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Comet Assay to Assess the Genotoxicity of Persian Walnut (*Juglans regia* L.) Husks with Statistical Evaluation

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Abstract The aim of this study was to confirm the utility of the Comet assay as a genotoxicity screening test for evaluating the impact of walnut husk aqueous extract. Phytotoxicity assays using diluted and undiluted walnut husk aqueous extracts were performed on young roots of *Raphanus sativus* (radish), and the Comet assay was used to evaluate DNA integrity in isolated radish radicle nuclei. The results reveal a dose-dependent accumulation of DNA damage in radish radicles treated with walnut husks water extract and that the Kolmogorov-Smirnov test combined with Johnson SB distribution was the best approach for describing Comet assay data.

Keywords Persian walnut · Comet assay · Kolmogorov-Smirnov test · Genotoxicity

The genus *Juglans* includes approximately 20 species of walnut with a natural distribution range across the Northern Hemisphere and extending into South America. Some species are commercially important as the source of edible walnuts, highly prized timber, and as ornamental trees. The Persian walnut (*Juglans regia* L.) is a traditional nut grown in old world agriculture. The Persian walnut is among the earliest recorded plants suggested to engage in allelopathy, defined as any direct or indirect harmful or beneficial effect of one plant on another through the production of chemical compounds (Petriccione and Aliotta 2006; Rice 1984). The allelopathic effect of walnut trees has been known since Pliny the Elder, whose *Naturalis Historia* declared that "the shade of the walnut is heavy and even causes headaches in men and injury to anything planted in its vicinity" (Pliny, Book XVII, 18) (Pliny Secundus, pp. 1938–1963). Although Pliny was referring to the species *J. regia,* American growers agree that *J. regia* is less toxic than *J. nigra*, also known as black walnut. Recently, the green husks of the Persian walnut have been found to contain high concentrations of phenolic compounds, including chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, catechin, epicatechin, myricetin and juglone, with higher concentrations of juglone than the other phenols (Radix et al. 1998; Stampar et al. 2006). There is significant evidence that polyphenols are the major compounds responsible for walnut phytotoxicity, reducing germination rates and seedling development in cultivated plants (Bonari et al. 1993) and inhibiting the growth of soil and water microorganisms (Della Greca et al. 2004; Fiorentino et al. 2003; Isidori et al. 2004; Obied et al. 2005).

However, there are very few reports on the potential genotoxicity of the walnut, even though genotoxic damage can have long-term effects in natural ecosystems. To date, genotoxicity in plant systems has been evaluated with a wide range of assays, including the micronucleus assay (MN) (El Hajjouji et al. 2007), Ames test (Lah et al. 2008), AO/EB double staining test (Ciniglia et al. 2010), and the Single Cell Gel Electrophoresis (SCGE) also known as Comet assay (Gichner et al. 2004). Comet assay is a very sensitive and simple technique for measuring primary DNA damage events, such as single-strand and double-strand breaks, the generation of alkali-labile sites and excision repair sites and changes in chromosomal structure (Singh

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et al. 1988, Collins 2004). One advantage of this technique is that it allows the collection of data at the individual cell level, allowing better statistical analyses. Another advantage is that only small numbers of cells per sample (<10.000) are required, and its sensitivity for detecting DNA damage in any eukaryotic cell population can be analysed. The present study aims to evaluate the proficiency of the Comet assay for the rapid assessment of the genotoxicity of walnut green husks to the radish *Raphanus sativus* L.

DNA damage can be evaluated through the analysis of several parameters in the Comet assay, such as tail length, % tail DNA and tail moment, which can be calculated using commercially available and public domain software. However, the histograms representing these data rarely fit a normal distribution, limiting the utility of common parametric statistical tools (Georgieva et al. 2010, Ejchart et al. 2003; Garcia et al. 2011); therefore, the selection of statistical models appropriate for these experimental data is crucial in Comet assay studies. To this end, the present work aimed to identify a distribution that accurately fits the Comet data obtained from the walnut green husk treatment of radish radicles by comparing the obtained data with non-Gaussian distributions. The Kolmogorov-Smirnov test, which compares an empirical distribution function with a hypothesised mathematical distribution, was applied to evaluate the goodness-of-fit.

Materials and Methods

Unripened walnut fruits were collected in June 2010 from *J. regia* L. cv Sorrento cultivated in the CRA experimental field (Caserta). The trees were 10 years old and had not received phytosanitary treatments. A total of 500 g of walnut husks, detached from the nutshells, were homogenised with 500 mL distilled water (1:1 w/v) and filtered through 0, 22 μm Whatman paper. The resulting aqueous husk extract was evaluated for its genotoxic effects on *R. sativus* L. cv Precoce a punta Bianca. Radish seeds were purchased from Ingegnoli (CAT no. 1315A). Assays were performed with undiluted and diluted (1:1, 1:2 and 1:4) walnut husk aqueous extracts (WHAEs).

