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Genotoxicity studies on plant growth promoting smoke-water and smoke-derived compounds using *Vicia faba* and *Persea americana* S10 metabolic activation

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ABSTRACT

The potential agricultural or horticultural use of 'smoke technology' has received considerable attention since plant-derived smoke-water or smoke-derived compounds can play an intriguing role in seed germination and seedling vigor. However, it is of major importance to investigate whether such treatments may play a detrimental role in the genetic health of future generations. Cytotoxic and genotoxic effects of smoke-water and isolated compounds have already been tested in the Ames assay with or without mammalian metabolic activation (S9). Yet, it is known that plant metabolism of agricultural stimulants can also produce harmful substances that may be introduced into the human food chain. Therefore, possible toxic and DNA damaging effects of smoke-water and isolated compounds were tested and compared this time with or without *Vicia faba* and *Persea americana* metabolic activation (S10) in the Ames assay. Additionally, potential (geno)toxic effects were also evaluated with or without S9 metabolic activation. Results of the presented study showed no mutagenic effect for both smoke-water and smoke-derived compounds at the concentrations tested, in the absence or presence of *V. faba* and *P. americana* metabolism. Authors confirmed previous findings that the use of 'smoke technology' can, at least according to the results of this genotoxicity evaluation, be regarded as being safe for use in agriculture, horticulture and other related sectors.

1. Introduction

In recent years, the need to reduce the input of chemical fertilizers has led to more research into environmentally friendly alternatives such as agricultural biostimulants. Plant-derived smoke is one such biostimulant that has received attention over the last 20 years. Since the early 1990s, it has become apparent that smoke or smoke-derived compounds play an intriguing role in the seed germination and seedling vigor of smoke-responsive plant species (Baxter et al., 1994; Jäger et al., 1996; Sparg et al., 2006). Thus, the application of smoke-water to crops may be a cost-effective technique for improving production and may be an interesting practice for agriculture, horticulture and other related sectors (Kulkarni et al., 2010). In addition, further studies have demonstrated the germination stimulatory activity of the highly active karrikinolide (3-methyl-2*H*-furo[2,3-*c*]pyran-2-one or KAR_1, Fig. 1A), isolated from plant-derived smoke (Flematti et al., 2004; Van Staden et al., 2004). Besides active compounds, an inhibitory compound was also found to be present in plant-derived smoke. In a study of Light et al. (2010), the inhibitory effect on germination of the related compound trimethylbutenolide (3,4,5-trimethylfuran-2(5*H*)-one or TMB, Fig. 1B) was demonstrated.

For smoke-water or smoke-derived compounds to be viable for use in agricultural systems, any possible DNA damaging effects of these compounds should be considered to ensure that such treatments are not potentially harmful. There have been a few studies examining cytotoxic and genotoxic effects of smoke-water and smoke-derived compounds with strains of *Salmonella typhimurium* in the Ames test (Verschaeve et al., 2006; Trinh et al., 2010; Light et al., 2015). Furthermore, in a study by Kulkarni et al. (2010), the DNA damaging effects of smoke and isolated compounds on onion plant cells were examined. To date, investigations on smoke-water and smoke-derived compounds using the *in vitro* Ames assay or other genotoxicity tests have shown no indication of genotoxicity or altered antigenotoxicity at the levels tested (Verschaeve et al., 2006; Kulkarni et al., 2010; Trinh et al., 2010; Light et al., 2015).

Abbreviations: 2-AF, 2-aminofluorene; 4-NQO, 4-nitroquinoline-oxide; CFU, colony forming units; EDTA, ethylenediaminetetraacetic acid; EPCT, ethylpentachlorothiophene; His^+, histidine prototophic; IF, induction factor; IR, inhibition rate; KAR, karrikin; Kb, *Themeda triandra* and *Passerina vulgaris*; NPDA, 4-nitro-*o*-phenylenediamine; PVPP, polyvinylpolypyrrolidone; RC, revertant colonies; S9, supernatant fraction 9; S10, supernatant fraction 10; S117, supernatant fraction 117; SA, sodium azide; SE, standard error of the mean; SW, smoke-water; TMB, trimethylbutenolide; Tt, *Themeda triandra*.

