Food and Chemical Toxicology 48 (2010) 120–128

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Genotoxicity studies of organically grown broccoli (*Brassica oleracea* var. italica) and its interactions with urethane, methyl methanesulfonate and 4-nitroquinoline-1-oxide genotoxicity in the wing spot test of *Drosophila melanogaster*

María Eugenia Heres-Pulido *, Irma Dueñas-García, Laura Castañeda-Partida, Luis Felipe Santos-Cruz, Viridiana Vega-Contreras, Rosa Rebollar-Vega, Juan Carlos Gómez-Luna, Ángel Durán-Díaz

Genetic Toxicology, Biology, FES Iztacala, Universidad Nacional Autónoma de México, 54090 Tlalnepantla, Estado de México, Mexico

ARTICLE INFO

Article history:
Received 25 March 2009
Accepted 22 September 2009

Keywords:
Ethyl carbamate
Synergy
Vitamin C
Modulation
SMART
Pesticides
Broccoli

ABSTRACT

Broccoli (*Brassica oleracea* var. italica) has been defined as a cancer preventive food. Nevertheless, broccoli contains potentially genotoxic compounds as well. We performed the wing spot test of *Drosophila melanogaster* in treatments with organically grown broccoli (OGB) and co-treatments with the promutagen urethane (URE), the direct alkylating agent methyl methanesulfonate (MMS) and the carcinogen 4-nitroquinoline-1-oxide (4-NQO) in the standard (ST) and high bioactivation (HB) crosses with inducible and high levels of cytochrome *P450s* (CYPs), respectively. Larvae of both crosses were chronically fed with OGB or fresh market broccoli (FMB) as a non-organically grown control, added with solvents or mutagens solutions. In both crosses, the OGB added with Tween–ethanol yielded the expected reduction in the genotoxicity spontaneous rate. OGB co-treatments did not affect the URE effect, MMS showed synergy and 4-NQO damage was modulated in both crosses. In contrast, FMB controls produced damage increase; co-treatments modulated URE genotoxicity, diminished MMS damage, and did not change the 4-NQO damage. The high dietary consumption of both types of broccoli and its protective effects in *D. melanogaster* are discussed.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

High dietary intakes of the *Brassicacea* family vegetables, like broccoli (*Brassica oleracea* var. italica) have been related to the reduction of certain types of cancer because they seem to possess cancer chemopreventive properties (Conaway et al., 2000) related to the effect of their constituents (Zhu and Loft, 2003) such as their water-soluble antioxidants (Podsędek, 2007). The anticarcinogenic effects of *Brassica* vegetables seem to depend on the biological species (Traka et al., 2008), the test system, the doses, the timing of the treatment, the target tissue, the genotoxic or anticarcinogenic compounds and many other factors (Verhoeven et al., 1997). Broccoli is chemically defined as a complex mixture containing among other compounds (Podsędek, 2007): carotenoids, phenols, vitamins C > E, Fe and glucosinolates (β-thioglucoside *N*-hydroxysulfates) (GLSs) which are isothiocyanates (ITCs) precursors, nitriles, epithionitriles and indoles (Hayes et al., 2008). Some of the GSLs are glucoraphanin (GRP), precursor of isothiocyanate sulforaphane (SF); sinigrin, precursor of allyl isothiocyanate (AITC); gluconasturtin, precursor of phenethyl isothiocyanate (PEITC) and glucobrassicin, precursor of indole-3-carbinol (I3C), that may react with ascorbic acid and generate indol ascorbigen, an inductor of cytochromes CYP1A1 and CYP1A2 enzymes (Stephensen et al., 2000). All the GSLs are hydrolyzed by myrosinase enzyme (β-thioglucoside glycohydrolase [TGG]; EC 3.2.3.1) when the vegetable is fragmented or chewed raw or cooked (Verhoeven et al., 1997). The action of the TGG is diminished because it is denatured during cooking or blanching prior to freezing (Conaway et al., 2000) or by other multiple factors (Rungapamestry et al., 2008) but ITCs

Abbreviations: AITC, allyl isothiocyanate; CYPs, cytochromes *P450*; GRP, glucoraphanin; GSH, glutathione; GSLs, glucosinolates (β-thioglucoside *N*-hydroxysulfates); GST, glutathione *S*-transferases; ITCs, isothiocyanates; I3C, indole-3-carbinol; MMS, methylmethanesulfonate; FMB, fresh market broccoli; NQO1, NAD(P)H: quinone oxidoreductase; 4-NQO, 4-nitroquinoline-1-oxide; OGB, organically grown broccoli; PEITC, phenethyl isothiocyanate; ROS, reactive oxygen species; SF, sulforaphane; TGG, myrosinase; Tw–EtOH, Tween® 80–ethanol 5%; UGT, UDP-glucuronosyltransferases; URE, urethane; VitC, vitamin C; XM, xenobiotic metabolism.

* Corresponding author. Address: Laboratorio de Genetica Toxicologica, FES Iztacala, Universidad Nacional Autónoma de México, Av. Los Barrios 1, 54090 Tlalnepantla, Estado de México, Mexico. Tel./fax: +52 55 56231197.
E-mail addresses: heres@campus.iztacala.unam.mx, meheres@hotmail.com (M.E. Heres-Pulido).

0278-6915/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2009.09.027

can be produced from the GSLs by the action of microbial enzymes in the gut (Rouzaud et al., 2003).

The protective action by GSLs and ITCs is related to their modulation of phase I (Zhou et al., 2007) and phase II (Juge et al., 2007) of the xenobiotic metabolism (XM). Phase I oxidases are cytochromes *P450s* (CYPs); phase II enzymes [*e.i.* UDP-glucuronosyltransferases (UGT; EC 2.4.1.17), glutathione *S*-transferases (GSTs; EC 2.5.1.18), NAD(P)H: quinone oxidoreductase (NQO1; EC 1.6.99.2)] catalyze conjugation reactions for the detoxification of electrophiles (Kensler, 1997). Nevertheless, some ITCs have shown potential genotoxicity in bacteria and mammalian cells (Verhoeven et al., 1997). The GRP can induce the phase I carcinogen-activating enzymes, generate oxidative stress and DNA damage (Paolini et al., 2004). It has also been demonstrated that AITC and PEITC are genotoxic (Kassie and Knasmuller, 2000) and PEITC is also clastogenic (Musk et al., 1995). CYP3A4 is responsible for the human hepatic and intestinal metabolism of protoxicants, pharmaceutical compounds and endogenous sterols (Zhou et al., 2007). *Drosophila melanogaster* has been shown to have CYPs similar to those found in the S9 fraction of mammalian liver (Hällström et al., 1984). Furthermore, Danielson et al. (1998) have proved that the CYP6 family of *D. melanogaster* shows strong regional homologies with the CYP3 family of vertebrates which, along with the CYP2 family are responsible for drug metabolism in vertebrates (Thomas, 2007). Finally, Saner et al. (1996) and Dunkov et al. (1997) functionally characterized the CYP6A2 gene of *D. melanogaster* responsible for XM in HB strains. Therefore, we would expect a CYPs metabolism of the broccoli constituents similar to that of human organs (Yang et al., 2007). It has also been demonstrated that SF inhibits CYP3A4 in human primary hepatocytes (Zhou et al., 2007) and induces phase II of the XM in *Drosophila* (Trinh et al., 2008) and in the human jejunum *in vivo* (Petri et al., 2003) by a process that implies the activation of the Nrf2/KEAP complex and also induces apoptosis (Gingras et al., 2004) and cell cycle arrest (Juge et al., 2007).

SMART is a sensitive *in vivo* assay that can determine genotoxicity of chemical agents by loss of heterozygosity of the two cell markers *multiple wing hairs* (*mwh*) and *flare* (*flr*) in chromosome 3 of wing imaginal disk cells of larvae fed with such compounds. This loss of heterozygosity produces single *mwh* spots (small and large) by point mutation, deletion, exchange between markers, or by non-disjunction. Small spots can originate by two possible mechanisms: (i) segmental aneuploidy, which results from non-disjunction and produces a reduced proliferation capacity (Frei et al., 1992) or (ii) late damage by the secondary metabolites of the parental compound (Graf and Singer, 1992). Large spots are produced by early damage in the wing imaginal disk cells of larvae and continuous mitosis of cells expressing the markers. Recombination proximal to the centromere, which involves exchange of the chromosomal fragment containing both markers, leads to the expression of the *mwh* and *flr* markers in adjacent cells as twin spots. The wing spot test uses the standard (ST) cross with regulated levels of CYPs (Graf et al., 1989) and the high bioactivation (HB) cross (Graf and van Schaik, 1992). The latter cross is highly sensitive to procarcinogens and promutagens because the strain *ORR*; *flr^3/In(3LR)TM3, ri ppsep 1(3) 89Aa bx34ee Bd^S* carries chromosomes 1 and 2 from a DDT-resistant Oregon R(R) line, which is characterized by high levels of CYPs.