Radish seeds were germinated under sterile conditions on filter paper disks embedded with distilled water. When the plants had 2 true leaves, the radicles of the radish seedlings were immersed in 2 mL of a defined WHAE dilution (in plastic vials). The plants were treated for 24 h at room temperature with a 16 h photoperiod.

After WHAE treatment, the excised radicles were placed in a Petri dish on ice and dissociated with cold 400 mM Tris buffer pH 7.5. Using a fresh razor blade, the radicles were gently sliced, and the isolated nuclei were collected in the buffer. The nuclei were then embedded in a three layered microgel (on a fully frosted microscopic slide) composed of the following: (1) a bottom layer of 1 % normal melting agarose; (2) a second layer of 0.5 % low-melt agarose containing 50 μL of tested and untested nuclear suspensions; (3) an upper layer of 0.7 % low-melt agarose [16]. The slides were dipped into a lysis solution containing 300 mM NaOH, 30 mM Na_2EDTA and 0.01 % sodium dodecyl sulphate (SDS) for 1 h and then dipped in an electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA, pH > 13) for 15 min at 4°C to allow the unwinding of the DNA. Electrophoresis was conducted using the same buffer at 4°C for 20 min at 25 V and 300 mA. Then, the gels were neutralised by two treatments with 400 mM Tris buffer (pH 7.5). The DNA molecules were stained with 80 μL ethidium bromide (20 μg/mL). The DNA images were observed using an epifluorescent microscope with an excitation filter of BP546/10 nm and a barrier filter of 590 nm (Nikon Eclipse E800) and equipped with a digital camera. Tail length, tail DNA % and tail moment were measured with an automatic analysis system (TriTek CometScore version 1.0.1.1). Three slides were evaluated per treatment, and each treatment was repeated twice; 50 nuclei from each treatment were scored. The average values from each replicate were used to calculate the median tail length, tail DNA % and tail moment values for each treatment group.

The frequency distributions of the % DNA in tail were analysed using EasyFit software (version 5.3, MathWave technologies). The most likely distributions were estimated using distribution fitting to generate estimates of variation (Georgieva et al. 2010). The Kolmogorov-Smirnov (KS) goodness-of-fit test is a non-parametric test for the equality of continuous, one-dimensional probability distributions that can be used to compare a sample to a reference probability distribution (one-sample KS test) or to compare two empirical samples (two-sample KS test). This test returns a value that reflects the distance between the empirical distribution function of the sample and the cumulative distribution function of the reference distribution (or between the empirical distribution functions of two samples):

$$Fn(x) = \frac{1}{n}\sum_{i=1}^{n} I_{X(i)\le x}$$

where $I_{X(i)\le x}$ represents the indicator function, equal to 1 if $X(i) \le x$ and equal to 0 otherwise;

$$D = \max_{1\le i\le n}\left(F(x_i) - \frac{i-1}{n}, \frac{i}{n} - F(x_i)\right)$$

represents the maximum distance between the empirical distribution and the cumulative distribution function of the reference distribution.

Results and Discussion

Because investigators disagree about which parameters of the Comet assay are most useful for evaluating DNA damage, we measured tail length, tail moment and % tail DNA (Table 1; Fig. 1). In all cases, strong correlations between the DNA damage observed in the radish radicles and the concentration of walnut husk extract used were observed, regardless of whether the DNA damage was expressed as tail moment ($r^2 = 0.867$), % of tail DNA ($r^2 = 0.982$) or tail length ($r^2 = 0.987$) (Fig. 2).

As shown in the histograms in Fig. 3, the frequency distributions of the % tail DNA data were highly asymmetric in both the control and in walnut aqueous extract-treated groups, indicating that the Comet data deviated from a normal distribution. The interpretation of the parameters of the Comet assay is often controversial because of the high dispersion of the values; this could be ascribed to the status of the nuclei after isolation, to the asynchronous developmental stages of the tested populations or to distinct individual responses to the studied agent (Georgieva et al. 2010). To accurately standardise the distribution and calculate a reliable mean, we compared different distribution types to the data distribution and verified the results using the Kolmogorov-Smirnov test to evaluate the goodness-of-fit. The best-fitted non-Gaussian distribution for our WHAE-treated samples was the Johnson SB distribution, as reported in other studies (Georgieva et al. 2010). The JSB is the distribution that best describes Comet assay data, allowing the comparison of the results of different treatments to the parameters of the fitted distribution.

