



## SELECTED NATURAL HUMIC MATERIALS INDUCE AND CHAR SUBSTRATES REPRESS A GENE IN *CAENORHABDITIS ELEGANS* HOMOLOG TO HUMAN ANTICANCER *P53*

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Received October 30, 2010; in final form January 7, 2011, accepted January 10, 2011

### ABSTRACT

Humic substances (HSs) and char substrates are major carbon compartments of contrasting origin, the latter being increasingly applied as an amendment of sandy soils. HSs are known to interact with exposed organisms and to induce transcriptionally controlled responses. Intrigued by anti-carcinogenic properties of HSs in human cell cultures, we checked whether tropical HSs from two Brazilian coastal lagoons modulate the gene (*cep-1*) in the invertebrate model *Caenorhabditis elegans*, which is a homolog to the human key anticancer gene *p53*. The modulation of this gene can also be indicative of effects caused by char substrates, because this carbon compartment originates from burning or pyrolysis and is suspected to contain non-natural xenobiotics. We tested *Terra Preta* soils from central Amazonia and a hydrothermal carbonization (HTC) product from poplar wood. HSs and char substrates modulated the *cep-1* gene, but in contrasting modes. Whereas HSs significantly induced it, the char substrates mainly repressed it. Our study begs for a more comprehensive effect evaluation when applying char substrates as soil amendments. It is recommended to include impact studies on non-target organisms.

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**Keywords:** *Caenorhabditis elegans*, transcription, char substrates, tropical humic substances, *cep-1*, *p53*, carcinogenic property

### 1. INTRODUCTION

In all ecosystems of the world, humic substances (HSs) are a major carbon compartment in the global carbon cycle [1]. Due to available low-molar mass building blocks [2], HSs, at least in part, are taken up directly or, in even larger quantities, indirectly via food by aquatic and soil organisms [3]. Once taken up, HSs are able to migrate to organs or organelles and may provoke stress response reactions [4, 5]. They have non-specific as well as specific effects. Non-specific effects are physical and chemical membrane irritation, induction and modulation of biotransformation activity, induction of chemical defense proteins (stress proteins, such as crystalline HSPs, HSP70) [6], and the development of internal oxidative stress by creating free radicals and reactive oxygen species (ROS) with subsequent lipid oxidation and induction of ROS defense enzymes [7]. Recent evidence showed that the stress response is transcriptionally regulated [4, 8]. Preliminary results with the clawed frog, *Xenopus laevis* (Daudin), hepatocytes even show that apoptotic (programmed cell death) reactions may be induced upon exposures to HSs [9]. Apoptosis may be considered as the final step in a complex cancer defense mechanism when the mutation or mutated cell is unrepairable. Very recently apoptosis has been observed with human cells upon exposure to HSs [10–15]. These findings even attribute anti-carcinogenic properties to HSs. It is currently unclear whether or not an anti-carcinogenic property is a general property of HSs.

Besides HSs, char substrates also may play a major role for the (bio)availability of carbon in soils, particularly in agriculture. The traditional practice to produce agricultural soil was to slash-and-burn plant material, which should be replaced by slash-and-char practice in order to reduce greenhouse gases and aerosol pollution. The application of char substrates (charcoal or biomass-derived black carbon) to soil is being proposed a novel approach to establish a significant, long-term sink for atmospheric carbon dioxide in terrestrial ecosystems. Furthermore, the anthropogenic char substrates increase soil fertility and crop production [16]. In addition to reducing greenhouse gas emissions, char applications to soil have the potential to decrease environmental pollution, such as the emission of ammonia and nitrous oxide.

Biological immobilization of inorganic N aids in retaining N and in decreasing ammonia volatilization. Further, biochars are very efficient adsorbers of dissolved ammonium, nitrate, phosphate, and other ionic solutes as well as hydrophobic organic pollutants [16]. In addition to the adsorption of organic environmental pollutants, anthropogenic xenobiotics may also be generated during the formation of biochars. Almost no information exists at present whether biochar and the organic pollutants have the potential to interact with soil organisms via direct membrane contact or via food uptake. To date only microorganisms and plant-microorganism interactions have been studied. One study showed that the addition of biochar led to a shift in the inherited soil microorganisms; yet, the microorganisms adapted to the new carbon source and utilized it [17]. The goal of an even more recent paper was to test whether or not hydrothermal carbonization material had adverse effects on plant growth or that of root associated symbionts such as arbuscular mycorrhizal fungi. The study clearly showed that symbionts and plant growth may respond in opposite ways to such soil additives [18]. Contaminants were considered in neither study.

