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The Investigation of the Cytotoxic Effects of Some Pesticides on Soybean (*Glycine max* L.)

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Summary In this study, the effects of the fungicide Pomarsol Forte WP 80, the insecticide Arrivo 25 EC and the herbicide The End EC were examined for seed germination percentages, the phases of mitosis and chromosomal abnormalities in soybean (*Glycine max* L.). Median EC (effective concentration) values were calculated according to relative reduction in root length (T/C%) after treatment for 72 h. Germination percentages of primary soybean roots decreased with increasing pesticide concentrations. Cytological observations showed that the mitotic frequency in root meristematic cells was decreased and that abnormality frequency was also decreased in parallel to the increase in concentrations for all pesticides, except for the 9.6 M concentration of Arrivo 25 EC. The obtained results indicate that these 3 pesticides had the ability to cause a reduction in the seed germination percentage in the number of different phases of mitosis and in the leaf pigment content of soybean (*G. max* L.).

Key words Soybean, Pesticide, Seed germination, Mitosis, Chromosomal abnormality.

Different pesticides and plant growth regulators are being used extensively in modern agriculture; although the use of these chemicals has become a necessity, their frequent and indiscriminate use has many undesirable consequences in culture plants (Aksoy *et al.* 2007). Pesticides from a broad range of classes are widely used in various combinations and at different stages of cultivation and during postharvest storage to protect crops against a range of pests and fungi, and/or to provide quality preservation. Pesticides can be carried to the final products, such as infant foods, even after food processing (Wang and Cheung 2006). The dithiocarbamate derivative thiram is currently in widespread use as a contact fungicide for the protection of vegetable crops and for mould control in a variety of fields (Villani *et al.* 1998). Cypermethrin is a synthetic pyrethroid used as an insecticide in large-scale commercial agricultural applications as well as in consumer products for domestic purposes. The major target sites of the cypermethrin are sodium channel and adenosine triphosphatase. In addition, it inhibits the monoamine oxidase and γ-aminobutyric acid receptor, causing excitability and convulsions (Ramadan *et al.* 1988, Rao *et al.* 1993). Toxic effects of the cypermethrin in *Allium cepa* L. cells were investigated by Çavuşoğlu *et al.* (2011). This study showd that cypermethrin has a mitodepressive action on mitosis. Quizalofop-*p*-ethyl is a post-emergence phenoxy herbicide which is used in lentil fields for weed control. It is absorbed rapidly through leaf surfaces and quickly hydrolyzes to fluazifop acid. The acid is transported primarily in the phloem and accumulates in the meristems where it disrupts the synthesis of lipids in susceptible species (Urano 1982, Erlingson 1988). Plants treated with herbicides that cause disruption of cell division have mitotic stages present, but sometimes 1 or more stages normally present will be absent or aberrant. Inhibition of cell division is a secondary effect caused by some disturbance of a plant's metabolic

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process (Hess 1983, Kim and Bendixen 1987). In a study, the phytotoxic and genotoxic effects of water samples taken from the Eastern Channel on *V. faba* and *Z. mays* were investigated and it was found that all the concentrations used caused several abnormalities in mitotic cell divisions and decreased the mitotic frequency in the root tip cells. Micronucleus, chromosomal bridges, laggard chromosome, polar slip and lack of cytokinesis and some other abnormalities were observed in different phases of mitosis (Aksoy *et al.* 2012).

According to our knowledge, seed germination and mitosis on soybean have not yet been examined in relation to the action of the these 3 different pesticides. The present investigation was conducted to understand the root growth inhibiting effect of these 3 different pesticides on soybean seeds. The objectives of this investigation were: to determine the effect of different concentrations of the pesticides that were used in the soybean plant cultivation on seed germination percentages and on the abnormalities on the phases of mitosis of soybean.

