NARDI FUNDULEA, ROMANIA
www.incda-fundulea.ro

ROMANIAN AGRICULTURAL RESEARCH, NO. 28, 2011
Print ISSN 1222-4227; Online ISSN 2067-5720

CYTOGENETIC EFFECTS INDUCED BY 2,4-D AND KINETIN IN RADISH AND COMMON BEAN ROOT MERISTEMS

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ABSTRACT

The wide utilization of plant growth regulators requires the knowledge of possible unwanted effects induced by uncontrolled administration of these chemicals, inclusively at genetic level. The cytogenetic effects induced by two plant growth regulators, an auxin (2,4-D) and a cytokinin (kinetin), in root meristems of plants belonging to two species of economic importance - *Raphanus sativus* L. and *Phaseolus vulgaris* L. - were studied. Mitotic index, rate and categories of ana-telophase chromosome aberrations, as well as the frequency and types of metaphase disturbances were comparatively analyzed after seed exposure to two concentrations (1 mg/L and 10 mg/L) for each plant hormone. 2,4-D had a slight cytotoxic effect in radish, but did not show genotoxic potential, whereas kinetin stimulated cell division. In common bean, the small concentrations of 2,4-D and kinetin enhanced mitosis, but the maximum tested concentrations had inhibitive effect on cell division. Generally, the rate of ana-telophase chromosome aberrations induced by 2,4-D and kinetin, at the tested concentrations and at the respective exposure, was not significant. Auxin caused increase of total abnormal metaphases (metaphases with expulsed chromosomes and colchicine-like metaphases) in both species, indifferently of concentration, whereas all the variants exposed to kinetin action showed values lower than control, in both species. Therefore, 2,4-D have aneugenic potential by acting as spindle poison and by disturbing the correct separation of the chromosomes to cell poles. The most complex pattern of ana-telophase and metaphase modifications was present in variant of common bean exposed to the action of 10 mg/L 2,4-D.

Key words: aneugenic effects, genotoxic potential, plant growth regulators, 2,4-D, kinetin, *Phaseolus vulgaris* L., *Raphanus sativus* L.

INTRODUCTION

Phytohormones have essentials roles in the regulation of plant physiological processes. Use in excess of some plant growth regulators and fertilizers in order to obtain higher yields of crops can cause genotoxic effects on living organisms (Ateeq et al., 2002), because they can accumulate in the food to a toxic level and consequently affect the animal and human health. Plant growth regulators are widely used in horticulture, floriculture, *in vitro* tissue and cell cultures, in control of growth of dwarf plant cultures etc. This large utilization requires the knowledge of possible unwanted effects induced by uncontrolled administration of growth regulators, inclusively at genetic level.

Several examples from literature confirm the effects of some plant growth regulators on genetic material in plants and animals. In some species, like *Nicotiana tabacum*, *Lens caulinaris*, *Helianthus tuberosus*, *Cucumis sativus,* growth regulators determined modification of labelled timidine incorporation in DNA, with the alteration of nucleic acid synthesis. Also, the addition of phytohormones in *Cymbidium* tissue cultures induced DNA modifications. Thus, auxins determined increases of A + T rich fraction, gibberellic acid increased G + C fraction, while the cytokinins had the lowest effect (Nagl and Rücker, 1976). In modern agricultural technology, 2,4-D is used in high concentrations as a herbicide to control weeds, but in low concentrations it is used as a growth regulator.

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Received 21 January 2011; accepted 20 June 2011

There are several reports on the genotoxicity of 2,4-D (Kumari and Vaidyanath, 1989). The cytotoxic and mutagenic effects of 2,4-D synthetic auxin were observed both in animals (on hamster fibroblasts, for example) and in root apical meristems (Pavlica et al., 1991), where it determined modifications of cell cycle and mitosis, changes in chromosome and chromatin structure, especially at concentrations higher than 5 µg/ml. At small doses, 2,4-D behaves like a systemic herbicide, having carcinogenetic, mutagenic, clastogen or neurotoxic effects. Mustonen et al. (1986) evidenced a significant increase of aberrations in *in vitro* human lymphocytes subjected to 2,4-D (0.125 – 1.250 mM). Also, it has been suggested that 2,4-D and other phenoxy herbicides may cause Non-Hodgkin's Lymphoma (NHL) and other cancers (Holland et al., 2002). Kinetin is a cytokinin that positively influences numerous aspects of plant growth, development and physiology, but which can also induce cytogenetic disturbances (Kallak and Vapper, 1985).

