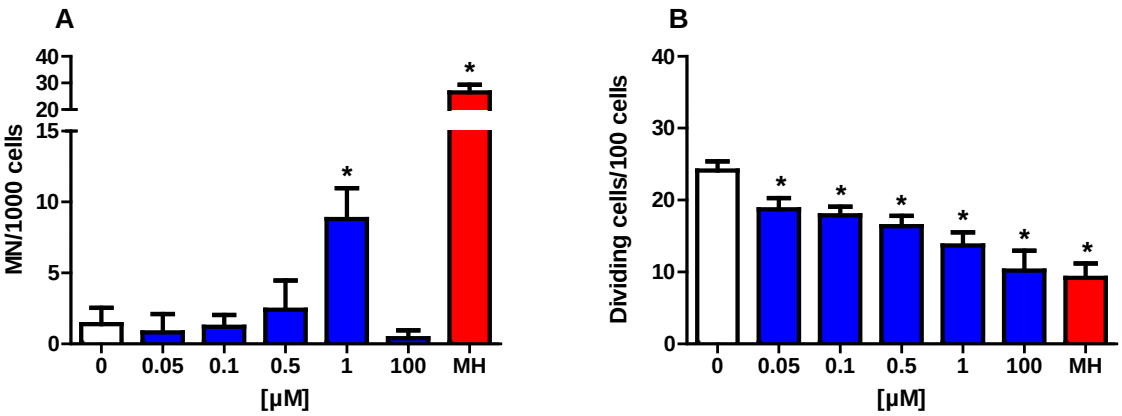

Figure 24: Induction of MN (Figure 24A) and MI (Figure 24B) in root tip cells of *Allium cepa* by IM 72/0h. Further details can be found in the legend of Figure 19.

3.2.3 Impact of the drugs on the root length of *Allium cepa* – toxic effects

The inhibition of root growth by the two drugs is shown in Figure 25. The highest concentration of CisPt (250µM) caused a 70% inhibition of the root growth after 72h (Figure 25A). The IC_{50} value for the drug is 15.34µM (95%, CI: 10.71 – 21.98µM). With IM (Fig. 25B) ca. 80% of the growth was inhibited by the highest concentration (500µM). A concentration of 250µM retarded the growth by 75%. The IC_{50} value for IM is 119.1µM (95%, CI: 97.1-146.2µM)

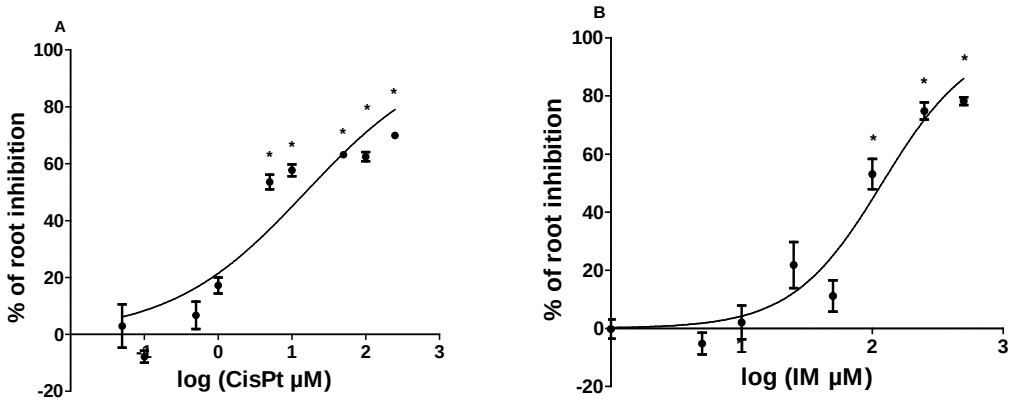

Figure 25: Impact of CisPt (Figure 25A) and IM (figure 25B) on the root growth. The onions were exposed to different concentrations of the drugs for 72h. Subsequently, the root lengths were measured. For each experimental point the longest roots were recorded in 10 treated plants. Each experimental point shows the means ±SD of 10 onions. Curves were calculated with the prism program. Stars indicate significance.

3.4 Pollen fertility (PF) assays with wildlife plants

Three different species, namely *Tradescantia paludosa* (clone #03), *Chelidonium majus* and *Arabidopsis thaliana* were used in the pollen fertility assays (PF assays). Morphological alterations of the grains were evaluated according to criteria described in the paper by Murin and Micieta [26]. Figure 26 depicts photographic images of typical aberrations which were commonly observed as well as mechanically destroyed grains which were not recorded as abortive pollen. The results are summarized in figures. The effects of each drug were monitored at 3-4 time points.

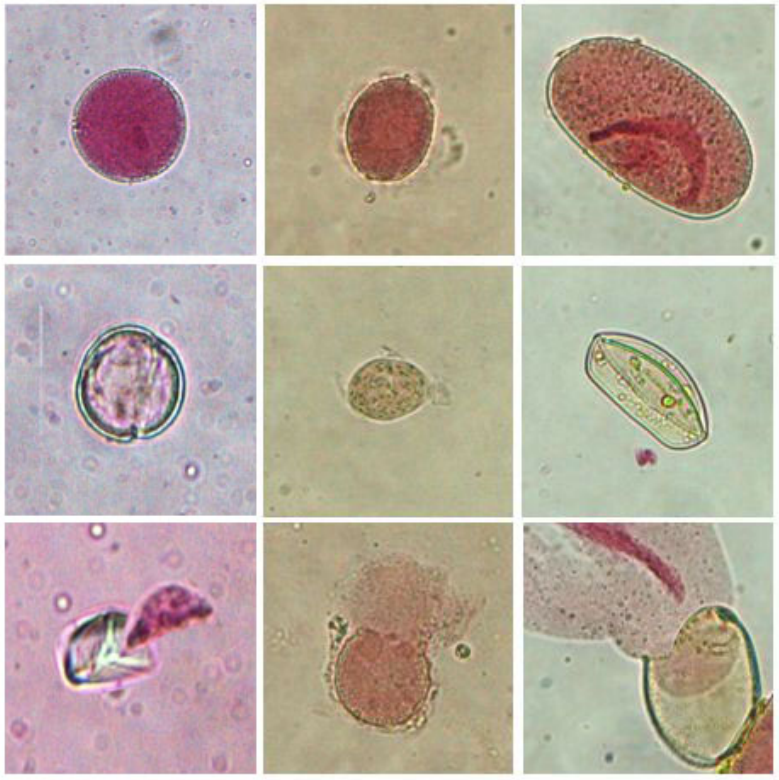

Figure 26: Pollen of *Chelidonium majus* (left), *Arabidopsis thaliana* (center) and *Tradescantia paludosa* (right). The top row shows healthy grains, in the middle row abortive pollen grains can be seen, the bottom row shows mechanical destroyed grains, which were not recorded. (400-fold magnification; staining with 2% acetocarmine).

3.4.1 Background frequencies of the wildlife plants

The background frequencies of the three species which were used varied quite strongly. In *Chelidonium majus* 10±SD abortive pollen grains per 100 pollen were found while the rates in *Arabidopsis thaliana* and *Tradescantia paludosa* were considerably lower (i.e. 1.9 ± SD and 2.9 ±SD respectively). The values were calculated on the basis of six separate experiments. Several plants were cultivated in pots and for each experiment 10 flowers were evaluated per time point .The values were calculated on the bases of the results of six individual studies (with 11 time points) which were conducted over a period of four months. It can be seen, that the standard deviations are quite low, indicating that the rates are stable. (Figure 27)

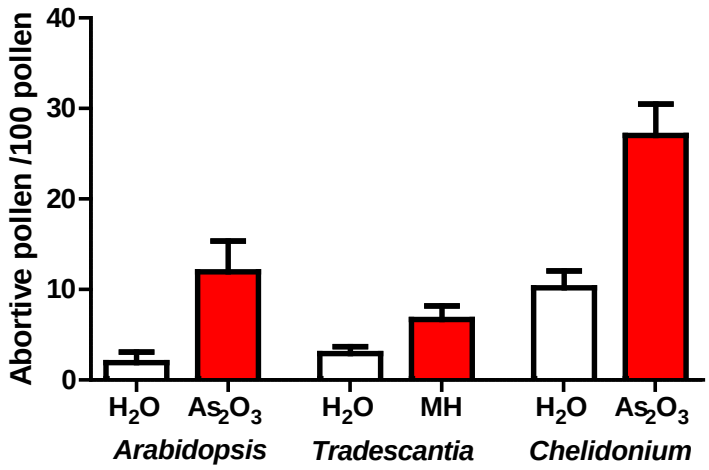

Figure 27: Shows the means ±SD of the rates of abortive grains in three indicator plants. The grains were scored in untreated plants (H_2O) and in the positive controls after exposure with 200.0ml of the solutions As_2O_3 20ml of 1.0mM/l in *Arabidopsis thaliana*/*Chelidonium majus* and MH 10.0mg/l in *Tradescantia paludosa* which were used in the experiments with the cytostatic drugs. In each experiment 10 flowers were evaluated and 300 cells were analysed per flower. The statistical analyses were proceeded with ANOVA, and Dunnett's multiple comparison test, p≤0.05).

3.4.2 Results of pollen fertility assays

The effects which were caused by both drugs in *Tradescantia* #03 are in the same range. Significant increases of the rates of abortive pollen were seen with IM at doses ≥62.5µM; CisPt was effective at a level of ≥125.0µM. The duration of the exposure had only a marginal impact on the toxic properties of the drugs.

Interestingly, a clear difference was seen in regard to the dose response relationships. In the case of CisPt, the highest frequencies of abortive grains were found with doses in the range between 62.5 and 250.0µM. The levels increased as a function of the exposure concentrations. However, at the highest dose (500.0µM), the effect decreased at all four time points. This may be, due to the acute toxicity of the cytostatic as decreased flower formation was observed with this dose. The ranking order of sensitivity was with the platinum compound in all experimental series *Tradescantia>Arabidopsis>Chelidonium*. The dose response effects which were caused by IM were under all experimental conditions more pronounced when an effect is seen at a lower dose; i.e. potent effects were already seen at the lowest dose. In most experiments maximal induction of abortion grains was seen with doses in the range between 62 and 125µM at higher concentrations abortion frequencies remained at identical levels.

Comparisons of the sensitivity of the three species show that they are more or less identical; i.e. with the lowest doses of the drugs the increase of the abortive pollen grains was with CisPt 3-fold and with IM 2-3-fold over the background. The most pronounced effects were seen with the low dose levels with *Arabidopsis thaliana*; i.e. the increases were up to 5-fold over the control values.