Materials and methods

Pesticides

Pomarsol Forte WP 80 (thiram), [bis(dimethylthiocarbamoyl) disulfide] is a type of sulfur fungicide, widely used to prevent fungal diseases in seed and crops. It is an 80% water-wettable powder produced by Bayer Company and applied for control fungal diseases in seed and crops at rate of 200 g (recommended dose) active ingredient per ha. Arrivo 25 EC (cypermethrin), [(*RS*)-*α*-cyano-3-phenoxybenzyl (1*RS*)-*cis*-*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate] is a synthetic pyrethroid widely used as an insecticide in large-scale commercial agricultural applications. It is a 25% emulsifiable solution produced by Hektaş Company and applied for control insects from crops at rate of 25 g (recommended dose) active ingredient per da. The End EC (Quizalofop-*p*-ethyl), [ethyl (2*R*)-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionate] is a post-emergence phenoxy herbicide widely used in soybean fields for weed control. It is a 5% emulsifiable solution produced by Agrogeneral Company and applied for control weeds in soybean fields at rate of 50 g (recommended dose) active ingredient per decare.

Determination of EC50

Clean and washed *G. max* seeds were allowed to produce roots in distilled water for 24 h, after which the 50 homogeneous seeds were transferred to distilled water and 10 different concentrations of Pomarsol Forte WP 80 (0.00125–0.64 M), Arrivo 25 EC (0.075–38.4 M) and The End (0.0125–6.4 M) for 72 h. For the preperation of the pesticide solutions, a stock solution of each pesticide was prepared in distilled water and the test concentrations were obtained by diluting the stock solution. The root lengths in the control and pesticide treated goups were measured and the relative reduction in root length was calculated (T/C%) after treatment for 72 h. Three replicates were made for each concentration.

Treatment of soybean seeds with different pesticides

Seeds of *G. max* were pretreated with 5% NaOCl for 10 min for seed surface sterilization, then placed under clean bench conditions in Petri dishes and treated with different concentrations of 3 different pesticides. The control groups were treated only with distilled water. Soybean seeds were treated with EC50 and EC50x2 concentrations of each pesticide. Root tips which were 0.5 cm and higher were determined as germinated. All experiments were done in 3 repetitions. The germination of seeds was observed in laboratory conditions in petri dishes for 5 d.

Mitotic analysis

The root tips were fixed in Carnoy solution (3:1, alcohol-asetic acid) and hydrolysed in 1 N

HCl at 60°C for 5–10 min followed by squashing in a 2% orcein stain in 45% acetic acid. Slides were examined immediately. The mitotic frequency was determined by the examination of 500 cells per slide and calculated as mitotic cells per approximately 1,000 cells. Three replicates were made for each concentration.

Statistical analysis

The statistical analysis of data was made by SPSS for Windows version 16.0 statistical software (SPSS Inc., Chicago, USA). Statistically significant differences between the groups were compared using one-way analysis of variance (ANOVA) and Duncan's test. The data are displayed as means ± standard deviation (SD), and *p*-values less than 0.05 are considered statistically significant.

Results and discussion

EC50 indicated the effective concentration for 50% growth inhibition. EC50 values were determined *ca.* 0.08 M for Pomarsol Forte WP 80 fungicide (Fig. 1), *ca.* 9.6 M for Arrivo 25 EC insecticide (Fig. 2) and *ca.*0.4 M for The End herbicide (Fig. 3). All experimental procedures were carried out using 0.08 M (EC50) and 0.16 M (2xEC50) Pomarsol Forte WP 80 concentrations, 9.6

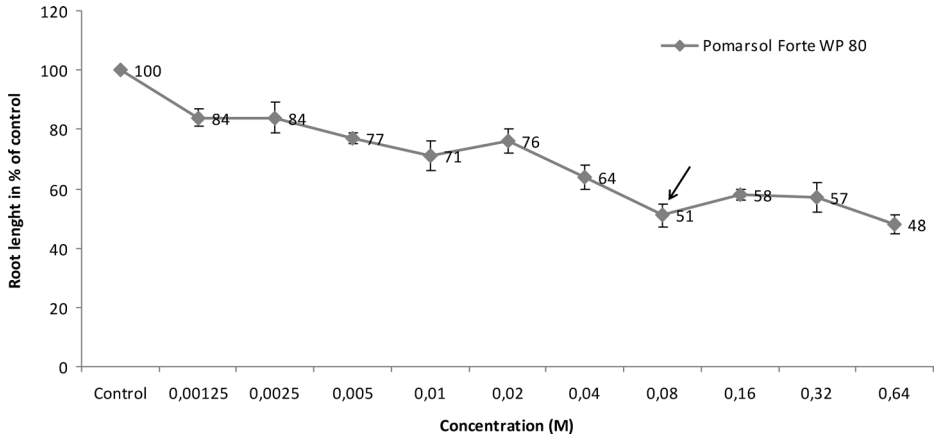


Fig. 1. Growth curve and EC50-value of Pomarsol Forte WP 80 fungucide.

