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Allium cepa chromosome aberration and micronucleus tests applied to study genotoxicity of pesticide-treated vegetable extracts

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1 ***Allium cepa* chromosome aberration and micronucleus tests applied to study**
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3 **genotoxicity of extracts from pesticide-treated vegetables and grapes**
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Abstract

The *Allium cepa* assay is an efficient test for chemical screening and *in situ* monitoring for genotoxicity of environmental contaminants. This test has been used widely to study genotoxicity of many pesticides revealing that these compounds can induce chromosomal aberrations in root meristems of *Allium cepa*. Pesticide residues can be present in fruit and vegetables and represent a risk for human health. The mutagenic and carcinogenic action of herbicides, insecticides and fungicides on experimental animals is well known. Several studies have shown that chronic exposure to low levels of pesticides can cause birth defects and that prenatal exposure is associated with carcinogenicity. In this study we evaluated the potential application of plant genotoxicity tests for monitoring mutagens in edible vegetables. The presence of pesticides and genotoxic compounds extracted from twenty-one treated vegetables and eight types of grapes sampled from several markets in Campania, a region in Southern Italy, was monitored concurrently. The extracts were analysed for pesticides by gas chromatography and high-performance liquid chromatography, and for genotoxicity using two plant tests: the micronucleus test and the chromosomal aberration test in *Allium cepa* roots. Thirty-three pesticides were detected, some of which are not approved. Genotoxicity was found in some of the vegetables and grapes tested. *Allium cepa* tests proved to be sensitive in monitoring genotoxicity in food extracts. The micronucleus test in interphase cells gave a much higher mutagenicity than the chromosomal aberration test in anaphase-telophase cells.

Keywords: *Allium cepa* test; genotoxicity; pesticides; vegetables.

Introduction

Many studies have shown that air, water, soil and food are frequently contaminated with mutagens and carcinogens, which can reach humans and increase environmental carcinogenic hazards. For this reason the monitoring of genotoxic compounds in the environment has become an important objective of public health, with the intention of avoiding or minimizing direct and indirect human exposure to these toxic substances.

Plant tests have been widely used for detecting the genotoxicity of chemical compounds and for *in situ* monitoring of environmental genotoxic contaminants, without submitting samples to preliminary concentration or to a very long, difficult and expensive preparation phase (Grant, 1994, 1999). Among the genotoxicity plant tests the *Allium cepa* assay is well known and commonly used in many laboratories with a wide area of applications and with a good agreement with the results from other established test systems using eukaryotic as well prokaryotic cells. *Allium cepa* roots chromosomal aberration (AL-RAA) and micronuclei (AL-MCN) tests are widely employed to evaluate the genotoxicity of many chemical compounds and environmental pollutants. These assays are good and sensitive methods for monitoring clastogenic effects (Grant, 1982; Ma et al., 1995; Rank and Nielsen, 1997). Not only known chemicals but also water-soluble compounds (e.g. salt solutions), heavy metals and complex environmental mixtures are studied by the *Allium cepa* test: river and lake waters, waters of well, chlorinated drinking water, domestic and industrial wastewaters, leachate of landfill, industrial waste, soil samples and soil extracts have been studied using this test (Fiskesjö, 1985; Smaka-Kincl et al., 1996; Steinkellner et al., 1998; Cabrera et al., 1999a, 1999b; Grover and Satwinderjeet Kaur, 1999; Monarca et al., 2000, 2002a, 2002b, 2003).

The advantages of the *Allium cepa* test are that it is a fast and inexpensive method, easy to handle, gives reliable results, comparable with other tests performed in mammalian systems, e.g. with high concordance with the chromosomal aberration assay in bone marrow cells in rats (Grover et al.,