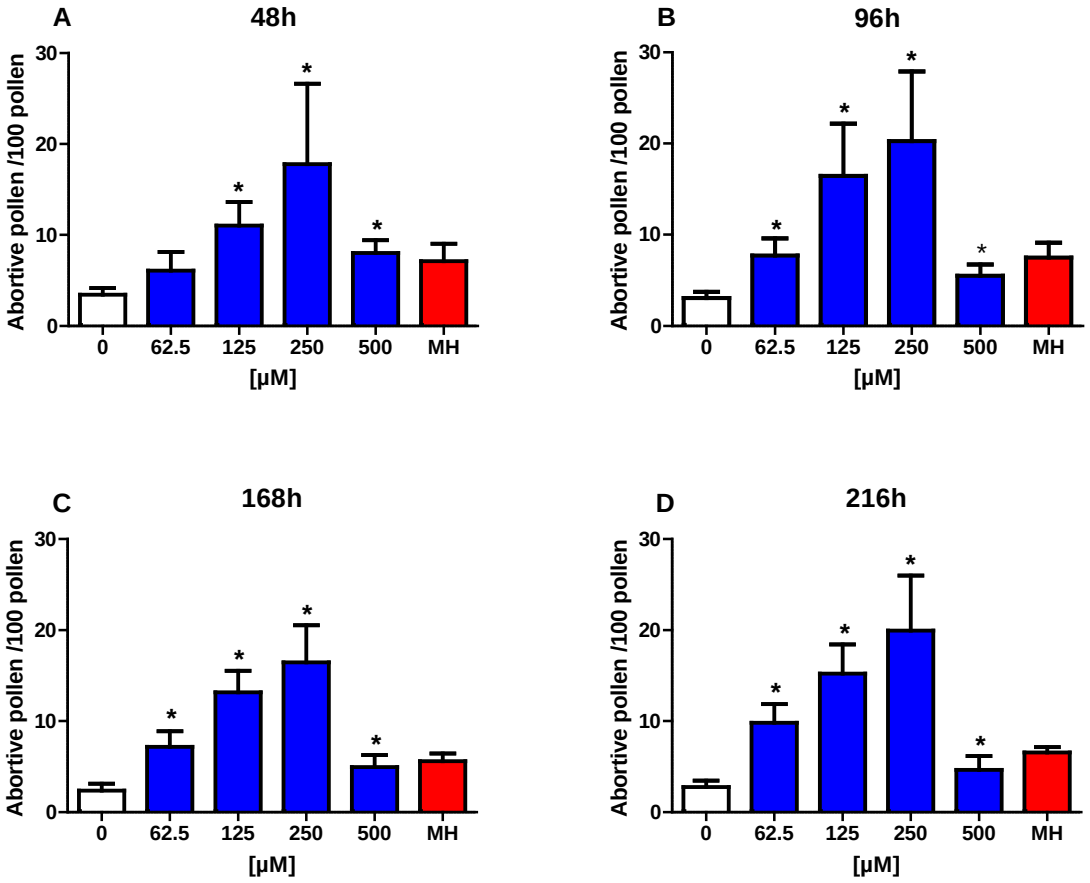

Figure 28: Induction of abortive pollen grains in *Tradescantia paludosa* by CisPt. Per experimental point at least 8 plants were treated. Maleic hydrazide (MH) 10.0mg/l was used as a positive control. Details see materials and methods section. After different treatment periods, the frequencies of abortive pollen grains were determined under a light microscope (400-fold magnification). From each plant, 10 closed flowers were collected and 300 grains were analysed per flower; (in total 3000 grains). Bars indicate means ± SD values. Stars indicate statistical significance (Kruskal-Wallis and Dunn's multiple comparison p ≤0.05).

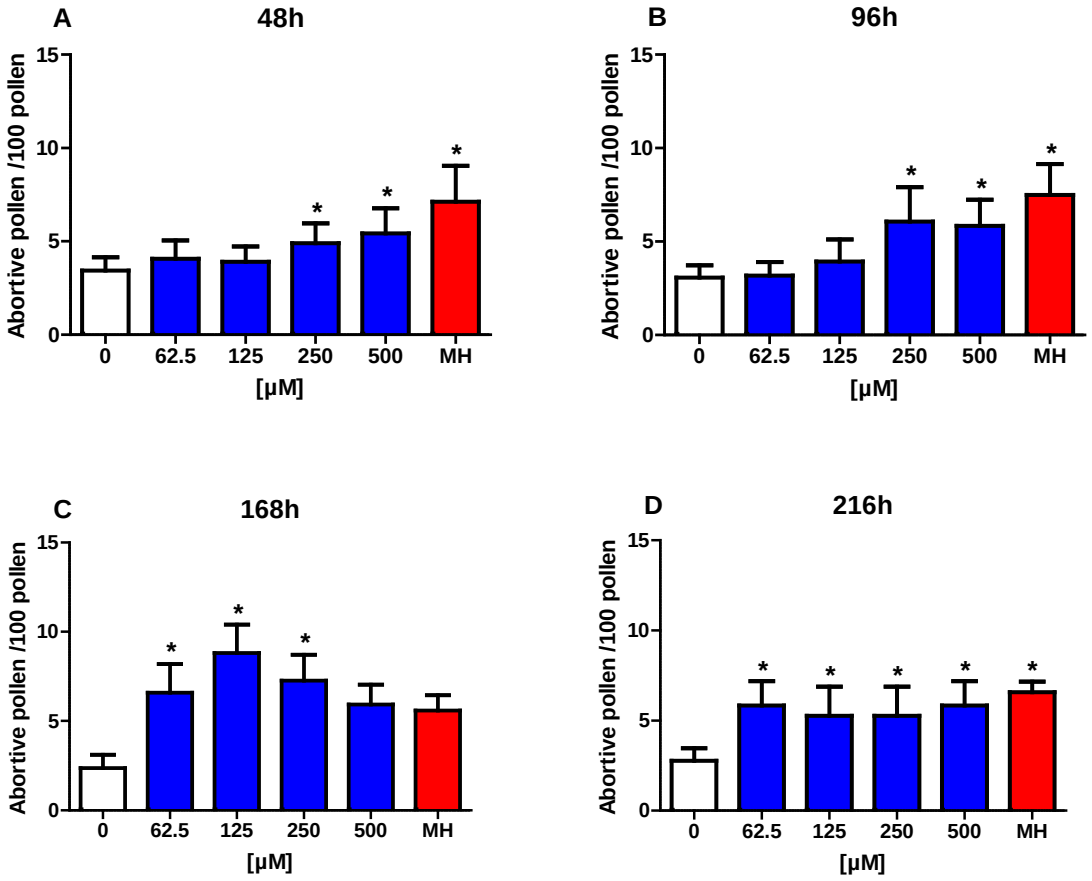

Figure 29: Induction of abortive pollen grains in *Tradescantia paludosa* by IM. The experiment was conducted as described in the legend of Figure 28.

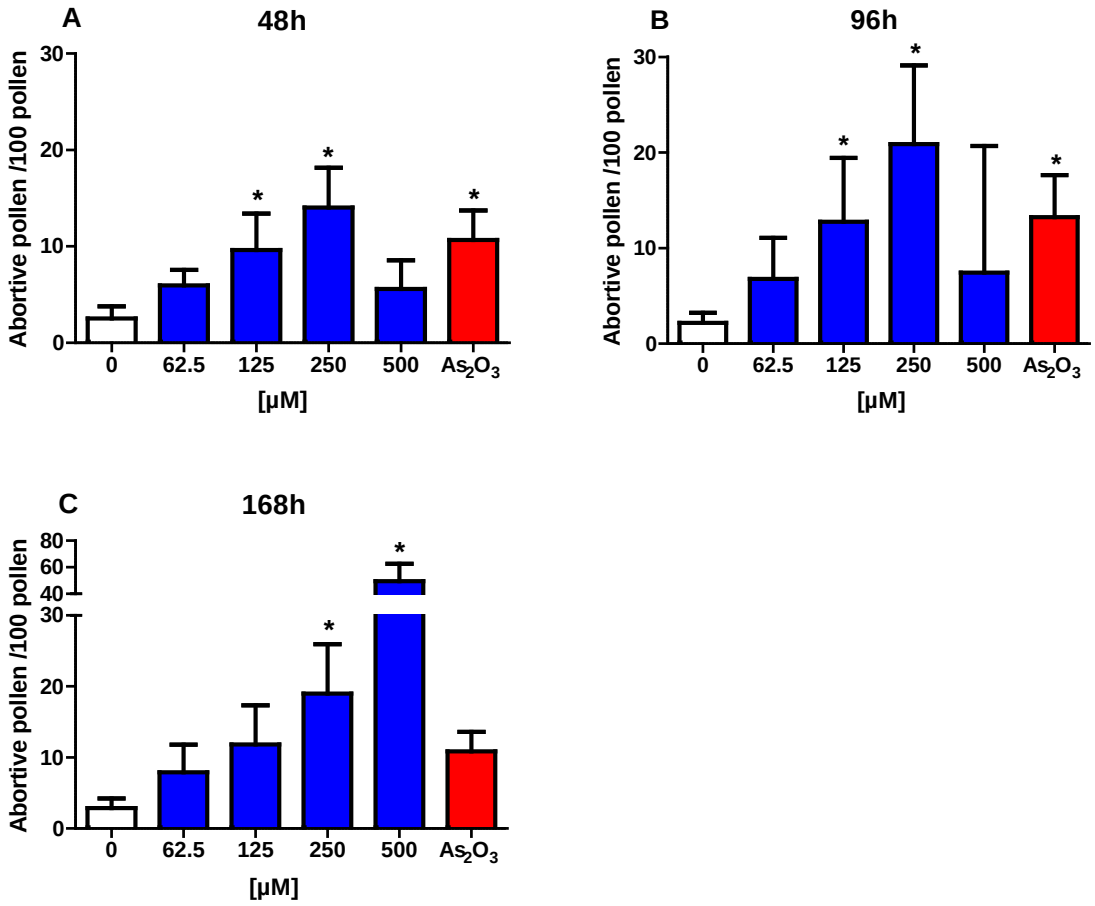

Figure 30: Induction of abortive pollen grains in *Arabidopsis thaliana* by CisPt. Per experimental point at least 8 plants were treated. Arsenic trioxide (As_2O_3) 20ml of 1.0mM was used as a positive control. Details see materials and methods section. After different treatment periods, the frequencies of abortive pollen grains were determined under a light microscope (400-fold magnification). From each plant, 10 closed flowers were taken and 300 pollen grains were analysed per flower; (in total 3000 grains). Bars indicate means ± SD values. Stars indicate statistical significance (Kruskal-Wallis and Dunn's multiple comparison p ≤0.05).

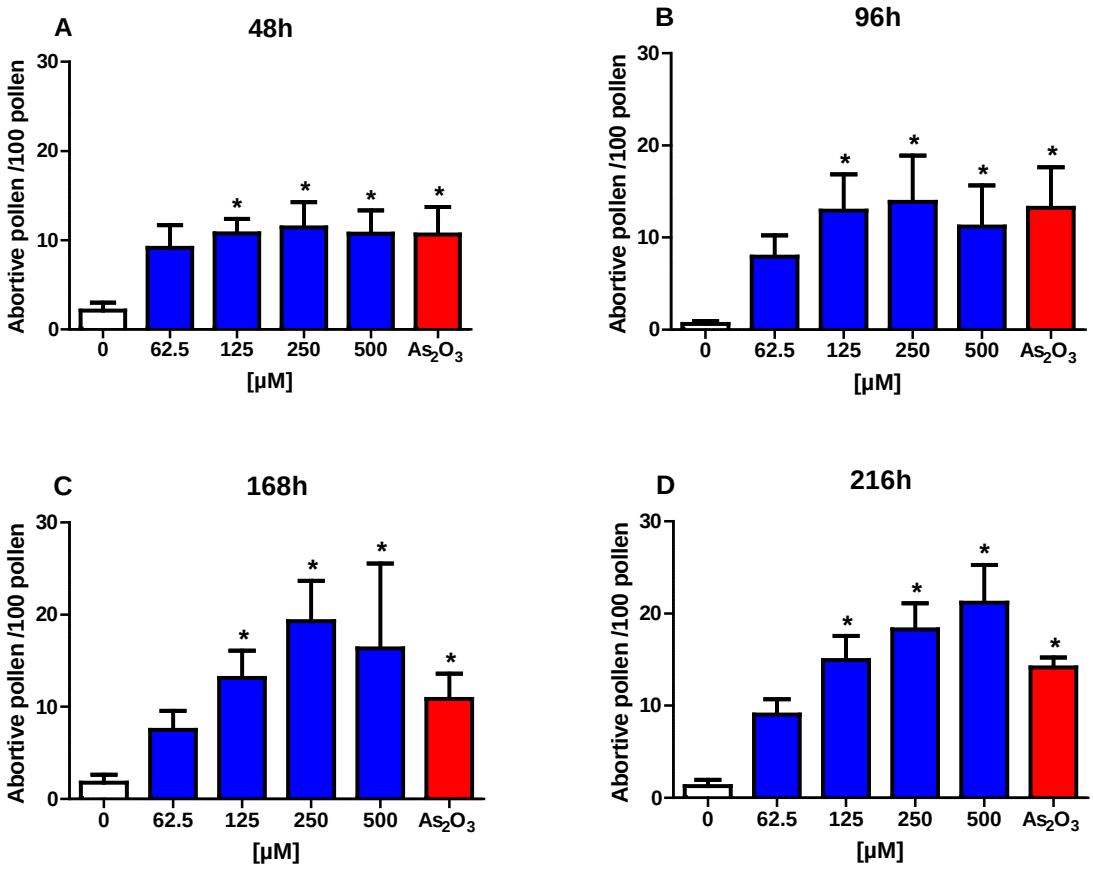

Figure 31: Induction of abortive pollen grains in *Arabidopsis thaliana* by IM. The experiment was conducted as described in the legend of Figure 30.

