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Comparative effect of ZnO NPs, ZnO bulk and $ZnSO_4$ in the antioxidant defences of two plant species growing in two agricultural soils under greenhouse conditions

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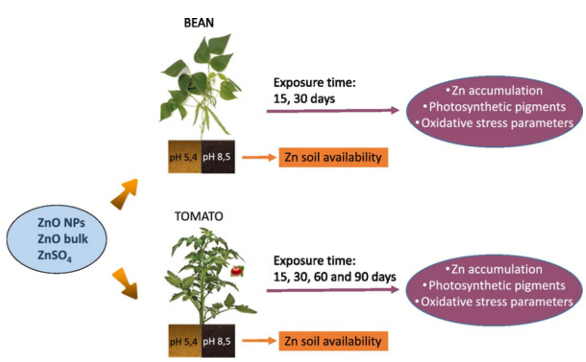
HIGHLIGHTS

- Long-term phytotoxicity of ZnO NPs in two natural soils under greenhouse conditions
- pH is a driving factor for ZnO NPs toxicity but its effect depends on plant species.
- Overall, ZnO NP uptake, toxicity and stimulation are similar to ion Zn and ZnO bulk.
- Under our experimental conditions ZnO NPs do not constitute nanospecific risk.

GRAPHICAL ABSTRACT



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ABSTRACT

The present study has investigated the toxicity of ZnO NPs to bean (*Phaseolus vulgaris*) and tomato (*Solanum lycopersicon*) crops grown to maturity under greenhouse conditions using an acidic (soil pH 5.4) and a calcareous soil (soil pH 8.3). The potentially available Zn in the soils and the Zn accumulation in the leaves from NPs applied to the soil (3, 20 and 225 mg Zn kg^{-1}) and changes in the chlorophylls, carotenoids and oxidative stress biomarkers were measured at 15, 30, 60 and 90 days and compared with those caused by bulk ZnO and $ZnSO_4$. The available Zn in the soil and the leaf Zn content did not differ among the Zn chemical species, except in the acidic soil at the highest concentration of Zn applied as Zn ions, where the highest values of the two variables were found. The ZnO NPs showed comparable Zn toxicity or biostimulation to their bulk counterparts and Zn salts, irrespective of certain significant differences suggesting a higher activity of the Zn ion. The treatments altered the photosynthetic pigment concentration and induced oxidative stress in plants. ROS formation was observed at Zn plant concentrations ranging from 590 to 760 mg kg^{-1}, but the effects on the rest of the parameters were highly dependent on the plant species, exposure time and especially soil type. In general, the effects were higher in the acidic soil than in the calcareous soil for the bean and the opposite for the tomato. The similar uptakes and toxicities of the different Zn forms suggest that the Zn ions derived from the ZnO NPs

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exerted a preferential toxicity in plants. However, several results obtained in soils treated with NPs at 3 mg Zn kg^{-1} soil indicated that may exist other underlying mechanisms related to the intrinsic nanoparticle properties, especially at low NP concentrations.

1. Introduction

The application of nanoparticles (NPs) in agriculture is just emerging as an effective strategy to improve agricultural practices. The use of NPs aims to reduce the applied amounts of plant protection products, minimise nutrient losses in fertilisation and increase yields through optimised nutrient management (Das et al., 2015; Rizwan et al., 2016). There is growing interest in the use of ZnO NPs in agricultural formulations, taking advantage of their properties as UV blockers or as fertilisers (Ishaque et al., 2009; Milani et al., 2012; Sabir et al., 2014; Wang et al., 2013). In addition, ZnO NPs have widespread uses in cosmetics, pharmaceuticals and photocatalyst pigments, and as a consequence, they are found in sewage water and may be integrated into sewage sludge (Keller et al., 2013; Ma et al., 2013; Sajid et al., 2015). Land applications of these residues may be an important pathway for the introduction of ZnO NPs into soils. (Fernandez et al., 2014; Rico et al., 2011). Therefore, much attention is being paid to the potential risks arising from the introduction (unintentional or deliberate) of these materials to agricultural soils.

In recent years, numerous studies on the phytotoxicity of ZnO NPs have been performed, but most of them were conducted using alternative exposure media (aqueous suspensions, nutrient solutions and agar) in place of soil (Kouhi et al., 2015; Lee et al., 2013; Lopez-Moreno et al., 2010; Pokhrel and Dubey, 2013). These studies allow for the investigation of the underlying mechanisms and the relevant physicochemical and biological parameters, but they do not mimic field conditions and hence may overestimate the toxicity (Arruda et al., 2015). Although some studies have examined the toxicity of ZnO NPs in artificial or modified soils (Manzo et al., 2011; Yoon et al., 2014; Zhao et al., 2013), studies of their phytotoxicity under relevant exposure conditions are scarce (Du et al., 2011; Garcia-Gomez et al., 2015; Liu et al., 2015; Wang et al., 2016; Watson et al., 2015). NPs are likely to react with the constituents of environmental matrices, which may modify their availability and thereby their toxicity and the metal concentrations in plants. The soil pH is a key driver of the Zn solubility (Zhao et al., 2012), and its influence on the availability and toxicity of ZnO NPs has been little addressed (Arruda et al., 2015). Therefore, additional research examining the phytotoxicity of NPs to plants grown in soil media is necessary (Watson et al., 2015). Moreover, phytotoxicity studies are usually performed over short time periods, so studies covering the whole cycle of a plant are desirable to determine the long-term effects of nanoparticle toxicity on plants (Kouhi et al., 2015; Yoon et al., 2014).