1 1990), in human lymphocytes, in V79 cell line of Chinese hamster and in other organism tests such
2
3 as fish, crustacea and unicellular algae (Fiskesjö, 1985). Moreover the test can be used to measure
4
5 also toxicity, studying macroscopic parameters as length of roots, variations in form, colour and
6
7 consistency of roots, presence of broken root tips, tumors and hooks (Fiskesjö, 1985).
8
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10 Many mutagenic/carcinogenenic compounds can be present in food and some epidemiological
11
12 studies have indicated that many dietary factors are related with the development of human cancer
13
14 (Doll and Peto, 1981). Also pesticide residues can be present in fruit and vegetables and represent a
15
16 risk for human exposure. Children in particular can be exposed to pesticides by dietary ingestion
17
18 because they eat more food per body mass than an adult, and their diets differ from those of adults.
19
20 The children's diet is often rich in foods containing higher levels of pesticide residue, such as
21
22 juices, fruits and vegetables (Curl et al., 2003). The mutagenic and carcinogenic action of
23
24 herbicides, insecticides and fungicides on experimental animals is well known. Several studies have
25
26 shown that chronic exposure to low levels of pesticides can cause birth defects and that prenatal
27
28 exposure is associated with carcinogenicity (Heeren et al., 2003). Carcinogenicity and neurotoxicity
29
30 in adults related to pesticides exposure are also reported (Alavanja et al., 2004). Some of these
31
32 substances have been associated with neuro-developmental toxicity and thyroid hormone
33
34 imbalance. Many pesticides are recalcitrant and bioaccumulative compounds and they are endocrine
35
36 disruptors (Vogel, 2005). Toxicological properties, such as mutagenicity, embryotoxicity,
37
38 teratogenesis and cancerogenesis, are well known for many pesticides (Cueto, 1980; Saleh, 1980;
39
40 Al-Saleh, 1994; Leiss and Savitz, 1995; De Bertoldi, 1996). Human exposure to agricultural
41
42 chemicals has been associated with an increase in cancer incidence (Saftlas et al., 1987; Brown et
43
44 al., 1990; Blair and Zahm, 1991; Cerhan et al., 1998; Settini et al., 2003), even if in some cases
45
46 only a moderate statistically association is reported (Abdalla et al., 2003). On the other hand studies
47
48 on the risk assessment for pesticide residues in food suggest a low importance in cancer risk and
49
50 adverse health effects in humans. The estimate of human risk related to fruit and vegetables
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1 consumption must consider that this food is protective for cancer (Block et al., 1992; Swirsky Gold
2 et al., 1997) and concurrently can contain natural substances with genotoxic activity (Ames et al.,
3 1987).
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8 The extensive use of pesticides can entail risk not only to human health, but also to the environment
9 and non-target organisms. For this reason, according to the European Directive 91/414, the
10 registration of pesticides is regulated in order to avoid harmful effects on human or animal health,
11 and adverse effects on the environment. Moreover pesticides can be move by a diffusive transport
12 which enables their adsorption by soil and a flow transport which cause the movement of
13 compounds from soil surface to groundwater, determining an higher risk for groundwater and
14 drinking water contamination (Guzzella et al., 1996).
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23 The Joint FAO/WHO Meeting on Pesticide Residues (JMPR) has established for food commodities
24 the maximum residue limits (MRLs) of pesticides that can be ingested daily without appreciable
25 human health risk (Environmental Health Criteria, EHC 104, 1990). In Italy, the MRLs are
26 regulated by European Directives 99/65/EC and 99/71/EC. Nevertheless, large quantities of
27 pesticides are released daily into the environment with potential hazards to human genetic material.
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35 Most pesticides were found capable of inducing mutations in short-term tests (Tezuka et al., 1980;
36 Waters et al., 1982; Plewa et al., 1984; Garrett et al., 1986; Panneerselvam et al., 1995; Ribas et al.,
37 1996; De Marco et al., 2000; Kaya et al., 2000; Zang et al., 2000), although genotoxicity was only
38 present at much higher doses than those utilized in agricultural activities (Rodrigues et al., 1998).
39 140 organophosphate compounds were tested for mutagenic activity in bacteria (*Salmonella*
40 *typhimurium* and *Escherichia coli*) by Hanna and Dyer (1975), who found that 20% of them gave
41 positive mutagenic responses and that this group of chemicals produced base substitutions rather
42 than frame-shift mutations. Out of 228 pesticides under study, 50 were found to be mutagenic in
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1 bacterial reversion-assay systems with 5 strains of *Salmonella typhimurium* (TA100, TA98,
2 TA1535, TA1537 and TA1538) and one strain of *Escherichia coli* (WP2 hcr) (Moriya et al., 1983).
3 Hour et al. (1998) carried out a comparative investigation on the mutagenicity of several pesticides
4 using the Ames test (TA100, TA98 and TA1535 strains) and *Salmonella* lactam test (JK947, JK3
5 and JK1 strains). Six out of 14 pesticides evaluated for genotoxicity using the new strains were
6 highly mutagenic in strain JK3. Dolara et al. (1993, 1994) carried out some studies in Central Italy
7 to determine the toxicological effects of a complex mixture of pesticide residues in common foods.
8 They mixed 15 pesticides, usually detected in food at high concentrations, and then tested this
9 mixture using the *Salmonella*-microsome assay, with and without metabolic activation. No
10 mutagenic activity was observed at concentrations up to 500 mg/plate; however, when they tested
11 the same mixture at concentrations ranging from 0.1 to 20 mg/L on human lymphocytes *in vitro*,
12 they observed a slight but statistically significant increase in sister chromatid exchanges at 1 mg/ml.
13 Bianchi-Santamaria et al. (1997) performed the human lymphocyte micronucleus test on single
14 pesticides and on mixtures of pesticides usually found in the environment and found a weak
15 genotoxicity for benomyl and for three of the four organophosphorous insecticides. However, the
16 various mixtures did not have any synergistic effects.

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34 The cytological effects of individual pesticides have been studied in plants (Ahmed and Grant,
35 1972) and some studies have been conducted by means of genotoxicity tests: in *Tradescantia*
36 flowers using the *Tradescantia*/stamen hair test and the *Tradescantia*/micronucleus test (Ma et al.,
37 1984; Rodrigues et al., 1998; Mohammed and Ma, 1999), in *Vicia faba* roots (micronuclei and root
38 tip length in *Vicia faba*) (De Kergommeaux et al., 1983; De Marco et al., 2000) and in *Allium cepa*
39 roots (chromosomal aberration in *Allium cepa*) (Fiskesjö et al., 1981; Grant, 1982; Fiskesjö, 1985;
40 Rank and Nielsen, 1993, 1997; Rank et al., 1993; Grover and Satwinderjeet Kaur, 1999). Higher
41 plants provide a valuable genetic assay system for screening and monitoring environmental
42 pollutants (Grant et al., 1994) and comparable results, in term of genetic abnormalities, are obtained

