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ANALYSIS OF SOIL GENOTOXICITY IN THE CITY OF IVANO-FRANKIVSK USING NICOTIANA TABACUM SU/+ PLANTS

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Abstract: Soil contamination is the result of rapid urbanization. Since plants are used as food and feed, it is important to analyze the effect of soil contaminants on plant growth. Moreover, plants can also be used to detect genotoxic effects of potential mutagens. Here, we analyzed whether *Nicotiana tabacum Su/+* plants that are heterozygous for the *Sulfur* gene can be used for biomonitoring of potential soil pollution. Leaves of these plants are pale green with occasional appearance of dark green spots. It was previously suggested that the appearance of these spots is the result of DNA damage at the *Sulfur* locus. Plants were germinated and grown on soil taken from five different areas of one of the Ukrainian cities, Ivano-Frankivsk. The areas were designated as clean (Plot #1 and Plot #2) and contaminated (Plot #3, 4 and 5). We analyzed the dynamics of stem elongation and changes in crown diameter. The analysis showed that plants grown on soil from Plot#5 had the slowest dynamics of both stem and crown growth. The dynamics of stem growth was statistically similar among other four samples. In contrast, the dynamics of crown growth was significantly slower in plants grown on soil from Plot#3 and #1, as compared to Plot#4 and #2. The analysis of spot appearance showed that the highest number of spots and the largest total area of spots were observed in plants from Plot#3. Analysis of index of mutation intensity (IMI) that reflects the percentage of leaf area occupied by mutation spots showed the lowest IMI in plants grown on soil from Plot#1 and 2 and the highest in plants from Plot #3, 4 and 5.

Keywords: transgenic *Nicotiana tabacum* Su/+ plants, contaminated soil, mutation frequency, plant growth

1. INTRODUCTION

Soil pollution is a common reality in most cities. Chemical analysis of soil for the identification of potential toxic compounds is costly and not always practical. The analysis of potential genotoxicity of contaminated soils is perhaps more important. The analysis of genotoxicity is typically done by using biological organisms, and thus it is called biomonitoring.

Plants are considered to be the best biomonitors since they can be grown on contaminated sites, they can absorb and accumulate contaminants, and they are consumed by human and animals (Kovalchuk et al., 2001; 2003). In spite of the fact that plants have rather complex mechanisms of perception, accumulation, compartmentalization and metabolism of various pollutants, plants start to get widely used for monitoring potential ecological risks of agricultural and industrial chemicals, food additives, as well as chemically/radioactively contaminated soil and water.

Usage of plant biosensors for testing environmental mutagenicity requires the ability to detect gross chromosomal aberrations that disrupt mitotic process; this is indeed possible to observe using *Allium cepa, Tradescantia, Vicia faba* and other plants (Ichikawa, 1992; Kanaya et al., 1994; Fiskesjo, 1995; Ma et al., 2005). Various types of other transgenic plants carrying a visible marker, such as GUS or luciferase have also been used for biomonitoring the environmental pollution (Kovalchuk et al., 2001, 2003; Ilnytskyy et al., 2004). The assays are designed in such a way that the transgene activity can only be restored after DNA damage at the transgene locus and subsequent repair of this damage (Ilnytskyy et al., 2004; Filkowski et al., 2004; Boyko et al., 2006 a,b). The use of these assays has some down sides: they typically require chemical processing, and since they

rely on the use of transgenic plants, these assays cannot be freely used for accessing mutagenicity of the open environment.