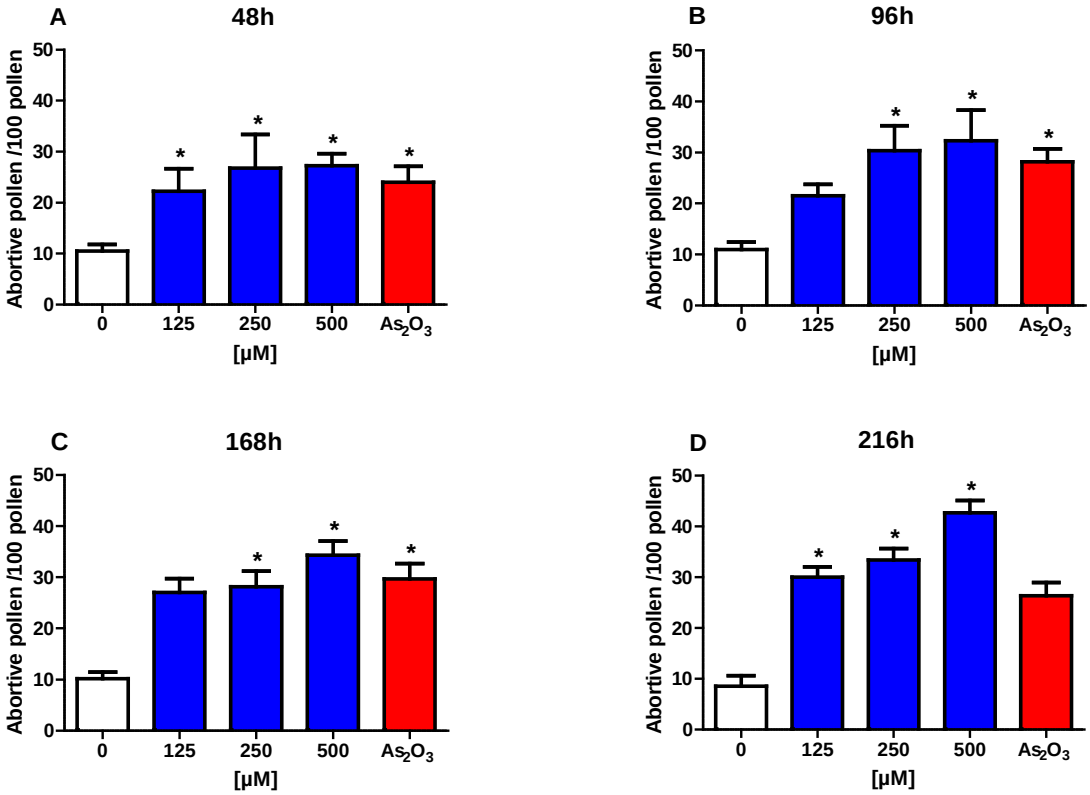

Figure 32: Induction of abortive pollen grains in *Chelidonium majus* by CisPt. Per experimental point at least 8 plants were treated. Arsenic trioxide (As_2O_3) 20ml of 1.0mM was used as a positive control. Details see materials and methods section. After different treatment periods, the frequencies of abortive pollen grains were determined under a light microscope (400-fold magnification). From each plant 10 closed flowers were taken and 300 pollen grains were analysed per flower; (in total 3000 grains). Bars indicate means ± SD values. Stars indicate statistical significance (Kruskal-Wallis and Dunn's multiple comparison p ≤0.05).

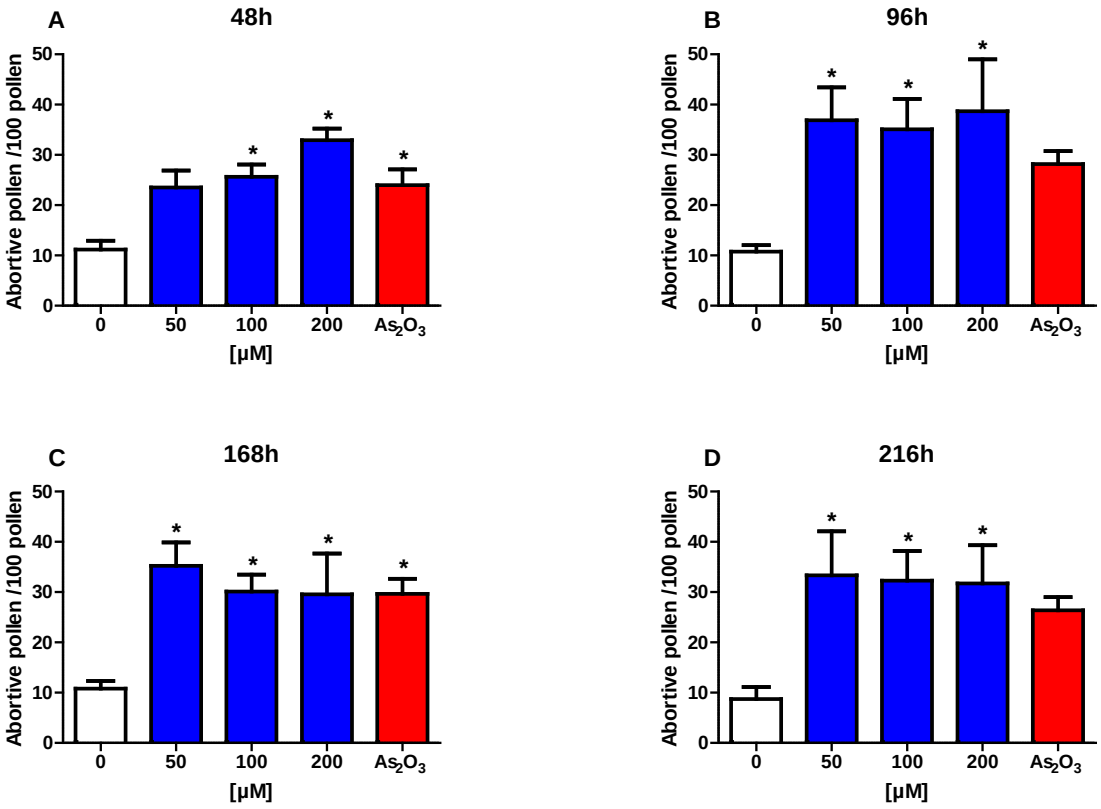

Figure 33: Induction of abortive pollen grains in *Chelidonium majus* with IM. The experiment and conducted as described in the legend of Figure 32.

4 Discussion

The discussion section comprises three subchapters. The first concerns results which were obtained with untreated controls and with the positive control maleic hydrazide in the different experimental models. The second paragraph describes the findings which were obtained with the two cytostatics in comparison to results caused by other types of important genotoxic carcinogens in plant bioassays and other genotoxicity tests. Finally, attempts were made to assess the consequences of the release of the drugs into the environment on the basis of data which were obtained in acute toxicity and genotoxicity experiments and on the consumption of the drugs in EU countries.

4.1 Results obtained with untreated plants and with Maleic hydrazide in earlier investigations

The MN frequencies which were found in the present study with untreated inflorescences of *Tradescantia* varied between 9 and 29 per 300 tetrads. The mean values of the different experimental series, which were recorded in the frame of the present study, were 11 and 23 per 300 tetrads. These values are similar to those obtained in earlier investigations with *Tradescantia* clone #4430 in Vienna and in other labs ([53], [54], [55]).

Also the results which we obtained in the MN experiments with *Allium cepa* roots are in the expected range. In our experiments the frequencies varied between 16 and 41 (mean of 6 individual experimental series) and in earlier studies similar values were obtained ([56], [57])

Likewise, also the MI rates are in a tolerable range. In the present study they were between 5 and 13 per 100 cells. In an earlier investigation 2-10 per 100 cells were reported [58]. It is notable that the relatively wide range maybe due to the use of different cultivars in different experimental series.

As described in detailed in the results section, positive findings were obtained in all experimental series with MH. The induction of MN in *Tradescantia* and *Allium*

cepa was similar to that which was seen in earlier investigations in Austria [31]. Ma, T.H. et al. found in a detailed study a clear induction over the background in *Allium cepa* after treatment [17].

4.2 Comparison of the genotoxic activities of the two drugs (CisPt and IM) with the effects of other genotoxic carcinogens

The LOEL-values which were obtained in earlier plant studies with different environmentally relevant compounds and the values we determined in the frame of the CytoThreat project with four cytostatic drugs are listed in Table 4. It can be seen, that the LOEL-values which were obtained with CisPt and IM were in both plant test systems substantially lower or similar as those of other toxins such as heavy metal compounds (e.g. As_2O_3, $Pb(C_2H_3O_2)_4$) which are among the most dangerous environmental toxins.

The genotoxic and acute toxic effects of two further cytostatic (etoposide and 5-fluorouracil) drugs have been investigated in the same set of plant bioassays as IM and CisPt. The results of these findings have not been published yet and are described in a master thesis by Rainer, B [59].

It is notable that the LOEL of Etoposide, in regard of induction of MN, in *Tradescantia* was 1.0µM (24/24h and 30/0h) and in the case of *Allium* MN 50.0µM (24/24h) and 0.1µM (72/0h). The values for 5-FU are in *Tradescantia* 100µM (24/24h) and 25.0µM (30/0h). In *Allium* the values are 100.0µM (24/24h) and 50.0µM (72/0h). These comparisons show that the genotoxic potencies of the different cytostatic drugs decline in the order CisPt>Etop>IM>5-FU.

Furthermore, we calculated also the rad equivalent values for the mutagenic action of both cytostatics in *Tradescantia* and *Allium*, on the basis of earlier x-radiation experiments ([9], [17], [60]). The CisPt doses which caused a 5-fold increase of the MN formation over the background frequencies were 1.0µM (24/24h) and 0.1µM (30/0h) in the Trad MN assay. In the *Allium* root tip assay the dose for a 10-fold increase with CisPt was 0.5µM in the 24/24h and 72/0h

treatment. Values for a 5-fold increase with IM are 25.0µM (24/24h) and 10.0µM (30/0h) in Trad Mn assay and in *Allium* 1.0µM (24/24h and 72/0h) respectively.

The x-ray dose which causes a 5-fold increase an identical effect in *Tradescantia* (i.e. 5-fold increase over the background) was detected with 0.10Gy. In *Allium* values of 0.125Gy or 0.25Gy cause a 5-fold or respectively a 10-fold increase of the effect (increase over the background). A dose of 1.0Gy would cause an effect in *Tradescantia* which is induced by 10.0µM of CisPt and 250.0µM of IM on the basis of data obtained with the 24h treatment protocols. In *Allium* in a dose of 1.0Gy would cause an effect which is induced by 2.0µM of CisPt and 8.0µM of IM.

In this context it is notable that the acceptable daily exposure with ionising radiation (such as gamma-radiation or x-radiation) for humans is at present 0.003Gy and the effective doses for intervention is 1.0mSv (1.0Gy) according to the Austrian law [61].

Table 4: LOEL doses of the four tested cytostatic drugs and other substances of earlier investigations.

Substance	*Tradescantia* LOEL[1)]	*Allium* LOEL[1)]	Ref.
CisPt	1.0 (24/24)	0.5µM (24/24)	present study
	0.1 (30/0h)	0.1µM (72/0)	
IM	10.0 (24/24)	1.0µM (24/24)	present study
	10.0 (30/0h)	1.0µM (72/0)	
5FU	100.0 (24/24)	100.0µM (24/24)	[59]
	25.0 (30/0)	50.0µM (72/0)	
Etop	1.0 (24/24)	50.0µM (24/24)	[59]
	1.0 (30/0)	0.1µM (72/0)	
$Pb(C_2H_3O_2)_4$	2.12		[62]
As_2O_3	10.0		[62]
		1250.0	[10]
$NaAsO_2$		7.6	[63]
Simazine	24.0		[64]
Dicamba	226.24		[64]
Chlorpyrifos	142.0		[65]
Cyazine	46.5		[65]
Metolachlor	100.0		[65]
Anthracene	70.13		[62]
Atrazine	46.29		[64]
		13.9	[66]
Chlorite	5.90	0.3	[67]
Chlorate	4.79M	1.79	[67]
Formaldehyde		25000.0	[17]

[1)] LOEL in µM

4.3 Comparison of the present findings with results of earlier genotoxicity-experiments with cytostatics

CisPt has been studied in numerous mutagenicity test systems and consistently positive results were obtained. Representative results are shown in Table 5. It can be seen, that gene mutations as well as chromosomal mutations were indicated by the drug in different tests. Also in humans evidence for induction of DNA instability was detected in several trials. The results of these investigations can be not directly compared to the findings of the present study in which the intact plants were exposed to aqueous solutions of the cytostatic drugs. According to our knowledge, the only investigation which concerned effects in higher plants was published by an Indian group (who reported on induction of chromosomal aberrations, which were observed after exposure in *Allium cepa* roots for 24h at dosis >1µM) [68].