1 in plant and animal systems (Grant et al., 1981). These studies were performed on single or
2
3 complex mixtures of pesticides, but a few data on the genotoxicity of residues extracted from fruit
4
5 and vegetables are yet available.
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8 The aim of this research was to evaluate the potential application of plant genotoxicity test on
9
10 monitoring of mutagens in food vegetable. The mutagenic activity of extracts of fruit and
11
12 vegetables used for human consumption was studied using two plant tests in *Allium cepa* roots, the
13
14 *Allium cepa* micronucleus test, and the *Allium cepa* chromosome aberration test and concurrently
15
16 the presence of pesticides in these residues by gas-chromatography or high-performance liquid
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18 chromatography (HPLC) was analysed.
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20

21 22 **Materials and methods**

23
24 We investigated 21 samples of vegetables (beet, cucumber, tomato, cherry tomato, lettuce, round
25
26 aubergine, pepper) and 8 types of grapes grown in Campania, a region in Southern Italy, and bought
27
28 from several greengrocer's shops. The samples were extracted for pesticide analyses and for
29
30 genotoxicity tests.
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32

33 *Fruit and vegetable sampling and pesticide extraction*

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35 The samples for the pesticide analyses and for the genotoxicity tests were prepared following two
36
37 different methods. The method used in the pesticide analyses consisted of the following stages:

- 38
39 1. Sample preparation. Unwashed parts of vegetables and grapes were sliced and whisked to
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41 obtain a homogeneous mixture.
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- 43
44 2. Residue extraction. A total of 10 g of homogenized sample was mixed with 12 g of
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46 diatomaceous earth and placed in an empty 60 ml column. After 10 minutes the column was
47
48 eluted with 100 ml of dichloromethane.
- 49
50 3. Extract purification or, if chlorophyll was present, on SCX (ion-exchange column). The
51
52 eluates dried by rotary evaporation at 40°C were re-suspended in 2 ml of

1 dichloromethane/acetone solution (80:20) and charged on a cartridge of Florisil (60 ml)
2
3 previously washed with 5 ml of dichloromethane/acetone solution (80:20) for activation,
4
5 and the eluates were recharged twice on the same type of cartridge (Dagna et al., 1993).
6

- 7 4. Extract purification in presence of chlorophyll. The extracts dried by rotary evaporation and
8
9 re-suspended in 2 ml of methanol were charged on a cartridge of SCX (60 ml) previously
10
11 washed with 5 ml of methanol for activation, and the eluate was recharged twice on the
12
13 same type of cartridge.
- 14 5. Gas chromatography and HPLC analysis of the residues. The final eluates were dried by
15
16 rotary evaporation and re-suspended in 2 ml acetone/hexane solution (1:1) for gas
17
18 chromatography analysis or in 2 ml water/acetonitrile solution (70:30) for HPLC analysis.
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22 Sample extraction of samples for genotoxicity test was performed as follows:
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- 24 1. Sample preparation. Unwashed parts of vegetables and grapes were sliced and whisked to
25
26 obtain a homogeneous mixture.
27
- 28 2. Sample extraction with diatomaceous earth in dichloromethane. 10 g of homogenized
29
30 sample were mixed with 12 g of diatomaceous earth and then placed in an empty 60 ml
31
32 column. After 10 minutes the column was eluted with 100 ml of dichloromethane and the
33
34 eluates were then dried by rotary evaporation at 40°C (Dagna et al., 1993). The extraction
35
36 was carried out on a total of 50 g of sample.
- 37 3. Genotoxicity tests on the extracts. The dried extracts were dissolved in dimethylsulphoxide
38
39 (DMSO) and diluted in distilled water and analysed for mutagenicity by means of plant
40
41 tests.
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44

45 *Analysis of the extracts*

46 *Pesticide analysis.*

47 Instrumental analysis of the extracts was performed by gas chromatography (Manninen et al., 1987)
48
49 and by HPLC (Ambrus et al., 1981). Gas chromatography analysis was performed using a
50
51

1 Shimadzu GC17A system (Shimadzu Corporation, Tokyo, Japan) which operates with a bi-channel
2
3 system: after filtering on a 0.45 µm filter, each extract was injected (1 µl) simultaneously into two
4
5 columns with different polarities (ZB-5 30 m x 0.32 mm ID x 0.25 µm film and ZB-1701 30 m x
6
7 0.32 mm ID x 0.25 µm film) (Phenomenex, Torrance, CA, USA) and connected with electron
8
9 capture detector (ECD) and nitrogen-phosphorus detector (NPD). This allowed one pesticide to be
10
11 detected by both columns at the same time, thus avoiding the possibility of overlapping of different
12
13 pesticides. An ECD detector was used to identify organochloride pesticides, pyrethroids,
14
15 dicarboximides and thiophthalimides; an NPD detector was used to identify phosphorates, triazoles
16
17 and acylanines. All these analyses were performed using pesticide standards (Ehrenstorfer GmbH,
18
19 Augsburg, Germany).

20
21
22 Quantitative gas chromatography was performed using a multiple internal standard (Series M). The
23
24 temperature programme consisted of an initial temperature of 50°C held for 2 minutes, followed by
25
26 an increase to 150°C at a rate of 25°C/min and to 270°C at 4°C/min (10 minutes) (Manninen et al.,
27
28 1987).