Nicotiana tabacum plants heterozygous for the sulfur (*Su*) nuclear gene is much more rarely used for biomonitoring. Su is a nuclear encoded, semi-dominant aurea mutation in *Nicotiana tabacum*. Homozygous plants (*Su/Su*) are pale yellow in color, and they are not able to perform photosynthesis. In contrast, heterozygous (*Su*/+) plants can carry on the photosynthesis process, although not to the full extent; as a result, plants appear to be yellow-green in color. Normal wild-type plants (+/+) are green (Burk & Menser, 1964; Kawata & Cheung, 1990). *Su*/+ plants were previously used to analyze the potential genotoxicity of ionizing radiation and toxic chemicals; a strong increase in the number of dark green spots on light-green leaves was observed (Baburek et al., 1997). Previously, it was suggested that dark-green sectors appeared as a result of various mutations and thus could serve as an indication of potential mutagenicity (Baburek et al., 1997). In contrast, the appearance of twin-spots of darker green and albino indicated a homologous recombination event (Baburek et al., 1997). These plants have the great advantage for being used as biomonitors as they allow visual analysis of occurring mutations, and they are not transgenic.

Here, we analyzed the potential genotoxicity of soils collected from clean (Plot#1 and #2) and contaminated (Plot#3, #4 and #5) areas of the city of Ivano-Frankivsk. We showed that soil from five different plots differently influenced the dynamics of plant growth and the appearance of dark-green spots in *Su*/+ plants. We found that changes in dynamics of plant growth correlated with the appearance of dark spots, that is, plants with less efficient growth had larger areas occupied by mutation spots.

2. MATERIALS AND METHODS

2.1 Soil sampling

The subject of investigation was samples of soil from various areas of the Ivano-Frankivsk city: ecologically clean (by convention) areas of the City park (designated as Plot#1) and commercial greenhouses (Plot#2) and chemically contaminated areas – Pasichna district (Plot#3), the park area near the Medical University (Plot#4) and Puluy Street (Plot#5) (soil contamination as per (Adamenko, 2000, 2005). Soil samples were taken in the following manner: 10 cm of the surface area was removed, and then 30 cm of the inner layer was sampled.

2.2 Plant growth

For the analysis, we used seeds of *Nicotiana tabacum* plants heterozygous for Sulfur gene (*Su*/+) (Kawata & Cheung, 1990; Kovalchuk et al., 2003). Heterozygous plants (*Su*/+) are photosynthetic and have a yellow-green phenotype since mutations in *Nicotiana tabacum Sulfur(Su)* plants are inherited in a semidominant manner. Wild-type *Nicotiana tabacum* plants (+/+) are green, whereas homozygous plants (Su/Su) – pale yellow and non-photosynthetic. The gene segregates in a normal Mendelian manner, and plants are propagated through the heterozygous stage (*Su*/+). The selfed progeny segregates in such a way that 25% of plants are wild-type (+/+), 50% are heterozygous (*Su*/+), and 25% are homozygous for mutations in the Sulfur gene (*Su*/*Su*). For the analysis, we used heterozygous (Su/+) plants. Mutations in cells of *Su*/+ result in the appearance of two types of sectors: light green (albino) or dark (dark green) spots against the background of yellow-green leaves. There exist some combinations of albino (*Su*/*Su*) and dark green (+/+) spots on the leaves of heterozygous *Nicotiana tabacum* plants known as "twin" spots (Friedlender et al., 1996; Baburek et al., 1997).

Plants were germinated and grown on soil taken from the aforementioned city areas. Plants were grown in standard pots under the following conditions: at a temperature of 22°C and under the light regime of 16h day and 8h night.

2.3 Analysis of dynamics of plant growth and mutations at the sulfur locus

On average, five plants were taken for the analysis. Two parameters of plant growth have been analyzed: the dynamics of stem elongation and the dynamics of the enlargement of crown diameter. The data points were taken every two weeks for approximately 4 months.

For the analysis of the appearance of dark spots, the following parameters were measured: the time of sport appearance, the percentage of plants with spots, the average number of spots per plant, the average area that spots occupy per plant, and the index of mutation intensity. Spots were counted at the last day of measuring stem height. The average area of spots per plant was calculated by measuring the size of each spot, summing it for each plant, and then averaging it for all plants grown on a particular soil sample. The index of mutation intensity (IMI) was measured by relating the average size of the total area that spots occupy in a single plant to the total area of leaves in a single plant.