The aim of this contribution was to evaluate the effects of natural HSs and char substrates with respect to cancer on the molecular biological level. Therefore, the model animal *Caenorhabditis elegans* Maupas whose genome is fully sequenced was applied. The worm possesses the *cep-1* gene (*C. elegans* p53-like protein) which encodes an ortholog of the human tumor suppressor *p53* that promotes DNA damage-induced apoptosis. In more detail, p53 (also known as protein 53 or tumor protein 53), is a tumor suppressor protein. It is important in multicellular organisms, where it regulates the cell cycle and thus functions as a tumor suppressor that is involved in preventing cancer. In *C. elegans*, it is required for normal meiotic segregation in the germ line, and affects sensitivity to hypoxia-induced lethality and longevity in response to starvation; *cep-1* is expressed ubiquitously in embryos and in the nucleoli of a subset of pharyngeal cells, expression levels are highest in the mitotic and meiotic regions of the germline [19]. The p53 protein has been described as “the guardian of the genome”, the “guardian angel gene”, and the “master watchman”, referring to its role in conserving stability by preventing genome mutation [20]. Consequently, the modulation of this key gene in *C. elegans* by exposure to char substrates or HSs gives an indication of the carcinogenic or anticarcinogenic potentials of the exposed material, although the function of *cep-1* in *C. elegans* itself is not yet fully understood.

## 2. MATERIALS AND METHODS

*Caenorhabditis elegans* var. Bristol, strain N2, was maintained as stocks of dauer larvae on nematode growth medium (NGM) agar (17 g bacto agar, 2.5 g bacto peptone and 3 g NaCl per Liter: after autoclaving, add 1 mL 1 M CaCl<sub>2</sub>, 1 mL 1 M MgSO<sub>4</sub>, 25 mL 1 M KH<sub>2</sub>PO<sub>4</sub> and 1 mL of 5 mg mL<sup>-1</sup> cholesterol solution in ethanol) according to standard procedures [21–23]. The animals were fed with 0.5 mL of *Escherichia coli* (OP50, approximately 10<sup>10</sup> cells mL<sup>-1</sup>) suspended in M9-medium as the food supply [21].

Exposure of the worms to the test substances took place on single large Petri dishes with approximately 3,000 synchronized individuals from the egg-stage up to young adult (52 h) at 20°C. In order to extract RNA for the quantitative analysis, worms, once grown, were washed with M9 buffer into falcon tubes. The total worm pellet of approximately 1 mL volume was homogenized with 4 times volume of Trizol<sup>®</sup>.

The total RNA was isolated according to the Trizol<sup>®</sup> extraction protocol with addition of 1/5 volume of glass beads and by using the corresponding homogenizer 'SpeedMill P12' (AnalytikJena, Germany). cDNA synthesis followed accurately the protocol published recently [24]. Quantitative real-time PCR was performed in a MyiQ single color qPCR detection system (Bio-Rad, Germany) using the double-stranded DNA intercalating fluorescent agent EvaGreen for amplicon detection. Each well contained the qPCR Green Core Kit (Jena Bioscience, Germany), 200 nM of each primer pair, and cDNA template equivalent to 5 ng RNA starting material. All sample and primer combinations were assessed in triplicate.  $\beta$ -Actin (*act-1*) served as reference gene. The relative expression of the target genes was calculated using the comparative 2<sup>- $\Delta\Delta C_t$</sup>  method [25]. The gene expression profiles were done with four replicates with the humic materials, and in triplicate with the char substrates. Data were considered significant if the expression/suppression differed from the control by at least the factor of 1.7. The two genes which were subjected to qPCR and their primers are listed in Table 1. Humic substances were obtained from two humic-rich Brazilian coastal lagoons (Atoleiro, Comprida) in the vicinity of Macaé, Rio de Janeiro State [26, 27]. Depending on the water table and the concerning degree of evaporation, the water of the lagoons contains up to 200 mg L<sup>-1</sup> dissolved organic carbon (DOC); hence, it does not need pre-concentration, but rather dilution with distilled water. Exposure

concentrations were 5, 25, and 50 mg L<sup>-1</sup> DOC. HSs of Atoleiro had a life-extending but offspring reducing effect on a Brazilian clone of the cladoceran *Moina macrocopa* Straus [28]. DOC concentrations were determined by means of a TOC-505 A analyzer (Shimadzu, USA). The experiments were conducted in the Macaé laboratories of the Universidade Federal do Rio de Janeiro.