In order to contribute to the elucidation of the processes that confer broccoli characteristics (Verhoeven et al., 1997; Jeffery and Keck, 2008) and confirm in this model the protective effects of chronic feeding with broccoli, we conducted experiments to screen for broccoli genotoxicity and/or compound interaction in the ST and HB crosses of the *Drosophila* wing spot test (SMART). We used lyophilized broccoli chronic treatments, and co-treatments with three well known genotoxic compounds that have different mechanisms of action: ethyl carbamate or urethane (URE), a promutagen that does require biotransformation by CYPs to produce DNA adducts (Guengerich et al., 1991); the direct alkylant methyl methane sulfonate (MMS) that does not require biotransformation to produce damage to DNA (Jenkins et al., 2005); 4-nitroquinoline 1-oxide (4-NQO), a pluripotent carcinogen that increases radical oxygen species (ROS), is a UV-mimetic agent (Kohda et al., 1991) and produces purine adducts by CYPs metabolism (Mirzayans et al., 1999).

2. Materials and methods

2.1. Chemical compounds and media

Ethyl carbamate or urethane (URE, CAS 51-79-6, purity 99%), methyl methanesulfonate (MMS, CAS 66-27-3, purity 98%) and Tween® 80 (CAS 9005-65-6) were purchased from Sigma–Aldrich (St. Luis, MO, USA); ethanol (analytical grade) was obtained from Merck (Darmstadt, Germany); 4-nitroquinoline-1-oxide (4-NQO, CAS 56-57-5, purity ⩾97.0%, HPLC) was purchased from Sigma–Fluka (St. Luis, MO, USA). To avoid toxicity and selection of resistant flies in the co-treatments with 4-NQO, MMS and URE, we proposed the convenience that experimental concentrations should be close or below the LC_{50} values reported in Dueñas-García et al. (2005): 4-NQO did not show statistically significant differences between LC_{50}, strain (*flare* and Oregon-*flare*) and sensitivity (origin ordinate) ($p > 0.05$). On the other hand, significant differences were found for potency (lineal slope) ($p < 0.05$) between strains showing that mortality increases in the *flare* strain ($y = -1.4214x^2 + 22.894x + 8.9515$) with a potency higher than that in the Oregon-*flare* strain ($y = -0.7772x^2 + 15.897x + 8.8812$). The polynomial curves and toxicity parameters determined for promutagen URE showed no significant differences between strains. The parameters values for the *flare* ($y = 0.0817x^2 - 0.4974x - 2.0582$) and Oregon-*flare* ($y = 0.0794x^2 - 0.5555x + 0.4124$) strains were similar. The LC_{50} values were slightly below the concentration of 30 mM reported as toxic by Frölich and Würgler (1990) for *D. melanogaster* larvae from the HB and ST crosses of the wing spot test fed with URE for 48 h. All these data were taken into consideration to use the 4-NQO and URE LC_{20}. Mortality percentages of all three mutagens fitted a polynomial dose–response relationship. The LC_{50} for MMS was 0.90 mM in the Oregon-*flare* strain ($y = -4.2431x^2 + 38.871x + 19.168$) and 0.87 mM in the *flare* strain ($y = 6.3138x^2 + 52.338x + 10.768$). One-way ANOVA results showed no significant differences between LC_{50}, strain, potency and sensitivity ($p > 0.05$). However, we found a great dispersion in both strains and in each one of the three independent experiments for the lower concentrations. It is noteworthy that regardless this dispersion the LC_{50} values were similar in both strains. In order to obtain a slightly less dispersion we increased the MMS concentration to LC_{40}, unfortunately toxicity increased. All solutions were prepared just before use; we used distilled water as solvent for URE and MMS; 4-NQO was dissolved in Tween® 80–ethanol 5% (Tw–EtOH). Drosophila Instant Medium (Formula 4-24) (DIM) was purchased from Carolina Biological Supply (Burlington, North Carolina, USA).

2.2. Broccoli

Only organically grown vegetables have data on the crop origin. The organically grown broccoli (OGB) floret/stem was cultivated according to the Oregon Tilh Certification (OTCO, 2009). It was kindly donated by Mar Bran Co. which is located in Irapuato (State of Guanajuato, Mexico). After hand made harvest and transportation it was cooled at 5–8 °C for no more than 12 h; cut in big fragments, blanched at 153 °C for 2 min 30 s to inactivate enzyme systems (Podsędek, 2007) and fast cooled in water (10 °C), frozen at −17 to −30 °C, stored at −30 °C, packaged later in a polymer-bag and stored at −3 °C until lyophilization. The freezing and packing of the OGB followed the NOM-Codex Stan 110-1981 and the NOM 081 FITA (NOM = Mexican Official Norm). MarBran Co. determined the following nutrient values (wet weight): water (90.72 g), protein (3.10 g), total lipid (0.11 g), carbohydrate, by difference (5.35 g), fiber, total dietary (3.00 g), iron, Fe (0.61 mg), vitamin C, total ascorbic acid (40.10 mg) and vitamin E (α-tocopherol) (1.32 mg) and these are similar to those provided by USDA SR-21 for broccoli (CND, 2009). Broccoli's nutrition values can vary between batches, localities, the part of the vegetable and type of process (NutritionData.com, 2009). Other components, such as selenium, glucosinolates, isothiocyanates, phenolic acids and vitamins vary depending on the ground (Jeffery, 2005), selenium fertilization, organic farming and water stress (Robbins et al., 2005) or on the conditions of packing (in stock market of plastic or without it), storage (temperature), transport, mechanical stress, cuts and sale. We used OGB to avoid the presence of pesticides residues and control the deterioration of some components, such as GSLs and vitamins, due to transport at room temperature, the mechanical damage and the dehydration. We used two samples of OGB (each poly-bag containing 500 g of frozen broccoli florets and stems) and mixed it before and after lyophilize it. As a non-organically broccoli control, 1 kg of fresh tightly packed florets and stems, deep green in color (FMB) was purchased

from a supermarket in Mexico City (Mexico). We do not know the FMB origin since the supermarket employees could not give us precise information on that. It was grown in Mexico because it did not have a tag indicating it was imported from any other country. Guanajuato is the main broccoli-producing state in Mexico (60%) followed by Michoacan (8%), Sonora (6.4%), Jalisco (5.2%), Puebla (4.6%) and Baja California (4.1%). Other 14 states grow broccoli too with a production below 2.7%. Farmers that follow international guidelines to grow organic broccoli export a part of the production and sell the other to local supermarkets and consumers (Fundacion Guanajuato Produce AC, 2003). Farmers that follow the Guide of Pesticides (2007) sell the production in the local market. FMB was stored in a misted cabinet display (18 ± 2 °C); we do not know when it was harvested but broccoli remains green up to two weeks after harvest. Broccoli stored at 5 °C can have a useful life of 14 days but only of 5 days if stored at 10 °C. Therefore, we deduce it had been harvested 5–14 days before we bought it. In the same way regular consumers do, FMB was transported to the laboratory without temperature control and stored (~4 °C) until lyophilization. We mixed all the FMB before and after lyophilization. The frozen OGB floret/stem or the raw FMB were cut in smaller pieces, lyophilized in vacuum 10^{-3} mbar [LabConco, Freeze Dry System/Freezore 4.5] during 24–72 h and −50 °C. After freeze drying they were separately pulverized in a food processor, and stored in a fresh and dry site by ~15 days until use. The same sample of lyophilized OGB or FMB was used throughout the study.

2.3. Somatic mutation and recombination test (SMART)

2.3.1. Strains and crosses

The standard (ST) and high bioactivation (HB) crosses of the wing spot test uses two markers: *multiple wing hairs* (*mwh*, 3–0.3) and *flare*^{3} (*flr*^{3}, 3–38.8) located in the left arm of chromosome 3. For the ST cross, virgin females of the strain *flr*^{3}/*In(3LR)TM3, ri* p^p *sep* bx^{34e} e^s Bd^S were mated to *mwh/mwh* males (Graf et al., 1989). For the HB cross, virgin females of the strain *Oregon-flare* (*ORR(1)*; *ORR(2)*; *flr*^{3}/*In(3LR)TM3, ri* p^p *sep* bx^{34e} e^s Bd^S) were mated to *mwh/mwh* males (Graf and van Schaik, 1992). Originally, the *D. melanogaster* stocks were kindly donated by Dr. Ulrich Graf of the ETH, from the University of Zurich, Switzerland. Two types of progeny are produced in both crosses, which differ phenotypically based on the Bd^S marker: (i) MH, marker-heterozygous flies (*mwh*+/+*flr*^{3} or *ORR*; *mwh*+/+*flr*^{3}): wild-type wings; (ii) BH, balancer-heterozygous flies (*mwh*+/*TM3*, Bd^S or *ORR*; *mwh*+/*TM3*, Bd^S): serrate wings. Eggs from the ST and HB crosses were collected at 25 °C, dark conditions and 60–80% humidity for 8 h in glass bottles containing a thick layer of fermenting live baker's yeast supplemented with sucrose. Three days later, the larvae (72 ± 4 h) were washed out of the bottles with tap water (25 °C) through a fine meshed stainless steel strainer.