2.4 Statistical analysis

Statistical analysis was performed using a Student's t-test and a Spearman's and Pearson rank correlation test.

3. RESULTS

Three months' observations of growth dynamics of *Nicotiana tabacum* plants grown on soil samples from different areas of the Ivano-Frankivsk city showed a gradual increase in stem height (Fig. 1). The slowest stem growth was observed for plants grown on soils samples from Plot#5; the mean value of the maximum height for these plants was 1.9 times lower than that in plants grown on soil samples from other areas. Plant growth on soil from other four plots was statistically similar to each other.

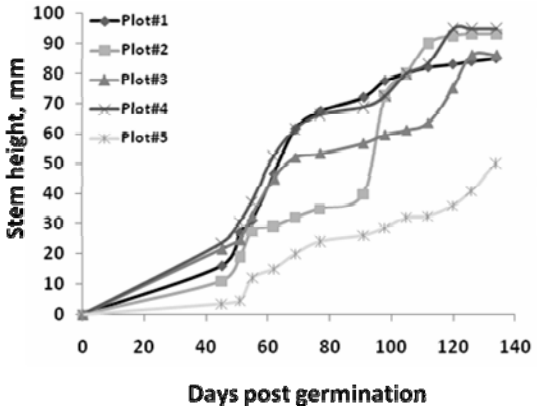

Figure 1. Dynamics of stem growth of *Nicotiana tabacum Su/+* plants cultivated on soil samples from different areas of the Ivano-Frankivsk city
Plants were germinated and grown on soil samples collected from five designated areas, labelled as Plot#1, 2, 3, 4 and 5. Five to ten plants were used for each experimental group, and the experiment was repeated three times. Stem size was measured every week, starting from day 45 post germination and ending with day 134 post germination.

A three-month study on crown diameter of *Nicotiana tabacum* plants grown on soil samples from different areas of the Ivano-Frankivsk city has revealed a tendency similar to the one observed for stem growth (Fig. 2). A positive correlation has been established between the results obtained as to growth dynamics of stem and crown ($r > 0.9$). Once again, on average, plants grown on soil from Plot#5 had the smallest crown diameter (119.5 mm). Plants grown on soil from Plot#1 and Plot#3 had larger crown diameters than plants from Plot#5 but much smaller crown diameters than plants from Plot#2 and Plot#4.

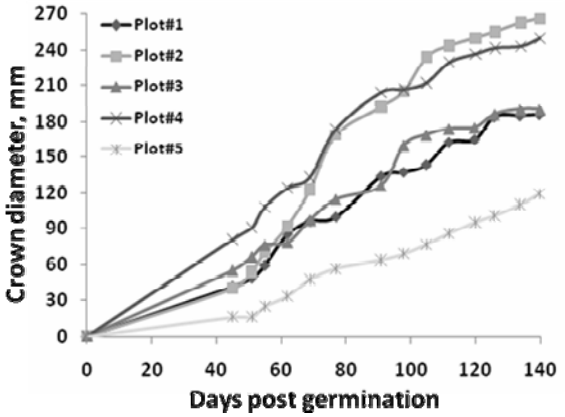

Figure 2. Dynamics of crown growth of *Nicotiana tabacum Su/+* plants cultivated on soil samples from different areas of the Ivano-Frankivsk city
Plants were germinated and grown on soil samples collected from five designated areas, labelled as Plot#1, 2, 3, 4 and 5. Five to ten plants were used for each experimental group, and the experiment was repeated three times. Crown diameter was measured every week, starting from day 45 post germination and ending with day 140 post germination.

Next, we analyzed the frequency of mutations at the Sulfur locus by analyzing the appearance of dark green spots. Examples of dark green and twin spots are shown in figure 3.