Phaseolus vulgaris L. (*Leguminosae)* (2n=22) is a highly polymorphic species, an annual plant with special economical features. Beans produce a high nutritive, relatively low-cost protein food. They are an important and inexpensive source of protein, dietary fibre and starch for a large part of the world's population, mainly in developing countries. For example, in Mexico, Brazil, Burundi, Rwanda, Kenya, Tanzania, beans are the primary source of protein in human diets. This plant contains important levels of protein, lipids, carbohydrates, minerals (Ca, P, Fe, and Zn), vitamins (thiamine, riboflavin, nicotinic acid, ascorbic acid), crude fibre (5-7 g/100 g, depending on bean variety) etc. Common bean represents one of the best non-meat sources of iron, providing 23-30% of daily recommended levels of this element from a single serving (Shimelis and Rakshit, 2005). The medicinal uses of beans are multiple: in bronchitis, as adjuvant in diabetes or as kidney antiseptic, in the treatment of acne, eczema, burns, cardiac troubles, rheumatism, arthritis and sciatica, because of the emollient, carminative and depurative properties of this plant. Recently, its use has focused on its ability to block starch absorption.

Raphanus sativus L. (*Cruciferae*) (2n=18) is an herbaceous vegetable, cultivated especially for its edible roots which in fresh state are used in food. Root contains 5-10% dry matter, 0.8-4.0% sugars, 0.8-1.3% proteins, amino acids, vitamins C, B_1, carotene, essential oils, and glycosides giving specific pungency**.** Raphanin is a radish component with antibacterial and antifungal properties. The leaves, seeds and old roots are used in the treatment of asthma, while the juice of the fresh leaves is diuretic and laxative. The seeds are carminative, diuretic, expectorant, laxative and stomachic, and the root has antiscorbutic, antispasmodic, astringent, cholagogue, digestive and diuretic effects. If crushed can be used as a poultice for burns, bruises and smelly feet.

Due to the great economic value of common bean and cultivated radish, it is necessary to deeply know the consequences of in excess utilization of some growth regulators, fertilizers or hormone-containing herbicides on genetic apparatus and on phenotype expression. For this reason, the objective of the present study was to analyze the amplitude of effects induced by 2,4-D and kinetin on cell division and nuclear chromosome material, by evaluation of main cytogenetic parameters (mitotic index, rate of ana-telophase aberrations, incidence of metaphase disturbances) in order to detect the possible harmful effects of respective chemicals.

MATERIAL AND METHODS

Seeds of commercial varieties of cultivated radish (*Raphanus sativus* L.) and common bean (*Phaseolus vulgaris* L.) were immersed for 3 h in solutions of 2,4-D (2,4-dichlorophenoxy acetic acid) (MW = 221.04 g/mol, Merck) and kinetin (6-furfuryl-aminopurine) (MW = 215.2 g/mol, SIGMA). Two concentrations were used for both tested chemicals: 1 mg/L and 10 mg/L. Controls were set up by seed immersion in distilled water.

Seed germination took place in Petri dishes, on moist filter paper, in dark. The fixa-

tion of root tips (10-15 mm in length) was done for about 24 hours in ethylic alcohol/acetic acid, 3:1, at room temperature. After 10 min of hydrolysis in 50% HCl, the plant material was stained in modified charbol fuchsin solution. For each variant, five slides (n = 5) were prepared according squash method, in 45% glacial acetic acid, and 10 microscopic fields were microscopically analyzed on every slide. A Nikon Eclipse 600 light microscope was used for this analysis. Photos were taken with a Nikon Cool Pix 950 digital camera (Nikon Eclipse 600 microscope) at 1600 x 1200 dpi resolution. The different phases of mitosis were counted to calculate the mitotic index (MI) and phase indices, as following:

Mitotic index = TDC x100/TC; PI% = prophase cells x 100/TDC; MeI% = metaphase cells x 100/TDC; AI% = anaphase cells x 100/TDC; TC% = telophase cells x 100/TDC, where TC = total cells (dividing and non-dividing), and TDC = total dividing cells. The rates of ana-telophase aberrations (A-T_{abr}%) and of metaphase abnormalities (M_{abn}%) were also calculated: A-T_{CA}% = A-T_{CA} x 100/TDC; M_{abn} % = M_{abn} x 100/TDC.

Statistical analysis

The data were expressed as mean ± standard error of the means for all groups of investigated parameters. To calculate and to graphically represent the statistical parameters, the Microsoft Office Excel 2003 software of Windows XP operating system was used.