This paper is the first report to evaluate the genotoxicity from walnut husks. The results of the Comet assays performed here indicate that WHAE causes DNA damage in radish radicle apical cells. Radicle cells were exposed to WHAE for 24 h; after treatment, major nuclear alterations were observed in a dose-dependent manner. As shown in Fig. 4, the frequency distributions of the % tail DNA are shifted to higher values with decreasing WHAE dilutions, as expected. The observed dilution-dependent response to

Table 1 Parameters measured in the Comet assay test

Treatment	Tail DNA (%)	Tail lenght (μm)	Tail moment
Negative control	3.27 ± 2.34	3.11 ± 2.23	0.15 ± 0.18
1:4	5.15 ± 3.52	5.03 ± 3.45	0.37 ± 0.47
1:2	17.70 ± 4.99	19.47 ± 5.49	3.71 ± 2.06
1:1	40.91 ± 4.26	49.09 ± 7.12	22.48 ± 5.21
Undiluted WHAE	93.72 ± 3.29	98.41 ± 1.76	93.12 ± 3.89

Results (mean ± standard deviation) of the Comet assay on radish radicles kept for 24 h in distilled water (negative control) and in different walnut husks aqueous extract dilutions

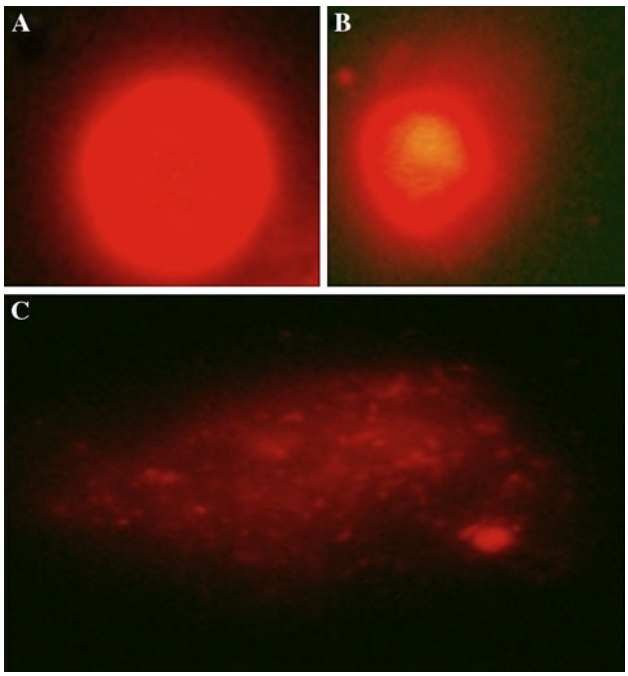

Fig. 1 Representative Comet images showing different levels of damage in radish nuclei inferred by walnut husks aqueous extracts (WHAE). **a** undamaged nuclei; **b** low-damaged nuclei with 1:4 WHAE; **c** high damaged nuclei, with undiluted WHAE

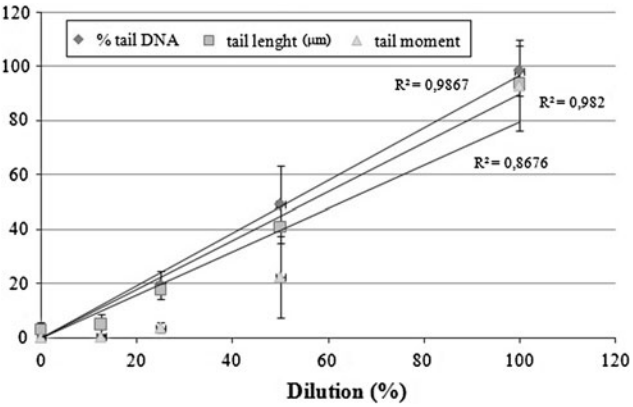

Fig. 2 Dose response curves of the DNA damage in nuclei of radish radicles as a function of walnut husks aqueous extract dilutions treatment for 24 h at room temperature. DNA damage expressed by tail moment (*filled triangle*), % tail DNA (*filled diamond*) and tail length (*filled square*). The error bars represent the standard error of the averaged medians