Table 5: Studies concerning the effect of CisPt.

Organisms	Test [1)]	Duration	LOEL	Ref.
Prokaryotes				
Escherichia coli	DNA-SB	1h	50.0µM	[69]
S.typhimurium TA100	RM	48h	0.01µM+/-S9	[70]
Lower Eucaryotes				
Saccharomyces cervisiae	RNA-AF	6h	100.0µM	[71]
Saccharomyces cervisiae	GI	6h	200.0µM	[71]
Plants				
Allium cepa	CA	24h	1.0µM	[68]
Allium cepa	RGI	24h	1.0µM	[68]
Insects				
Drosophila melanogaster	DNA-AF	24h	250.0µM	[72]
Drosophila melanogaster	GM	Larva	3.0µM	[73]
In vitro				
Chinese hamster ovary	MD	1h	0.5µg/ml	[74]
Chinese hamster ovary	CA	1h	0.5µg/ml	[75]
Rat PC12 cells	CF	2h	0.1µg/ml	[76]
Rat PC12 cells	MN	24h	0.1µg/ml	[76]

Continuing of Table 5: Studies concerning the effect of CisPt

Organisms	Test [1)]	Duration	LOEL	Ref.
Human lymphocytes	CA	24h	5.0µM +/- S9	[70]
Human lymphocytes	SCE	24h	1.0µM	[70]
Human lymphocytes	MN	48h	2.5µM	[70]
Human leukocytes	CF	1h	10.0µM	[77]
In vivo				
Rat bone marrow cells	MN	96h	6.0mg/kg IP	[78]
Rat bone marrow cells	CA	96h	6.0mg/kg IP	[78]
Rat bone marrow cells	CF	96h	6.0mg/kg IP	[78]
Rat astrocytes	MN	72h	2.5mg/kg	[79]
Female A/Jax mouse	Car	8 months	108.0µM	[80]

[1)]DNA strand breaks (DNA-SB), reverse mutation (RM), RNA adduct formation (RNA-AF), growth inhibition (GI), chromosomal aberration (CA), root growth inhibition (RGI), DNA adduct formation (DNA-AF), gene mutation (GM), mitotic delay (MD), Comet formation (CF), micronuclei formation (MN), sister chromatid exchange (SCE), carcinogenicity (Car), intra peritoneal (IP)

With IM only few studies have been published which concern the impact on genetic stability. The results are shown in table 6. Fabarius et al. reported an induction of chromosomal aberrations in Chinese hamster embryo fibroblasts, in Indian muntiac fibroblasts and in human dermal fibroblasts. The effects were seen with all types of indactor cells with doses > 0.5µM.

Table 6: Studies of the effect of IM

Organisms /cell line	Test [1)]	Duration	LOEL	Ref.
In vitro				
Chinese hamster EF	CA	21 days	5.0µM	[81]
Indian muntjac fibroblasts	CA	21 days	5.0µM	[81]
***In vitro* human**				
Dermal fibroblasts	CA	21 days	5.0µM	[81]
Dermal fibroblasts	CA	140 days	5.0µM	[81]
In vivo				
Immature male rats SD	DGCF	3 days	150.0mg/kg IP	[82]
Mouse C3H/HeJ strain	fertility	60days	150.0mg/kg/day	[83]

[1)]Chromosomal aberration (CA), Delay of germ cell formation (DGCF), embryonal fibroblasts, intra peritoneal (IP), Sprague-Dawley Rats

Also in the case concerning acute toxic effects data it is only meaningful to compare our results with those of other aquatic experiments in *Daphnia magna* and Algae. In the frame of the CytoThreat project toxicity was monitored in *Pseudokirchneriella subcapicata* by Metka Filipič National Institute for Biology Slovenia. Results have not been published so far. The IC_{50} doses which were found when the experiments were conducted under standardized conditions according to international (OECD) guidelines. In the algae experiments the IC_{50} for CisPt and IM were about 5.0µM (personal communication). According to the Novartis material and safety data sheet the EC_{50} for Algae is 4.2µM; detailed information is listed in Table 7.

Table 7: Effective and lethal concentrations in different Organisms[1)]

Organisms	concentrations mg/l	
Toxicity in Bacteria (activated sludge)	EC10:	65.0
	EC50:	232.0
	EC80:	605.0
Fish toxicity (*Cyprinus carpio*)	LC0:	56.0
	LC50:	82.0
Daphnia toxicity (*Daphnia magna*)	EC50:	80.0
	NoEC:	32.0
Toxicity in Algae (*Selenastrum capricornutum*)	EbC50:	2.5
	EbC10:	1.1
	NoEC:	0.96

[1)]Data are taken from Cancer Therapy Evaluation Program[84]

4.4 Mechanistic concentrations

The positive findings, which were obtained in the MN experiments with CisPt in the present study, were not unexpected since it is known that the drug caused DNA crosslinks which leads to gene mutations and chromosomal aberrations. See chapter 1.3.1., page 9.

On the contrary, IM does not cause direct DNA damage and negative results were obtained in a number of test systems e.g. in gene mutation

experiments with *Salmonella*/microsome assays [50], in the mouse lymphoma test and *in vivo* rat micronucleus assay [84]

While positive findings were detected in Chromosomal aberration experiments with Chinese Hamster ovaries [50] and fibroblasts [81, 85]. The hypothesis of the authors is that in DNA repair process and the control of cell cycle progression one or more ABL-related tyrosine kinase is involved by IM. They included that inhibition of c-ABL may affect the ubiquination of centrosomal components leading to extra centrosomal duplication and mitotic spindle catastrophy (data not published, paper in preparation).

4.5 Possible environmental impact of the release of CisPt and IM on higher plants

CisPt and IM are at present among the most widely used cytostatics. In 2006 the overall annual use was for CisPt in Germany estimated 46.0kg and for IM 806.0kg, in France the annual consumption of platinum compound was two years later 22.74kg and in the case of IM 873.9kg. Comparisons with the use of other cytostatic drugs are shown in figure 34.; it can be seen that CisPt and IM are at very high positions in regard of the annual use. Also in Slovenia both drugs are used for the treatment of cancer patients (Filipič, personal communication).

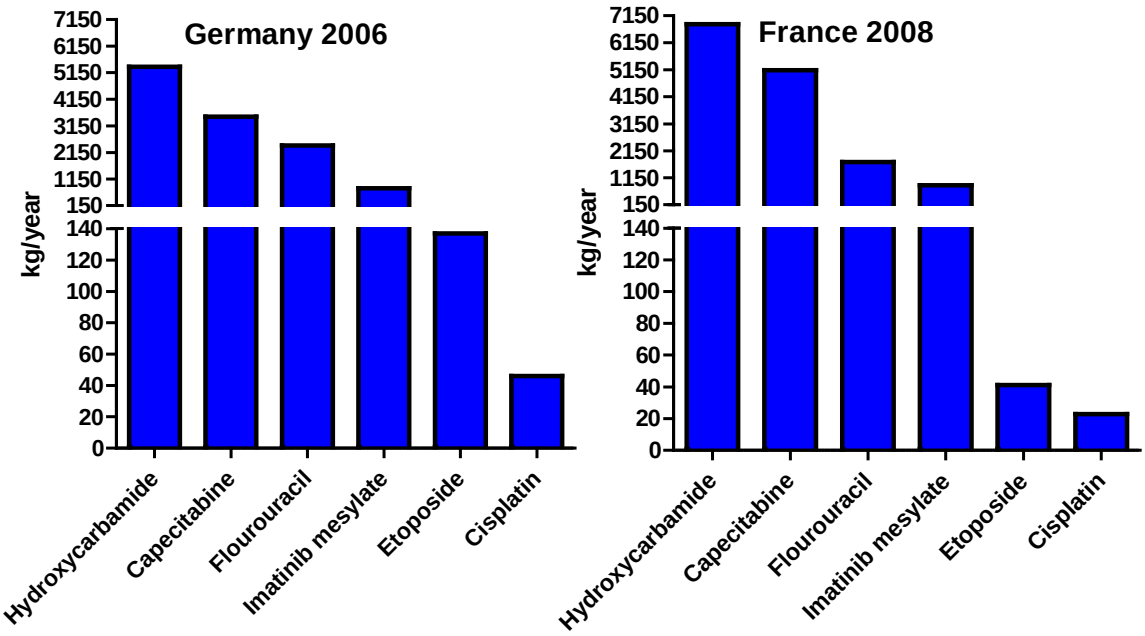

Figure 34: Comparisons of the annual use of different cytostatic drugs in Germany and in France. (Umweltrisikobewertung von Zytostatika [38], Besse et al. [86]).

The release of CisPt in the environment has been investigated in several studies. Relatively high levels 4.7–145µg/l [87] and 3.2-266µg/l [88]were found in wastewater from an oncological ward in in Vienna. According to our knowledge no data on the levels of the concentration of IM in surface and wastewaters have been published so far. According to a recent study of a Spanish group the analytical detection limits have not been established so far.

It is possible to use the annual consumption data to assess potential adverse effects in higher plants on the basis of the results of the present experiments. Relevant data are summarized in Table 8.

Table 8: Environmental concentrations of CisPt and IM.

	Country			
	Slovenia[1)]		France[2)]	
Cytostatic	**g/year (2011)**	**PEC (ng/l)**	**g/year (2008)**	**PEC (ng/l)**
CisPt	107.80	5.03	22740	0.52
IM	7350.0	0.07	873900	19.95

[1)] Filipič et al., 2012; [2)] Besse, et al., 2012

It can be seen in Table 8 the PEC-values (Predicted Environmental Concentrations) are several orders of magnitude lower than the amounts which caused significance effects in the present plant assays. In other words, the actual levels which may occur in waters are approximately several orders of magnitudes lower as the doses which are required to cause induction of DNA-damage or toxic effects in the plant bioassays. In the case of CisPt it is notable that the concentration which was detected in the water which came directly from an oncological ward was sufficiently high to cause effects in the plants.

Taken together, the comparisons between the biologically active concentrations and the environmental levels indicate, that it is unlikely that the concentrations of CisPt and IM in surface waters causes adverse effects in higher plants. However, this conclusion is only relevant when the drugs are removed from hospital waste waters by effective purification processes.

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5 Appendix

Raw data, of presented figures of the result section are shown in the appendix as tables.

Tradescantia micronuclei assay

The results of the Trad MN assay are shown in Tables 9-12.

Table 9: Induction of MN in *Tradescantia* clone #03 by CisPt (exposure time 24/24h).[1)]

		Concentration in µM			
Slide Nr.	**-C**	**1.0**	**10**	**100**	**+C**
1	6	21	13	32	26
2	4	22	26	24	20
3	5	23	29	33	21
4	4	11	26	23	19
5	3	16	25	31	22

[1)]Numbers indicate the Micronuclei which were found in 300 tetrads. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Table 10: Induction of MN in *Tradescantia* clone #03 by CisPt (exposure time 30/0h).[1)]

		Concentration in µM			
Slide Nr.	**-C**	**0.1**	**0.5**	**1.0**	**+C**
1	2	20	36	38	11
2	5	15	38	21	26
3	2	17	31	26	29
4	3	17	39	58	25
5	4	13	29	36	28

[1)]Numbers indicate the Micronuclei which were found in 300 tetrads. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Table 11: Induction MN in *Tradescantia* clone #03 by IM (exposure time 24/24h).[1)]

		Concentration in µM						
Slide Nr.	**-C**	**1**	**5**	**10**	**25**	**50**	**100**	**+C**
1	1	6	11	10	14	8	24	15
2	0	4	8	7	12	13	22	9
3	0	8	10	15	15	18	46	11
4	2	9	12	12	13	15	16	8
5	1	6	7	9	15	21	44	10

[1)]Numbers indicate the Micronuclei which were found in 300 tetrads. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Table 12: Induction MN in *Tradescantia* clone #03 by IM (exposure time 30/0h).[1)]

		Concentration in µM					
Slide Nr.	**-C**	**0.1**	**1**	**10**	**50**	**100**	**+C**
1	3	2	3	9	14	41	14
2	7	0	9	16	19	21	11
3	6	4	6	18	17	36	16
4	5	3	12	19	15	20	11
5	2	2	7	14	18	26	9

[1)]Numbers indicate the Micronuclei which were found in 300 tetrads. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Allium cepa micronuclei assay and mitotic index assay

The results of the *Allium cepa* MN assay and MI assay are shown in Tables 13-24.