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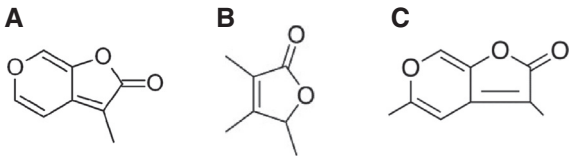

Fig. 1. Chemical structure of (A) 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (KAR_1), (B) 3,4,5-trimethylfuran-2(5*H*)-one (TMB) and (C) 3,5-methyl-2*H*-furo[2,3-*c*]pyran-2-one (KAR_3).

Plants also have metabolic systems with the capability to carry out biotransformation of chemical substances. Several studies have shown that synthetic compounds can be bioactivated by plants (Takehisa et al., 1988; Gómez-Arroyo et al., 1995; Calderón-Segura et al., 1999; Gómez-Arroyo et al., 2000; Calderón-Segura et al., 2007). Such examples of plants transforming promutagens into mutagens raise concern around the bioactivation of agricultural substances used in food crop production and the introduction of mutagens into the human food chain (Plewa and Gentile, 1982). However, the number of investigations where S10 or alternative plant biotransformation systems are used is rather limited. The S10 fraction, obtained from a plant homogenate by centrifuging at 10,000 ×*g* for 15–20 min in a suitable medium, contains enzymes specifically involved in the metabolism of xenobiotics.

A few studies have investigated the *in vitro* promutagen activation of insecticides, herbicides and pesticides using the S10 enzymatic system from the roots of *Vicia faba* L. (broad bean) (Takehisa et al., 1988; Calderón-Segura et al., 1999; Gómez-Arroyo et al., 2015). Calderón-Segura et al. (1999, 2007) investigated *V. faba* S10 activation of ametryn, metribuzin, EPTC thiocarbamate, molinate and butylate, in human lymphocyte culture. The results showed that all herbicide products required *V. faba* root metabolism to be activated to produce DNA damage in human lymphocyte culture. Similarly, Chiapella et al. (1995) demonstrated *in vitro* promutagen activation by the microsomal fraction S117 of *Persea americana* Mill. (avocado) mesocarp, obtained by ultracentrifugation at 117,000 ×*g* for 60 min.

The aim of the current study was, therefore, to provide a genotoxicity profile for plant-derived smoke-water and smoke-derived compounds after metabolization by S10 enzymatic fractions from *V. faba* and *P. americana*. The findings of this study may contribute to a better understanding of the role of plant metabolism in the activity of chemical substances. To complete previous findings, we also evaluated (geno)toxicity effects of smoke-water Kb and 3,5-dimethyl-2*H*-furo[2,3-*c*]pyran-2-one or KAR_3 (Fig. 1C) with and without S9 metabolic activation.

2. Materials and methods

2.1. Source materials

Vicia faba L. cv. Aquadulce (broad bean) seeds were obtained from McDonalds Seeds, Pietermaritzburg, South Africa. *Persea americana* Mill. cv. Hass (avocado) fruits were purchased from a local grocery store in Pietermaritzburg, South Africa.

Seeds of *V. faba* were soaked in tap-water for 1 h and rinsed. The seeds were placed on a double layer of germination paper moistened with tap-water and covered with two layers of wet germination paper. To prevent excessive dehydration, the tray was covered by a layer of heavy foil. Germinated seedlings were watered daily with 5 ml of tap-water. After 7 days at 25 ± 2 °C, seedlings with primary roots of ± 5 cm length were carefully selected and washed with distilled water. The primary root tips were cut at 2 cm length and kept on ice for preparation of the S10 fraction.

Fruits of *P. americana* were ripened at 25 ± 2 °C. For the extraction of the S10 enzymatic fraction from *P. americana*, a developmental stage of avocado fruits between unripened and fully ripened was selected based on subjective firmness determinations.

