

ECOTOX - BRASIL

Ecotoxicol. Environ. Contam., v. 12, n. 1, 2017, 25-31
doi: 10.5132/eec.2017.01.04

EEC

Toxicity assessment of silica nanoparticles on *Allium cepa*

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(Received October 31, 2016; Accept March 08, 2017)

Abstract

Nanotechnology is rapidly expanding, and several new nanoparticle products with improved performance have been developed. The environmental fate and toxicity of nanoparticles (NP) is poorly understood and should be studied to enable the safe use of this technology. This study aims to investigate genotoxic and phytotoxic impacts of silica nanoparticles (SiNP) using root tip cells of *Allium cepa* as an indicator system. *A. cepa* root tip cells were exposed to different concentrations (1.82 to 0.54 g L⁻¹; dilution factor of 1.5x) of three engineered dispersions of SiNP (TM40 – 22 nm; HS30 – 12 nm; SM30 – 7 nm). The following endpoints were measured: mitotic index, different types of chromosomal aberrations, germination index and root length. A decrease in the mitotic index was observed with increasing NP concentration, and significant decreases in the MI for all tested NP were found. For all NP cytological effects including chromosomal aberrations were observed in treated cells. Phytotoxic effects were also observed as germination rate and root growth were significantly reduced ($p < 0.05$). Plants are an essential component of ecosystems and these findings suggest that they should be included when evaluating the toxicological impact of NP on the environment.

Keywords: Nanomaterial. Genotoxicity. DNA damage. Size influence. Aggregation.

INTRODUCTION

Nanotechnology has been growing exponentially and there have been studies in all areas, including the physical, chemical, environmental and medical fields. Despite the wide range of research about nanoparticles (NP) and nanotechnology there are few studies on the effects of these nanomaterials on health and especially on environmental impacts. Silica nanoparticles (SiNP) are extensively used in manufactured products, for example in additives to drugs, in the chemical industry, in printer toners, in cosmetics and in food (Gordon *et al.*, 2009; Van Hoecke *et al.*, 2011; Fede *et al.*, 2012; Izak-Nau *et al.*, 2014; Pisani *et al.*, 2015). Nanoparticle materials that are between 1 and 100 nanometres are usually more toxic than bulk materials of larger size (Sager *et al.*, 2008; Kim *et al.*, 2012). Their adverse effects depend on a large number of factors, such as their size, shape, surface/volume ratio, stability, chemical composition, and others, which may be influenced by several environmental parameters (Maurer-Jones *et al.*, 2013).

Regarding the genotoxicity and cytotoxicity of SiNP most studies have found negative results using several cell lines, and the comet assay (Gehrke *et al.*, 2013; Gonzalez *et al.*, 2014; Tavares *et al.*, 2014). Also, some authors have found chromosomal damage in mouse and human cells (Sergent *et al.*, 2012; Lankoff *et al.*, 2013). As these studies have produced conflicting results, more research is needed to elucidate why and how these particles interact with cells and organisms. It is expected that smaller NP exert higher toxicity, because they may cross biological barriers more easily than larger NP. Some of the controversial results may therefore be explained by differences in nanoparticle size. In addition, nanoparticles are extremely reactive so different types of media and reagents may interact with them and consequently produce different results, making it difficult to compare studies. Not all studies support a relationship between size and nanoparticle toxicity theory. For example, Greish *et al.* (2012) observed that larger SiNP induced higher toxicity in CD-1 mice than their smaller counterparts. Nevertheless, Park *et al.* (2013) reported that negatively surface charged 20 nm SiNP exhibited higher toxicity than the equivalent 100 nm SiNP in keratinocytes.

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Deeper understanding of the influence of size on SiNP ecotoxicological potential is essential and further research is needed. Furthermore, the effects of SiNP have been studied on human cells, algae, and fish (Van Hoecke *et al.*, 2008; Fent *et al.*, 2010; Fede *et al.*, 2012; Gonzalez *et al.*, 2014; Silva, 2015) but data on the potential toxicity of NP in terrestrial ecological test species is still limited and only a few plants have been used as indicator organisms. Therefore, this work aimed to assess whether SiNP influence the cytotoxicity, genotoxicity and phytotoxicity in *Allium cepa*.

