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Triggered antioxidant defense mechanism in maize grown in soil with accumulation of Cu and Zn due to intensive application of pig slurry

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ABSTRACT

The present study investigated changes in both the growth parameters and the enzymatic and non-enzymatic antioxidant systems of maize (*Zea may* L.) plants grown in Typic Hapludalf soil containing an accumulation of Cu and Zn. This accumulation developed because the soil received nineteen applications of pig slurry in no-tillage system over seven years. In this study, the maize plants were grown for fifteen and 25 days after emergence (DAE) in pots containing undisturbed and disturbed soil samples collected from a field experiment that received the rates 0, 20, 40 and 80 $m^3\ ha^{-1}$ of pig slurry, which totalized the amount of 0, 380, 760 and 1520 $m^3\ ha^{-1}$ of pig slurry in seven years, respectively, and phosphorus (P) +potassium (K) treatment (in disturbed soil samples). The maize plants grown in the undisturbed soil samples with an accumulation of Cu and Zn did not indicate an apparent decrease in growth. However, when compared to the treatment with PK fertilization, the maize plants grown in the disturbed soil with pig slurry treatments indicated higher lipid peroxidation and a number of senescent leaves, as well as a significant decrease in plant height. Additionally, when compared to the PK treatment, the leaf superoxide dismutase and ascorbate peroxidase activities decreased and increased, respectively, with the addition of pig slurry treatments in the disturbed soil at 25 DAE. In general, when compared to the treatments with 20 $m^3\ ha^{-1}$ of pig slurry and PK at fifteen and 25 DAE, the leaf ascorbic acid and non-protein thiol groups concentrations decreased with the addition of 40 and 80 $m^3\ ha^{-1}$ of pig slurry. This result suggests that the excess of Cu and Zn in the pig slurry significantly changed the antioxidant system of the maize plants.

1. Introduction

Intensive pig production, which generates a large volume of pig slurry that is disposed of on agricultural land, has become a major environmental problem in countries with high densities of intensive pig farms, such as France (L'Herroux et al., 1997), the Netherlands (Esselink et al., 1995), Denmark (Bak et al., 1997), the USA (Novak et al., 2004) and, recently Southern Brazil, where the problem is especially evident in the States of Rio Grande do Sul (RS) and Santa Catarina (SC) (Girotto et al., 2010; Mattias et al., 2010). The application of pig slurry (PS) has raised serious environmental concerns because of the presence of Cu^{2+} and Zn^{2+} ions. These ions are abundantly used as pig feed additives, and they might reach excessive amounts in PS-amended soils, thus endangering the soil and water quality (Berenguer et al., 2008). As a result, over many years, the excessive accumulation of these metals in the soil might produce phytotoxic effects that lead to a reduction in the yield of sensitive crops (L'Herroux et al., 1997).

At a plant's cellular level, Cu is a structural and catalytic component of many proteins and enzymes that are involved in a variety of metabolic pathways (Pilon et al., 2006). Cu participates in many physiological processes because it is able to exist in multiple oxidation states in vivo (Yruela, 2005). However, the same redox properties that make Cu an essential element also contributes to the inherent toxicity of Cu. Through the Fenton reaction, redox cycling between Cu^{2+} and Cu^{+} catalyzes the production of hydroxyl radicals from superoxide and hydrogen peroxide and enhances the production of reactive oxygen species (ROS) (Briat and Lebrun, 1999). Thus, Cu has the capacity to initiate oxidative damage in plant tissues, which inhibits plant growth by interfering with important cellular processes, such as membrane

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permeability, chromatin structure, protein synthesis, enzyme activities, photosynthesis and respiratory processes (Yruela, 2005; Gratão et al., 2005).

However, Zn is a nonredox-metal that is not able to generate ROS directly through Fenton reaction, but it can generate oxidative stress by interfering with the antioxidant defense system of plants (Gratão et al., 2005). Zn toxicity can reduce rooting capacity, decrease chlorophyll content and even cause leaf chlorosis (Castiglione et al., 2007), which contributes to the inhibition of growth. Furthermore, Zn toxicity might negatively affect the membrane permeability, electron transport chain (De Magalhaes et al., 2004), and the uptake and translocation of nutrients (Jiang and Wang, 2008; Wang et al., 2009). Hence, the excessive uptake of Cu and Zn by plants can cause oxidative stress because of an imbalance between the antioxidant responses and the increased ROS production.