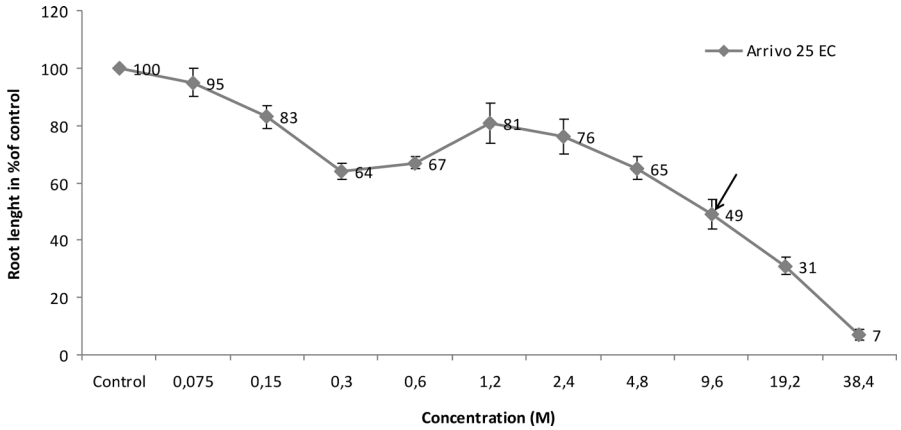


Fig. 2. Growth curve and EC50-value of the Arrivo 25 EC insecticide.

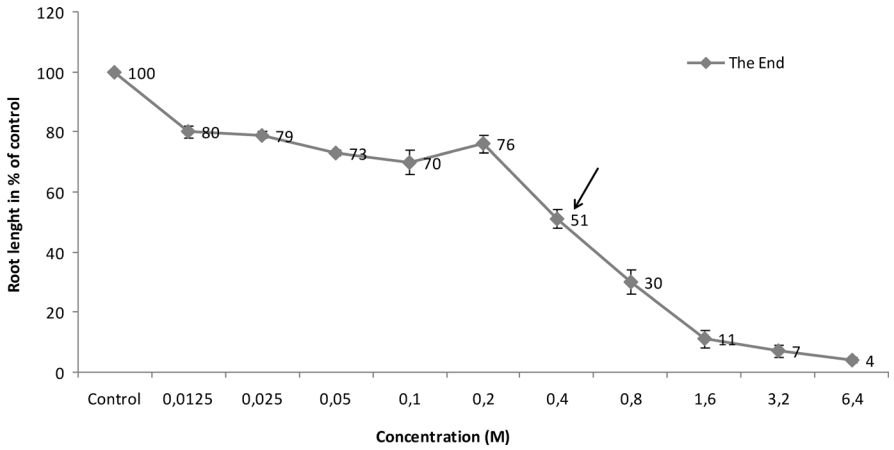


Fig. 3. Growth curve and EC50-value of The End herbicide.

Table 1. The effect of different pesticide concentrations on seed germination percentage (Values are presented as mean±standard deviation. The exposure time of different pecticides was 72 h).

Pesticide concentration	Seed germination percentage (%)±SD		
	2nd day	4th day	5th day
Control	78.16±0.4	90.34±0.4	90.91±0.3
Pomarsol Forte WP 80 (0.08 M)	48.56±0.3*	80.59±0.2	80.34±0.3*
Pomarsol Forte WP 80 (0.16 M)	38.34±0.4	72.01±0.3*	72.88±0.2
Arrivo 25 EC (9.6 M)	76.75±0.4	90.29±0.4	90.73±0.1*
Arrivo 25 EC (19.2 M)	42.69±0.2*	80.64±0.1	80.22±0.3
The End (0.4 M)	30.45±0.5	82.91±0.3*	82.85±0.1
The End (0.8 M)	24.92±0.6*	70.48±0.4	70.71±0.2*

* Means significantly different at the $p<0.05$ level compared to control.

M(EC50) and 19.2 M (2xEC50) Arrivo 25 EC concentrations and 0.4 M (EC50) and 0.8 M (2xEC50) The End concentrations.