2.3.2. Chronic treatments and co-treatments

For chronic treatments (~48 h until pupation) equal batches of larvae from the ST or the HB crosses were added to glass vials containing 0.5 g of OGB (0%, 25%, 50% and 100% w/DIM w) with 2 mL of distilled water or Tw–EtOH. We inferred that the three concentrations could represent the daily intake (100%), 3.5 times a week intake (50%) and at least 1.75 times a week intake (25%). FMB (0% and 100%) controls were done with water or Tw–EtOH. Chronic co-treatments were separately prepared with larvae from both crosses and 0.5 g of OGB (0%, 25%, 50% and 100% w/DIM w) or FMB (0% and 100% w/DIM w) plus 2 mL of fresh solutions of URE (20 mM), MMS (0.5 mM), or 4-NQO (2 mM). We used distilled water and Tw–EtOH as negative controls. All treatments were tested by triplicates in three independent experiments. The pooled results are shown in Tables 1, 2 and 4.

2.3.3. Wing spot analysis

After treatments, the emerging flies were collected and stored in 70% ethanol. Wings from MH flies (*mwh*+/+*flr*^{3} or *ORR*; *mwh*+/+*flr*^{3}) of both sexes were mounted on slides and both surfaces of the wings were analyzed microscopically (Graf et al., 1984) at 40× magnification; more than 55 flies were scored for each treatment whenever possible, because of toxicity, in order to get the optimal sample size and avoid inconclusive results (Frei and Würgler, 1995).

2.3.4. Data and statistical analysis

For the statistical assessment of genotoxicity, the frequencies of each type of spot per fly were compared pair wise with the corresponding solvent or mutagen control (Frei and Würgler, 1988, 1995). The proportion of somatic recombination against mutation was calculate based on the clone-size correction of clone induction frequency (per 10^{-5} cells per cell division) by regression curves and interpolation of the dose–response relationships obtained for the two types of wings: MH and BH (Rodriguez-Arnaiz et al., 1996). Based on the spot frequencies the percentages of inhibition or induction were calculated for the different categories of wing spots (Abraham, 1994).

Table 1
Summary of significant treatments results obtained in marker-heterozygous wings (MH) of flies, from the *Drosophila* wing spot test. Methyl methanesulfonate (MMS, 0.5 mM), organically grown broccoli (OGB), fresh market broccoli (FMB) and urethane (URE, 20 mM) added with water.

Compound cross[b]	Conc. (%)	Number of flies	Spots per fly (number of spots) statistical diagnosis[a]			
			Small single spots (1–2 cells) $m = 2$	Large single spots (>2 cells) $m = 5$	Twin spots $m = 5$	Total spots $m = 2$
Organically grown broccoli (OGB)						
ST						
MMS–OGB	0	60	6.78(407)	5.38(323)	3.65(219)	15.82(949)
MMS–OGB	25	60	23.68(1421)+	22.43(1346)+	4.75(285)+	50.87(3052)+
MMS–OGB	50	60	24.33(1460)+	24.75(1485)+	6.33(380)+	55.42(3325)+
MMS–OGB	100	60	23.73(1424)+	21.52(1291)+	4.27(256)+	49.52(2971)+
HB						
MMS–OGB	0	60	9.15(549)	8.88(533)	3.72(223)	21.75(1305)
MMS–OGB	25	60	13.58(815)+	15.37(922)+	5.33(320)+	34.28(2057)+
MMS–OGB	50	60	14.25(855)+	18.07(1084)+	4.87(292)+	37.18(2231)+
MMS–OGB	100	60	18.68(1121)+	19.50(1170)+	1.22(73)+	39.40(2364)+
Fresh market broccoli (FMB)						
ST						
Water	0	60	0.40(28)	0.05(3)	0.00(0)	0.52(31)
FMB	100	57	0.70(40)+	0.12(7)–	0.02(1)–	0.84(48)+
URE–FMB	0	58	2.43(141)	1.17(68)	0.17(10)	3.78(219)
URE–FMB	100	14	5.29(74)+	1.07(15)–	0.00(0)–	6.36(89)+
MMS–FMB	0	55	34.40(1892)	34.56(1901)	1.82(100)	70.78(3893)
MMS–FMB	100	55	30.04(1652)–	23.16(1274)↓	3.35(184)–	56.55(3110)–
HB						
Water	0	60	0.63(38)	0.22(13)	0.00(0)	0.85(51)
FMB	100	57	1.44(82)+	0.16(9)–	0.00(0)–	1.60(91)+
URE–FMB	0	68	4.46(303)	2.18(148)	0.15(10)	6.78(461)
URE–FMB	100	64	5.47(350)–	0.52(33)↓	0.00(0)–	5.98(383)–
MMS–FMB	0	11	15.55(171)	30.09(331)	0.00(0)	45.64(502)
MMS–FMB	100	35	12.34(432)–	19.69(689)↓	0.00(0)–	32.03(1121)↓

[a] Statistical diagnoses according to Frei and Würgler (1988, 1995). m: minimal risk multiplication factor for the assessment of negative results.
[b] ST, standard cross; HB, high bioactivation cross. For the final statistical diagnoses of all outcomes: positive (+) and negative (−) with the standard SMART software based in the conditional binomial test according to Kastenbaum–Bowman significance levels ($\alpha = \beta = 0.05$) (Frei and Würgler, 1988). The non-parametric Mann–Whitney and Wilcoxon U-test with significance levels ($\alpha = \beta = 0.05$, one-sided) was used in order to exclude false positive or negative diagnoses (Frei and Würgler, 1995). ↓ values statistically ($p < 0.05$) below the control.

Table 2
Summary of significant treatments results obtained in marker-heterozygous wings (MH) of flies, from the *Drosophila* wing spot test. Organically grown broccoli (OGB), 4-nitroquinoline-1-oxide (4-NQO, 2 mM) and fresh market broccoli (FMB) added with Tween–ethanol (5%).

Compound cross[b]	Conc. (%)	Number of flies	Spots per fly (number of spots) statistical diagnosis[a]			
			Small single spots (1–2 cells) $m = 2$	Large single spots (>2 cells) $m = 5$	Twin spots $m = 5$	Total spots $m = 2$
Organically grown broccoli (OGB)						
ST						
Tw–EtOH	5	60	0.28(17)	0.10(6)	0.05(3)	0.43(26)
OGB	100	59	0.17(10)↓	0.03(2)–	0.00(0)–	0.20(12)↓
4-NQO–OGB	0	60	0.40(24)	0.08(5)	0.02(1)	0.50(30)
4-NQO–OGB	50	60	0.58(35)+	0.20(12)–	0.08(5)–	0.87(52)+
4-NQO–OGB	100	60	0.80(48)+	0.22(13)–	0.02(1)–	1.03(62)+
HB						
Tw–EtOH	5	60	0.62(37)	0.33(20)	0.08(5)	1.03(62)
OGB	100	59	0.14(8)↓	0.03(2)↓	0.00(0)↓	0.17(10)↓
4-NQO–OGB	0	60	0.70(42)	0.20(12)	0.05(3)	0.95(57)
4-NQO–OGB	100	60	0.23(14)↓	0.17(10)–	0.03(2)–	0.43(26)↓
Fresh market broccoli (FMB)						
ST						
Tw–EtOH	5	60	0.27(16)	0.08(5)	0.00(0)	0.35(21)
FMB	100	55	1.85(102)+	0.29(16)+	0.00(0)–	2.15(118)+
HB						
Tw–EtOH	5	50	0.38(19)	0.08(4)	0.00(0)	0.46(23)
FMB	100	52	1.56(81)+	0.35(18)–	0.00(0)–	1.90(99)+

[a] Statistical diagnoses according to Frei and Würgler (1988, 1995). *m*: minimal risk multiplication factor for the assessment of negative results.
[b] ST, standard cross; HB, high bioactivation cross. For the final statistical diagnoses of all outcomes: positive (+) and negative (–) with the standard SMART software based in the conditional binomial test according to Kastenbaum–Bowman significance levels ($\alpha = \beta = 0.05$) (Frei and Würgler, 1988). The non-parametric Mann–Whitney and Wilcoxon *U*-test with significance levels ($\alpha = \beta = 0.05$, one-sided) was used in order to exclude false positive or negative diagnoses (Frei and Würgler, 1995). ↓ values statistically ($p < 0.05$) below the control.