The *Allium cepa* root chromosomal aberration assay is a reliable tool for the prediction of the environmental impact of disposed chemicals and nanomaterials. It has been validated by the International Programme on Chemical Safety (IPCS, WHO) and by the United Nations Environmental Programme (UNEP) as an efficient and standard test for the chemical screening and in situ monitoring for the genotoxicity of environmental substances (Cabrera & Rodriguez, 1999). In addition, *Allium cepa* is a sensitive, abundant species, with a large geographical distribution and wide availability making it suitable for the evaluation of nanoparticle toxicity (Pakrashi *et al.*, 2014). Many studies have shown important correlations between tests with plants and mammals (Fachinnetto *et al.*, 2007; Ray *et al.*, 2013). Rank & Nielsen (1994) observed a correlation of 82% between *Allium cepa* tests and carcinogenicity tests on rats. These findings strengthen the assumption that tests with plants can be robust and expose the dangerous effects that chemicals such as NP can have, not just on the environment but also on human health.

MATERIALS AND METHODS

Characterisation of nanoparticle suspensions

For this study, silica nanoparticles were purchased from Sigma-Aldrich (St Louis, USA), as aqueous suspensions with a concentration of 400 g L⁻¹ for TM40 and 300 g L⁻¹ for HS30 and SM30. They were electrostatically stabilised with negative charges, with sodium as counterion and were of different sizes: TM40 (size: 22 nm and surface area: 140 m² g⁻¹), HS30 (size: 12 nm and surface area: 220 m² g⁻¹) and SM30 (size: 7 nm and surface area: 320–400 m² g⁻¹). In order to obtain a serial dilution to which the seeds of *Allium cepa* were exposed, the stock solution was diluted with distilled water.

To characterise the NP suspensions a series of physical characterisation experiments were carried out (at the concentration of 0.01 g L⁻¹ of SiNPs). In order to measure the hydrodynamic diameter of the particles in suspension (NP size), the aggregation index and the polydispersity index (PDI), dynamic light scattering (DLS) was used. To measure the zeta potential (measure of charge) electrophoretic light scattering (ELS) was performed (Sayes & Warheit, 2009; Card & Magnuson, 2010). Information about the stability and size variability of the NP in the medium used for all tests

was provided by the zeta potential and polydispersity index. To categorize NP suspensions, the ASTM D4187-82 method (1985), to classify the stability behaviour of particles (i.e. classifying the zeta potential as a high or low value), was used. A Malvern Instrument Zetasizer Nano-ZS instrument (Malvern Instruments Ltd, Worcestershire, UK) was used to measure the parameters mentioned above at 20 °C, in backward scattering at 173°.

Allium cepa assay

The *A. cepa* assay was performed according to a modified version of Grant's protocol (Grant, 1982). All assays were carried out with only one kind of seed of *A. cepa* (variety Baia Periforme), to avoid different responses in the several stages of the process.

The *A. cepa* seeds were continuously exposed to different concentrations of SiNP: 1.82 to 0.54 g L⁻¹ with a dilution factor of 1.5 x. These concentrations were selected in order to obtain a stress concentration so that we were able to observe effects and correlate them with the influence of size on geno-, cyto- and phytotoxicity. The tests were conducted at 20 ± 5 °C and 12:12 h light/dark, in covered Petri dishes containing filter paper soaked with the NP solution. Four replicates per treatment, each with 100 seeds were used. A control using distilled water as a negative control was also used (Leme & Marin-Morales, 2008). The seeds were exposed to NP suspensions until the roots in the negative control reached a length of two centimetres (96 hours). At this stage, the germination index, in percentage, was calculated as the proportion of the seeds with visible radicle protrusion. Afterwards 30 randomly selected roots were picked from each replicate of each treatment, measured, collected (only those longer than two centimetres) and fixed in Carnoy 3:1 (ethanol: acetic acid, v/v) for 6–12 h. They were then transferred to a new Carnoy's fixative and stored at 4 °C before further analysis.