Table 13: Induction of MN in *Allium cepa* by CisPt (exposure time 24/24h).[1)]

		Concentration in µM			
Slide Nr.	**-C**	**0.5**	**1**	**5**	**+C**
1	1	2	11	7	3
2	0	5	19	5	15
3	1	9	9	5	0
4	0	20	10	6	3
5	0	12	11	10	0
6	0	10	7	10	0
7	1	7	12	1	7
8	0	12	8	3	7
9	1	6	12	3	33
10	1	13	14	8	12

[1)]Numbers indicate the Micronuclei which were found in 500 cells. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Table 14: Results of the MI in *Allium cepa* by CisPt (exposure time 24/24h).[1)]

		Concentration in µM			
Slide Nr.	**-C**	**0.5**	**1**	**5**	**+C**
1	14	17	4	2	0
2	16	12	3	2	2
3	9	12	3	1	0
4	11	10	5	2	1
5	14	10	3	6	0
6	17	8	3	3	0
7	8	9	3	1	0
8	17	10	2	1	2
9	13	5	3	0	2
10	15	12	3	2	1

[1)]Numbers indicate the dividing cells which were found in 100 cells. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Table 15: Induction of MN in *Allium cepa* by CisPt (exposure time 72/0h). [1)]

		Concentration in µM				
Slide Nr.	**-C**	**0.1**	**0.5**	**1**	**5**	**+C**
1	0	2	6	15	14	16
2	2	1	7	8	11	11
3	0	2	16	13	17	14
4	0	3	14	9	13	10
5	1	7	12	14	12	11
6	1	3	15	9	12	9
7	0	2	11	9	8	11
8	2	7	13	11	5	12
9	3	6	11	17	7	7
10	4	7	9	12	8	9

[1)]Numbers indicate the Micronuclei which were found in 500 cells. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Table 16: Results of the MI in *Allium cepa* by CisPt (exposure time 72/0h). [1)]

		Concentration in µM				
Slide Nr.	**-C**	**0.1**	**0.5**	**1**	**5**	**+C**
1	18	10	10	9	11	11
2	21	9	9	13	8	12
3	13	7	10	9	13	16
4	15	7	13	14	9	13
5	17	7	12	10	7	9
6	16	9	10	8	7	13
7	13	6	9	7	5	9
8	14	9	11	12	5	7
9	15	7	9	9	4	8
10	14	11	16	11	8	9

[1)]Numbers indicate the dividing cells which were found in 100 cells. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Table 17: Induction of MN in *Allium cepa* by CisPt (exposure time 72/0h).[1)]

		Concentration in µM					
Slide Nr.	**-C**	**0.05**	**0.1**	**0.5**	**1**	**5**	**+C**
1	0	1	4	5	9	8	14
2	1	3	3	9	13	9	17
3	0	1	1	8	9	7	12
4	0	1	2	11	8	6	16
5	0	2	3	8	6	8	19
6	1	0	2	11	9	7	13
7	1	2	2	13	4	9	14
8	0	1	2	14	5	8	16
9	3	0	0	22	6	5	19
10	1	1	2	21	9	9	21

[1)]Numbers indicate the Micronuclei which were found in 500 cells. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control

Table 18: Results of the MI in *Allium cepa* by CisPt (exposure time 72/0h).[1)]

		Concentration in µM					
Slide Nr.	**-C**	**0.05**	**0.1**	**0.5**	**1**	**5**	**+C**
1	13	16	20	11	10	9	7
2	14	28	18	12	7	7	8
3	12	28	20	10	7	8	11
4	13	24	17	11	9	7	10
5	14	19	16	17	8	6	9
6	10	27	20	18	11	5	8
7	13	22	23	14	4	8	11
8	11	19	21	16	4	7	10
9	12	17	21	18	7	5	9
10	10	27	18	16	11	9	12

[1)]Numbers indicate the dividing cells which were found in 100 cells. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Table 19: Induction of MN in *Allium cepa* by IM (exposure time 24/24h).[1)]

		Concentration in µM				
Slide Nr.	**-C**	**0.1**	**1**	**10**	**100**	**+C**
1	0	2	3	1	5	14
2	0	0	5	1	4	35
3	1	1	4	0	2	16
4	0	1	3	2	4	15
5	0	10	6	2	3	21
6	1	1	4	3	1	17
7	0	1	3	4	2	25
8	0	3	4	3	3	23
9	0	1	3	2	2	24
10	0	2	6	4	2	18

[1)]Numbers indicate the Micronuclei which were found in 500 cells. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control

Table 20: Results of the MI in *Allium cepa* by CisPt (exposure time 24/24h).[1)]

		Concentration in µM				
Slide Nr.	**-C**	**0.1**	**1**	**10**	**100**	**+C**
1	15	12	13	10	6	8
2	14	11	12	11	5	10
3	16	12	12	9	4	7
4	14	13	11	10	5	9
5	13	14	13	9	7	12
6	15	11	10	9	9	8
7	12	16	12	10	7	13
8	14	12	11	9	6	11
9	13	11	12	9	5	10
10	16	10	11	8	7	7

[1)]Numbers indicate the dividing cells which were found in 100 cells. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Table 21: Induction of MN in *Allium cepa* by IM (exposure time 72/0h).[1)]

		Concentration in µM							
Slide Nr.	**-C**	**1**	**5**	**10**	**25**	**50**	**100**	**NaOH**	**+C**
1	0	1	0	3	3	1	6	1	15
2	0	1	2	4	2	2	2	0	19
3	0	0	3	3	1	1	2	2	21
4	0	6	5	2	2	1	1	0	18
5	0	2	4	3	2	3	5	0	16
6	0	3	2	4	2	3	3	1	19
7	0	4	0	2	1	2	2	5	21
8	1	2	5	1	3	1	3	4	23
9	0	3	4	5	2	3	2	3	22
10	0	2	3	4	3	2	2	1	18

[1)]Numbers indicate the Micronuclei which were found in 500 cells. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control

Table 22: Results of MI evaluation in *Allium cepa* by IM (exposure time 72/0h).[1)]

		Concentration in µM							
Slide Nr.	**-C**	**1**	**5**	**10**	**25**	**50**	**100**	**NaOH**	**+C**
1	10	13	11	7	6	6	7	7	7
2	12	11	13	8	5	5	6	8	9
3	11	14	10	6	7	5	6	8	10
4	13	12	11	9	5	6	7	9	12
5	12	13	9	7	9	7	5	8	8
6	10	10	12	8	8	6	7	7	7
7	12	12	13	9	6	6	5	7	9
8	11	14	9	9	7	7	6	8	10
9	12	11	12	6	8	5	7	9	12
10	10	14	11	7	6	7	6	7	11

[1)]Numbers indicate the dividing cells which were found in 100 cells. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Table 23: Induction of MN in *Allium cepa* by IM (exposure time 72/0h). [1)]

		Concentration in µM					
Slide Nr.	**-C**	**0.05**	**0.1**	**0.5**	**1**	**100**	**+C**
1	1	0	0	2	3	0	10
2	1	0	0	3	4	0	12
3	3	0	1	0	4	1	15
4	0	0	0	0	3	0	13
5	0	0	0	0	6	0	11
6	1	1	2	2	2	0	14
7	0	2	0	3	9	1	12
8	0	1	2	1	3	0	16
9	0	0	1	0	4	0	14
10	1	0	0	1	6	0	15

[1)]Numbers indicate the Micronuclei which were found in 500 cells. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control

Table 24: Results of MI evaluation of *Allium cepa* by IM (exposure time 72/0h).[1)]

		Concentration in µM					
Slide Nr.	**-C**	**0.05**	**0.1**	**0.5**	**1**	**100**	**+C**
1	24	18	20	16	14	12	8
2	23	20	18	17	15	9	10
3	26	19	19	14	12	10	7
4	24	20	17	16	13	16	9
5	25	18	17	18	14	13	8
6	24	17	18	19	18	10	7
7	22	18	19	16	13	8	9
8	24	20	16	17	12	8	13
9	26	16	18	15	12	9	12
10	23	21	17	16	14	7	9

[1)]Numbers indicate the dividing cells which were found in 100 cells. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Pollen abortion assay with wild life species

The results of the pollen abortion assay are shown in tables 25-30.

Table 25: Induction of pollen abortion in *Tradescantia paludosa* by CisPt.

			Concentration in µM				
Time h	Slide	Tap water	62.5	125	250	500	As_2O_3
48	1	15	23	22	45	21	30
48	2	9	19	42	24	25	16
48	3	10	22	34	90	22	22
48	4	10	20	33	54	18	23
48	5	9	25	21	32	23	17
48	6	13	11	29	28	31	22
48	7	9	27	39	39	27	12
48	8	9	14	45	43	20	25
48	9	11	10	31	87	24	29
48	10	8	12	35	92	30	18
96	1	8	19	38	69	13	27
96	2	11	27	44	78	9	29
96	3	7	22	39	35	19	21
96	4	8	22	42	42	20	20
96	5	13	33	60	31	17	31
96	6	11	15	64	38	14	18
96	7	8	20	32	83	18	19
96	8	9	27	87	97	15	21
96	9	10	29	33	72	21	16
96	10	7	18	54	63	19	23
168	1	6	19	34	38	12	14
168	2	5	25	41	41	18	15
168	3	6	29	28	44	9	19
168	4	9	23	53	60	18	16
168	5	8	18	39	42	13	18
168	6	9	15	37	56	14	17
168	7	11	30	47	64	11	12
168	8	4	19	35	41	22	19
168	9	5	17	37	37	17	20
168	10	8	21	44	71	15	18
216	1	2	21	49	58	61	44
216	2	1	18	52	63	73	38
216	3	5	27	61	71	65	40
216	4	8	34	47	58	75	43
216	5	5	24	39	41	71	46
216	6	2	28	34	48	62	41
216	7	5	29	40	53	49	41
216	8	3	32	45	49	81	46
216	9	3	27	39	51	41	38
216	10	4	31	43	57	57	47

[1)]Numbers indicate the Micronuclei which were found in 300 pollen. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Table 26: Induction of pollen abortion in *Tradescantia paludosa* by IM.

			Concentration in µM				
Time h	Slide	Tap water	62.5	125	250	500	As_2O_3
48	1	15	45	39	39	39	30
48	2	9	48	27	54	42	16
48	3	10	36	48	36	48	22
48	4	10	36	33	51	27	23
48	5	9	27	30	42	66	17
48	6	13	21	24	42	45	22
48	7	9	36	39	27	63	12
48	8	9	48	39	39	60	25
48	9	11	30	30	57	45	29
48	10	8	39	42	54	54	18
96	1	8	11	8	24	12	27
96	2	11	11	11	11	22	29
96	3	7	13	14	21	21	21
96	4	8	7	15	28	15	20
96	5	13	10	9	15	20	31
96	6	11	6	16	18	18	18
96	7	8	7	17	21	12	19
96	8	9	9	12	13	13	21
96	9	10	10	8	19	19	16
96	10	7	11	8	12	23	23
168	1	6	21	31	29	20	14
168	2	5	15	29	21	19	15
168	3	6	18	25	19	16	19
168	4	9	24	21	19	22	16
168	5	8	22	32	15	18	18
168	6	9	11	25	28	22	17
168	7	11	17	33	21	11	12
168	8	4	19	27	19	18	19
168	9	5	27	19	25	17	20
168	10	8	24	22	22	15	18
216	1	11	23	17	17	23	19
216	2	8	19	12	12	19	19
216	3	8	24	22	22	24	20
216	4	5	21	21	21	21	18
216	5	10	16	10	10	16	22
216	6	11	14	14	14	14	22
216	7	7	14	9	9	14	22
216	8	10	12	13	13	12	19
216	9	6	15	19	19	15	17
216	10	7	17	21	21	17	19

[1)]Numbers indicate the Micronuclei which were found in 300 pollen. Maleic hydrazide 10.0mg/l was used as positive control, tap water as negative control.