3. Results

In this work we tested for effects of broccoli in the *Drosophila* wing spot test using the following strategies: (i) chronic treatments: three concentrations of OGB, and one of FMB as non-organically grown control, looking for a possible change in the rate of spontaneous mutation of the markers *mwh* and flr^3 in the ST and HB crosses with inducible and high levels of CYPs, respectively; and (ii) chronic co-treatments: larvae from both crosses fed with lyophilized OGB or FMB added with URE, MMS or 4-NQO fresh solutions in order to observe possible broccoli interactions (Tables 1 and 2). The inhibition and induction percentages for each compound and every type of spot were done (Table 3). Recombination percentages were calculated only for MMS–OGB co-treatments because they showed positive differences between twin spots and control (Table 4). They were not calculated for URE and 4-NQO treatments because when the frequencies of induced twin spots per fly obtained in the MH wings are rather low, it is very difficult to determine these frequencies in the BH wings in a precise manner, as the frequencies will never be higher.

3.1. Broccoli

In both crosses the OGB treatment added with water did not change the spontaneous mutation and recombination rate (data not shown). When Tw–EtOH was added, an inhibition in small and total spots was observed in the ST cross and in all types of spots in the HB cross (Table 2). On the contrary, feeding of larvae with FMB added with water or Tw–EtOH produced an induction in genotoxicity (Tables 1 and 2). In the HB cross, the FMB added with water yielded two fold the frequencies in the small and total spots than in the ST cross (Table 1); the FMB with Tw–EtOH yielded an increase in small, large and total spots in the ST cross, and in small and total spots in the HB cross (Table 2). Estimation of the inhibition and induction percentages exemplifies this (Table 3).

3.2. URE

The URE–OGB co-treatments did not change the spontaneous spot frequencies. The URE–FMB co-treatments yielded an increase of small and total spots in the ST cross, but the HB cross showed a decrease of large spots (data not shown). Inhibition or induction results were not consistent for the URE co-treatments (Table 3).

3.3. MMS

The MMS–OGB co-treatments gave total frequencies ~3.3-fold in the ST cross and ~1.7-fold in the HB cross (Table 1) with high induction values in both crosses (Table 3). Spot frequencies seem different between both crosses, but the *U*-test (data not shown) proved they are statistically similar, therefore as expected the DNA damage was direct and was not modulated by the XM. We obtained synergy in the MMS–OGB co-treatments that showed a polynomial dose–response (data not shown) in the clone-size correction of clone induction frequencies (Table 4); with these data the proportion of somatic recombination versus mutation was calculated for the two types of wings: MH and BH, yielding 22% of recombination in the ST cross and 34% of recombination in the HB cross (Table 4). The MMS–FMB co-treatments yielded a decrease of large spots in the ST cross and of large and total spots in the HB cross (Table 1) that are represented in the inhibition values (Table 3).

3.4. 4-NQO

In the case of 4-NQO–OGB co-treatments, the OGB [50%, 100%] yielded increases in small and total spots and a slight dose–response in the ST cross (Table 2). In the HB cross, frequencies

Table 3
Percentages of inhibition (values underlined) or induction (values not underlined) observed for the different categories of wing spots after administration of broccoli or co-administration with mutagens.

Compound Cross[a]	Conc. (%)	Small single spots	Large single spots	Twin spots	Total spots
Organically grown broccoli (OGB) + water					
ST					
OGB	100	10.5	0.0	0.0	8.3
URE–OGB	25	54.1	63.0	15.2	53.9
URE–OGB	50	19.3	45.9	81.8	02.3
URE–OGB	100	34.5	79.3	118.2	49.5
MMS–OGB	25	249.3	316.9	30.1	221.6
MMS–OGB	50	258.8	360.0	73.4	250.3
MMS–OGB	100	250.0	300.0	17.0	213.0
HB					
OGB	100	9.5	22.2	100.0	13.0
URE–OGB	25	17.8	0.6	34.6	15.1
URE–OGB	50	37.8	1.2	17.8	25.2
URE–OGB	100	00.5	19.0	24.3	6.4
MMS–OGB	25	48.4	73.1	43.3	57.6
MMS–OGB	50	55.7	103.5	30.9	70.9
MMS–OGB	100	104.2	119.6	67.2	81.1
Fresh market broccoli (FMB) + water					
ST					
FMB	100	75.0	140.0	–	61.5
URE–FMB	100	117.7	8.5	100.0	68.3
MMS–FMB	100	12.7	33.0	84.1	20.1
HB					
FMB	100	128.6	27.3	0.0	88.2
URE–FMB	100	22.6	76.1	100.0	11.8
MMS–FMB	100	20.6	34.6	0.0	29.8
Organically grown broccoli (OGB) + Tw–EtOH (5%)					
ST					
OGB	100	39.3	70.0	100.0	53.5
4-NQO–OGB	25	20.0	62.5	100.0	10.0
4-NQO–OGB	50	45.0	150.0	300.0	74.0
4-NQO–OGB	100	100.0	175.0	0.0	106.0
HB					
OGB	100	77.4	90.9	100.0	83.5
4-NQO–OGB	25	57.1	60.0	60.0	57.9
4-NQO–OGB	50	28.6	15.0	140.0	10.5
4-NQO–OGB	100	67.1	15.0	40.0	54.7
Fresh market broccoli (FMB) + Tw–EtOH (5%)					
ST					
FMB	100	585.2	262.5	0.0	514.3
4-NQO–FMB	100	52.0	07.7	100.0	31.9
HB					
FMB	100	310.5	337.5	0.0	313.0
4-NQO–FMB	100	45.2	24.2	0.0	9.4

Based on the spot frequencies obtained in the wing spot test, the percentage of inhibition (values underlined) or induction (values not underlined) was calculated as follows: (genotoxin alone − broccoli and genotoxin/genotoxin alone) × 100 (Abraham, 1994).
[a] ST, standard cross; HB, high bioactivation cross.

showed a tendency to decrease with a dose–response effect inverse to that in the ST cross, but there are only significant differences for small single spots in the 4-NQO–OGB-100% treatment. Spot frequencies in co-treatments 4-NQO + FMB [0%, 100%] in the ST cross and HB crosses, did not show differences between them. Inhibition events were more frequent in the 4-NQO–OGB co-treatments in the HB cross (Table 3).

4. Discussion

4.1. Broccoli

In both crosses, the OGB added with water gave negative results; we propose that this could occur because only the polar components of broccoli were accessible in the culture media. The OGB added with Tw–EtOH yielded a clear reduction in spots frequencies. We infer this solution produced cell membrane lysis which led to a higher release of OGB constituents; GSLs could have been partially hydrolyzed by plant myrosinase residues or by yeasts in the DIM (Brabban and Edwards, 1995) and yielded the expected reduction in the spontaneous genotoxicity rate. The highest inhibition percentage was observed in all types of spots of OGB added with Tw–EtOH in the following order for total spots: OGB–HB/Tw–EtOH > OGB–ST/Tw–EtOH > OGB–HB/water (Table 3); this supports the hypothesis that OGB's non-polar compounds release contributed to the antigenotoxic effect. On the contrary, in both crosses the FMB treatments added with water or Tw–EtOH, produced an increase in the total spots frequency; these results must be related with the non-organic culture, usually with pesticides,

Table 4
Summary of results obtained in marker-heterozygous wings (MH) and balancer-heterozygous wings (BH) of flies, from the *Drosophila* wing spot test, treated with organically grown broccoli (OGB) and methyl methanesulfonate (MMS, 0.5 mM) added with water.