Disturbance of seed germination and mitosis could produce severe consequences for root growth and development. This was clearly demonstrated for the roots of *Pisum sativum* and *Zea mays* with different concentrations of the sulfonylurea herbicides chlorsulfuron and metsulfuron methyl which caused severe injuries of the root growth (Fayez *et al.* 1994). Ruiz-Santaella *et al.* (2003) found that the herbicide cyhalofop-butyl from the family of phenoxy acetic acid decreases seed germination by 50% in *Echinochloa muricata*. Sasaki *et al.* (1968) reported that the use of herbicide 2,4-dicloro-phenoxy acetic acid (2,4-D) in Pinus decreased the seed germination percentage and similar results were found by Chopra and Singh (1978) in Guizotia which was treated with the same herbicide 2,4-D. Also, studies with different pesticides showed that seed germination percentages decreases parallel to the increse in concentrations. In *Lens culinaris* (Aktaç *et al.* 1994) the use of insecticide endosulphan and in *Allium cepa* (Dane and Dalgıç 2005) the use of fungicide benomyl decreased seed germination percentages with increasing concentrations. After the treatment with different concentrations of pesticides, we observed that the percentages of seed germination decreased while the concentrations increased for each concentration of Pomarsol Forte WP 80 fungucide and The End EC herbicide. However, there was no change in seed germination in 9.6 M concentration of Arrivo 25 EC insecticide when we compared the control group (Table 1). On the second day, the difference between the concentrations can be seen easily. After treatment with dif-

ferent concentrations of different pesticides germination of soybean seeds on the fifth day is seen in Fig. 4.

Mitotic index (MI), which reflects the frequency of cell division, is an acceptable measure of cytotoxicity for all living organisms. If the mitotic index decreases below 22% of the control, it can cause lethal effects on test organism (Çavuşoğlu *et al.* 2011). Also in this investigation it was observed that the use of higher concentrations of each different pesticide decreased the number of phases of mitosis. As seen in Table 2, all the pesticide concentrations used had a negative effect on mitotic division. The mitotic frequency was reduced as concentration was increased when we compare the control group (Table 2). The mitotic frequency was 5.90% in the control group and decreased to 5.00% at 0.08 M of Pomarsol Forte WP 80 concentration and 2.21% at 0.16 M of Pomarsol Forte WP 80 concentration. During our observations of seed germination for 5 days, there was also the most decrease in root growth at 0.16 M of Pomarsol Forte WP 80 concentration. In this concentration, mitotic frequency was reduced to almost half of that of the control and determined to be 2.21%.

Different concentrations of pesticides almost reduced the number of phases of mitosis when compared with the control. In particular, when we compare the concentration 0.16 M of Pomarsol Forte WP 80, and both of the 2 Arrivo 25 EC concentrations, there was a significant reduction and there was a slight reduction in the concentration of 0.08 M of Pomarsol Forte WP 80 in all phases of mitosis. Also, in the concentration of 0.08 M of Pomarsol Forte WP 80, there was no reduction on the prophase stage of mitosis (Fig. 4).

In this study, the commercial form of the pesticide was tested because this is the form that is utilized in agriculture and introduced into the environment. *G. max* was used as the test system because pesticides are applied on plants in agriculture and plants may produce unique genotoxic me-

Table 2. The effect of pesticide concentrations on mitotic frequency percentage.

Pesticide concentration	The number of total counted cells	The number of divided cells	Mitotic frequency (%)
Control	2101	124	5.90
Pomarsol Forte WP 80 (0.08 M)	2138	107	5.00
Pomarsol Forte WP 80 (0.16 M)	1678	37	2.21
Arrivo 25 EC (9.6 M)	1962	51	2.59
Arrivo 25 EC (19.2 M)	1678	42	2.50
The End (0.4 M)	1581	84	5.31
The End (0.8 M)	1766	61	3.45