RESULTS AND DISCUSSION

Mitotic index (MI) and incidence of mitotic phases

Many authors reported on the cytogenetic effects of 2,4-D that induced chromosome abnormalities into the meiosis or mitosis of *Vicia faba, Hordeum vulgare*, *Triticum aestivum* (Gul et al., 2006).

The individuals belonging to the two species displayed different profiles of the mitotic index. In *R. sativus*, auxin decreased mitotic index at both tested concentrations, comparatively to control mean (5.00 ± 0.69 %), while kinetin had a stimulant effect on cell division, for both concentrations, but in a more significant manner at 1.0 mg/L, where MI was with 33.0% higher than control (Table 1, Figure 1).

In *Ph. vulgaris*, the small concentrations of 2,4-D and kinetin (1 mg/L) exerted positive effects on cell division (MI average value was with 12.5%, respectively 16,2% higher than control). The 10 mg/L concentration proved to be inhibitive both for 2,4-D and cytokinin (with 35%, for auxin, and 23%, for kinetin, comparatively to control) (Table 2, Figure 1).

Table 1. Effects induced by 2,4-D and kinetin on cell division in root meristems of radish, depending on hormone concentration

Variant	Analyzed cells*	MI (%)*	PI%*	MeI%*	AI%*	TI%*
Control	1115.0±182.80	5.00±0.69	50.09±3.89	24.15±3.30	14.71±1.64	11.02±2.18
1 mg/L 2,4-D	1449.8±264.16	4.08±0.60	41.57±4.72	33.01±5.01	12.36±1.69	13.04±2,44
10 mg/L 2,4-D	1007.2±87.75	4.59±0.51	46.90±2.70	21.55±0.58	16.97±2.71	14.55±2.00
1 mg/L kinetin	1273.8±155.28	6.67±0.78	59.03±2.58	16.82±3.08	13.25±0.42	10.87±1.92
10 mg/L kinetin	1040.4±126.56	5.95±1.66	49.26±3.57	20.38±2.80	14.20±2.53	16.13±4.58

*mean ± standard error of the means; MI = mitotic index;
PI% = prophase index; MeI% = metaphase index;
AI% = anaphase index; TI% = telophase index.

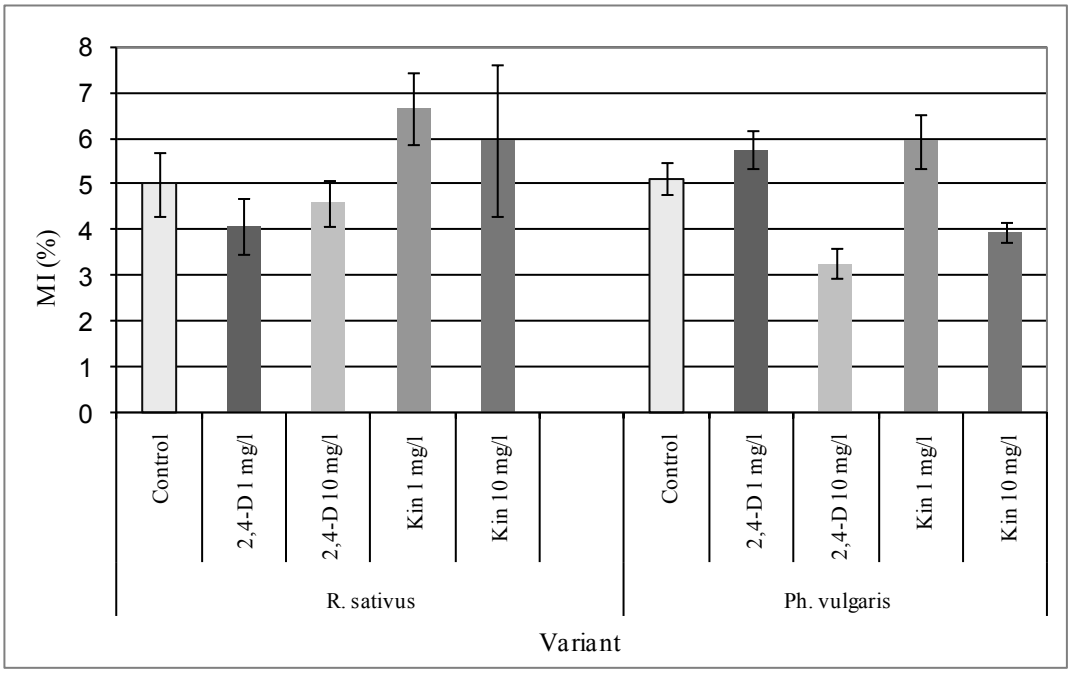


Figure 1. Mitotic index in *Raphanus sativu*s and *Phaseolus vulgaris*, after treatment with 2,4-D and kinetin (bars represent standard error of the mean)