29
30
31
32 High-performance liquid chromatography is the most appropriate method to determine certain types
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34 of compounds, such as ureic compounds, growth regulators, benzimidazole and carbamates. Most
35
36 of these compounds present heat sensitivity and low volatility due to their high molecular weight
37
38 and/or polarity, leading to long retention on GC columns. Hence, these molecules are not amenable
39
40 to analysis by gas chromatography. HPLC analysis was performed on a Shimadzu LC10 system
41
42 (Shimadzu Corporation, Tokyo, Japan) equipped with reverse-phase separation columns packed
43
44 with 5 µm particles and with pH stability from 2 to 10. The latter parameter is particularly
45
46 important considering the wide range of molecules used nowadays as pesticides.

Analyses of benzimidazole and other pesticides were carried out with 250 x 4.6 mm ID Luna 5 µm Phenyl-Hexyl and 5 µm C₁₈(2) columns (Phenomenex, Torrance, CA, USA) and a DAD detector, whereas for carbamate analysis, separation on 250 x 4.6 mm ID 5 µm C₈(2) Luna columns (Phenomenex, Torrance, CA, USA) was followed by post-column derivatization with NaOH and o-phthalaldehyde and fluorometric detection. The flow rate was 1 ml/min and quantitative analysis was performed using an external standard. The samples were extracted, purified and dried, and then suspended in a 70:30 water-acetonitrile solution, filtered using a 0.45 µm cellulose filter and injected (100 µl) in HPLC.

Toxicity and genotoxicity tests on *Allium cepa* roots.

A phytotoxicity assay was performed prior to the genotoxicity tests on some extracts in order to determine the non-cytotoxic doses. The assay consisted of a 96-hour root toxicity test using *Allium cepa* bulbs grown in extract solutions in distilled water of three kinds of samples (tomato, aubergine, grapes) at concentrations of 225 mg/L, 75 mg/L and 25 mg/L.

Commercial onion bulbs of *Allium cepa* of equal size were carefully cleaned and washed without destroying the primordial roots. For each sample, six onions were exposed to different concentrations of extracts, and natural mineral water known without mutagenic activity was used as a negative control. On three consecutive days the lengths of the roots were measured and macroscopic parameters were considered. The concentrations used caused no visible cytotoxic effects and therefore they were also used for the genotoxicity tests (Fiskesjö, 1985, 1995).

For the plant genotoxicity tests, dried extract equivalent to 50 g of vegetables or grapes was dissolved in 2 ml of DMSO and diluted with distilled water to 225 mg/L, 75 mg/L and 25 mg/L. These solutions were used to analyse micronucleus frequency in interphase and also chromosome aberrations in anaphase-telophase *Allium cepa* root tip cells (Grant, 1982; Rank and Nielsen, 1993,

1 1997; Rank et al., 1993; Ma et al., 1995; Grover and Satwinderjeet Kaur, 1999). The *Allium cepa*
2
3 bulbs were put in natural mineral water for pre-germination until the root length was 1.5-2 cm; the
4
5 roots were then exposed to the extract solutions for 48 hours. Each extract was tested with a series
6
7 of five onions. After exposure, the root tips were fixed in Carnoy's solution (ethyl alcohol and
8
9 acetic acid, 3:1) for 24 hours, immediately for chromosome aberrations or after 48 hours' recovery
10
11 time in mineral water for micronuclei analysis (Ma *et al.*, 1995). After fixation, the roots were
12
13 transferred to 70% ethyl alcohol and stored in a refrigerator until examined. The root tips were
14
15 placed in 1N HCl at 60°C for 10 minutes and stained with Feulgen reagent for 1 hour. The
16
17 microscopic analysis included mitotic index and the recording of micronuclei in interphase cells and
18
19 of aberrant cells in anaphase-telophase cells. For each extract, the mitotic index was determined by
20
21 examining 5000 cells from 5 separate slides and calculated as mitotic cells per 1000 cells. The
22
23 micronuclei were scored in 4000 cells per onion from 4 separate slides and expressed as
24
25 micronuclei/1000 cells (data were reported as mean \pm standard deviation). Analysis of variance
26
27 (ANOVA) and Dunnett's *t* test were used to determine the significance of the difference between
28
29 the samples and negative control at the 5% significance level.
30

31
32 For the *Allium cepa* aberration test, bridges, fragments, laggards, c-mitosis and multipolar
33
34 anaphases were scored. For each slide, 40 cells in anaphase-telophase were examined, and with 5 of
35
36 the onions 800 cells per concentration were scored (data were expressed as percentages on 800 cells
37
38 at least). Statistical analysis was performed by means of a test for the linear trend and the Fisher
39
40 test.
41

42
43 Each plant test had a negative control with natural mineral water and DMSO 1% and a positive
44
45 control with potassium dichromate ($K_2Cr_2O_7$, 1.8 μ l/ml) for the *Allium cepa* micronuclei test and
46
47 sodium azide (NaN_3 , 0.3 μ g/ml) for the aberration test with a 48-hours exposure.
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Results

Chemical analyses of the pesticides

The chemical analyses enabled us to identify pesticides with a wide-polarity range in the samples of the vegetables and grapes examined (Table I). The pesticides found in the extracts consisted of various chemical compounds: carbamates (carbaryl, propamocarb), organophosphate compounds (acephate, chlorpyrifos, ethopropos, dimethoate, parathion-methyl, omethoate, azinphos-methyl), benzonitriles (chlorothalonil), amides (iprodione, vinclozolin), acylalanines (methalaxyl), phthalates (folpet) and pyrethroids (fluvalinate, cypermethrin).

