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# Influence of exposure to pesticides on telomere length in tobacco farmers: A biology system approach

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ABSTRACT

Various pesticides in the form of mixtures must be used to keep tobacco crops pest-free. Recent studies have shown a link between occupational exposure to pesticides in tobacco crops and increased damage to the DNA, mononuclei, nuclear buds and binucleated cells in buccal cells as well as micronuclei in lymphocytes. Furthermore, pesticides used specifically for tobacco crops shorten telomere length (TL) significantly. However, the molecular mechanism of pesticide action on telomere length is not fully understood. Our study evaluated the interaction between a complex mixture of chemical compounds (tobacco cultivation pesticides plus nicotine) and proteins associated with maintaining TL, as well as the biological processes involved in this exposure by System Biology tools to provide insight regarding the influence of pesticide exposure on TL maintenance in tobacco farmers. Our analysis showed that one cluster was associated with TL proteins that act in bioprocesses such as (i) telomere maintenance via telomere lengthening; (ii) senescence; (iii) age-dependent telomere shortening; (iv) DNA repair (v) cellular response to stress and (vi) regulation of proteasome ubiquitin-dependent protein catabolic process. We also describe how pesticides and nicotine regulate telomere length. In addition, pesticides inhibit the ubiquitin proteasome system (UPS) and consequently increase proteins of the shelterin complex, avoiding the access of telomerase in telomere and, nicotine activates UPS mechanisms and promotes the degradation of human telomerase reverse transcriptase (hTERT), decreasing telomerase activity.


## 1. Introduction

Brazil is the second largest tobacco producer worldwide, right after China [1]. Although they make a high financial contribution to the country, tobacco farms present several problems regarding worker health, involving the high demand for manual labor, the occupational exposure to nicotine (a natural pesticide in tobacco) during leaf harvesting, and also to pesticides throughout almost the entire production cycle [2].

Recent studies have shown that occupational exposure to pesticides in tobacco fields is related to an increase in micronuclei, nuclear buds and binucleated cells in buccal cells [3], as well as micronucleus in lymphocytes [2], including an association with *GSTM1* and *CYP2A6* polymorphisms [4]. Increased cell death and DNA damage were also observed in these workers [2,4]. Plasma levels of acetylcholinesterase and trace elements, proving exposure to pesticides, and plasma levels of cotinine, the main metabolite of nicotine, were also elevated [2–6]. Tobacco farmers also showed higher levels of oxidative stress when compared to the control group. Among those parameters, thiobarbituric acid reactive substances, total antioxidant activity, catalase and superoxide dismutase were found significantly elevated [6,7]. Others studies have focused on demonstrating the increase of mental health diseases [8], as well as green tobacco sickness (GTS) [9] in tobacco farmers.

Tobacco production requires the use of several pesticides to keep the crop pest-free, and they are used in the form of mixtures. The combined toxicological effects of these products are very difficult to assess [10,11], but it is known that pesticide mixtures may act in three molecular forms in the human body: independent, dose addition or interaction [11].

As to telomere length (TL), some studies indicate that TL may be influenced by exposure to pesticides [12,13]. Studies found a shorter TL for agricultural workers occupationally exposed to alachlor, 2,4-D, diazinon, butylate, metolachlor, trifluralin, perme-

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thrin, malathion and toxaphene [12,13], while alachlor was also found related to longer TL after recent use [13]. Although some studies indicate that exposure to pesticide mixtures interferes with TL biology [14,15], just recently our group found that pesticides used specifically for tobacco crops (which do not include any of the pesticides cited above) shorten TL 14-fold when compared to the control group [6].

Human telomeres are the DNA repetition of the hexamer TTAGGG [16]. A protein complex called shelterin, composed by TRF1, TRF2, POT1, RAP1, TIN2 and TPP1, is one of the factors responsible for maintaining telomere integrity and for protecting chromosomes against nucleolytic degradation, end-to-end fusion and breakage-fusion-bridge-cycle [17]. Due to the semi-conservative replication of DNA, in each cell division, a portion of telomeres at 5′-end is not replicated [16], and therefore, telomeres are naturally shortened during aging in somatic tissues [18]. In germinative and neoplastic tissues, telomeres are elongated by telomerase, an enzyme that has a catalytic subunit that acts as reverse transcriptase, the hTERT [19].