Table 2. Effects induced by 2,4-D and kinetin on cell division in root meristems of common bean, depending on hormone concentration

Variant	Analyzed cells*	MI (%)*	PI%*	MeI%*	AI%*	TI%*
Control	1894.0±192.12	5.11±0.35	42.03±3.24	27.39±2.64	19.63±1.8	10.92±1.32
1 mg/L 2,4-D	1650.2±67.21	5.75±0.42	36.91±3.11	30.47±2.16	22.03±1.78	10.56±2.63
10 mg/L 2,4-D	2288.6±191.50	3.26±0.34	36.07±1.64	34.57±1.36	20.03±0.81	9.31±1.73
1 mg/L kinetin	2131.6±202.00	5.94±0.60	46.02±1.25	21.65±1.70	18.89±1.70	13.43±2.00
10 mg/L kinetin	2171.2±169.36	3.95±0.21	40.69±2.34	25.62±1.71	21.64±2.84	12,40±1.59

*mean ± standard error of the means; MI = mitotic index;
PI% = prophase index; MeI% = metaphase index;
AI% = anaphase index; TI% = telophase index.

Mitotic index is not only a parameter of intensity of cell division, but also an indicator of cytotoxicity. According to Marcano et al. (2004), cytotoxicity is defined as a decrease in mitotic index and as an increase in the fraction of cells with c-mitosis, multi-polar anaphase, sticky chromosomes and laggards. The decline of the mitotic activity, evidenced in above-mentioned variants from radish and common bean, could be due to the inhibition of DNA synthesis. On the other hand, the inhibition of certain specific proteins of cell cycle remains as a possible target site of 2,4-D-containing herbicides. Inhibition of DNA-polymerase, necessary for the synthesis of DNA precursors, as well as of other enzymes more directly involved with spindle assembly or orientation, could explain the mitodepressive effect (Hidalgo et al., 1989).

Kinetin is known as a strongly stimulating factor of mitotic division in plant and animal cells. Olszewska (1958) showed that kinetin induces an increase of number of dividing cells in *Allium cepa* L., after 5 h action. Mitotic stimulation and

the increase of the total nitrogen content suggest an augmentation of the protein synthesis in kinetin-treated individuals and, thus, the stimulation of turnover or even of nucleic acid synthesis.

Concerning the frequency of mitotic phases (Tables 1 and 2; Figure 2), the decreasing order generally was the following: prophases>metaphases>anaphases>telophase; only in radish, the variants exposed to 2,4-D – 1 mg/L and kinetin – 10 mg/L showed a slightly increased value of telophases comparatively to that of anaphases. Prophases surpass control mean in 1 mg/L treated variant, both in radish and common bean, and the metaphases are in higher percent in 1 mg/L 2,4-D, in radish, and also in bean, at both auxin concentrations.

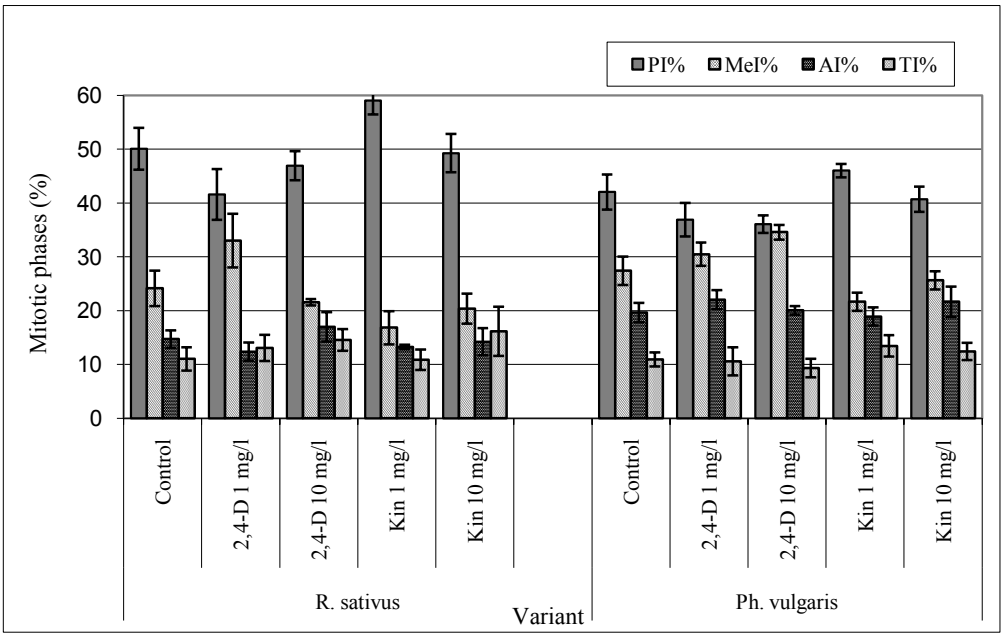


Figure 2. Incidence of mitotic stages in radish and common bean root tip meristems, after treatment with 2,4-D and kinetin