2.2. Preparation of the V. faba and P. americana S10 fraction

The S10 enzymatic fraction of *V. faba* was prepared according to the methods described in Takehisa et al. (1988), with minor changes (Calderón-Segura et al., 1999). Roots of *V. faba* were macerated and subsequently homogenized with a pre-cooled pestle and mortar in 0.1 M sodium phosphate buffer solution (pH 7.4), containing 1 mM dithiothreitol, 1 mM EDTA, and 0.6 M mannitol (Sigma-Aldrich, Co., St. Louis, MO, USA). A ratio of 1:1 of volume of buffer solution in ml to the fresh mass of root cuttings in grams was used, unless otherwise stated. Immediately before homogenization, polyvinylpolypyrrolidone (PVPP, Sigma-Aldrich, Co., St. Louis, MO, USA) was added to the buffer at a ratio of 10% to the fresh mass of root cuttings. Thereafter, the homogenate was centrifuged for 15 min at 10,000 ×*g* at 4 °C. Finally, the supernatant was filtered using a sterile 0.45 μm Millipore filter (Durapore®, Merck KGaA, Darmstadt, Germany), yielding the S10 fraction.

For the extraction of the enzymatic fraction of *P. americana*, the mesocarp was used. A similar procedure as described above was used to extract S10 from *P. americana* fruit.

2.3. Biochemical analysis

Total protein content of *V. faba* and *P. americana* S10 fractions was determined according to the method of Bradford (1976).

2.4. Preparation of test substances

Smoke-water extracts from two different sources were used in this experiment. Smoke-water Tt (*Themeda triandra*) and Kb (*T. triandra* and *Passerina vulgaris*) were prepared according to the method of Baxter et al. (1994). Different dilutions of smoke-water Tt and Kb were prepared by diluting stock solutions with sterile distilled water. Smoke-derived compounds (3-methyl-2*H*-furo[2,3-*c*]pyran-2-one or KAR_1; 3,5-dimethyl-2*H*-furo[2,3-*c*]pyran-2-one or KAR_3; and 3,4,5-trimethylfuran-2(5*H*)-one or TMB) used in this study were synthesized according to the methods outlined in Flematti et al. (2004) and Light et al. (2010). Smoke-water Tt and Kb were both tested at doses of 0.1, 1, 10 and 100 μl/plate and KAR_1, KAR_3 and TMB were tested at concentrations ranging from 1×10^{-4} M to 1×10^{-7} M (*i.e.*, 1.5 μg to 1.5 ng/plate, 1.64 μg to 1.64 ng/plate, and 1.26 μg to 1.26 ng/plate, respectively).

2.5. Mutagenicity testing

To test for mutagenicity, the Ames assay was performed using the method of plate incorporation as previously described by Maron and Ames (1983), and as modified by Mortelmans and Zeiger (2000). For this study, three *S. typhimurium* tester strains were used: TA98 (which tests for frameshift mutation), TA100 (which tests for base pair substitution) and TA102 (which tests for transitions/transversions). Prior to use, the bacterial strains were tested on the basis of associated genetic markers as defined by Maron and Ames (1983).

For each assay, overnight fresh cultures of the *S. typhimurium* tester strains were prepared from frozen stocks [100 μl of the culture to 10 ml of Nutrient Broth No. 2 (Oxoid Ltd., Hampshire, England)] and incubated in a water bath with an orbital shaker for 16 h at 37 °C to obtain a density of 2×10^9 colony forming units (CFU/ml).

For the experiments without metabolic activation, 100 μl of the sterile test solutions (smoke-water or smoke-derived compounds, and negative or positive control chemicals) were placed in a test tube and 500 μl of 0.1 M sodium phosphate buffer (pH 7.4) was added. Before addition of 100 μl overnight bacterial cultures, the tubes were allowed to stand for 3 min and 2 ml of top agar containing 0.05 mM histidine-biotin was added to the mixture. The mixture was then poured over

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