Compound cross[b]	Conc. (%)	Number of flies	Spots per fly (number of spots) statistical diagnosis[a]					Mean number of cell division cycles	Clone induction frequency of per **10^5 cells per cell division[c,d]	
			Small single spots (1–2 cells) $m = 2$	Large single spots (>2 cells) $m = 5$	Twin spots[e] $m = 5$	Total spots $m = 2$	Spots with *mwh* clones		Without size correction (n/NC)	With clone-size correction
Organically grown broccoli (OGB)										
ST MH										
MMS–OGB	0	60	6.78(407)	5.38(323)	3.65(219)	15.82(949)	730	2.60	24.93[0.00]	37.89[0.00]
MMS–OGB	25	60	23.68(1421)+	22.43(1346)+	4.75(285)+	50.87(3052)+	2253	2.40	76.94[52.01]	101.74[63.85]
MMS–OGB	50	60	24.33(1460)+	24.75(1485)+	6.33(380)+	55.42(3325)+	2370	2.45	80.94[56.01]	110.33[72.44]
MMS–OGB	100	60	23.73(1424)+	21.52(1291)+	4.27(256)+	49.52(2971)+	1987	2.36	67.86[42.93]	87.14[49.25]
ST BH										
MMS–OGB	0	25	1.92(48)	0.20(5)		2.12(53)	49	1.39	4.01[0.00]	2.62[0.00]
MMS–OGB	25	11	11.00(121)+	13.27(146)+		24.27(267)+	264	2.72	54.15[50.14]	83.01[80.39]
MMS–OGB	50	23	15.43(355)+	5.57(128)+		21.00(483)+	482	1.88	42.94[38.93]	39.39[36.77]
MMS–OGB	100	22	7.05(155)+	1.32(29)+		8.36(184)+	170	1.59	15.83[07.91]	11.90[09.28]
HB MH										
MMS–OGB	0	60	9.15(549)	8.88(533)	3.72(223)	21.75(1305)	1172	2.76	40.02[0.00]	67.63[0.00]
MMS–OGB	25	60	13.58(815)+	15.37(922)+	5.33(320)+	34.28(2057)+	1502	2.57	51.30[11.28]	76.15[8.52]
MMS–OGB	50	60	14.25(855)+	18.07(1084)+	4.87(292)+	37.18(2231)+	1396	2.49	47.68[7.66]	67.02[−0.61]
MMS–OGB	100	60	18.68(1121)+	19.50(1170)+	1.22(73)+	39.40(2364)+	1662	2.40	56.76[16.74]	74.96[07.33]
HB BH										
MMS–OGB	0	20	0.90(18)	0.55(11)		1.45(29)	29	2.62	2.97[0.00]	4.57[0.00]
MMS–OGB	25	25	8.84(221)+	1.76(44)+		10.60(265)+	256	1.58	20.98[18.01]	15.66[11.09]
MMS–OGB	50	24	5.62(135)+	3.04(73)+		8.67(208)+	206	2.18	17.59[14.62]	19.92[15.35]
MMS–OGB	100	22	2.82(62)+	0.77(17)+		3.59(79)+	62	1.48	5.77[2.80]	4.04[−0.53]

[a] Statistical diagnoses according to Frei and Würgler (1988, 1995). *m*: minimal risk multiplication factor for the assessment of negative results.
[b] ST, standard cross; HB, high bioactivation cross.
[c] Clone frequencies per fly divided by the number of cells examined per fly (48,800) gives an estimate of formation frequencies per cell and per cell division in chronic exposure experiments (Frei and Würgler, 1995).
[d] Values in brackets are control corrected.
[e] Only *mwh* single spots can be recovered in *mwh*/*TM3* balancer-heterozygotes as *TM3* does not carry flr^3; for the final statistical diagnoses of all outcomes: positive (+) and negative (−) with the standard SMART software based in the conditional binomial test according to Kastenbaum–Bowman significance levels ($\alpha = \beta = 0.05$) (Frei and Würgler, 1988); the non-parametric Mann–Whitney and Wilcoxon *U*-test with significance levels ($\alpha = \beta = 0.05$, one-sided) was used in order to exclude false positive or negative diagnoses (Frei and Würgler, 1995).

and the release of different broccoli constituents. The highest induction percentage was observed in all types of spots of FMB with the following order for total spots: FMB–ST/Tw–EtOH > FMB–HB/Tw–EtOH > FMB–HB/water > FMB–ST/water (Table 3). Since FMB was not organically grown, it is inferred it was exposed to several compounds; also it was not frozen or packed in a plastic polymer, it was exposed to temperatures higher than −4 °C and lost humidity which could leads to a decrease in VitC content (Barth et al., 2006). Furthermore, the cutting and fragmentation of FMB by manipulation and transportation without temperature control could have activated the myrosinase residues and generate certain hydrolysis of GSLs and production of their metabolites (Hayes et al., 2008). OGB was organically grown and kept frozen until lyophilization; it did not contain pesticides and therefore its compounds presented the least changes after blanching, which could explain its effect in the reduction of spontaneous mutation and recombination rates we observed (Table 2) when Tw–EtOH was added. Opposite to this, we infer that FMB constituents must have been modified by transport and marketing activities; also it could have contained pesticide residues, as some of them have been detected by other authors in cultures of this vegetable (Pérez et al., 2009). In Mexico, the non-organically culture of broccoli for national production and consumption, implies the use of several pesticides as methyl-parathion, metamidophos, diazinon or endosulfan/methyl-parathion mixtures (Guide of Pesticides, 2007) to control the high incidence of plagues such as: *Brevicoryne brassicae*, *Trichoplusia ni*, *Copitarcia consueta*, *Artogeia rapae*, *Plasmodiophora brassicae*, *Plutella xylostella*, *Trialeurodes* spp. and *Bemisia tabaci* (Barrios et al., 2004; Fundacion Guanajuato Produce AC, 2003) which usually makes it necessary to spray the crops with pesticides 2–10 times during farming. All the pesticides authorized in the Guide of Pesticides (2007) had shown genotoxic effects *in vivo* and *in vitro* protocols (data not shown), specially methyl-parathion, endosulfan (Hreljac et al., 2008) and diazinon (Salazar-Arredondo et al., 2008); this could explain the obtained genotoxicity (Tables 1 and 2).

4.2. URE

Urethane has proved to be a clear genotoxic promutagen in the *D. melanogaster* wing spot test (Frölich and Würgler, 1990). It has been demonstrated in this bioassay the protective effect of extracted essential oils from three medicinal plants (Idaomar et al., 2002), bell pepper (*Capsicum annuum*) and black pepper (*Piper nigrum*) (El-Hamss et al., 2003). Surprisingly, the URE–OGB co-treatment did not change the spontaneous mutation and recombination frequencies in both crosses; on the contrary, URE–FMB showed a modulation response (Table 1). We propose that the URE–OGB results could be due to various hypothesis (i) the putative absence of GSLs metabolites and therefore no ITCs effects on XM in both crosses; (ii) metabolism of some ITCs and URE competed for detoxification by GSH (Kemper et al., 1995); (iii) putative ITCs inhibited one or some CYPs (Morris et al., 2004) diminishing damage by URE, but VitC high concentration showed a pro-oxidant activity (Cooke et al., 2003) that increased oxidative stress and lipids peroxidation generating etheno-adducts (Bartsch and Nair, 2000) which could contribute to the total genotoxic effect. These hypothesis are supported by Tiku et al. (2008) who fed mice with mustard leaf extract

(*Brassica campestris*) that contained GSLs and then exposed them to URE observing modulation of lipid peroxidation, an increase in GSH and GST, although they also observed a decrease in micronuclei. Modulation in URE–FMB co-treatments could be explained assuming that FMB must have some pesticides residues and low VitC concentration, as a result of marketing and transportation procedures. It is also possible that GSLs were hydrolyzed by events inherent to its marketing, causing that some of its compounds induced one or more CYPs in the ST cross, since the small spots frequency increased to values similar to those found in the HB cross (Table 1). To explain the effects that diminished early events (large spots) in the HB cross (Table 1) we could consider that URE is biotransformed in mammals by CYP2E1 and it has been reported that PEITC (Morris et al., 2004) and SF inhibit this cytochrome (McCarty, 2001). Future research is needed to determine the genotoxic interactions of URE with specific compounds present in broccoli.