Table 27: Induction of pollen abortion in *Arabidopsis thaliana* by CisPt.

			Concentration in µM				
Time	**Slide**	**Tap water**	**62.5**	**125**	**250**	**500**	**As_2O_3**
48	1	6	22	36	51	20	25
48	2	5	15	18	50	16	17
48	3	7	9	8	36	7	29
48	4	6	22	36	47	9	35
48	5	8	18	31	32	10	39
48	6	7	26	14	57	8	23
48	7	12	16	34	38	16	47
48	8	3	18	36	59	24	36
48	9	16	14	42	27	23	28
48	10	6	19	34	24	35	41
96	1	5	5	8	41	70	132
96	2	6	6	12	38	77	8
96	3	4	4	35	75	65	0
96	4	7	7	6	36	89	7
96	5	11	11	33	49	51	11
96	6	4	4	10	56	42	36
96	7	4	4	29	41	92	12
96	8	3	3	25	10	9	6
96	9	9	9	38	29	55	4
96	10	12	12	7	8	76	7
168	1	13	13	23	17	72	149
168	2	6	6	7	27	51	130
168	3	5	5	27	69	55	152
168	4	2	2	35	35	58	228
168	5	6	6	30	33	73	206
168	6	9	9	41	11	102	122
168	7	12	12	17	42	33	135
168	8	8	8	16	29	38	120
168	9	11	11	8	49	51	141
168	10	15	15	34	43	37	102

[1)]Numbers indicate the Micronuclei which were found in 300 pollen. Arsenic trioxide (As_2O_3) 20ml of 1.0mM was used as positive control, tap water as negative control.

Table 28: Induction of pollen abortion in *Arabidopsis thaliana* by IM.

			Concentration in µM				
Time h	**Slide**	**Tap water**	**62.5**	**125**	**250**	**500**	**As_2O_3**
48	1	8	36	28	27	26	25
48	2	6	31	37	35	19	17
48	3	4	21	31	40	27	29
48	4	4	23	25	29	36	35
48	5	12	37	33	33	29	39
48	6	4	18	37	36	35	23
48	7	7	23	31	28	39	47
48	8	9	22	29	23	28	36
48	9	6	25	41	41	45	28
48	10	4	39	32	52	39	41
96	1	1	38	58	22	22	32
96	2	3	20	51	61	65	25
96	3	2	24	21	30	31	39
96	4	1	18	45	29	42	26
96	5	3	18	27	39	23	30
96	6	2	32	41	42	34	48
96	7	3	28	32	33	23	66
96	8	1	19	49	37	22	50
96	9	2	18	33	59	33	32
96	10	1	23	30	65	41	49
168	1	6	23	43	54	35	28
168	2	4	31	38	78	48	31
168	3	2	18	25	62	50	40
168	4	6	22	47	51	25	20
168	5	7	22	44	67	42	39
168	6	5	15	31	32	21	28
168	7	11	23	28	69	60	25
168	8	5	17	51	54	78	38
168	9	5	35	48	47	109	30
168	10	2	19	39	65	22	47
216	1	2	21	49	58	61	44
216	2	1	18	52	63	73	38
216	3	5	27	61	71	65	40
216	4	8	34	47	58	75	43
216	5	5	24	39	41	71	46
216	6	2	28	34	48	62	41
216	7	5	29	40	53	49	41
216	8	3	32	45	49	81	46
216	9	3	27	39	51	41	38
216	10	4	31	43	57	57	47

[1)]Numbers indicate the Micronuclei which were found in 300 pollen. Arsenic trioxide (As_2O_3) 20ml of 1.0mM was used as positive control, tap water as negative control.

Table 29: Induction of pollen abortion in *Chelidonium majus* by CisPt.

			Concentration in µM			
Time h	Slide	Tap water	125	250	500	As_2O_3
48	1	31	81	80	73	77
48	2	29	78	89	79	61
48	3	35	56	56	83	73
48	4	29	45	66	69	68
48	5	31	51	113	86	80
48	6	26	58	107	92	72
48	7	32	83	68	84	59
48	8	29	71	71	77	65
48	9	33	69	59	84	91
48	10	40	75	93	89	73
96	1	27	64	97	81	78
96	2	34	71	93	96	72
96	3	28	67	95	87	83
96	4	41	61	89	98	79
96	5	36	68	79	97	88
96	6	31	57	86	91	81
96	7	29	69	105	135	83
96	8	32	75	96	86	89
96	9	38	59	59	121	98
96	10	32	54	112	78	93
168	1	31	87	78	98	87
168	2	35	91	86	105	73
168	3	33	67	74	91	95
168	4	37	73	79	97	89
168	5	29	86	84	106	106
168	6	27	81	97	109	89
168	7	32	83	76	93	90
168	8	25	79	85	105	79
168	9	29	91	83	108	88
168	10	27	73	103	118	94
216	1	25	78	95	136	79
216	2	16	86	102	121	69
216	3	34	95	93	119	71
216	4	29	89	98	132	81
216	5	15	98	109	139	78
216	6	26	91	105	118	97
216	7	24	97	97	129	76
216	8	28	92	108	125	85
216	9	33	86	89	134	80
216	10	27	88	106	127	75

[1)]Numbers indicate the Micronuclei which were found in 300 pollen. Arsenic trioxide (As_2O_3) 20ml of 1.0mM was used as positive control, tap water as negative control.

Table 30: Induction of pollen abortion in *Chelidonium majus* by IM.

			Concentration in µM			
Time h	**Slide**	**Tap water**	**125**	**250**	**500**	**As_2O_3**
48	1	29	62	78	99	77
48	2	26	56	75	105	61
48	3	36	78	62	88	73
48	4	31	71	79	104	68
48	5	28	83	87	109	80
48	6	33	74	81	101	72
48	7	35	59	67	89	59
48	8	42	68	79	93	65
48	9	33	86	82	102	91
48	10	41	69	79	97	73
96	1	37	83	94	151	78
96	2	28	76	129	108	72
96	3	32	103	135	94	83
96	4	31	119	84	101	79
96	5	28	127	96	94	88
96	6	38	131	107	110	81
96	7	29	103	115	91	83
96	8	33	125	112	85	89
96	9	37	107	79	157	98
96	10	29	132	101	169	93
168	1	35	89	96	131	87
168	2	32	96	101	86	73
168	3	33	103	79	121	95
168	4	36	109	90	91	89
168	5	29	90	81	101	106
168	6	31	115	89	62	89
168	7	41	97	93	79	90
168	8	25	135	110	93	79
168	9	28	110	78	60	88
168	10	34	113	86	63	94
216	1	25	68	92	112	79
216	2	38	68	87	125	69
216	3	36	82	79	115	71
216	4	15	131	139	119	81
216	5	27	91	78	105	78
216	6	18	115	109	69	97
216	7	24	92	91	73	76
216	8	29	122	103	87	85
216	9	24	143	98	63	80
216	10	26	87	92	84	75

[1)]Numbers indicate the Micronuclei which were found in 300 pollen. Arsenic trioxide (As_2O_3) 20ml of 1.0mM was used as positive control, tap water as negative control.

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Chapter 22

Micronucleus Assay with Tetrad Cells of *Tradescantia*

Miroslav Mišík, Clemens Pichler, Bernhard Rainer, Armen Nersesyan, and Siegfried Knasmueller

Abstract

The *Tradescantia* micronucleus assay is being used since almost 50 years for the detection of genotoxins (including carcinogens) in the environment. A large database on the effects of individual compounds and of complex environmental mixtures (soil, air, and water) has accumulated. In contrast to other mutagenicity test systems, the effects of low concentrations of heavy metals, radionuclides, certain herbicides and pesticides, and gaseous mutagens can be detected and it is also possible to use the test for in situ biomonitoring studies. The test system has been validated, and standardized protocols have been developed for laboratory experiments and for field studies, which are described in this chapter.

Key words *Tradescantia*, Tetrads, Genotoxicity studies, Micronucleus

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1 Introduction

Already in the 1960s and 1970s, a variety of protocols for mutagenicity assays with higher plants were developed, which were based on the detection of mutations in the offspring, somatic mutations, or on the evaluation of chromosomal aberrations [1]. Most of the protocols were time consuming and labor intensive; therefore, only few models survived and are used at present, in particular for environmental monitoring.

The most frequently employed approaches are micronucleus (MN) assays with mitotic root tip cells of *Vicia faba* and *Allium cepa* and experiments with meiotic tetrad cells of *Tradescantia* (*Commelinaceae*), which grows in tropical and subtropical areas. A description of the methods used in root assays and the current databases can be found in the publications of Leme and Marin-Morales [2], Foltete et al. [3], and White and Claxton [4]. Micronucleus was first described in *Tradescantia* in the 1950s by Steffensen [5]. The most frequently used clone for genotoxicity experiments is #4430 (Fig. 1), hybrid between *T. subacaulis* and *T. hirsutifolia*, which was developed in early 1960s in the Brookhaven

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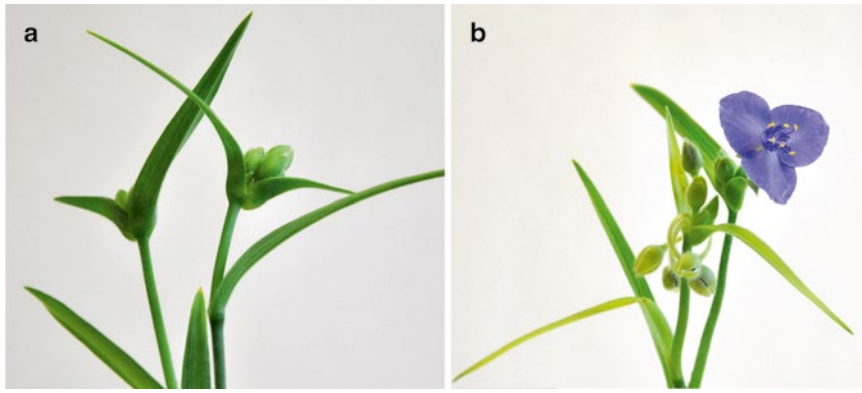

Fig. 1 Inflorescences of *Tradescantia* #4430 suitable (**a**) and not suitable (**b**) for treatment and subsequent evaluation of MN

laboratories [6]. Another clone, *T. paludosa* #03, was frequently used in radiation experiments. In the 1990s, Brazilian groups started to use *T. pallida*, a wild plant, which grows in several South American countries for experiments [7, 8].

Hundreds of studies have shown that plant bioassays, in particular the *Tradescantia* micronucleus (Trad MN) test, are valuable tools for the detection of genotoxic carcinogens in the environment. This assay is highly sensitive towards compounds which cause negative or moderate effects in other widely used systems. For example, heavy metals cannot be detected in bacterial mutagenicity assays, which are also not sensitive towards ionizing radiation. Furthermore, it is important to note that, due to the insensitivity of most mutagenicity assays, concentration procedures are required which may lead to loss of active compounds [9, 10]. Such procedures can be avoided in Trad MN experiments. Another point, which argues for the use of Trad MN assays, concerns the fact that more than 120 individual compounds and about 100 complex environmental mixtures have been tested [11–13], and therefore, the sensitivity of the assay to different environmental toxicants and to environmental pollution is well known. On the basis of the currently available data it can be concluded that the Trad MN bioassay is complementarily used to other current methods for the detection of environmental genotoxins. Therefore the assay should be included in test batteries that are used in studies concerning environmental pollution such as radioactive materials, heavy metals, and air pollution. The test system is based on the detection of micronuclei which refer to clastogenic (chromosome breaking) and aneugenic effects in meiotic pollen tetrad cells. The MN frequencies are scored in buds, which contain early tetrads and are highly synchronized. Plants can be exposed to test substances as stems either in aqueous solutions or in intact form with the roots in soil (in the case of soil experiments). [AU1]

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The results which were obtained with *Tradescantia* are summarized in two comprehensive reviews: the article of Rodrigues et al. [14] covers papers which appeared before 1996, while the update by Mišík et al. [15] concerns later publications.