Slide preparation with the meristematic root cells followed the procedure described by Leme & Marin-Morales (2008). Fixed roots were washed with distilled water and hydrolysed in 1 N HCl in a 60 °C bath for 8–11 min. They were then washed in distilled water again and stained with Schiff's reagent for two hours in the dark, before washing one more time with distilled water. For slide preparation, the meristematic regions were cut onto the slide into a drop of 2% acetic carmine solution, to increase the cytoplasm contrast, and covered with a cover slip and carefully squashed. Afterwards the cover slip was removed with liquid nitrogen and the slides were mounted in synthetic resin (Enthellan, Merck) for further analysis. In order to evaluate cell damage, ten slides per treatment were prepared and 500 intact cells were analysed per slide, under an optical microscope (1000x), totalling 5000 cells per test for each treatment.

Genotoxic potential was determined for all treatments according to the observation and quantification of any chromosomal and nuclear abnormalities in the meristematic cells, in all phases of the cell cycle, such as abnormal

telophases and anaphases (e.g. multipolar, with bridges, delayed), fragmented or lost chromosomes, or C-metaphases. Chromosomal aberrations index (CA) was calculated as the ratio between the number of the cells with chromosomal aberration and the total number of cells scored and expressed as percentage. The evaluation of mutagenic effects was carried out by scoring micronucleated cells of meristematic regions in all slides of all treatments. The micronuclei index (MN) was scored as the ratio between the number of micronucleated cells and the total number of cells scored and expressed as percentage. We also analysed the mitotic index (MI) i.e. the number of cells in division. The MI was calculated as the ratio between the number of mitotic cells and the total number of cells scored and expressed as percentage.

DATA ANALYSIS

All data were analysed using statistical analysis One Way ANOVA (Dunnett test) to determine treatment effects, and then to compare each treatment to the control using the software SAS 9.1 for Windows. All statistical analyses were based on a 0.05 significance level.

RESULTS

Characterisation of silica nanoparticles

Through characterisation of the nanoparticles we could evaluate the stability, agglomeration, size and heterogeneity of all SiNP in the distilled water used for the dilutions. Zeta potential and polydispersity index provided information about stability, agglomeration and size heterogeneity of NP in the medium used for all assays. Polydispersity values equal to or lower than 0.2 indicate adequate uniformity of size distribution (Kovacevic *et al.*, 2011), and zeta potential values higher than -30 mV indicate adequate stability. All of the NP were influenced by the medium used (Table 1). The stability as measured by the zeta potential and polydispersity index was different for each type of particle. TM40 NP were more stable, presenting the lowest polydispersity index (0.122), and the highest zeta potential (-25.99). The polydispersity index for HS30 was 0.251 and its zeta potential was -0.38; for SM30 the values were 0.262 and -1.65, respectively. SM30 particles showed the largest change compared to the primary size reported by Sigma-Aldrich, their diameter changing

from 7 nm to 17.1 nm. For the TM40 NP the average size increased from 22 nm to 37.2 nm when diluted, and for HS30 the average size increased from 12 nm to 19.1 nm.

Allium cepa assay

The results of the tests with SiNP showed that the percentage of mitotic cells (prophase, metaphase, anaphase and telophase) decreased when exposed to SiNP, however these results were not dose dependent. Also, the results shown an increase of micronucleus, for all nanoparticles, when compared to the control, but the number of micronucleus did not pass 10% in any of the treatments, therefore, there was no toxicity relevance. The same was observed for chromosomal aberration (Fig. 1).

Phytotoxic effects of SiNP in *A. cepa* were also observed. The germination rate of the seeds exposed to the studied NPs and the results for the root length after exposure to the NP are shown in Fig. 2. Compared to control, the germination rate presented significantly reduced values ($p < 0.05$) for all particles and for all concentrations, except for TM40 and SM30 at 1.2 g L⁻¹. The germination results seem to be dose dependent for HS30, but not for TM40 or SM30. Root length was significantly reduced for NP treatments.