**Table 1** Genes tested by quantitative RT-PCR and used primers

Gene code	Gene name	Primer sequence (5' - 3')
T04C12.6	<i>act-1</i>	TCC AAG AGA GGT ATC CTT AC
		CGG TTA GCC TTT GGA TTG AG
F52B5.5	<i>cep-1</i>	AAC GCT CAC TCT GTC GAC TG
		GCG GTG AGG AAT CTT TCA AGT C

Char substrates were: (1) *Terra Preta* from the Amazon basin and (2) the hydrothermal carbonization (HTC) product from poplar wood. *Terra Preta* soils of central Amazonia exhibit approximately three times more soil organic matter, nitrogen and phosphorus, and 70 times more charcoal compared to adjacent infertile soils. The *Terra Preta* soils were generated by pre-Columbian native populations by chance or intentionally adding large amounts of charred residues (charcoal), organic wastes, excrements and bones [29]. Chemical characteristics are given in Table 2.

**Table 2** Chemical characteristics of dry matter of char substrates

	Loss on ignition %	C <sub>tot</sub> %	DOC mg L <sup>-1</sup>	N <sub>tot</sub> %	P <sub>tot</sub> %
<i>Terra Preta</i>	10.0	4.4	405	0.28	0.156
HTC biochar	99.5	56.9	727	0.12	0.002

HTC biochar was derived from carbonization of biomass in water under autogenous pressure and temperatures at the lower region of liquefaction process [30]. This wet pyrolysis process was run at 210°C for 4 hours and yielded a total carbon content of 57% (Table 2). The char substrates were prepared by grinding the material finely. Later they were dissolved in 100mM NaOH and shaken overnight.

After centrifugation and filtering, the DOC concentrations were determined with a TOC-505 A (Shimadzu, Europe) and adjusted to the same exposure concentrations as with the HSs.