[insert Table I here]

Eleven out of twenty-one (52.2%) vegetable samples did not comply with the current law on pesticide residues; eight (38.1%) contained concentrations of pesticides over the MLRs, one (4.8%) contained pesticides not allowed for the type of crop, and one (4.8%) was in breach of the law for both reasons.

Five out of eight grape samples (62.5%) contained concentrations of pesticides over the MLRs.

Only two samples (beet and pepper) contained no pesticide residues.

Allium cepa tests

Table II shows the micronuclei frequencies ($\% \pm SD$) in *Allium cepa* root tip cells after 48 hours of exposure to three doses of vegetable and grape extracts (225 mg/L, 75 mg/L and 25 mg/L) versus a negative control. Only two vegetable samples (9.5%) were negative in the *Allium cepa* micronuclei test and were concurrently not contaminated by pesticides, whereas nineteen vegetable samples (90.5%) were positive in this test. 73.7% of the vegetable samples showed a positive response at all three doses, 15.8% showed a positive response at high and medium doses, and 10.5% were positive only at highest doses.

[insert Table II here]

1 All the grape samples gave positive results in the micronuclei test at all three tested doses at
2
3 the 1% significance level ($p < 0.01$).

4
5 Tables IIIa, IIIb and IIIc show the frequency of aberrant cells (%) and the mitotic index in
6
7 *Allium cepa* root tip cells exposed for 48 hours to the vegetable and grape extracts (225 mg/L, 75
8
9 mg/L, 25 mg/L) versus the negative control.

10
11 [insert Table IIIa here]

12
13 According to the Fisher test, seven vegetable samples (33.3%) were positive using the
14
15 chromosomal aberration test: two were positive at all tested doses, one at high and medium doses,
16
17 and four at the highest dose only (Tables IIIa and IIIb).

18
19 [insert Table IIIb here]

20
21 About the grapes only one was not able to induce chromosomal aberrations in root cells of
22
23 *Allium cepa*. Seven grape samples (87.5%) gave positive results inducing an increase in the
24
25 frequency of aberrations: one at all tested doses, three at high and medium tested doses, and three at
26
27 the highest dose (Table IIIc).

28
29 [insert Table IIIc here]

30
31 In the linear trend analysis, five vegetable samples (sample 8 at $p < 0.001$, samples 5 and 17
32
33 at $p < 0.01$ and samples 4 and 6 at $p < 0.05$) and only one grape sample (sample 2 at $p < 0.05$) gave a
34
35 positive response, showing a clear dose-response relation.

36 37 **Discussion and conclusions**

38
39 The presence of pesticides and genotoxic compounds extracted from 21 treated vegetables and 8
40
41 grape samples bought in the markets of Campania, a region in Southern Italy, was assessed by
42
43 means of chemical and genotoxicological analysis. Gas chromatography and HPLC analysis
44
45 showed the presence of many pesticides in the vegetable and grape extracts. Sometimes the
46
47 pesticide residues exceeded the maximum concentrations allowed by Italian law. Many vegetable
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49

1 extracts showed chromosomal aberrations and/or micronuclei formation in *Allium cepa* roots,
2
3 presumably due to the presence of pesticide residue and/or other unknown components.
4
5

6
7 As previously reported, many pesticides are effective clastogens in plant cells. *The Allium cepa*
8
9 tests have been successfully carried out in previous research for *in situ* monitoring water samples.
10
11 This is the first application of the *Allium cepa* tests in food extracts, and the results suggest their
12
13 applicability for this purpose, especially when using the micronucleus test in interphase cells, which
14
15 gave much higher mutagenicity than the chromosomal aberration test in anaphase-telophase cells.
16
17 Micronucleus frequency was much higher compared to the negative control, with a dose-response
18
19 relation in some cases. The lowest dose of extracts (25 mg/L) induced an increase of micronuclei in
20
21 most of the vegetable samples and in all the grape samples. Fewer samples showed chromosomal
22
23 aberrations. Some positive samples (vegetable samples 4, 5, 6, 8 and 17 and grape sample 2)
24
25 showed a dose-response relationship. The only two vegetable samples resulted without pesticide
26
27 residues have not induced increase of micronuclei frequency and chromosomal aberrations too
28
29 (samples 1 and 14).
30
31

32 The higher sensitivity of the *Allium cepa* micronuclei test compared to the chromosomal aberration
33
34 test in *Allium cepa* can be explained by the fact that chromosomal aberrations are more closely
35
36 associated with DNA damage (bridges, laggards), whereas the induction of micronuclei in root
37
38 meristems of *Allium cepa* is the manifestation of chromosome damage and disturbance of the
39
40 mitotic process. The micronucleus is formed by a new membrane developing around the chromatin
41
42 matter that failed to move to either pole during the anaphase of mitosis. Such chromatin matter
43
44 arises either from anomalous disjunction of chromosomes due to spindle abnormalities or from
45
46 breakage of chromosomes resulting in the formation of acentric fragments, dicentric chromosomes
47
48 and chromatin bridges. Therefore, the induction of micronuclei suggests that the pesticide residues
49
50 in the vegetables may have been either spindle inhibitors or clastogens. Recently a research has
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52

1 shown in about 7,000 subjects that an increase of micronucleus frequency in lymphocytes predicts
2
3 the risk of carcinogenesis in human (Bonassi et al., in press).
4