4.3. MMS

We observed an unexpected synergy with MMS–OGB co-treatments that showed a polynomial dose–response caused by the lower total frequencies in MMS–OGB [100%] in the ST cross MH and BH wings and HB cross BH wings; these reductions could be related with toxicity and apoptosis induction. All results, spot frequencies and recombination percentages show this synergy is not related to CYPs levels, meaning that broccoli compounds that caused synergy do not require biotransformation. In most events, the MMS–OGB synergy is represented by a higher induction (Table 3) in the ST cross than the HB cross, although when statistically compared, the frequencies between both crosses showed no significant differences. Nivard et al. (1992) treated *D. melanogaster* with MMS and showed that uncompleted excision repair caused a higher chance for induction of a deletion. They proposed that at higher damage the repair systems fails to fully remove all lesions due to saturation of enzymatic activity resulting in a genotoxicity increase. The observed synergy with lyophilized OGB could be explained by high VitC + Fe [~401 mg + ~6.1 mg Fe/100 g dry weight] concentrations, which could be pro-oxidant and modulate DNA repair. Cooke et al. (2003) showed the *in vivo* generation of glyoxal (gdC), after the supplementation of volunteers with 400 mg/d VitC, and the reduction in gdC levels correlated with the up regulation of nucleotide excision repair (NER) enzymes. Also, the unrepaired or misrepaired DNA damage by inefficient base excision repair (BER) and NER mechanisms increase free radicals and reactive oxygen species (ROS) in yeast (Salmon et al., 2004). On the other hand, interaction between VitC and MMS or Fe has shown modulation in DNA damage: Franke et al. (2005a) treated mice orally with MMS and orange juice; they conclude that in pre-treatment, VitC could have competed as target site for alkylation; in post-treatment it could have influenced the kinetics of repair. Also, Franke et al. (2005b) applied a single treatment with VitC after administration of ferrous sulfate or MMS; VitC enhanced DNA damage caused by the ferrous sulfate, and double treatment with VitC (at 0 and 24 h) induced a cumulative genotoxic response more intense for the higher dose of MMS. On the other hand, Rowe et al. (2008) found that a major activator of the oxidative stress response, Yap1, relocalizes to the nucleus following exposure to MMS. Considering MMS mainly produces N7G and N3A adducts, which in *D. melanogaster* are repaired by glycosylases independent DNA repair systems (Dusenbery and Smith, 1996) and that there is a putative oxidant effect derived from MMS damage (Rowe et al., 2008) we propose that the increase in the spots/fly frequencies found in MMS–OGB co-treatments could have been mainly produced by the lyophilized OGB, which with a pro-oxidant activity could stimulate the repair pathway, inducing a high number of unrepaired AP sites, and an increase in DNA damage. Decrease in MMS–FMB co-treatment results could occur because this control sample was not frozen or packed with polymeric film after the harvest, and was transported at uncontrolled temperature (Podsędek, 2007), therefore, VitC concentration in the FMB must be lower than in the OGB and it would let other broccoli compounds, such as SF, diminish the spots/fly frequencies by apoptosis induction. More studies are needed to investigate VitC + Fe and the SF effect with MMS in both crosses.

4.4. 4-NQO

The mutagen 4-NQO has been studied in the wing spot test before (Heres-Pulido et al., 2004). It is a potent mutagen (Nagao and Sugimura, 1976), carcinogenic (Nakahara et al., 1957) and UV-mimetic agent that is metabolized to 4-hydroxyaminoquinoline 1-oxide (4-HAQO) that, indirectly, causes G → T and A → C sustitutions (Cheng et al., 1992). The nitroso anion radical 4-NQOQO$^{\cdot-}$ is generated by non-enzymatic reduction (Fann et al., 1999). Also, 4-NQO forms hydrogen peroxide (H_2O_2) and hydroxyl radicals that yield 8-OHdG and deplete GSH (Arima et al., 2006). It induces cell cycle arrest (G_1) to repair DNA or apoptosis by the p53-dependent mitochondrial signaling pathway (Han et al., 2007). 4-NQO reductions by microsomal NADPH–CYP reductase and cytosolic xanthine oxidase generate free radicals as superoxide radical (Fann et al., 1999); XM of 4-NQO produces acetoxy-amino-quinilone that yields purine adducts (N^2G > *C*8G > N^6A) repaired by the excision repair pathway (Mirzayans et al., 1999). Therefore, the action of 4-NQO is direct and indirect. Our results in the ST cross show that the 4-NQO–OGB [50%, 100%] co-treatments yielded an increase and a slight dose–response, but in the HB cross spot frequencies showed a tendency to decrease with a dose–response effect inverse to that in the ST cross (Table 2). The 4-NQO–OGB interactions show more inhibition events in HB than in ST (Table 3); this must be related to the higher levels of CYPs in the HB cross.

These results support some hypothesis: (i) VitC + Fe in high concentrations can have pro-oxidant effects and could modulate the NER system (Cooke et al., 2003); (ii) since 4-NQO produces oxidative stress, and 8-oxo-2′-deoxyguanosine (8-oxodG) (Cheng et al., 1992) the increase of the damage in the ST cross could be the result of the 4-NQO and VitC + Fe pro-oxidant effects, and an override of the NER system (Lunec et al., 2002). Kaya et al. (2002) performed co-treatment experiments with 4-NQO and VitC (25, 75 and 250 mM) in the ST cross. Non-antigenotoxic effect of 4-NQO damage was observed, but their results with VitC and $CoCl_2$ agree with the hypothesis that the oxidant/pro-oxidant activity of this vitamin is dependent on the presence of transition metals that could increase the production of OH$^{\cdot}$ from H_2O_2 via the Fenton reaction. We propose that results in the 4-NQO–OGB co-treatments, in both crosses, could be due to an increase of the pro-oxidant effect of VitC + Fe, besides the influence of 4-NQO which induces apoptosis (Han et al., 2007), especially in the HB cross. This explanation is supported by 4-NQO–FMB results, where no modification of 4-NQO genotoxicity was obtained. This agrees with the assumption of VitC depletion by transportation and marketing methods. Nevertheless, it is also possible that high genotoxicity exerted by putative FMB pesticides residues or GSLs metabolites could hide the 4-NQO damages modulation by other broccoli components. Several explanations can be put forward, but future investigations are necessary to definitely identify the VitC + Fe and 4-NQO effects in the *Drosophila* wing spot test. In order to answer these experimental evidences further experiments with VitC, Fe and VitC + Fe at concentrations equivalent to their content in OGB (100%) and co-treatments with different concentrations of the three mutagens must be done.

Our results confirm that broccoli effects in the eukaryote *D. melanogaster* depend on multiple events: growing (organic or

non-organic), type of solutions (polar or no polar), CYPs levels (inducible or high) and processing-marketing events. As many of these factors can not be controlled by the consumer, they should be considered to modify the general belief that is enough to eat broccoli frequently to prevent the occurrence of cancer.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We thank Dr. Ulrich Graf for critical reading of the manuscript. Also, to Fermín Vaca-Delgado and Martha Ramírez for their kind donation of the OGB and information related with its growing, harvest, conservation procedures and nutrient values. The authors wish to acknowledge the technical assistance of María del Rocío Arellano Llamas.

References

Abraham, S.K., 1994. Antigenotoxicity of coffee in the *Drosophila* assay for somatic mutation and recombination. Mutagenesis 9, 383–386.

Arima, Y., Nishigori, C., Takeuchi, T., Oka, S., Morimoto, K., Utani, A., Miyachi, Y., 2006. 4-Nitroquinoline 1-oxide forms 8-hydroxydeoxyguanosine in human fibroblasts through reactive oxygen species. Toxicol. Sci. 91, 382–392.

Barrios, D.B., Alatorre, R.R., Bautista, M.N., Calyecac, C.G., 2004. Identificación y fluctuación poblacional de plagas de col (*Brassica oleracea* var. capitata) y sus enemigos naturales en Acatzingo. Puebla. Agrociencia 38, 239–248.

Barth, M.M., Kerbel, E.L., Broussard, S., Schmidt, S.J., 2006. Modified atmosphere packaging protects market quality in broccoli spears under ambient temperature storage. J. Food Sci. 58, 1070–1072.

Bartsch, H., Nair, J., 2000. Ultrasensitive and specific detection methods for exocyclic DNA adducts: markers for lipid peroxidation and oxidative stress. Toxicology 153, 105–114.

Brabban, A.D., Edwards, C., 1995. The effects of glucosinolates and their hydrolysis products on microbial growth. J. Appl. Bacteriol. 79, 171–177.

Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S., Loeb, L.A., 1992. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G–T and A–C substitutions. J. Biol. Chem. 267, 166–172.

CND, 2009. NutritionData.com©. Condé Nast Digital 20 August 2008 (on line). <http://www.nutritiondata.com/facts/vegetables-and-vegetable-products/2361/2> (accessed 02.06.2009).

Conaway, C.C., Getahun, S.M., Liebes, L.L., Pusateri, D.J., Topham, D.K., Botero-Omary, M., Chung, F.L., 2000. Disposition of glucosinolates and sulforaphane in humans after ingestion of steamed and fresh broccoli. Nutr. Cancer 38, 168–178.

Cooke, M.S., Mistry, N., Ahmad, J., Waller, H., Langford, L., Bevan, R.J., Evans, M.D., Jones, G.D., Herbert, K.E., Griffiths, H.R., Lunec, J., 2003. Deoxycytidine glyoxal: lesion induction and evidence of repair following vitamin C supplementation *in vivo*. Free Radic. Biol. Med. 34, 218–225.