NPs have properties linked to their low size and high specific surface that confer upon them characteristics different from those of other presentations of the same substance. Previous studies on ZnO NPs suggest two main mechanisms of action, the dissolution of the nanoparticles to ionic zinc and the intrinsic nanoparticle properties (particle size, surface area, etc.), as well as a combination of the two modes of action (Arruda et al., 2015; Fernandez et al., 2013; Judy and Bertsch, 2014; Ma et al., 2013). One the most discussed mechanisms by which NPs exert toxic effects on organisms is the generation of reactive oxygen species (ROS) due to their redox activity (Du et al., 2016; Ma et al., 2013; Sajid et al., 2015). ROS are partially reduced forms of atmospheric oxygen, such as superoxide radical $O_2{\bullet}^-$, H_2O_2, singlet oxygen $^1O_2$ and hydroxyl radical OH•, which are highly reactive and can result in oxidative stress to organisms. Their accumulation induces lipid peroxidation, membrane and DNA/RNA damage and metabolic imbalance (Bartosz, 1997; Gill and Tuteja, 2010). Plants have evolved various protective mechanisms to limit oxidative damage by the production of antioxidants, such as ascorbate, carotenoids, thiols and antioxidant enzymes. The enzymatic system operates through the actions of a set of enzymes, among them ascorbate peroxidase (APX), guaiacol peroxidase (GPOD) and catalase (CAT). Nevertheless, when the defences of an organism can no longer eliminate the undesired ROS, oxidative stress occurs, causing a marked reduction in growth and ultimately plant death. Several studies have shown evidence of stress in plants exposed to soils contaminated with ZnO NPs (Hernandez-Viezcas et al., 2011; Kim et al., 2012; Liu et al., 2015; Mukherjee et al., 2014), but more information is necessary, particularly under realistic exposure conditions.

In this paper, the effects of ZnO NPs on two plant species, the bean (*Phaseolus vulgaris* var. *contender*) and the red cherry tomato (*Solanum lycopersicon* var. *cerasiforme*), were studied. Two agricultural soils with different physicochemical characteristics, especially in terms of soil pH, were used to evaluate the influence of the soil type on the availability of the ZnO NPs and their toxicity. The experiment was conducted in a greenhouse to provide more realistic conditions of exposure, and the responses were measured several times along of the whole cycle of the plant life. The effects studied were the changes in the chlorophyll (CHL) and carotenoids and parameters related to the oxidative stress in the plants: the changes in ROS levels, the activity of the antioxidant enzymes (APX, GPOD and CAT), the levels of proteins and the effects on lipid peroxidation. In addition, the potential Zn availability in the soil and the Zn accumulation in the leaves were studied. To determine the relative degrees of importance of the release of ions and the nanoparticle-specific properties in the toxicity of the ZnO NPs, a comparative experiment was conducted simultaneously with bulk ZnO and $ZnSO_4$.

The main objectives of this study were i) to investigate the responses of the oxidative stress parameters in two plant species exposed to ZnO NPs in two agricultural soils and their evolution along the cycle of plant life, ii) to determine the influence of the soil type on the potential availability, leaf accumulation and toxicity of ZnO NPs and iii) to compare the effects of ZnO NPs with those of other Zn species to elucidate the mechanisms of NP toxicity.

2. Materials and methods

2.1. Chemicals

ZnO NPs (<100 nm) were obtained from Sigma-Aldrich (Germany) with a nominal primary particle size of <100 nm (i.e., rp ≤ 50 nm). The ZnO bulk form was purchased from Sigma–Aldrich (Germany), and the $ZnSO_4.7H_2O$ was from Panreac (Spain). The size and shape of the nanoparticles were determined previously by the authors with a transmission electron microscope (TEM) (Fernandez et al., 2013).

2.2. Soils and treatments

Soils were collected from the surface layer (0–20 cm) of two agricultural fields located in Madrid (Spain): an acidic soil (pH 5.4) and a calcareous soil (pH 8.5). The soils were air-dried and sieved (2-mm mesh) prior to characterisation and use in a plant growth experiment. Selected physical and chemical properties were measured in both soils, and the analytical results are shown in Table 1. According to Lindsay and Norvell (1978), DTPA-TEA-extractable Zn is adequate for