5
6 The micronuclei test in *Allium cepa* was positive for both the vegetable and grape extracts
7
8 contaminated by pesticides outlawed in Italy and for samples contaminated by legal pesticides in
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10 Italy. This agrees with the hypothesis of potential synergistic activity between the components of a
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12 chemical mixture used in agriculture. The only two negative vegetable samples were not
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14 contaminated by pesticides. This is in agreement with considerations by Ennever et al. (1988), who
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16 describe the *Allium* test as one of the most sensitive tests as it produces low percentages of false
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18 negatives. Like all other tests on plants, it seems to be less specific because it gives a high degree of
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20 false positives. With reference to the significance of micronuclei test in plants for human health
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22 hazards it is recognized that if a chemical compound causes damage to chromosomes in plant cells,
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24 it can be potentially dangerous also for the chromosomes of mammalian cells.
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27
28 The genotoxic effects detected may be the result of the synergistic activity of the different pesticide
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30 residues present in the vegetable and grape extracts, even if many natural substances in plant
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32 material might be genotoxic (Ames et al., 1987). In this study unwashed vegetables and grapes were
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34 used, but it would be useful to test thoroughly washed vegetables and grapes to evaluate if the
35
36 genotoxicity is really related to pesticide residues in food. In fact washing processes remove a large
37
38 amount of residues, and ensure a large margin of safety (Jayakrishnan et al., 2005; Radwan et al.,
39
40 2005; Lentza-Rizos et al., 2006).
41

42
43 Moreover gas-chromatography and HPLC analyses looked for only some pesticides in vegetables
44
45 and grapes, usually utilized in agricultural practice, and this fact can explain the variability of the
46
47 results because other compounds not considered here could be present in the extracts which were
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49 tested.
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3 The *Allium cepa* test appears to be a fast, inexpensive and easy to handle method, and, as other
4 short-term mutagenicity tests, is particularly suitable to analyze complex mixtures of contaminants
5 and to reveal synergistic effects. For these reasons this chemical/biological approach permits to
6 study different but complementary aspects of food contamination and could be helpful to better
7 evaluate cancer hazards related to diet.
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11

12 13 14 **Acknowledgements**

15
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Table I. Pesticides found in the vegetable and grape samples.

Sample no	Vegetables	Pesticides (mg kg ⁻¹) ^a
1	Beet	nd
2	Beet	Endosulfan (0.87); Methalaxyl (1.02); Deltamethrin (0.12)
3	Cucumber	Dichloran (0.6); Procymidone (0.02); Folpet (0.07)
4	Lettuce	Dichloran (0.1); Endosulfan (0.03); Propamocarb (3.69); Chlorothalonil (0.12)
5	Lettuce	Dichloran (0.04); Procymidone (0.01); Vinclozolin (0.02); λ-Cyhalothrin (0.04)
6	Lettuce	Dichloran (0.02); Endosulfan (0.04); Propamocarb (12.6)
7	Roman lettuce	Procymidone (0.04); Delthamethrin (0.01); Endosulfan (0.08); Chlorothalonil (0.44)
8	Roman lettuce	Dichloran (0.24); Dichlofluanid (0.03); Iprodione (2.97); Chlorothalonil (0.08); Bifenthrin (1.10)
9	Roman Lettuce	Propamocarb (5.93); Iprodione (4.83); Cymoxanil (5.69)
10	Round aubergine	Dichloran (0.01); Iprodione (0.11); Chlozolinat (0.01)
11	Round aubergine	Dichloran (0.12); Procymidone (0.03); Ethoprophos (1.35)
12	Round aubergine	Dichloran (0.02); Iprodione (0.73); Azinphos-methyl (0.04); Acrinathrin (0.05)
13	Pepper	Acephate (0.85); Chlorothalonil (0.09)
14	Pepper	Nd
15	Pepper	Fenarimol (0.01); Fluvalinate (0.02); Chlorothalonil (0.10)
16	Sweet pepper	Procymidone (0.40); Ethoprophos (0.64)
17	Sweet pepper	Dimethoate (0.17); Acephate (15.3); Omethoate (0.71); λ-Cyhalothrin (0.11)
18	Sweet pepper	Endosulfan (0.07); Iprodione (1.16)
19	Cherry tomato	Chlorothalonil (0.04); Dichlofluanid (0.02); Procymidone (0.02)
20	Tomato	Dichloran (0.09); Procymidone (0.03); Vinclozolin (0.09)
21	Tomato	Procymidone (0.16); Iprodione (1.51); Fenarimol (0.10); Fluvalinate (0.41)

Sample No	Grapes	Pesticides (mg kg ⁻¹)
1	Italian grapes	Dimethoate (0.10); Vinclozolin (0.92); Procymidone (1.44); Fenarimol (0.03); Hexaconazole (0.12); Myclobutanil (0.27)
2	Italian grapes	Parathion-ethyl (0.06); Folpet (0.10); Procymidone (0.44); Cypermethrin (0.04)
3	Italian grapes	Procymidone (0.03); Endosulfan (0.07)
4	Italian grapes	Parathion-methyl (0.16); Procymidone (1.58); Endosulfan (0.24); Bromopropylate (0.03); Iprodione (7.55); Pyrimethanil (3.10); Fenithrothion (0.75)
5	Italian grapes	Parathion-ethyl (0.16); Folpet (1.10); Procymidone (0.17); Acephate (0.04); Myclobutanil (0.56)
6	Italian grapes	Parathion-ethyl (0.11); Folpet (1.03); Procymidone (0.14); Acephate (0.05); Myclobutanil (0.49)
7	Italian grapes	Pyrimethanil (0.30); Parathion-ethyl (0.05); Procymidone (0.25); Bromopropylate (0.22); Omethoate (0.26)
8	Palierian grapes	Omethoate (0.19); Carbaryl (0.57); Parathion-ethyl (0.07); Iprodione (0.44); Procymidone (0.92); Chlorpyrifos-ethyl (0.23)

^aThe values in bold are the residues found in concentrations exceeding the maximum values permitted by Italian law or the pesticides not allowed for that food product under the same law (99/65/EC and 99/71/EC)

nd = not detected

Table II. Mean frequency ($\% \pm \text{SD}$) of micronuclei in *Allium cepa* root tips exposed to pesticide-treated vegetables and grapes for 48 hours.