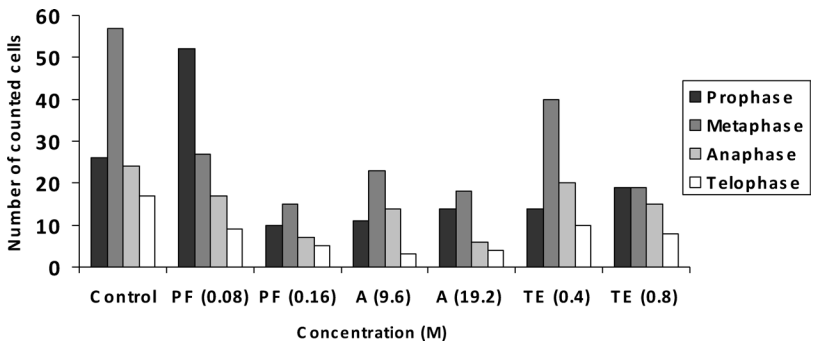


Fig. 4. The effect of pesticide concentrations on phases of mitosis (PF: Pomarsol Forte WP 80, A: Arrivo 25 EC, TE: The End).

Table 3. The effect of pesticide concentrations on mitotic abnormality percentage.

Pesticide concentration	Prophase			Metaphase			Anaphase			Telophase		
	N	A	%	N	A	%	N	A	%	N	A	%
Control	26	–	–	57	–	–	24	–	–	17	–	–
Pomarsol Forte WP 80 (0.08 M)	35	17	32.69	20	7	25.92	11	6	35.29	7	2	22.22
Pomarsol Forte WP 80 (0.16 M)	9	1	10.00	9	6	40.00	6	1	14.28	4	1	20.00
Arrivo 25 EC (9.6 M)	6	5	45.45	17	6	26.08	12	2	14.28	3	–	–
Arrivo 25 EC (19.2 M)	37	2	5.12	15	3	16.66	9	1	10.00	4	1	20.00
The End (0.4 M)	9	5	35.71	27	13	32.50	17	3	15.00	8	2	20.00
The End (0.8 M)	16	3	15.78	10	9	90.00	12	3	20.00	3	5	62.50

tabolites. In this study, the pesticides changed the mitotic cycle and decreased the mitotic index in *G. max* root tip cells. This decrease was significant in all concentrations when compared to the control. These results show that concentrations of pesticides used were cytotoxic in *G. max*. A similar result in mitosis has been observed from the treatment of the herbicides racer (Yüzbaşıoğlu *et al.* 2003), atrazine (Bolle *et al.* 2004), and arsenal (Grisolia *et al.* 2004). There are some possible mechanisms for the chemically decreased mitotic index in plant cells. The first is that a decrease in MI could be due to a blocking of G1, the suppressing DNA synthesis (Schneiderman *et al.* 1971). The second possible mechanism is a blocking of G2, thereby preventing the cell from entering mitosis. The lowering of the mitotic index might have been achieved by the inhibition of DNA synthesis at the S-phase (Sudhakar *et al.* 2001).

Pesticides increased the percentage of abnormal cells in *G. max* (Table 3). This increase was significant in all concentrations applied when compared to the control and was dose dependent. Common abnormalities were micronucleus formations in anaphase, vacualisation, c-mitosis, stickness, vagrant chromosome and shift in the equatorial plane in metaphase (Fig. 6). Chromosomal stickiness was recorded in both mitotic and meiotic division after most treatments. Stickiness has been attributed to the improper folding of chromosomal fibers that makes the chromatids connected by means of subchromatid bridges (Badr and Ibrahim 1987). Stickiness has been also attributed to the reaction of the applied pesticide with DNA, proteins or both of them forming inter- and intrachromosomal cross links (Patil and Bhat 1992). Chauhan *et al.* (1986) reported that subchromatid connections were seen under an electron microscope after deltamethrin treatments. Also, the sticky chromosomes result from the defective functioning of some specific nonhistone proteins which are needed for chromosome organization. The altered functions of these proteins are caused by mutations in the structural genes or by direct action with toxic agents (Türkoğlu 2007). The induction of vagrant chromosomes leads to the separation of unequal numbers of chromosomes in the daughter nuclei and subsequently to the formation of daughter cells with unequal sized or irregularly shaped nuclei at interphase (El-Ghamery *et al.* 2003). Levan (1938) described colchicine mitosis (c-metaphase or c-anaphase) as an inactivation of the spindle followed by a random scattering of the condensed chromosomes in the cell. It is reported that the immediate effect of all the pesticide treatments was the partial or entire inactivation of the spindle mechanism followed by a scattering of chromosomes (Jain and Sarbhoy 1987). c-Metaphase indicates that pesticides act as potent spindle inhibitors. On the other hand, micronuclei are the results of chromosome breaks (or mitotic anomalies) that require a passage through mitosis to be recognisable. The molecular mechanism of DNA breakage is not yet clearly understood. Rahoui *et al.* (2008) concluded that the germinating *Vicia faba* seeds showed a differential behaviour to cadmium stress with respect to cultivars. MN size can be an effective parameter to assess the clastogenic and aneugenic effects of chemicals. Namely, large MN would indicate an aneugenic effect resulting from a chromosome loss, whereas small MN may indicate a clastogenic action resulting from chromosome break (Çavuşoğlu *et al.* 2011). The