DISCUSSION

Some studies have investigated the genotoxic and cytotoxic effects of differently sized SiNP at several concentrations and on different organisms, with both positive and negative results (Kim *et al.*, 2012; Liang *et al.*, 2014; Kasper *et al.*, 2015). Conflicting results have also been recorded for phytotoxic effects on different plants (Nair *et al.*, 2011; Slomberg & Schoenfish, 2012; Suriyaprabha *et al.*, 2012). In the present study, high doses of SiNP induced cytotoxic but not genotoxic effects in meristematic cells of *A. cepa*. The cytotoxicity was verified by the decreased of the mitotic index. The cytotoxicity level of a test compound can be determined based on the increase or decrease in the mitotic index (MI), which can be used as a parameter of cytotoxicity in studies of environmental biomonitoring (Fernandes *et al.*, 2007). Reduction in cell activity could be due to changes in the mitotic cycle duration, which can be attributed to the increase in S phase duration (Kumari *et al.*, 2011). Therefore, the SiNP may have a mitodepressive effect on the *A. cepa* roots, effect that could interfere with normal mitosis, preventing cells from entering

Table 1 Physical parameters measured at the highest tested concentration of the three SiNP, using dynamic and electrophoretic light scattering.

	SiNP	Concentration (g L ⁻¹)	Size (nm)	Polydispersity index	Zeta Potential (mV)
H ₂ O dest.	TM40 (22 nm)	0.01	37.20	0.12	-25.99
H ₂ O dest	HS30 (12 nm)	0.01	19.10	0.25	-0.38
H ₂ O dest	SM30 (7 nm)	0.01	17.10	0.26	-1.65