Plants possess several potential cellular mechanisms that might be involved in the detoxification of heavy metals (Gratão et al., 2005). To control the level of the ROS and to protect the cells, plants possess low molecular weight antioxidants, such as ascorbic acid, glutathione and carotenoids, and antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), that scavenge the ROS (Gratão et al., 2005). Antioxidant responses have been observed in leaves and roots that are both Cu and Zn concentration dependent and time-dependent. Additionally, these responses have been observed in leaves and roots that are dependent on the plant species or ecotype (Cuypers et al., 2002).

Many previous studies have explored how Cu and Zn interfere with a variety of physiological processes. Additionally, many of these studies have been concerned with how the antioxidant systems in plants respond to metal stress in nutrient solutions (Cuypers et al., 2002; Tewari et al., 2006; Li et al., 2012). However, there is little available information about maize grown in soil that has accumulated Cu and Zn because of extended applications of pig slurry. Because of this lack of information, the objective of the present study was to evaluate the possible changes in both the growth parameters and the enzymatic and non-enzymatic antioxidant systems of maize plants grown in soil that has accumulated Cu and Zn because of nineteen applications of pig slurry over seven years.

2. Material and methods

2.1. Soil

Disturbed and undisturbed top of the Typic Hapludalf soil (Soil Survey Staff, 2006) samples (0–20 cm) were collected from a field experiment conducted at the Department of Soil Science at Federal University of Santa Maria (UFSM), Rio Grande do Sul (RS) State (29°41′11.46″S and 53°43′8.28″W), Southern Brazil. Prior to the year 2000 (when the field experiment began), the area had been kept under a no-tillage system for eight years. This experiment was initiated in 2000 to evaluate the response of crop cultures to pig slurry application rates of 0, 20, 40 and 80 m^3 ha^{-1} in a no-tillage system.

As of May 2000, the experimental field was managed under the following crop sequence: black-oats (*Avena strigosa* Schreb.), maize (*Zea mays* L.) and forage turnips (*Raphanus sativus* L.) in 2000/2001 and 2001/2002; black-oats, pearl millet (*Pennisetum americanum* L.) and black beans (*Phaseolus vulgaris* L.) in 2002/2003; black-oats/common vetch (*Vicia sativa* L.) and maize in 2003/2004 and 2004/2005; black-oats, black beans and sunn hemp (*Crotalaria juncea* L.) in 2005/2006 and, finally, black-oats and maize in 2006/2007. Maize and black beans are grown in the spring and summer as cash crops. In addition, common vetch and black-oats are grown in the winter, and sunn hemp and pearl millet are grown in the spring, as cover crops. All the stubble produced was left on the soil surface.

First, pig slurry rates of 0, 20, 40 and 80 m^3 ha^{-1} were applied in each plot with a total area of 12 m^2 (4 m × 3 m) in May 2000. A randomized block experimental design with three replicates for each treatment was used. After grain harvest in summer crops, or flowering in winter crops, plants were desiccated and, on the crop residues, pig slurry was applied for the next crop. Pig slurry was spread on the soil surface one day before the sowing of each crop, without incorporating it into the soil. Over the 2000–2007 period, nineteen pig slurry applications were performed. The total amount of Cu applied was 0, 16.5, 33.0 and 66.0 kg ha^{-1}; for the Zn was applied a amount of 0, 20.4, 40.8 and 81.6 kg ha^{-1}; for the N was applied a amount of 0, 951.0, 1902.0 and 3804.0 kg ha^{-1}; for the P was applied a amount of 0, 624.3, 1248.5 and 2497.0 kg ha^{-1} and for the K was applied a amount of 0, 363.7, 727.4 and 1454.8 kg ha^{-1} for the pig slurry application rates of 0, 20, 40 and 80 m^3 ha^{-1}, respectively. The chemical attributes of these soils are indicated in Tables 1 and Supplementary material 1.