Danielson, P.B., Foster, J.L.M., McMahill, M.M., Smith, M.K., Fogleman, J.C., 1998. Induction by alkaloids and phenobarbital of family 4 cytochrome *P450s* in *Drosophila*: evidence for involvement in host plant utilization. Mol. Gen. Genet. 259, 54–59.

Dueñas-García, I.E., Vega-Contreras, V., Gómez-Luna, J.C., Santos-Cruz, L.F., Castañeda-Partida, L., Durán-Díaz, A., Heres-Pulido, M.E., 2005. Toxicity parameters of 4-nitroquinoline-1-oxide, urethane and methyl methanesulfonate in the *flare* and Oregon-*flare* strains of *Drosophila*. Dros. Inf. Serv. 88, 83–89.

Dunkov, B.C., Guzov, V.M., Mocelin, G., Shotkoski, F., Brun, A., Amichot, M., Ffrench-Constant, R.H., Feyereisen, R., 1997. The *Drosophila* cytochrome *P450* gene Cyp6a2: structure, localization, heterologous expression and induction by phenobarbital. DNA Cell Biol. 16, 1345–1356.

Dusenbery, R.L., Smith, P.D., 1996. Cellular responses to DNA damage in *Drosophila melanogaster*. Mutat. Res. 364, 133–145.

El-Hamss, R., Idaomar, M., Alonso-Moraga, A., Muñoz-Serrano, A., 2003. Antimutagenic properties of bell and black peppers. Food Chem. Toxicol. 41, 41–47.

Fann, Y.C., Metosh-Dickey, C.A., Winston, G.W., Sygula, A., Ramakrishna, R., Kadiiska, M.B., Mason, R.P., 1999. Enzymatic and nonenzymatic production of free radicals from the carcinogens 4-nitroquinoline *N*-oxide and 4-hydroxylaminoquinoline *N*-oxide. Chem. Res. Toxicol. 12, 450–458.

Franke, S.I., PrÁ, D., Erdtmann, B., Henriques, J.A., da Silva, J., 2005a. Influence of orange juice over the genotoxicity induced by alkylating agents: an *in vivo* analysis. Mutagenesis 20, 279–283.

Franke, S.I., Prá, D., da Silva, J., Erdtmann, B., Henriques, J.A., 2005b. Possible repair action of vitamin C on DNA damage induced by methyl methanesulfonate, cyclophosphamide, $FeSO_4$ and $CuSO_4$ in mouse blood cells *in vivo*. Mutat. Res. 583, 75–84.

Frei, H., Würgler, F.E., 1988. Statistical methods to decide whether mutagenicity test data from *Drosophila* assays indicate a positive, negative, or inconclusive result. Mutat. Res. 203, 297–308.

Frei, H., Würgler, F.E., 1995. Optimal experimental design and sample size for the statistical evaluation of data from somatic mutation and recombination tests (SMART) in *Drosophila*. Mutat. Res. 334, 247–258.

Frei, H., Clements, J., Howe, D., Würgler, F.E., 1992. The genotoxicity of the anti-cancer drug mitoxantrone in somatic and germ cells of *Drosophila melanogaster*. Mutat. Res. 279, 21–33.

Frölich, A., Würgler, F.E., 1990. Genotoxicity of ethyl carbamate in the *Drosophila* wing spot test: dependence on genotype-controlled metabolic capacity. Mutat. Res. 244, 201–208.

Fundacion Guanajuato Produce, AC, 2003. ITESM, Querétaro, México, May 2003 (on line). <http://www.snitt.org.mx/pdfs/demanda/brocoli.pdf>. (accessed 22.05.2009)

Gingras, D., Gendron, M., Boivin, D., Moghrabi, A., Theoret, Y., Beliveau, R., 2004. Induction of medulloblastoma cell apoptosis by sulforaphane, a dietary anticarcinogen from *Brassica* vegetables. Cancer Lett. 203, 35–43.

Graf, U., Singer, D., 1992. Genotoxicity testing of promutagens in the wing somatic mutation and recombination test in *Drosophila melanogaster*. Rev. Int. Contam. Ambient. 8, 15–27.

Graf, U., van Schaik, N., 1992. Improved high bioactivation cross for the wing somatic mutation and recombination test in *Drosophila melanogaster*. Mutat. Res. 271, 59–67.

Graf, U., Würgler, F.E., Katz, A.J., Frei, H., Juon, H., Hall, C.B., Kale, P.G., 1984. Somatic mutation and recombination test in *Drosophila melanogaster*. Environ. Mutagen. 6, 153–188.

Graf, U., Frei, H., Kägi, A., Katz, A.J., Würgler, F.E., 1989. Thirty compounds tested in the *Drosophila* wing spot test. Mutat. Res. 222, 359–373.

Guengerich, F.P., Kim, D.H., Iwasaki, M., 1991. Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. Chem. Res. Toxicol. 4, 168–179.

Guide of Pesticides, 2007. SNSICA (Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria) Dirección General de Inocuidad Agroalimentaria, Acuícola y Pesquera, Mexico, July 2007, pp. 25–37 (on line). <http://148.245.191.4/guiaplag/(S(tosky0qc04uoo1zyfevhq145))/Documentos/Hortalizas.pdf> (accessed 22.05.2009).

Hällström, I., Blanck, A., Atuma, S., 1984. Genetic variation in cytochrome *P450* and xenobiotic metabolism in *Drosophila melanogaster*. Biochem. Pharmacol. 33, 13–20.

Han, H., Pan, Q., Zhang, B., Li, J., Deng, X., Lian, Z., Li, N., 2007. 4-NQO induces apoptosis via p53-dependent mitochondrial signaling pathway. Toxicology 230, 151–163.

Hayes, J.D., Kelleher, M.O., Eggleston, I.M., 2008. The cancer chemopreventive actions of phytochemicals derived from glucosinolates. Eur. J. Nutr. 47, 73–88.

Heres-Pulido, M.E., Dueñas-García, I., Castañeda-Partida, L., Sánchez-García, A., Contreras-Sousa, M., Durán-Díaz, A., Graf, U., 2004. Genotoxicity of tamoxifen citrate and 4-nitroquinoline-1-oxide in the wing spot test of *Drosophila melanogaster*. Mutagenesis 19, 187–193.

Hreljac, I., Zajc, I., Lah, T., Filipic, M., 2008. Effects of model organophosphorous pesticides on DNA damage and proliferation of HepG2 cells. Environ. Mol. Mutagen. 49, 360–367.

Idaomar, M., El-Hamss, R., Bakkali, F., Mezzoug, N., Zhiri, A., Baudoux, D., Muñoz-Serrano, A., Liemans, V., Alonso-Moraga, A., 2002. Genotoxicity and antigenotoxicity of some essential oils evaluated by wing spot test of *Drosophila melanogaster*. Mutat. Res. 513, 61–68.

Jeffery, E., 2005. Component interactions for efficacy of functional foods. J. Nutr. 135, 1223–1225.

Jeffery, E.H., Keck, A.S., 2008. Translating knowledge generated by epidemiological and *in vitro* studies into dietary cancer prevention. Mol. Nutr. Food Res. 52, S7–S17.

Jenkins, G.J., Doak, S.H., Johnson, G.E., Quick, E., Waters, E.M., Parry, J.M., 2005. Do dose–response thresholds exist for genotoxic alkylating agents? Mutagenesis 20, 389–398.

Juge, N., Mithen, R.F., Traka, M., 2007. Molecular basis for chemoprevention by sulforaphane: a comprehensive review. Cell Mol. Life Sci. 64, 1105–1127.

Kassie, F., Knasmuller, S., 2000. Genotoxic effects of allyl isotiocyanate (AITC) and phenethyl isothiocyanate (PEITC). Chem. Biol. Interact. 127, 163–180.

Kaya, B., Creus, A., Velázquez, A., Yanikoğlu, A., Marcos, R., 2002. Genotoxicity is modulated by ascorbic acid. Studies using the wing spot test in *Drosophila*. Mutat. Res. 520, 93–101.

Kemper, R., Myers, S., Hurst, H., 1995. Detoxification of vinyl carbamate epoxide by glutathione: evidence for participation of glutathione S-transferases in metabolism of ethyl carbamate. Toxicol. Appl. Pharmacol. 135, 110–118.

Kensler, T.W., 1997. Chemoprevention by inducers of carcinogen detoxification enzymes. Environ. Health Perspect. 105, 965–970.

Kohda, K., Kawazoe, Y., Minoura, Y., Tada, M., 1991. Separation and identification of N4-(guanosin-7-yl)-4-aminoquinoline 1-oxide, a novel nucleic acid adduct of carcinogen 4-nitroquinoline 1-oxide. Carcinogenesis 12, 1523–1525.