2 Materials

1. Indicator plants: *Tradescantia* clones #4430, #02, and *T. paludosa* #03 should be used for genotoxicity experiments (*see* **Note 1**).
2. Preparation of plant material: Glacial acetic acid, 70 % ethanol, and pure ethanol.
3. Dilution of the test compounds: 1 % dimethyl sulfoxide (DMSO, an organic solvent) can be used in stem absorption experiments.
4. Positive controls: 1.0 mM ethyl methanesulfonate (EMS) or 0.5 mM arsenic oxide (As_2O_3) or 0.2 mM maleic hydrazide (MH) can be used. These are direct acting mutagens.
5. Staining agent: 1–2 % acetocarmine (Carmine). Heat 50 mL of 45 % acetic acid solution (45 mL glacial acetic acid and 55 mL of distilled water) to boiling. Add 500 mg of carmine and continue heating for 15–20 min while stirring. Cool the resulting solution and filter to remove any precipitate. The solution can be stored in a dark place for several years at room temperature.
6. Pots: The plants should be cultivated in 15 cm diameter plastic pots in pesticide- and herbicide-free soil, or in the case of hydroponic culture, in smaller pots (13/12 cm diameter).
7. Soil/nutrient: 2 parts sand, 4 parts soil, and 1 part peat moss constitute the soil for greenhouse propagation. Two applications of any liquid fertilizer per month are needed (*see* **Note 2**). Standard NPK (nitrogen, phosphor, potassium) liquid fertilizer can be used for hydroponic cultivation.

3 Methods

3.1 Cultivation of Plants

1. Light: If the plants are cultivated in a growth chamber with artificial light, the intensity of the fluorescent light should be about 378 E/m²/s (1,800 ft candles) and the incandescent light should be around 38 E/m²/s (180 ft candles) at the tip of the plants. Use of an 18/6-h (light/dark) period is essential for flowering of the plants.
2. Temperature: The daytime temperature should be around 21–25 °C, and the nighttime temperature around 16 °C. An increase over 30 °C may have an impact on the background MN frequencies [16].

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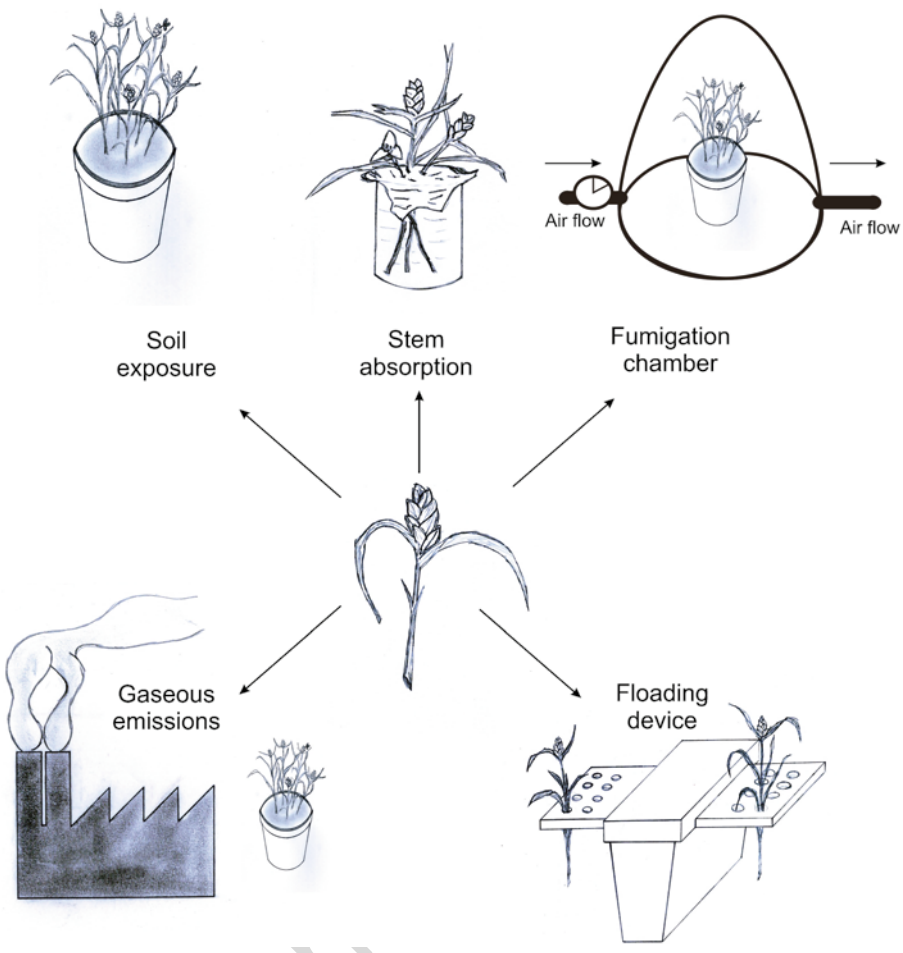

Fig. 2 Different forms of in situ experiments and design of laboratory experiments

3. Propagation of the plants: After 8–12 weeks of cultivation when the pots are full, the plants should be divided and plant rhizomes with leaves are transferred to new pots (*see* **Note 3**).

3.2 Exposure to Chemicals and Complex Mixtures

At least 15 cuttings per experimental group with immature inflorescences (not with blooming flowers; refer to Fig. 1) should be treated. Various types of exposures to the plant are depicted in Fig. 2.

1. Treatments of the plants in the laboratory: In stem absorption experiments, expose the plant cuttings to liquid solution in 250 mL beakers, which are covered with aluminum foil.
2. Testing of pure solid or liquid chemicals: The "classical" approach is stem absorption experiment, in which the test compounds are diluted with tap water or an organic solvent (1 % DMSO, maximum final concentration).
3. Testing of soils contaminated with heavy metals: Expose the roots of whole plants in the soils.

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4. Testing of gases: Expose the plant cuttings in water to the gaseous agent in fumigation chambers.
5. Testing of contaminated air: It is recommended to expose the plants, at different distances from the source of the emissions, in the direction of the wind.
6. Treatments of the plants in situ: Expose the plants as cuttings or as intact plants growing in pots.
7. Testing of surface waters: Use floating devices (perforated swimming plastic plates) with plants (Fig. 2).

3.3 Exposure Time and Recovery Time

1. Exposure time depends on the type of experiment performed.
2. For pure substances (e.g., aqueous solution of herbicides, insecticides, industrial chemicals) in the laboratory, high concentrations can be included and it is suggested to use an exposure time between 3 and 12 h followed by a 24-h recovery period (*see* **Note 4**).
3. For complex mixtures (e.g., polluted water, air, and soil) a continuous exposure up to 30 h is recommended with no recovery periods, since the complex mixtures often contain only low concentrations of mutagens (*see* **Note 5**).

3.4 Fixation

1. Prepare the fixative liquid *ex tempore* (*see* **Note 6**).
2. Fix the inflorescences in 3:1 ratio (v/v) of pure ethanol and acetic acid for 24 h.
3. Subsequently store the fixed tissue in 70 % ethanol (we recommend the use of scintillation tubes; all inflorescences of a single treatment dose can be stored in the same tube in an appropriate volume of fixative, i.e., material should be submerged completely in the liquid).

3.5 Slide Preparation

The different steps are shown in Fig. 3.

1. Only an early tetrad stage is suitable for scoring of MN.
2. First examine one bud of an inflorescence with one to two drops of 1–2 % acetocarmine stain for 5–8 min to see if an early tetrad stage is found.
3. If so, remove the debris using a botanical needle and subsequently put a 24 × 24 mm coverslip.
4. Slides should be evaluated under a light microscope with 400-fold magnification.
5. Heat slides over a flame for 2–4 s and gently press the coverslips after this treatment so that older tetrads burst and only early ones remain intact.

3.6 Scoring

1. For each experiment, examine slides from at least five inflorescences, which are precursors of buds in the early tetrad stage.

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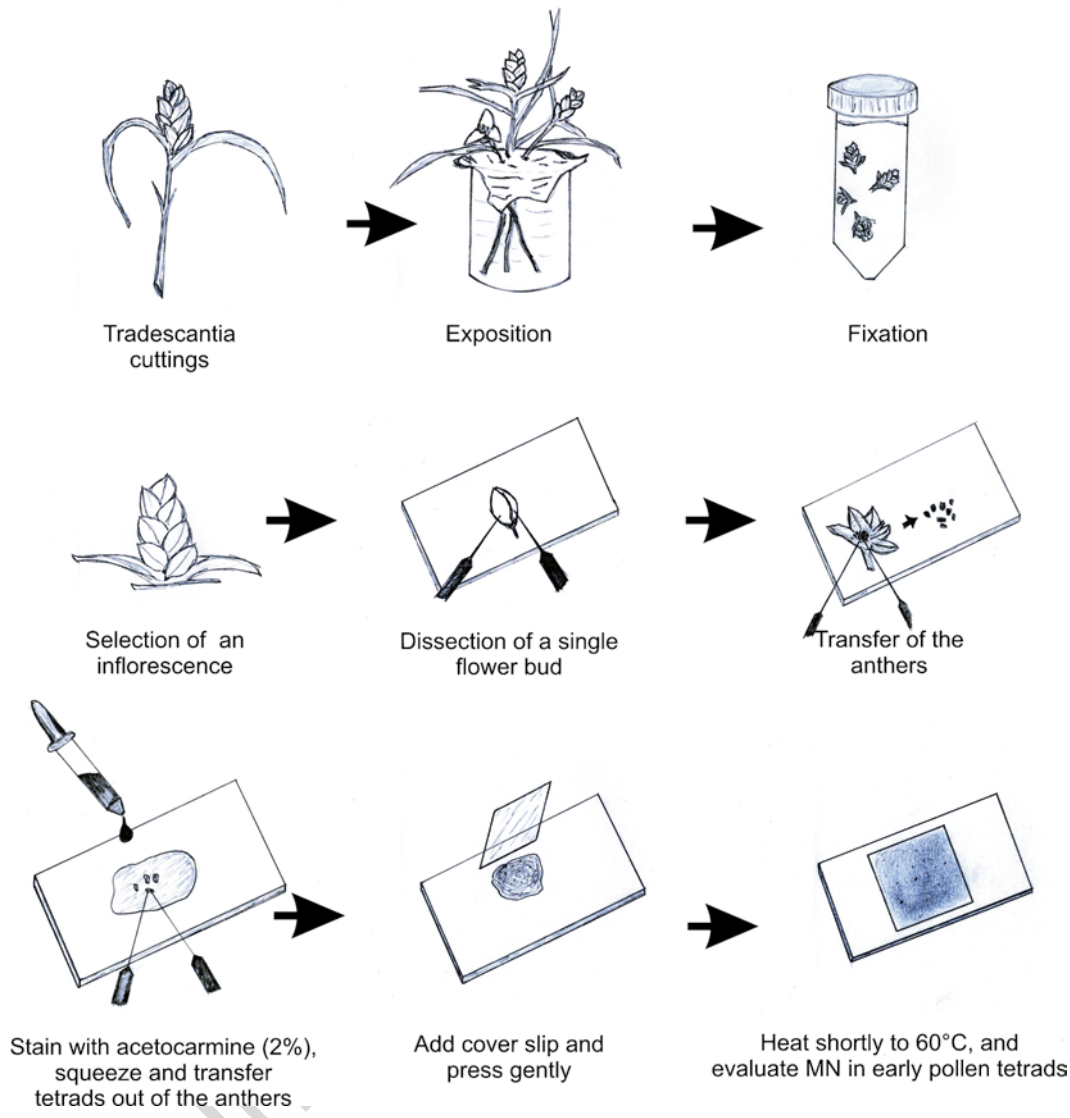

Fig. 3 Different steps of the slide preparation

From each slide evaluate at least 300 tetrads (300 tetrads per slide).