Ana-telophase chromosome aberrations

It is known that, depending on concentration, 2,4-D can be used as a growth-promoting auxin or as an effective herbicide. The reports on the cytogenetic effects of 2,4-D are rather contradictory. For example, according to Singh and Harvey (1975), 2,4-D does not induce mitotic irregularities in plant tissue cultures, but the investigations of Bayliss (1977) revealed an opposite tendency: the higher doses of 2,4-D (>5 mg L^{-1}) induced chromosome damage in root cells of plants and the impairment of spindle function. For example, the main abnormalities induced by the herbicide Avenoxan (active substance is 2,4-D) in *Allium cepa* and *Allium sativum* were c-mitoses, chromosome stickiness, bridges, laggards, multipolar cells. The inhibition of the mitotic index was dependent on the concentration and time of treatment (Gul et al., 2006). The increase in kinetin concentration above the growth promoting level did not only produce growth inhibition, but also significant cytogenetic changes in treated callus cells. At a concentration of 1.8×10^{-4} M, kinetin induced nearly three times more aberrant ana- and telophases, compared with 2,4-D (Kallak and Vapper, 1985).

In this study, bridges, expelled chromosomes, and laggards were the most encountered aberration types in mitotic ana-telophases (Table 3, Figure 5). Since bean and radish chromosomes are small, the structural variations of chromosomes are difficult to characterize. Laggards may be attributed on

the failure of spindle apparatus to organize in a normal way. They lead to genetic disequilibriums between daughter cells. Multipolar anaphases indicate the inhibition of cytokinesis. Multipolar segregation can also result in aneuploidy, whereas the bridges are the consequence of the fusion of two chromosomes, being visible between the two chromatidic groups separated to the cell poles.

Table 3. Effects induced by 2,4-D and kinetin on number and types of ana-telophase and metaphase disturbances, in radish and common bean root tip meristems, depending on concentration

Species	variant	Ana-telophase aberrations						Metaphase disturbances		
		$A\text{-}T_{CA}$ %	type (%)					M_{abn} %	type (%)	
			B	exp	lag	C	M		exp	C-like
R. sativus	Control	4.27	1.78	0.71	1.06	0.35	0.35	6.04	4.26	1.06
	1 mg/L 2,4-D	4.74	3.16	0.00	0.32	0.95	0.32	9.49	6.64	2.84
	10 mg/L 2,4-D	4.70	1.70	1.28	1.28	0.42	0.00	8.11	4.27	3.84
	1 mg/L kin	4.80	1.92	1.20	0.48	0.96	0.24	2.64	1.44	1.20
	10 mg/L kin	7.03	1.85	3.33	1.48	0.37	0.00	4.81	1.85	2.96
P. vulgaris	Control	7.38	2.95	1.47	1.05	0.82	0.41	8.22	5.90	2.32
	1 mg/L 2,4-D	6.78	2.75	1.43	0.85	1.27	0.21	8.89	2.97	5.92
	10 mg/L 2,4-D	9.43	4.31	2,43	1.61	0.00	0.81	10.78	4.04	6.73
	1 mg/L kin	4.91	2.78	0.33	0.82	0.65	0.33	6.39	3.77	2.13
	10 mg/L kin	6.35	3.06	1.41	1.41	0.23	0.23	3.52	2.82	0.70

$A\text{-}T_{CA}$% = rate of ana-telophase aberrations; B = bridges; exp = expelled chromosomes; lag = lagging chromosomes; C = complex aberrations; M = multipolar segregation; M_{abn} % = rate of metaphase disturbances; C-like = colchicine-like metaphases.

In *R. sativus*, a significant increment of total number of ana-telophase aberrations (64% more aberrations than in control) was present only in 10 mg/L kinetin-treated variant; low increases were registered in variants subjected to auxin action (approximately 10%) (Figure 3a). In *Ph. vulgaris*, except 10 mg/L 2,4-D variant, all the others had smaller levels of ana-telophase aberrations than control average (Figure 3b).