Lunec, J., Holloway, K.A., Cooke, M.S., Faux, S., Griffiths, H.R., Evans, M.D., 2002. Urinary 8-oxo-2′-deoxyguanosine: redox regulation of DNA repair in vivo? Free Radical. Biol. Med. 33, 875–885.

McCarty, M.F., 2001. Inhibition of CYP2E1 with natural agents may be a feasible strategy for minimizing the hepatotoxicity of ethanol. Med. Hypotheses 56, 8–11.

Mirzayans, R., Bashir, S., Murray, D., Paterson, M.C., 1999. Inverse correlation between p53 protein levels and DNA repair efficiency in human fibroblast strains treated with 4-nitroquinoline 1-oxide: evidence that lesions other than DNA strand breaks trigger the p53 response. Carcinogenesis 20, 941–946.

Morris, C.R., Chen, S.C., Zhou, L., Schopfer, L.M., Ding, X., Mirvish, S.S., 2004. Inhibition by allyl sulfides and phenethyl isothiocyanate of methyl-n-pentylnitrosamine depentylation by rat esophageal microsomes, human and rat CYP2E1, and rat CYP2A3. Nutr. Cancer 48, 54–63.

Musk, S.R., Smith, T.K., Johnson, I.T., 1995. On the cytotoxicity and genotoxicity of allyl and phenethyl isothiocyanates and their parent glucosinolates sinigrin and gluconasturtiin. Mutat. Res. 348, 19–23.

Nagao, M., Sugimura, T., 1976. Molecular biology of the carcinogen, 4-nitroquinoline 1-oxide. Adv. Cancer Res. 23, 131–169.

Nakahara, W., Fukuoka, F., Sugimura, T., 1957. Carcinogenic action of 4-nitroquinoline-*N*-oxide. Gann 48, 129–137.

Nivard, M.J.M., Pastink, A., Vogel, E.W., 1992. Molecular analysis of mutations induced in the vermilion gene of *Drosophila melanogaster* by methyl methanesulfonate. Genetics 131, 673–682.

NutritionData.com, 2009. Condé Nast Digital (on line). <http://www.nutritiondata.com/foods-broccoli011000000000000000000.html> (accessed 20.02.2009).

Oregon Tilth Certified Organic (OTCO), 2009 (on line). <http://www.tilth.org/certification/certification-spotlights-folder/organic-valley> (accessed 20.02.2009).

Paolini, M., Perocco, P., Canistro, D., Valgimigli, L., Pedulli, G.F., Iori, R., Croce, C.D., Cantelli-Forti, G., Legator, M.S., Abdel-Rahman, S.Z., 2004. Induction of cytochrome *P450*, generation of oxidative stress and *in vitro* cell-transforming and DNA-damaging activities by glucoraphanin, the bioprecursor of the chemopreventive agent sulforaphane found in broccoli. Carcinogenesis 25, 61–67.

Pérez, M.A., Segura, A., García, R., Colinas, T., Pérez, M., Vázquez, A., Navarro, H., 2009. Residuos de plaguicidas organofosforados en cabezuela de brócoli (*Brassica oleracea*) determinados por cromatografía de gases. Rev. Int. Contam. Ambient. 25, 103–110.

Petri, N., Tannergren, C., Holst, B., Mellon, F.A., Bao, Y., Plumb, G.W., Bacon, J., O'Leary, K.A., Kroon, P.A., Knutson, L., Forsell, P., Eriksson, T., Lennernas, H., Williamson, G., 2003. Absorption/metabolism of sulforaphane and quercetin, and regulation of phase II, in human jejunum *in vivo*. Drug Metab. Dispos. 31, 805–813.

Podsędek, A., 2007. Natural antioxidants and antioxidant capacity of *Brassica* vegetables: a review. LWT Food Sci. Technol. 40, 1–11.

Robbins, R.J., Keck, A.S., Banuelos, G., Finley, J.W., 2005. Cultivation conditions and selenium fertilization alter the phenolic profile, glucosinolate, and sulforaphane content of broccoli. J. Med. Food 8, 204–214.

Rodriguez-Arnaiz, R., Soto, P.O., Oyarzún, J.C., Graf, U., 1996. Analysis of mitotic recombination induced by several mono- and bifunctional alkylating agents in the *Drosophila* wing-spot test. Mutat. Res. 351, 133–145.

Rouzaud, G., Rabot, S., Ratcliffe, B., Duncan, A.J., 2003. Influence of plant and bacterial myrosinase activity on the metabolic fate of glucosinolates in gnotobiotic rats. Br. J. Nutr. 90, 395–405.

Rowe, L.A., Degtyareva, N., Paul, W., Doetsch, P.W., 2008. DNA damage-induced reactive oxygen species (ROS) stress response in *Saccharomyces cerevisiae*. Free Radical. Biol. Med. 45, 1167–1177.

Rungapamestry, V., Duncan, A.J., Fuller, Z., Ratcliffe, B., 2008. Influence of blanching and freezing broccoli (*Brassica oleracea* var. italica) prior to storage and cooking on glucosinolate concentrations and myrosinase activity. Eur. Food Res. Technol. 227, 37–44.

Salazar-Arredondo, E., de Jesús Solís-Heredia, M., Rojas-García, E., Hernández-Ochoa, I., Quintanilla-Vega, B., 2008. Sperm chromatin alteration and DNA damage by methyl-parathion, chlorpyrifos and diazinon and their oxon metabolites in human spermatozoa. Reprod. Toxicol. 25, 455–460.

Salmon, T.B., Evert, B.A., Binwei Song, B., Doetsch, P.W., 2004. Biological consequences of oxidative stress-induced DNA damage in *Saccharomyces cerevisiae*. Nucleic Acids Res. 32, 3712–3723.

Saner, C., Weibel, B., Würgler, F.E., Sengstag, C., 1996. Metabolism of promutagens catalyzed by *Drosophila melanogaster* CYP6A2 enzyme in *Saccharomyces cerevisiae*. Environ. Mol. Mutagen. 27, 46–58.

Stephensen, P.U., Bonnesen, C., Schaldach, C., Andersen, O., Bjeldanes, L.F., Vang, O., 2000. *N*-Methoxyindole-3-carbinol is a more efficient inducer of cytochrome *P-450* 1A1 in cultured cells than indol-3-carbinol. Nutr. Cancer 36, 112–121.

Thomas, J.H., 2007. Rapid birth–death evolution specific to xenobiotic cytochrome *P450* genes in vertebrates. PLoS Genet. 3, e67.

Tiku, A.B., Abraham, S.K., Kale, R.K., 2008. Protective effect of the cruciferous vegetable mustard leaf (*Brassica campestris*) against *in vivo* chromosomal damage and oxidative stress induced by gamma-radiation and genotoxic chemicals. Environ. Mol. Mutagen. 49, 335–342.

Traka, M., Gasper, A.V., Melchini, A., Bacon, J.R., Needs, P.W., Frost, V., Chantry, A., Jones, A.M., Ortori, C.A., Barrett, D.A., Ball, R.Y., Mills, R.D., Mithen, R.F., 2008. Broccoli consumption interacts with GSTM1 to perturb oncogenic signaling pathways in the prostate. PLoS ONE 3, 1–14.

Trinh, K., Moore, K., Wes, P.D., Muchowski, P.J., Dey, J., Andrews, L., Pallanck, L.J., 2008. Induction of the phase II detoxification pathway suppresses neuron loss in *Drosophila* models of Parkinson's disease. J. Neurosci. 28, 465–472.

Verhoeven, D.T., Verhagen, H., Goldbohm, R.A., van den Brandt, P.A., van Poppel, G., 1997. A review of mechanisms underlying anticarcinogenicity by brassica vegetables. Chem. Biol. Interact. 103, 79–129.

Yang, J., McCart, C., Woods, D.J., Terhzaz, S., Greenwood, K.G., Ffrench-Constant, R.H., Julian, A.T., Dow, J.A.T., 2007. A *Drosophila* systems approach to xenobiotic metabolism. Physiol. Genomics 30, 223–231.

Zhou, C., Poulton, E.J., Grün, F., Bammler, T.K., Blumberg, B., Thummel, K.E., Eaton, D.L., 2007. The dietary isothiocyanate sulforaphane is an antagonist of the human steroid and xenobiotic nuclear receptor. Mol. Pharmacol. 71, 220–229.

Zhu, C.Y., Loft, S., 2003. Effect of chemopreventive compounds from *Brassica* vegetables on NAD(P)H:quinone reductase and induction of DNA strand breaks in murine hepa1c1c7 cells. Food Chem. Toxicol. 41, 455–462.