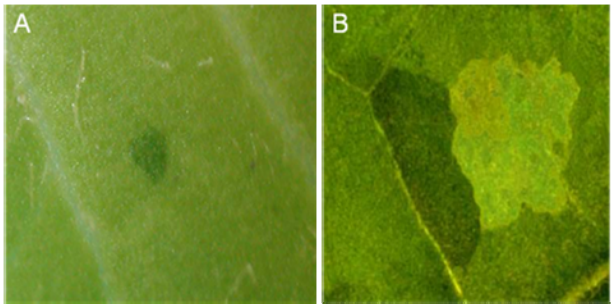

Figure 3. Examples of mutation spots in Su/+ tobacco plants
A. Example of dark green spot – mutation at one locus. B. Example of twin spot – mutation at two loci.

First, we analyzed the time of occurrence of these spots. The earliest occurrence of dark green (+/+) spots was observed on leaves of *Nicotiana tabacum* plants grown on soil samples from Plot#3 (69.75 ± 11.46 days after planting).

The latest appearance of these spots was observed on leaves of *Nicotiana tabacum* plants grown on soil from Plot#1 and Plot#2 (93, 67 ± 10.04 and 95, 33 ± 4.67 days of growth, respectively). Curiously, the time of appearance of mutations at the *Sulfur* locus in the slowest growing plants from Plot#5 was intermediate (85.00 ± 22.00 days of vegetation); there was also the largest range of fluctuations in the time of spot appearance in these plants.

Table 1. The analysis of dark green and twin spots visualized in leaves of *Nicotiana tabacum Su/+* plants cultivated on soil samples from different areas of the Ivano-Frankivsk city in Ukraine

The site of soil sample collection	Plot designation	Plants with spots, %	The number of spots per plant	Area of spots per plant, mm²	Index of mutation intensity, %
The City park	Plot#1	60	2.67±0.33	2.67±0.33	0.05±0.0
Commercial greenhouse	Plot#2	100	7.00 ±2.52	8.33±2.46	0.05±0.0
Pasichna street	Plot#3	80	5.25±1.89	248.88±242.8	2.64±2.47
Medical Academy	Plot#4	100	3.20±1.11	6.80±3.16	0.8±0.02
The flowerbed in Puluy Street	Plot#5	40	3.00± 0.0	3.25±0.25	0.22±0.04

The percentage of plants with spots was calculated by relating the total number of plants with spots to the total number of plants analyzed. The average number of spots was calculated by relating the total number of spots in the population to the total number of plants in the population. The area of spots per plant was calculated by measuring the size of each spot per plant (mm^2) and summing it up for each plant. Mutation index was calculated by relating the total area spots occupy on one plant to the total size of the leaves with these spots.

Next, we analyzed the mutation index by identifying the percentage of plants with spots. On Plot#2 and Plot#4, there were 100% of plants with spots, whereas on Plot#3 and Plot#1, there were 80% and 60% of plants with spots, respectively. The smallest percentage of plants with spots (40%) was recorded for soil samples from Plot#5. A high percentage of plants with spots found in Plot#2 and Plot#4 correlated well with the highest growth intensity observed in these plants ($r \geq 0.9$).

The analysis of the average number of spots per plant showed the largest number of 7.00 ± 2.52 spots in plants from Plot#2 and the lowest number of 2.67 ± 0.33 spots in plants from Plot#1. Plants from Plot#3 showed the second largest number of spots per plant, 5.25±1.89. The number of spots per plant grown on soil from Plot#5 was 3.00±0.00.

While analyzing the appearance of dark green spots, we noticed that spots had different sizes; we hypothesized that mutations which appeared earlier resulted in larger spots, whereas those which appeared later were smaller. Thus, measurements of spot area would serve as a reflection of how earlier mutations occurred in plants.