In radish, in 1 mg/L 2,4-D variant, 66.6% from total aberrant ana-telophases were represented by bridges, whereas in 10 mg/L kinetin-treated variant the expelled chromosomes, bridges and laggards surpassed the control average. Complex aberrations were noted in variants exposed to maximum tested concentration of auxin and cytokinin.

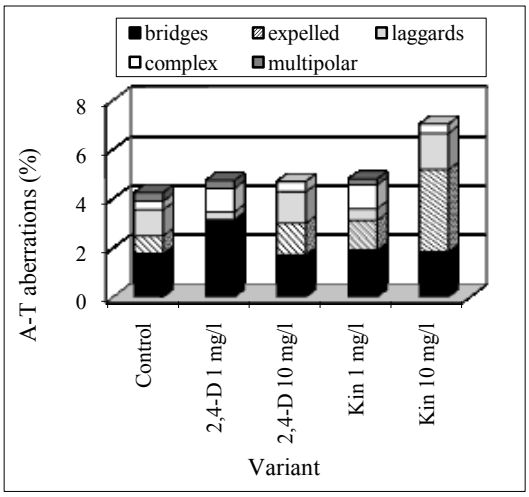
In common bean, 2,4-D 10 mg/l variant had the highest $A\text{-}T_{CA}$%, and all the aberration categories exceeded in this case the control average. This variant also showed the most numerous metaphase disturbances, and the lowest mitotic index.

It is plausible that the genotoxic and cytotoxic effects induced by 2,4-D are due to its action on nucleic acids (Mohandas and Grant, 1972) or to its capacity to combine with proteins to form chemical complexes (Butts and Fang, 1956, cited by Kumari and Vaidyanath, 1989).

Out of the above-mentioned aberrations, in common bean, numerical modifications (cells with tetraploidy state) were also evidenced in 2,4-D 1 mg/L and kinetin 10 mg/L treated variants, and ana-telophases with polar deviations in auxin-treated variants. Polar deviations, consisting in orientation fault of equatorial plate or transversally orientation of anaphase and telophase

configurations can be attributed to the hormone action on mitotic spindle components. Polyploids are probably formed because of respective growth regulator impairment in the regular disjunction of chromosomes due to its conjugation to spindle proteins (Tomkins and Grant, 1976). The maximum concentration of kinetin was also correlated with the presence of numerous like-apoptotic cells having large vacuoles and peripheral positioned nuclei. The apoptotic cells prove the cytotoxic effect of respective hormone concentration.

a) *Raphanus sativus*



b) *Phaseolus vulgaris*

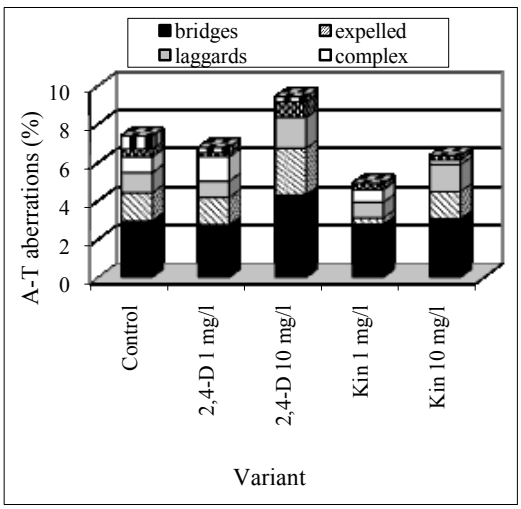


Figure 3. Rate of ana-telophase chromosome aberrations in radish (a) and common beans (b) root tip meristems, after 2,4-D and kinetin treatment and contribution of each category to total of aberrant ana-telophases

Metaphase disturbances

Seed treatments with the two growth regulators influenced the chromosome behaviour in mitotic metaphases (Table 3). The main metaphase disturbances found in root tip meristems of radish and common bean were C-like metaphases, result of the spindle inactivation and formation of chromosome configurations similar to those induced by colchicine, and metaphases with chromosomes expulsed from equatorial plate (Figure 5). Auxin caused increase of total aberrant metaphases in both species, indifferently of concentration, whereas all variants exposed to kinetin action showed values lower than control, in both species. Comparatively to control, the most numerous anomalous metaphases were present in radish root meristems after treatment with 2,4-D – 1 mg/L (57% more aberrant metaphases), followed by variants treated with 2,4-D – 10 mg/L, both in radish and common bean (approximately 30% over control) (Figures 4a, 4b).