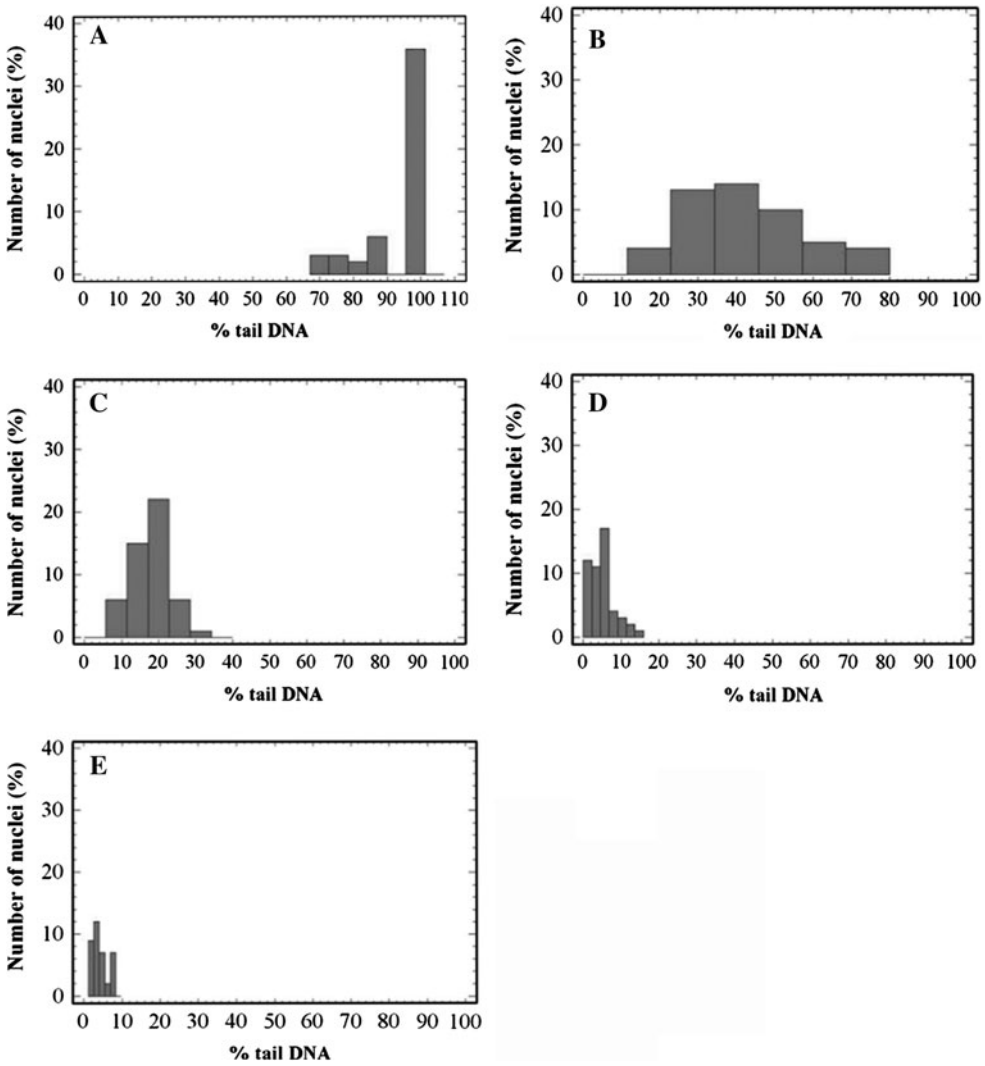

Fig. 3 Distribution of the percentage of migrated DNA among 50 nuclei of radish radicles, treated with undiluted (**a**), 1:1 (**b**), 1:2 (**c**) and 1:4 (**d**) walnut husks aqueous extracts and control (**e**)

walnut extract confirms that the Comet assay is a valuable test for the detection of genotoxicants. Although this test is applicable to all types of eukaryotic cells, it is rarely performed on plant cells due to the presence of thick cell walls, which are challenging to disrupt (Gichner 2003). Radish radicles were suitable for the assessment of genotoxicity via Comet assay because they are found in the meristematic zone; this tissue is characterised by cells with a high mitotic index, and the elongation and differentiation zones are the major targets of allelochemicals.

The Comet assay is a versatile and simple assay, and the number of publications reporting Comet assay results increases each year. However, the interpretation of the results of this assay is sometimes complex and unclear. Accurate and specific statistical analysis of the data is particularly important for this assay because the distribution of the data is strongly dependent on both the mutagenic agent and the target species.