In January of 2008 the disturbed and undisturbed soil samples were collected at a depth of 0–20 cm. The disturbed soil samples were dried, ground and reserved for the cultivation of maize (*Zea mays* L.). The undisturbed soil samples were collected using PVC tubes of 200 mm diameter and 220 mm depth, with approximate volume of 6283 cm^3 of soil. These samples were taken in such a way that the soil layers were preserved, simulating cultivation in no-tillage system.

The treatments consisted of disturbed soil samples collected in the field plots with application of 0, 20, 40 and 80 m^3 ha^{-1} of pig slurry, and phosphorus (P) +potassium (K) treatment. The soil without pig slurry application was divided into two parts. One part was fertilized with P and K, respectively 125 and 90 mg kg^{-1} of soil (PK treatment), and the other was not fertilized with P and K (negative control). The equivalent amount estimated for the area was 362 kg ha^{-1} of P applied as triple superphosphate and 232 kg ha^{-1} of K applied as chloride of K. The treatments for undisturbed soil consisted of soil samples collected using PVC tubes in the field plots with application of 0 (control), 20, 40 and 80 m^3 ha^{-1} per crop of pig slurry, totalized the amount of 0, 380, 760 and 1520 m^3 ha^{-1} of pig slurry in seven years, respectively. The chemical attributes of these soils are indicated in Tables 1 and Supplementary material 1.

2.2. Greenhouse experiment

The experimental unit for the disturbed soil consisted of a pot that contained 4 kg of air-dried soil (2 mm mesh) and four plants. Additionally, the pot was internally lined with a plastic bag to prevent both the loss of nutrients and water drainage. The experimental unit for the undisturbed soil was composed of a pot containing approximately 11 kg of soil and four plants. The experimental design consisted of randomized treatments with six and eight replications, respectively for the undisturbed and disturbed soils. Additionally, the pots received periodic rotations in the greenhouse to avoid the effects of a single location.

The N fertilization (45 mg kg^{-1} of soil) was similar for all treatments in both the disturbed (control, PK, 20, 40 and 80 m^3 ha^{-1} of pig slurry) and undisturbed soil samples (control, 20, 40 and 80 m^3 ha^{-1} of pig slurry). The equivalent amount estimated for the area was 130 kg ha^{-1} of N applied as urea. The fertilization of N was divided into two applications, at the sowing (15 mg) and at eight days after plant emergence (30 mg), when the plant was in V2.

The experiment was conducted in a greenhouse that had no automatic temperature control. However, ventilation and mist was used to partially control the temperature on warmer days. Using distilled water, daily irrigations were performed for both the disturbed and undisturbed soil samples. The soil moisture content was measured daily (by weighing) and maintained between 60 percent and 80 percent of field capacity.

On days fifteen and 25 after plant emergence, respectively related to the stage of growth V4 and V6, maize plants from the experimental units of four disturbed soil samples and three undisturbed soil samples were harvested. At harvest, the plants were divided into roots, stalk and leaves to determine the fresh weight, plant height, and the number of senescent leaves and green leaves. Additionally, biochemical and chemical analysis were performed.

2.3. Soil analysis

After plant harvesting at 25 days after plant emergence, soil samples were collected for the analysis of pH, exchangeable Ca and Mg, K and plant-available P concentrations, and plant-available Cu and Zn. After being shaken for 30 min with an end-over-end shaker at room temperature (20 °C), the soil was extracted using 0.1 mol L^{-1} of HCl with a soil/solution ratio of 1:5. At the end of the shaking period (15 h), the supernatant solutions were separated from the soil and then measured for Cu and Zn using AAS (Embrapa, 2009). The exchangeable Ca and Mg were extracted using 1.0 mol L^{-1} of KCl in a soil/solution ratio of 1:20. The soil K and plant-available P concentrations were extracted using Mehlich 1 in a soil/solution ratio of 1:10. The P concentration was determined using the Murphy and Riley (1962) method, and the K concentration was determined with flame spectrometry (B262 Micronal).

2.4. Chemical analysis of the leaves

Dried leaf tissues (0.1 g) were ground and digested in 3.0 mL of HNO_3 plus 1 mL of $HClO_4$ (Embrapa, 2009). The sample digestion was performed in an open system and utilized a block digester Velp Scientifica (Milano, Italy), which was heated at 130 °C over 4 h. The Cu, Zn, Ca and Mg concentrations were estimated using flame