In most cases, the metaphases with expulsed chromosomes constituted the major part in total aberrant metaphases; only in radish 2,4-D – 1 mg/L variant, and in common bean in both variants treated with auxin, C-metaphases were the predominant type of abnormalities. Comparatively to control, in radish all variants exceed the level of C-metaphases, the most important increases being registered in variants treated with 2,4-D – 10 mg/L (3.6 times more C-metaphases than in control) and 1 mg/L 2,4-D, respectively 10 mg/L kinetin (C-metaphase number was 2.7 times higher than control).

In *Ph. vulgaris*, only 2,4-D auxin induced increases of C-metaphases number, comparatively to control (2.5, respectively 2.9 times). Large number of c-mitoses indicates that the respective growth regulator acts as potent spindle inhibitor due to which the chromosomes lie on the metaphase plate instead of moving towards their respective poles. In radish, the highest number of metaphases with expulsed chromosomes was present at 1 mg/L 2,4-D.

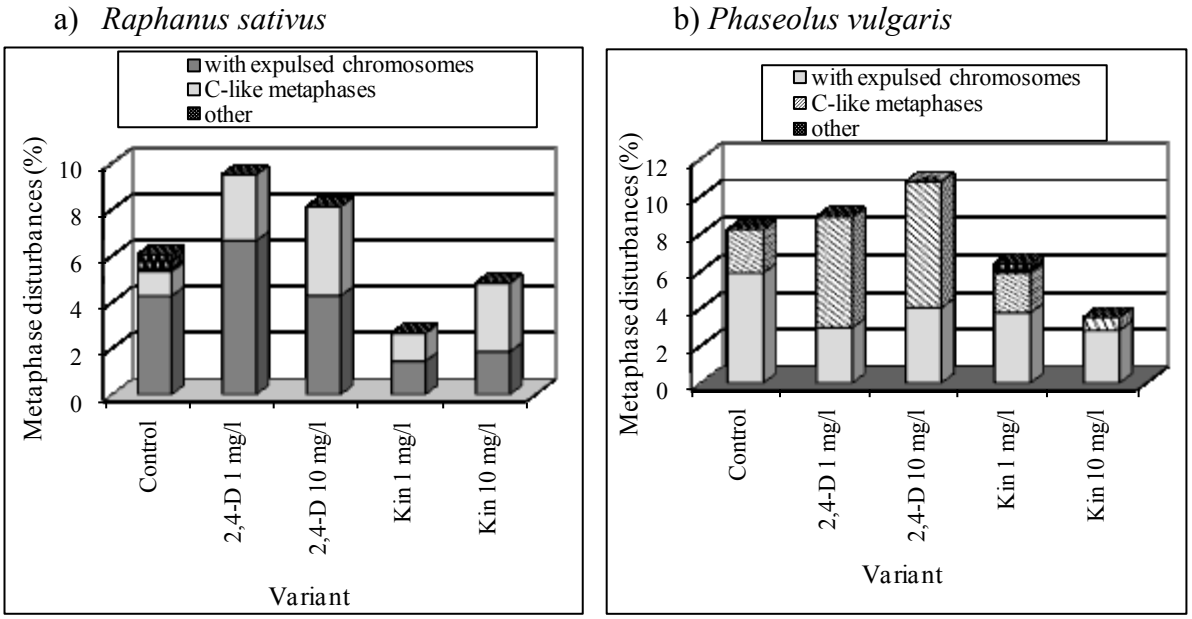
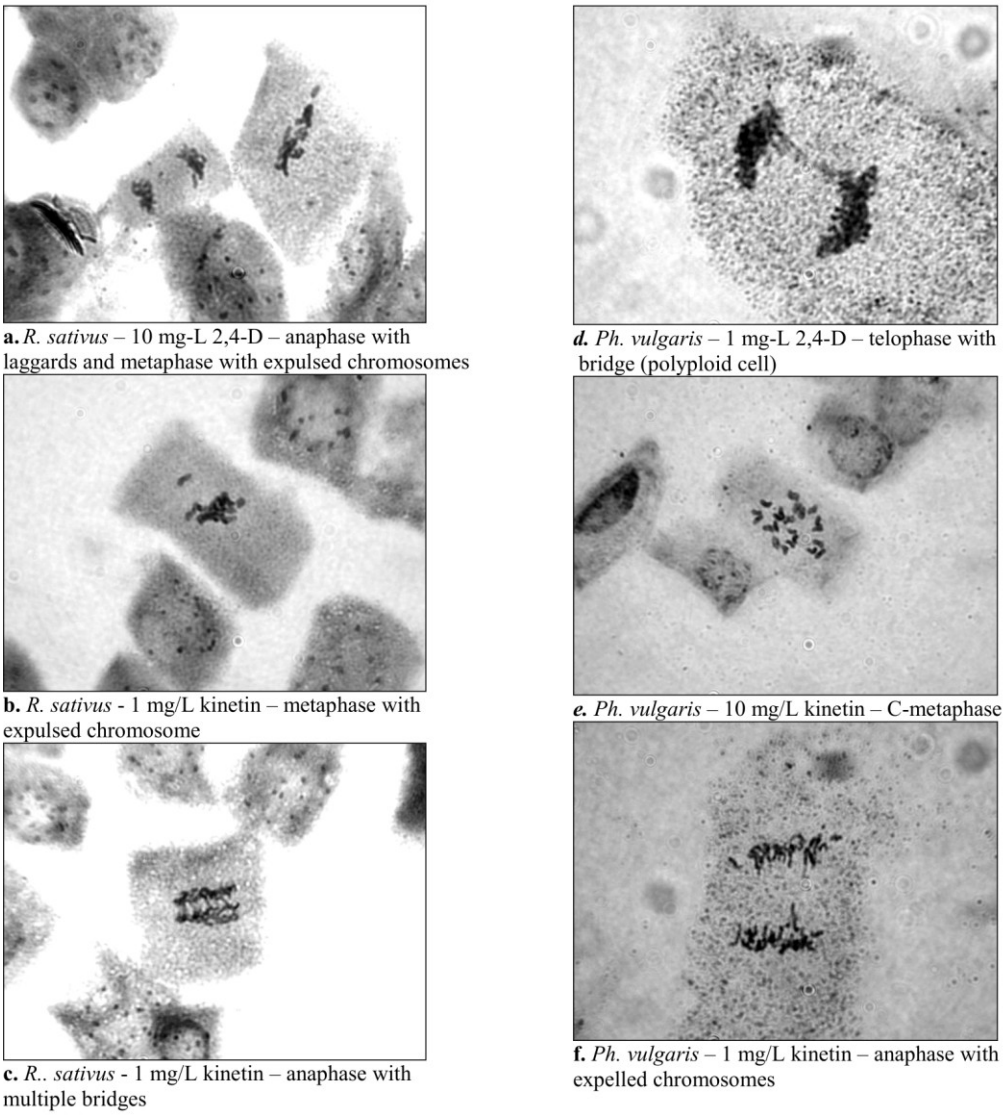