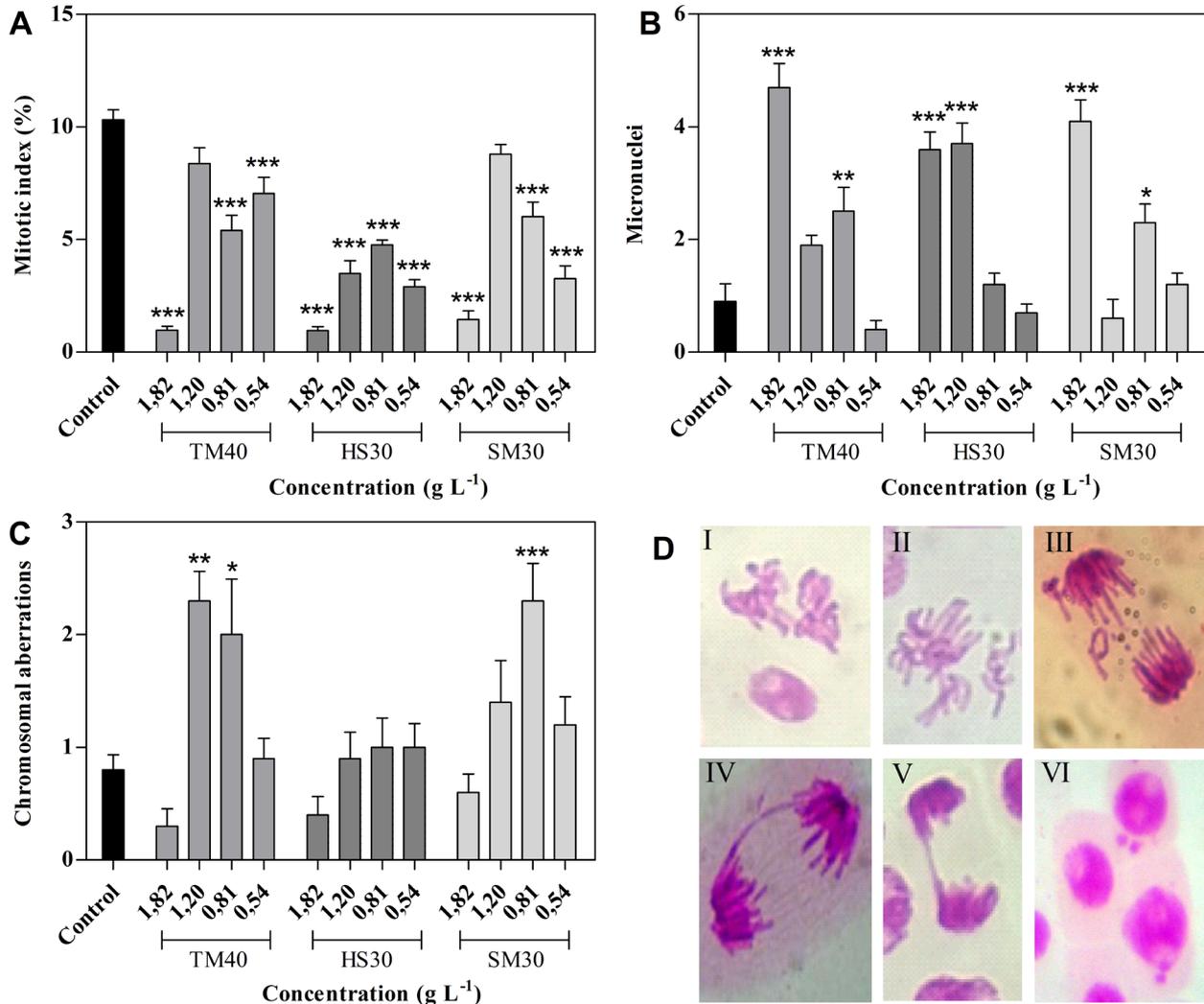


Fig. 1 Silica nanoparticles induced chromosomal aberrations and delays entry into mitosis in root tip cells of *A. cepa*. (A) Graph plotting the mitotic index following treatment of *A. cepa* with SiNP (B) Graph plotting the number of cells presenting micronuclei following treatment of *A. cepa* with SiNP (C) Graph plotting the number of cells presenting chromosomal aberration following treatment of *A. cepa* with SiNP (D) Representative images of (I) stickiness; (II) disturbed metaphase (III) anaphase with chromosome loss (IV) anaphase with bridge (V) telophase with bridge (VI) micronucleus. Magnification 40x.

Asterisks indicate significant differences relative to the negative control (* $p < 0.05$ /** $p < 0.001$ /***) $p < 0.0001$)

prophase and blocking the mitotic cycle during interphase, thus inhibiting dna/protein synthesis.

Different kinds of chromosomal aberrations were observed, such as chromosomal breaks, bridges, stickiness, and disturbed metaphase. Different chromosomal aberrations in metaphase and anaphase may be caused by the shifting of poles by the depolymerisation of spindle fibers (Darlington & McLeish, 1951), also stickiness may cause fragmentation of chromosomes and bridges, leading towards to structural chromosomal mutations (Panda *et al.*, 2011). Toxicity studies carried out by (Yu *et al.*, 2008; Napierska *et al.*, 2009) suggested that amorphous SiNP below 100 nm induced cytotoxicity, so the size of the particles is critical with respect to their biological effects.

Phytotoxic effects were observed for all SiNP studied, germination rate and root length were significantly reduced. One important parameter, that determines the rate of root

growth due cell proliferation, is the mitotic index, which is correlated with the frequency of cell division (d'Aquino *et al.*, 2009). Therefore, the low mitotic index observed in the cytotoxicity assay may have contributed to the short root length observed. Studies with SiNP (of size 14, 50 and 200 nm) in *A. thaliana* found that SiNP can interfere with plant development, and also accumulate in root cells in a size-dependent manner (Slomberg & Schoenfisch, 2012). Also Stampoulis *et al.* (2009) studied germination and root growth of *C. pepo* treated with SiNP (<100 nm). The SiNP completely inhibited germination at 1000 mg L⁻¹ and had a significant impact on root elongation. Those studies present similar results to our experiments on germination and root length in *A. cepa* exposed to SiNP.

Overall in this study was not observe a dose-dependent toxicity, and only a dependence on size towards to TM40 (being the least toxic, i.e. with bigger size). This may be explained