Samples	Negative control	Micronucleus frequency ($\% \pm \text{SD}$)		
		225 mg/L	75 mg/L	25 mg/L
Vegetables				
1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2	0.0 ± 0.0	3.9 ± 1.1**	2.6 ± 0.6**	1.5 ± 0.5**
3	0.2 ± 0.7	2.6 ± 1.2**	2.6 ± 1.2**	0.8 ± 0.7
4	0.1 ± 0.2	3.7 ± 0.9**	3.3 ± 1.2**	2.2 ± 1.4**
5	0.1 ± 0.3	4.4 ± 1.1**	3.5 ± 0.9**	1.9 ± 0.7**
6	0.5 ± 0.7	4.6 ± 3.8**	3.5 ± 1.4**	1.0 ± 0.9
7	0.0 ± 0.0	4.2 ± 2.0**	0.4 ± 0.6	0.3 ± 0.6
8	0.2 ± 0.4	2.6 ± 2.5**	0.7 ± 1.0	0.4 ± 0.7
9	0.2 ± 0.4	4.2 ± 2.2**	2.6 ± 1.4**	1.7 ± 1.4**
10	0.2 ± 0.4	5.4 ± 0.9**	4.2 ± 1.4**	2.3 ± 0.7**
11	0.5 ± 0.6	4.3 ± 1.8**	1.9 ± 0.9**	1.3 ± 0.7
12	0.0 ± 0.0	3.8 ± 0.7**	2.7 ± 0.9**	1.5 ± 0.6**
13	0.6 ± 0.6	4.7 ± 2.1**	4.1 ± 0.7**	2.7 ± 0.9**
14	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
15	0.2 ± 0.7	5.1 ± 1.2**	2.7 ± 0.9**	1.9 ± 0.9**
16	0.2 ± 0.7	3.8 ± 0.9**	3.7 ± 1.2**	2.6 ± 1.2**
17	0.1 ± 0.2	4.6 ± 0.8**	3.8 ± 0.8**	1.5 ± 0.6**
18	0.1 ± 0.2	7.4 ± 1.4**	4.3 ± 0.8**	2.3 ± 1.0**
19	0.1 ± 0.2	4.0 ± 0.7**	4.1 ± 1.0**	4.0 ± 0.7**
20	0.3 ± 0.6	5.8 ± 0.9**	3.6 ± 0.8**	1.8 ± 0.7**
21	0.2 ± 0.4	5.5 ± 0.9**	4.4 ± 1.2**	1.6 ± 0.7**
Grapes				
1	0.0 ± 0.0	4.0 ± 0.8**	3.8 ± 0.6**	1.4 ± 0.5**
2	0.0 ± 0.0	4.0 ± 0.6**	2.3 ± 0.9**	1.5 ± 0.5**
3	0.0 ± 0.0	4.3 ± 0.7**	2.2 ± 0.8**	1.5 ± 0.5**
4	0.0 ± 0.0	4.4 ± 0.7**	3.4 ± 0.5**	2.2 ± 0.8**
5	0.0 ± 0.0	4.7 ± 0.7**	3.5 ± 0.5**	1.1 ± 0.7**
6	0.0 ± 0.0	3.2 ± 0.8**	2.6 ± 0.7**	1.4 ± 0.5**
7	0.0 ± 0.0	3.5 ± 0.6**	3.5 ± 0.7**	1.1 ± 0.8**
8	0.0 ± 0.0	4.2 ± 0.7**	3.1 ± 0.7**	1.5 ± 0.6**

** Statistically significant versus negative control according to Dunnett's *t* test ($p < 0.01$)
 Positive control ($\text{K}_2\text{Cr}_2\text{O}_7$, 1.8 $\mu\text{g/ml}$): 6.3 ± 1.5%

Table IIIa. Mitotic index and anaphase aberrations in *Allium cepa* roots exposed to the vegetable extracts (samples 1-10) for 48 hours.