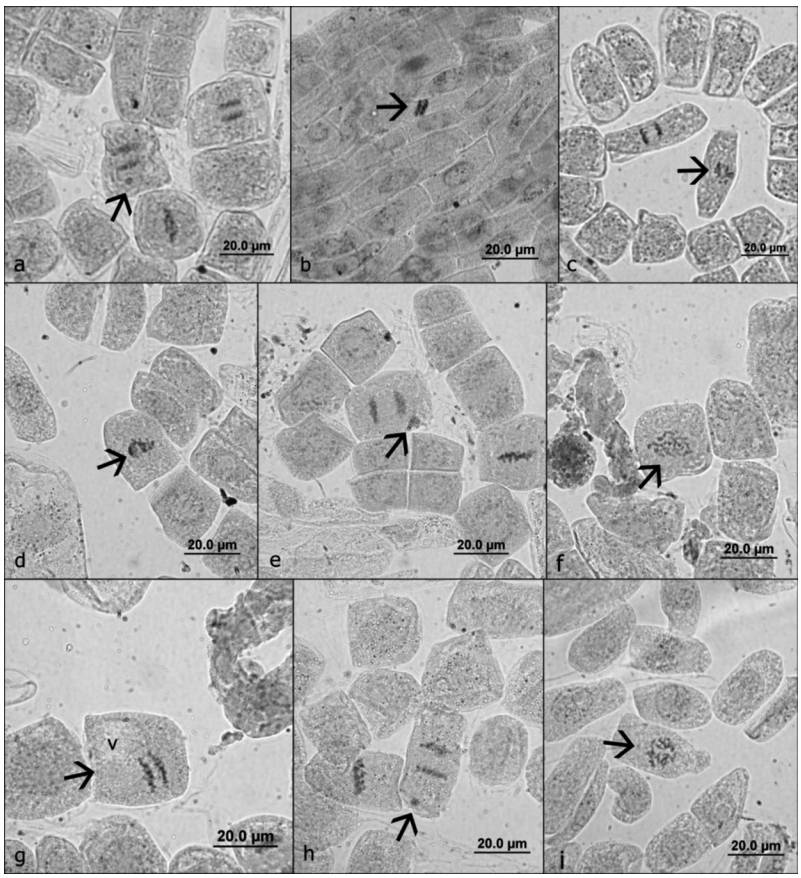


Fig. 5. The effect of pesticide concentrations on mitotic abnormalities. a) micronucleus formation in anaphase, b) shift in the equatorial plane in metaphase, c) and d) stickiness, e) vagrant chromosome, f) c-mitosis, g) vacualisation h) micronucleus formation in anaphase, i) c-mitosis. Arrows show the abnormalities.

different sizes of MN in meristematic cells of *G. max* roots are given in Fig. 5. The induction of these kinds of chromosomal aberrations was determined at different concentrations and exposure times of environmental pollutants (Chauhan *et al.* 1999, Amin 2002, Ateeq *et al.* 2002, Rank *et al.* 2002, El-Ghamery *et al.* 2000, Chandra *et al.* 2005, Saxena *et al.* 2005).

The pesticide treatment in root meristem cells of *G. max* with different concentrations resulted in the reduction in germination of seeds, the mitotic frequency of root tip cells of soybean seeds and the leaf pigment content of soybean leaves. As we know, mitotic frequency reflects cell division frequency and is used to determine the root growth ratio as a significant parameter. Regardless of which process is affected, both processes result in a reduced supply of new cells in the root meristem leading to an eventual inhibition of growth. It is vital to confirm that the usage of these pesticides should be subject to control since they may have a toxic effect on farmers and on humans consumed the plant.

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