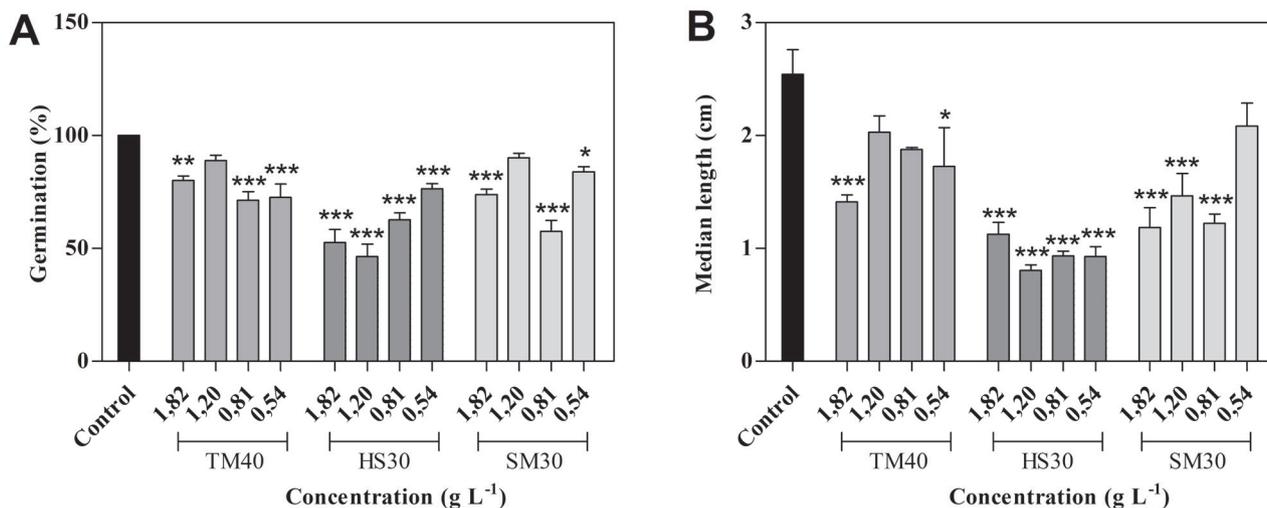


Fig. 2 Silica nanoparticles induced phytotoxicity in *Allium cepa*. (A) germination rate (ratio between the number of germinated seeds and the total number of seeds scored and expressed as percentage) after exposure to three types of silica nanoparticles at different concentrations. (B) Mean and SD of *Allium cepa* root length (in cm) after exposure to three types of silica nanoparticles at different concentrations. Asterisks indicate significant differences relative to the negative control (* $p < 0.05$ / ** $p < 0.001$ / *** $p < 0.0001$)

by the NP properties, which tend to form agglomerates. Agglomeration would decrease the size differences between the particles, possibly preventing such effect from being observed. This can be confirmed by the DLS results, which shows that the HS30 and SM30 dispersions present a very similar size (19.10 nm and 17.10 nm, respectively), therefore reinforcing the toxicity results. Fang *et al.* (2015) studied different primary sizes of TiO₂ NP and found a positive correlation between size and surface area or surface energy. Therefore, particles with large surface area or high surface energy usually have a more unstable thermodynamic system, and therefore tend to form aggregates. This may be an explanation for the low stability of the two smaller particles in our research. Also, there are many factors that can influence the aggregation of nanoparticles, including concentration (Narayanan & El-Sayed, 2005). Therefore, the toxicity results, in our study, may not be dose-dependent due to the fact that the NPs was not stable, thereby it may have a tendency to aggregate more at higher concentrations, causing more effect on toxicity at lower concentrations.

There are still a very limited information concerning the adverse effects of engineered nanomaterials on terrestrial plant systems. Although some studies corroborate our findings, it should be noted that phytotoxicity data for SiNP are still contradictory. While some studies present no phytotoxicity for SiNP, others present negative effects, as presented above, and some even present positive effects of SiNP on plant germination and root length (Nair *et al.*, 2011; Siddiqui *et al.*, 2015). The results presented in this paper provide complementary information regarding the ecotoxicity of SiNP in higher plant systems, and highlight the importance of assessing the nanomaterial properties, such as aggregation, to understand its toxicity and avoid underestimation of their environmental risks.

CONCLUSIONS

In summary, negative effects were observed after exposure of *Allium cepa* to high doses of SiNP. Those nanoparticles can penetrate the plant system causing effects on growth, germination and cell division. Our findings provide a complementary information regarding the toxicity of silica nanoparticles to higher plant systems and highlight the importance of the nanoparticles properties to understand their environmental risks. Plants are an important component of ecosystems and these findings suggest that they should be included when evaluating the toxicological impacts of nanoparticles on the environment.

ACKNOWLEDGMENTS

We would like to thank Dr. Amadeu Soares and Isabel Lopes from the Department of Biology & CESAM, University of Aveiro, Portugal, also Dr. Filipe Antunes and Dr. Claudia Duarte from the Department of Chemistry, University of Coimbra, Portugal. The authors are grateful to Proof-Reading Services, England.

This study was funded by CNPq and CAPES, Brazil. The author G. Silva acknowledges the support and scholarship provided by SANTANDER, Brazil.

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