Telomere proteins play an important role in the appropriate progression of the cell cycle [20]. When BUB1 is linked to telomeres, it recruits anaphase inhibitors in a kinase-dependent manner [21], while CENP-E regulates the location at the kinetochore of an interphase inhibitor telomerase, which when activated, causes chromatid bridges in anaphase and micronuclei in interphase [22]. A recent study demonstrated that CENP-A from fission yeast cells accumulates in telomeres through H3K9 methylation, influencing many aspects of the cell cycle, including centrosome location [23]. Chk2 is a downstream effector of the DNA damage response, which can be triggered by dysfunctional telomeres. Through a p53/p21 signaling pathway, Chk2 plays a role in the in vivo signaling of dysfunctional telomeres [24]. Although the specific functions of the six shelterin proteins are limited to telomeres [25], shelterin works as a rhythmic complex and, in any dysfunction, silencing and/or inhibition of one of its proteins, it may induce apoptosis or cell cycle arrest [26].

While some studies propose the pesticide mechanisms to modify the cell cycle) the cell cycle [27–29], few of them address this action in telomeres [12]. The molecular mechanism is not fully understood in either case. Therefore, our study evaluated the interaction between the complex mixture of chemical compounds (pesticides for tobacco crops plus nicotine) and proteins associated with TL maintenance, as well as the biological processes related to this exposure by System Biology tools to provide insight into the influence of pesticide exposure on TL maintenance in tobacco farmers.

## 2. Material and methods

### 2.1. Building an interactive network for tobacco crop pesticides and proteins associated with TL maintenance

In this study a chemo-biology interatomic network was built involving proteins associated with telomere length and pesticides used in tobacco cultivation to analyze this network regarding the occupational consequence for TL maintenance in tobacco farmers. For this, we used a list of major agrochemicals (including the natural pesticide, nicotine) that tobacco farmers are exposed to when farming tobacco in southern Brazil [2] and performed a systematic review of both original research articles and reviews by searching for literature associated with TL maintenance proteins in the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/). Then, pesticide–protein interactions were searched by utilizing the search tool for chemical interactions STITCH 4.0 (http://stitch.embl.de) and the search tool for protein interactions (related to TL maintenance) STRING 9.1 (http://string-db.org) [30,31]. In STITCH chemicals are linked to proteins by evidence derived from experiments, databases and the literature [30]. In STITCH 4.0, pesticide-protein interactions were downloaded using the following parameters: no more than 50 interactions; medium confidence score (0.400); and network depth equal to 2; active prediction methods all enabled except text mining. This initial pesticide list generated 17 small pesticide-protein subnetworks (data not shown). The systematic review generated a list of the 19 proteins related to TL maintenance. Protein interactions were predicted in STRING 9.1. This search tool predicted protein interactions that can be direct (physical) and indirect (functional) associations [31]. In STRING 9.1, protein–protein interactions were downloaded using the following parameters: no more than 50 interactions; medium confidence score (0.400); and network depth equal to 2; all active prediction methods enabled except text mining; each subnetwork had no more than 100 interactions. The different subnetworks generated from these screenings were combined in a single pesticide-protein interaction (PPI) network employing the union function of the Cytoscape 2.8.2 [32] plugin Advanced Merge Network.

### 2.2. Global topological analysis: evaluation of molecular complexes in the pesticide-protein interaction network

Next, PPI networks were analyzed with the Cytoscape 2.8.2 plugin Molecular Complex Detection (MCODE) [33] in order to detect modules/clusters (densely connected regions) that suggest functional protein complexes. In accordance with Rosado, Henriques and Bonatto [34] the parameters used in MCODE to generate the clusters were: loops included; cutoff degree 2; deletion of single connected nodes from cluster (haircut option enabled); expansion of cluster by one neighbor shell allowed (fluff option enabled); node density cutoff 0.1; node score cutoff 0.2; kcore 2; and maximum depth of network 100. A MCODE score was calculated for each protein/pesticide present in the PPI networks.

### 2.3. Gene ontology analysis

The main bioprocesses associated with the clusters generated from MCODE were analyzed by Cytoscape 2.8.2 plugin Biological Network Gene Ontology (BiNGO) [35]. The degree of functional enrichment for a given cluster and category was quantitatively computed ($p$ value) by hypergeometric distribution, and multiple test correction was also assessed by applying the false discovery rate (FDR) algorithm [36], which was fully implemented through BiNGO software with a significance level of $P < 0.05$.

### 2.4. Local topological analysis: evaluation of central nodes in the pesticide-protein interaction network

Node centrality analysis was computed using Cytoscape 2.8.2 plugin Centiscape 1.21 [37] to identify which nodes (proteins) have a central position within the networks. Centralities implemented were degree and betweenness, nodes with a relatively higher degree were termed hubs and nodes with higher betweenness were named bottlenecks. Hubs are highly connected nodes, whereas bottlenecks are nodes with a higher probability of joining different clusters [38,39]. Therefore a node hub-bottleneck (N-HB) can be considered a key regulator of biological processes and essential for the successful transfer of information through the network, where N-HB perturbations are more likely to cause communication network disruption.