Samples	Negative control (%)		Doses (mg/L)	Mitotic index (%)	Aberrant cells ^a (%)	Statistical tests	
	Mitotic index (%)	Aberrant cells (%)				Fisher's exact test	Linear trend
1	21.6	0.0	225	20.2	0.0	ns	ns
			75	20.8	0.0	ns	
			25	20.8	0.0	ns	
2	21.6	0.0	225	20.8	0.6	ns	ns
			75	20.4	0.6	ns	
			25	20.8	0.4	ns	
3	21.6	0.0	225	14.2	0.0	ns	ns
			75	15.6	0.0	ns	
			25	15.2	0.0	ns	
4	21.4	0.0	225	15.4	0.8	*	*
			75	13.8	0.6	ns	
			25	13.3	0.0	ns	
5	21.4	0.0	225	13.0	0.6	ns	**
			75	23.0	0.0	ns	
			25	13.7	0.0	ns	
6	19.7	0.0	225	18.2	2.5	***	*
			75	19.8	1.4	***	
			25	20.6	1.0	**	
7	19.7	0.0	225	14.8	1.1	**	ns
			75	17.0	0.8	*	
			25	14.6	0.8	*	
8	19.7	0.0	225	16.6	1.4	***	***
			75	16.8	0.5	ns	
			25	16.0	0.0	ns	
9	19.7	0.0	225	16.6	0.1	ns	ns
			75	16.9	0.0	ns	
			25	16.4	0.0	ns	
10	20.8	0.0	225	15.8	0.6	ns	ns
			75	16.0	0.5	ns	
			25	15.8	0.3	ns	

*Statistically significant versus negative control according to Fisher's exact and linear trend tests ($p < 0.05$)

**Statistically significant versus negative control according to Fisher's exact and linear trend tests ($p < 0.01$)

***Statistically significant versus negative control according to Fisher's exact and linear trend tests ($p < 0.001$)

ns = not significant

^aPositive control (NaN₃, 0.3 µg/ml): 5.5%

Table IIIb. Mitotic index and anaphase aberrations in *Allium cepa* roots exposed to the vegetable extracts (samples 11-21) for 48 hours.

Samples	Negative control (%)		Doses (mg/L)	Mitotic index (%)	Aberrant cells ^a (%)	Statistical tests	
	Mitotic index (%)	Aberrant cells (%)				Fisher's exact test	Linear trend
11	20.8	0.0	225	15.8	0.0	ns	ns
			75	15.6	0.0	ns	
			25	16.6	0.0	ns	
12	20.8	0.0	225	20.0	0.6	ns	ns
			75	20.0	0.5	ns	
			25	19.8	0.4	ns	
13	20.8	0.0	225	17.2	1.1	**	ns
			75	18.4	0.4	ns	
			25	18.4	0.5	ns	
14	20.8	0.0	225	19.4	0.0	ns	ns
			75	19.6	0.0	ns	
			25	19.4	0.0	ns	
15	20.8	0.0	225	15.8	0.0	ns	ns
			75	14.0	0.0	ns	
			25	15.6	0.0	ns	
16	22.0	0.0	225	18.2	0.0	ns	ns
			75	17.0	0.0	ns	
			25	18.2	0.0	ns	
17	22.0	0.0	225	16.0	0.9	*	**
			75	13.9	0.4	ns	
			25	16.0	0.0	ns	
18	22.0	0.0	225	17.0	0.0	ns	ns
			75	16.2	0.0	ns	
			25	15.6	0.0	ns	
19	22.8	0.0	225	11.3	0.4	ns	ns
			75	13.2	0.3	ns	
			25	11.6	0.0	ns	
20	22.8	0.0	225	16.4	1.3	*	ns
			75	16.3	0.8	*	
			25	13.8	0.5	ns	
21	22.8	0.0	225	20.2	0.5	ns	ns
			75	21.0	0.5	ns	
			25	21.0	0.1	ns	

*Statistically significant versus negative control according to Fisher's exact and linear trend tests ($p < 0.05$)

**Statistically significant versus negative control according to Fisher's exact and linear trend tests ($p < 0.01$)

***Statistically significant versus negative control according to Fisher's exact and linear trend tests ($p < 0.001$)

ns = not significant

^aPositive control (NaN₃, 0.3 µg/ml): 5.5%

Table IIIc. Mitotic index and anaphase aberrations in *Allium cepa* roots exposed to the grape extracts for 48 hours.

Samples	Negative control (%)		Doses (mg/L)	Mitotic index (%)	Aberrant cells ^a (%)	Statistical tests	
	Mitotic index (%)	Aberrant cells (%)				Fisher's exact test	Linear trend
1	20.6	0.0	225	20.4	0.6	ns	ns
			75	20.8	0.6	ns	
			25	20.8	0.5	ns	
2	20.6	0.0	225	21.6	0.8	*	*
			75	20.1	0.8	*	
			25	20.8	0.0	ns	
3	20.6	0.0	225	21.0	0.8	*	ns
			75	21.2	0.4	ns	
			25	20.5	0.3	ns	
4	20.6	0.0	225	20.2	1.0	**	ns
			75	19.6	0.8	*	
			25	19.6	0.8	*	
5	21.0	0.0	225	21.2	1.0	**	ns
			75	21.6	0.5	ns	
			25	19.6	0.5	ns	
6	21.0	0.0	225	21.0	0.8	*	ns
			75	20.8	0.6	ns	
			25	22.0	0.4	ns	
7	21.0	0.0	225	19.3	1.4	***	ns
			75	20.2	1.0	**	
			25	18.4	0.6	ns	
8	21.0	0.0	225	20.0	0.8	*	ns
			75	21.0	0.9	*	
			25	20.2	0.4	ns	

*Statistically significant versus negative control according to Fisher's exact and linear trend tests ($p < 0.05$)

**Statistically significant versus negative control according to Fisher's exact and linear trend tests ($p < 0.01$)

***Statistically significant versus negative control according to Fisher's exact and linear trend tests ($p < 0.001$)

ns = not significant

^aPositive control (NaN_3 , 0.3 $\mu\text{g/ml}$): 5.5%