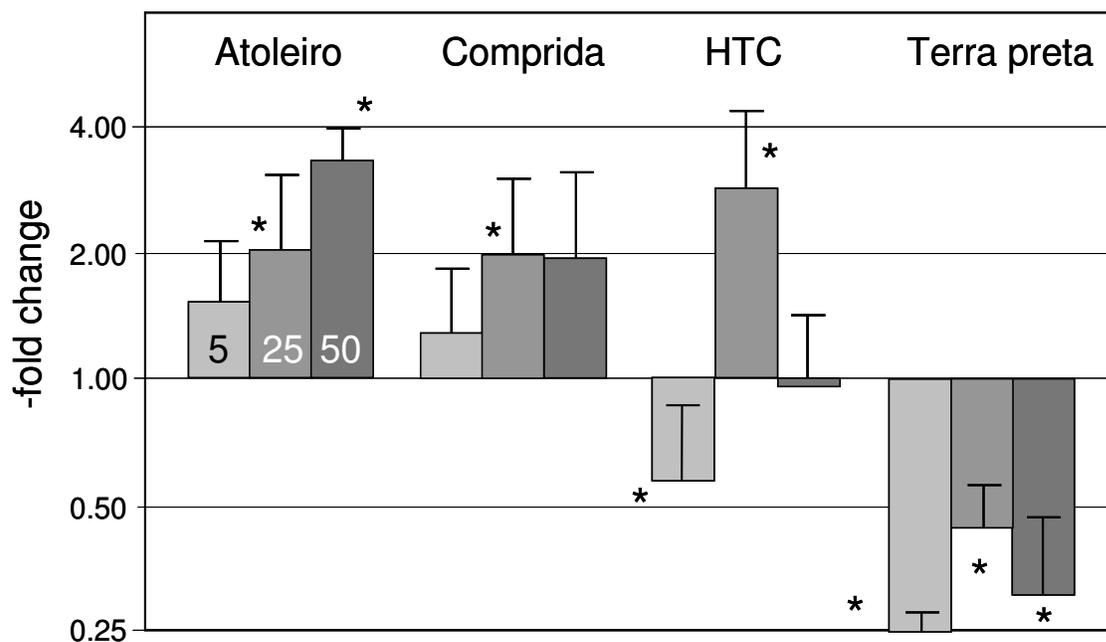
### 3. RESULTS AND DISCUSSION

Expression of the *cep-1* gene was modulated by all organic substances the worm was exposed to (Fig. 1). The two Brazilian HSs induced a significant expression of this gene if the concentrations were above 5 mg L<sup>-1</sup> DOC. With Atoleiro HSs there was even a clear dose-response relationship. The results with the HTC biochar are non-uniform. The 5 mg L<sup>-1</sup> DOC exposure repressed, whereas 25 mg L<sup>-1</sup> induced *cep-1*; the highest exposure concentration did not modulate this gene. In contrast to the HTC biochar, all concentrations of the *Terra Preta* exposure significantly suppressed the *cep-1* gene.

Given that *cep-1* is indicative of an anti-carcinogenic protein, a kind of “guardian of the genome”, both sources of organic carbon induced apparently contrasting pathways: the Brazilian HSs activate this “guardian of the genome”, whereas char substrates mainly repress it. This means the anti-carcinogenic property, so far only described with human cell cultures [10–15], could be confirmed with an invertebrate model animal and natural HSs from a tropical source of the southern hemisphere. One can assume that aquatic and soil organisms and HSs have co-evolved, since HSs are documented to be as old as biomolecules themselves [31], and due to the longtime of coexistence between HSs and organisms, the latter have not merely adapted to these natural xenobiotics, but organisms have instead developed biochemical and molecular biological strategies to convert an adverse stress into benefits for their individual integrity, for individual health, such as multiple stress resistance [32] and often – but not always – longevity [5, 28, 33].

Compared to HSs, char substrates are relatively young and man-made; non-target organisms did not co-evolve with this kind of carbon and, due to a much longer generation time and a lesser metabolic flexibility than bacteria, they did not adapt to biochars. In case of HTC biochar we found an indifferent pattern, which in part may reflect a relationship between the content of carbonaceous compounds and the expression of the *cep-1* gene.

Furthermore, several studies show that biochars of various origins contain contaminants, such as polycyclic aromatic hydrocarbons.



**Figure 1** Relative expression of the *cep-1* gene in *Caenorhabditis elegans*, exposed to 5, 25, and 50 mg L<sup>-1</sup> DOC of humic substances from two Brazilian coastal lagoons (Lagoa Atoleiro, Lagoa Comprida) and two char substrates (Amazonian *Terra Preta* and a HTC product from poplar wood). \* indicates differences in gene expression, induction >1.7- or repression <1.7-fold, respectively, as compared to the control gene *act-1*.

The majority of these have to be considered man-made xenobiotics. For instance, pyrolysis studies reveal that organic educts in the biomaterial can be decomposed to small molecules, such as the phenyl radical, the benzyl radical and C2- and C3-species, which are critical to the formation of polycyclic aromatic hydrocarbons, PAHs [34–38]. PAHs, in turn, have a co-carcinogenic or even carcinogenic potential [39, 40]. From this information, it is not surprising that char substrates may have a carcinogenic potential. In the process of biomass charring, primarily temperatures above 700°C are considered to form PAHs [41]. That might explain why the carcinogenic potential is less manifested with the HTC biochar, which is derived from low temperature pyrolysis compared to *Terra Preta* soils containing coal residues from thermal conversion at high temperatures. To date, this is only one hypothesis about the mechanistic background, which has to be confirmed in future studies; nevertheless, the current findings are a clear plea for a more comprehensive effect evaluation when applying biochars to soils, and also to study the impact on non-target organisms.

#### Acknowledgement

The work was supported by the Deutsche Forschungsgemeinschaft, Grant STE 673-16/1 to R. M., which is gratefully acknowledged. We owe thanks also to Albert Suhett, Universidade Federal do Rio de Janeiro, for helpful technical assistance with the field sampling and the DOC analysis in Macaé.

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AES 101030

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