Plants from Plot#3 had the largest total spot area of 248.88 ± 242.8 per plant. In contrast, the total average spot area in plants from Plot#1 and Plot#5 was 2.67 ± 0.33 and 3.25±0.25, respectively. Sometimes, spots with a large sector area occupying half of the leaf area were observed only in plants from Plot#3. Only one plant from Plot#4 had a single spot occupying 15 mm^2; all other individual spots were not larger than 2 ±1 mm^2.

We also observed a "twin" spot of a total area of 2.5 mm^2 which was found in a single plant grown on soil samples from Plot#4. Of all experimental plants of this sampling, it was a single example of visualization of simultaneous mutations in both alleles of the *Su* gene; mutations of *Su* to wild type (+) generates a +/+ genotype visualized as a dark green spot, whereas mutation of wild type (+) to *Su* generates a *Su/Su* genotype visualized as an albino spot.

Since plants grown on soil from Plot#5 were much smaller, it was important to prorate the differences in the plant size. Thus, we decided to relate the total area of spots to the total area of the leaf with these spots; we named this as an index of mutation intensity (IMI). The largest IMI of 2.64 ± 2.47% was observed in plants from Plot#3. The second largest IMI of 0.22 ± 0.04% was observed in plants from Plot#5. IMI in plants from Plot#1 and #2 were much smaller. High IMI observed in plants grown on soil collected from Plot#3 and Plot#5 correlated with inhibition of growth observed in these plants. Thus, IMI seems to be a good indication of potential mutagenicity.

Strong positive correlations were established between: i.) the stem height and the crown diameter of a plant ($r = 0.93$, $p<0.05$); ii.) the stem height and the index of plants with spots ($r = 0.96$, $p<0.01$); iii.) the index of mutation intensity and the area of spots per plant ($r = 1$, $p<0.01$).

5. DISCUSSION

Here, we analyzed the possibility of using *Nicotiana tabacum* plants heterozygous for the *Sulfur* gene for the analysis of potential mutagenicity of soil. The analysis showed that plants grown on soil samples collected from the busy street area (Plot#5)

indeed had the slowest stem growth and the smallest crown diameter; at the same time, these plants had the second highest IMI, the percentage of leaf area occupied by dark green spots. Plants grown on soil from another street area (Plot#3) also demonstrated one of the smallest crown diameters as well as the highest number of spots and the largest total area occupied by spots. At the same time, plants grown on soil samples from the city park (the ecologically clean zone by convention; Adamenko, 2000) had the fast stem growth, large crown diameter, the lowest number of spots per plant, the smallest area of spots per plant and the smallest IMI.

In the current study, we did not analyze the chemical composition of soil, and thus, we cannot comment whether there was a correlation between the aforementioned parameters of plant growth and the chemical contamination of soil. However, we assume that soil taken from the commercial greenhouse (Plot#2) and the city park area (Plot#1) was much less chemically contaminated than soil taken from two busy street areas (Plot#3 and Plot#5). Previous reports by Adamenko (2000; 2005) indeed suggested that soils from Plot#3, 4 and 5 are more contaminated than soil from Plot#1. A direct correlation between the chemical composition and the level of mutations in *Su*/+ plants still remain to be established. In the future, we would also like to analyze whether there is a concentration-dependent increase in mutations in *Su*/+ plants.

Our data showed that the number of mutational spots increase in plants grown on contaminated soil. Unfortunately, *Su*/+ plants have not been used for the analysis of any soil or water pollution, and thus, we cannot compare our data with the data from any other studies. At the same time, *Su*/+ plants were in past used to analyze the influence of radiation and toxic chemicals that were able to increase the frequency of dark green and twin spots.

6. CONCLUSIONS

We conclude that *Nicotiana tabacum Su*/+ plants indeed can be used for analysis of soil quality. We found that among all the parameters used for mutational studies, the index of mutation intensity that reflects the measure of the relative area occupied by mutated cells is one of the most ideal for analysis of potential soil mutagenicity.

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