The observations reported here showed that the radish is an ideal organism for use in the Comet assay, thus expanding the range of plant species that may be used for genotoxicity evaluations. Furthermore, the genotoxic potential of walnut extract has been confirmed by Comet assay. The standard parameters used as Comet assay results (% tail DNA, tail moment and tail length) are not normally distributed, as is frequently observed for plant Comet data; in this respect, our study confirms the necessity of the accurate and appropriate statistical evaluation of plant Comet results. The most appropriate distribution was found to be KS-JSB.

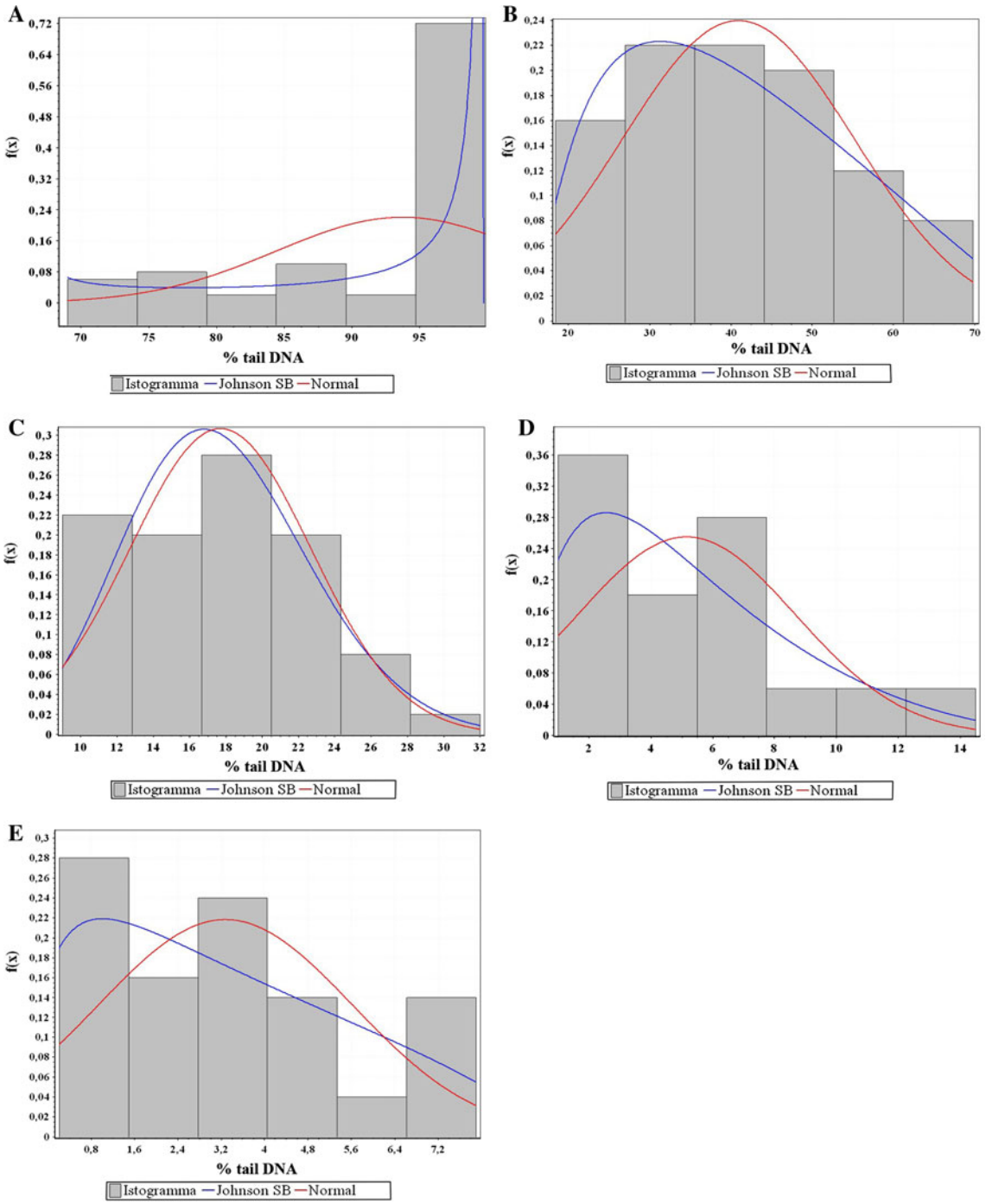

Fig. 4 Calculated distribution frequency by KS test compared to the real distribution of the observation of % DNA in tail among 50 nuclei of radish radicles treated with undiluted (**a**), 1:1 (**b**), 1:2 (**c**) and 1:4 (**d**) walnut husks aqueous extract and control (**e**). Normal distribution is presented only for comparison (*red line*) to Johnson SB distribution (*blue line*) (Color figure online)

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