Figure 4. Rate of metaphase disturbances in radish (a) and common bean (b) root tip meristems, after 2,4-D and kinetin treatment and contribution of each category to total of abnormal metaphases



a. *R. sativus* – 10 mg-L 2,4-D – anaphase with laggards and metaphase with expulsed chromosomes

b. *R. sativus* - 1 mg/L kinetin – metaphase with expulsed chromosome

c. *R.. sativus* - 1 mg/L kinetin – anaphase with multiple bridges

d. *Ph. vulgaris* – 1 mg-L 2,4-D – telophase with bridge (polyploid cell)

e. *Ph. vulgaris* – 10 mg/L kinetin – C-metaphase

f. *Ph. vulgaris* – 1 mg/L kinetin – anaphase with expelled chromosomes

Figure 5. Ana-telophase aberrations and metaphase disturbances in *Raphanus sativus* (**a, b, c**) and *Phaseolus vulgaris* (**d, e, f**), induced by exposure to 2,4-D and kinetin

The results of this study are generally in accordance with the data published for other species concerning the stronger cytogenetic effects induced by 2,4-D and the lower action of kinetin at genetic level. The amplitude of the responses of the studied genotypes to the action of tested plant growth regulators was large enough to justify detailed studies concerning the genetic risks of their use – especially for 2,4-D.

CONCLUSIONS

2,4-D had a slight cytotoxic effect in radish (but was not genotoxic), whereas kinetin stimulated cell division. In common bean, the small concentrations of 2,4-D and kinetin enhanced mitosis, but the maximum tested concentrations had inhibitive effect. Generally, the rate of ana-telophase chromosome aberrations induced by 2,4-D and kinetin, to the tested concentrations and to the respective hormone exposure was not significant. Auxin caused increase of total abnormal metaphases in both species, indifferently of concentration, whereas all the variants exposed to kinetin action showed values lower than control, in both species. These results indicate that auxin shows aneugenic potential by acting as spindle poison and disturbing the correct separation of the chromosomes at cell poles. The metaphases with expulsed chromosomes and C-like metaphases constituted the major part of total aberrant metaphases. The most complex pattern of ana-telophase and metaphase modifications was present in common bean exposed to the action of 10 mg/L 2,4-D.

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