2. Enter the results of the scoring in scoring sheets (to include the overall number of tetrads evaluated, tetrads with one MN, two MN, and three MN).
3. Furthermore, record other anomalies (*see* Fig. 4, *see* **Note 7**).
4. To avoid misinterpretations due to division delays, the synchronicity of the cells should also be taken into consideration. Under normal conditions, tetrads within one bud are highly synchronized, but asynchrony (presence of diads,

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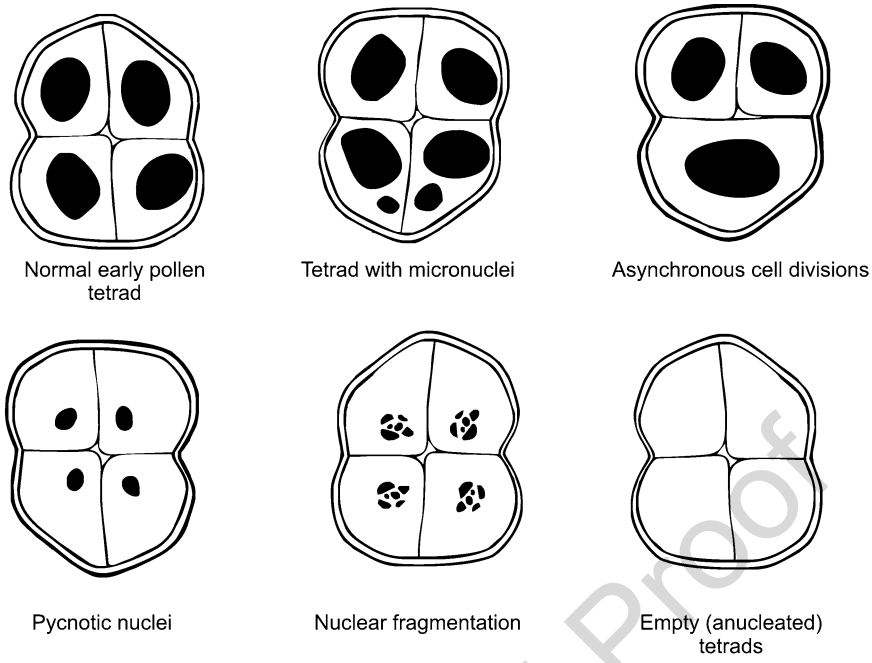

Fig. 4 Schematic representation of cells in different stages and with different nuclear anomalies

tetrads, and "triads") may be caused by chemical compounds and complex mixtures.

5. Scorers should also check the number of MN in diads, which may be indicative of retarded cell division (*see* **Note 7**).

3.7 Statistical Evaluation

Use one-way ANOVA with Dunnett's posttest for statistics in Trad MN assay, but other statistical methods can also be applied.

4 Notes

1. We do not recommend the use of *T. pallida* for several reasons: (a) it has never been validated with a sufficiently large number of model mutagens, (b) it is not known if it is as sensitive as the sterile clone #4430, and (c) as the plants are usually collected in the field it is unclear if specimens from different sites differ in their sensitivity towards genotoxins as a consequence of adaptive responses. Such an effect can be excluded when genetically stable sterile hybrids are used.
2. If pesticides or herbicides are used for hydroponic culture, we strongly recommend change of the nutrient solutions after their application. Parasite infections with *Parthenolecanium* species and aphids should be treated with paraffin-based sprays and emulsions. We recommend avoiding synthetic pesticides, which may increase the background MN frequencies.

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3. In order to conduct continuous experiments with one test compound per week, with three to four doses and positive and negative controls, 75–150 inflorescences are required which are usually obtained with 120–140 pots.
4. The exposure times and/or the recovery periods have to be sufficiently long to ensure that the cells undergo division which is a prerequisite for MN formation. Some types of exposure may cause inhibition of the cell division; in this case the majority of MN is found in the diad stage and a repetition of the experiment with extended exposure time is mandatory.
5. Negative and positive controls: Tap water can be used to set up the controls but it has to be tested before the main experiments, to ensure that it does not increase the mutation frequencies. As an alternative, Hoagland's nutrient solution [17] can be used. It is essential to test from each test compound at least three to four concentrations and to include negative (solvent) and positive controls. The background MN frequencies in #4430 vary over a broad range (i.e., between 0.6 and 5.0 MN/100 tetrads). For laboratory experiments, suitable positive controls are directly acting mutagens such as EMS, heavy metals compounds (e.g., As_2O_3), MH, and X-rays or γ-radiation [14, 15]. Results with positive controls according to our knowledge are as follows:

   0.2 mM MH (30-h exposure): 5–12.0 MN/100 tetrads

   2.5 mM As_2O_3 (6-h exposure, 24-h recovery): 5–10.0 MN/100 tetrads

   In environmental monitoring studies, dose–effect relationship should be monitored by collection of samples with different pollution levels or in the case of water or air experiments by exposing plants at different distances from the source of the pollution. The reproducibility of the results should be confirmed in follow-up experiments.
6. Fixed samples can be stored at room temperature up to several years before evaluation.
7. For the scoring of MN it is necessary to identify buds, which contain early-tetrad-stage cells in the inflorescence, by use of botanical needles. The procedure is shown in Fig. 3. When a bud is too old, no tetrads are found and younger, smaller buds have to be opened. The pollen cells, which are contained in the buds, represent different stages of meiosis. Buds with early stages are, in general, smaller and located in lower (proximal) parts of the inflorescence. Figure 5 depicts the different stages of microsporogenesis in *Tradescantia*. MN are formed when pollen mother cells are exposed to DNA-damaging compounds and undergo the first and second meiotic division. In order to form MN, the cells have to undergo one to two cell divisions.

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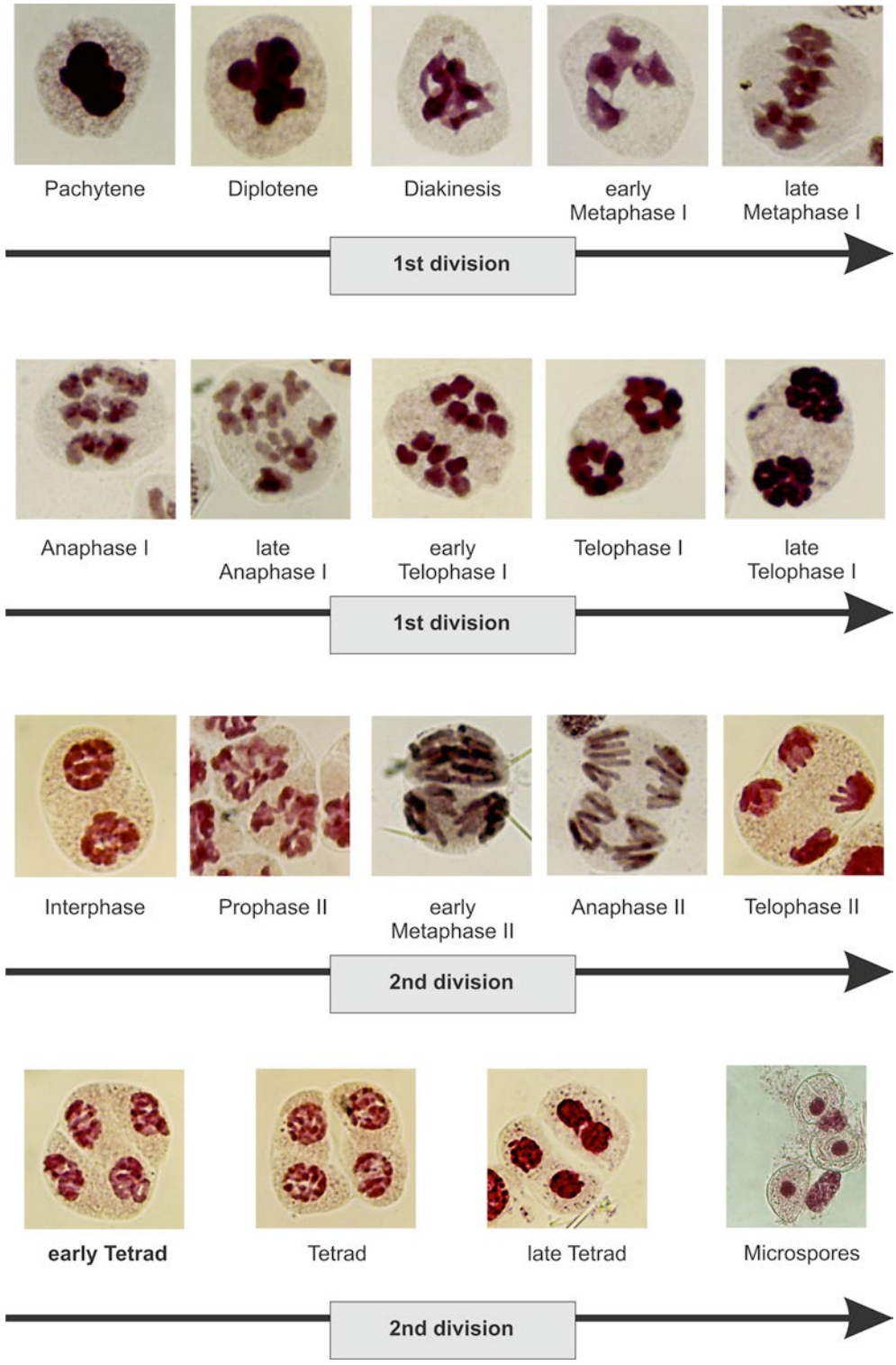

Fig. 5 Different stages of microsporogenesis in *Tradescantia*

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Therefore, the precise timing of the experiments is essential to ensure optimal sensitivity. The endpoints of the assay are MN, which are defined as extranuclear bodies containing DNA and a nuclear membrane. The criteria for the identification of MN are the same as described by Tolbert et al. [18] for the evaluation of mammalian cells, as given below:

(a) Rounded smooth perimeter suggestive of a membrane.
(b) Less than one-third of the diameter of associated nucleus, but large enough to discern shape and color.
(c) Similar staining intensity as the nucleus.
(d) Texture similar to nucleus.
(e) Same focal plane as nucleus.
(f) Absence of overlap with, or bridge to, nucleus.

Quite often, other types of nuclear aberrations can also be found in the experiments as a consequence of toxic exposure. Sometimes three cells instead of a tetrad are seen, which are indicative of meiotic disturbances; atypical nuclear aberrations are pyknotic cells with condensed (dead) nuclei, and sometimes anuclear cells as well as nuclear fragmentations are found (*see* Fig. 4).

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Acknowledgement

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Uncorrected Proof

Curriculum vitae

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Education

January 2012 – August 2013	Diploma thesis in the working group of Prof. Knasmüller at the Institute of Cancer Research in Vienna
September 2006 – 2013	University of Vienna – Studies of Ecology
September 2005 – Mai 2006	Military service – paramedic education
September 2000 – July2005	Tourism school in Bludenz
September 1995 – July 2000	Grammar school in Bludenz
September 1991 – July 1995	Primary school in Tschagguns

Languages

Mothertongue	German
Advanced level	English
School level	French

Work experience

July 2001 - September 2001	Training placement in the “Posthotel Rössle” in Gaschurn (kitchen)
June 2002 – September 2002	Training placement in the “Posthotel Rössle” in Gaschurn (service)
July 2003 – September 2003	Training placement in the “Posthotel Rössle” in Gaschurn (service)
July 2004 – September 2004	Waiter - Chef de rang - in the “Posthotel Rössle” in Gaschurn
September 2005 – Mai 2006	Military service
2000-2008	Skiing instructor at the “Ski-Region Golm”

October 2008-...	Zoom Kindermuseum – Exhibitionhall Team spokesman since Oct. 2010
July 2009	Training placement OMV Burghausen
August 2009	Seaturtles Project Yanniklar, Turkey
January 2012-…	Cancer Research Institute, Vienna

Publications

2013	Micronucleus Assay with Tetrad Cells of Tradescantia. Genotoxicity Assessment: Methods and Protocols, Methods in Molecular Biology, vol. 1044, DOI 10.1007/978-1-62703-529-3_22, © Springer Science+Business Media, New York 2013

Conferences

16.-18. Oct. 2012	The effects of residues of cytostatics and other pharmaceuticals on non-target organisms – 1st CytoThreat workshop Napoli/Italy

Vienna, 30.Aug. 2013 Clemens Pichler