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AMELIORATIVE EFFECT OF EXOGENOUSLY APPLIED OXALIC ACID ON NICKEL HEAVY METAL INDUCED STRESS IN *ZEA MAYS*

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Abstract

Oxalic acid (ethanedioic acid: OA) is the simplest organic acid occurring naturally in plants. It functions as a chelator of metals. The effects of the exogenous application of OA (1000 μM) on maize plants' (*Zea mays)* growth parameters and superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), reduced glutathione (GSH) and lipid peroxide contents were evaluated in the presence or absence of nickel (Ni) induced stress. The plants were treated with 200 μM and 400 μM of nickel sulphate ($NiSO_4$) solution. At the end of the 29-day treatment period, OA enhanced SOD, GSH and catalase activity. The level of lipid peroxidation as determined by TBARS (thiobarbiturate reactive species) was decreased significantly. OA improved the growth parameters of maize plants under nickel induced stress (that is: the root length, root fresh weight and root dry weight all were increased compared to the control). This is a clear indication that OA ameliorates heavy metal stress in maize via biochemical pathways in which it acts as an important chelator and detoxifier of nickel. It thereby depletes the pool of Ni available for the production of free radicals.

Key words: *Zea mays*, Heavy metals, Nickel, Oxalic acid, Reactive oxygen species.

Introduction

Heavy metals (HMs) are naturally occurring trace elements and at high concentrations they are very toxic (He *et al.,* 2005). They are considered toxic when they appear in unwanted areas, at concentration levels from 1 mg kg^{-1} to 100, 000 mg kg^{-1}. At such a high concentration HMs tend to affect the quality of the soil and, subsequently, crop yield (Yang *et al.,* 2005). HMs such as chromium, nickel, zinc, lead and iron frequently come from industrial effluents generated by different processes such as metal plating, metal smelting, combustion of fossil fuel, fertilizer production and mining. The HMs are then deposited in the environment as a result of these processes (Ekmekyapar *et al*., 2006; Goddard & Brown, 2014). Unlike most other organic pollutants, HMs tend not to be degradable, making them very dangerous to humans and to nature in general. When these HMs accumulate in the environment they cause chronic acute toxicity (Jung & Thornton, 1996; He *et al.,* 2005). The resulting toxicity tends to increase more in the presence of human civilization, fossil fuel development and mining (Teodosiu *et al.,* 2014; Lofty *et al.,* 2013).

HMs are emitted as gas in almost every process of combustion in industrial areas and find their way to into the environment through rainfall (Xiao *et al*., 2015). The release of heavy metal pollutants from mining industries, agricultural practices and improper water disposal methods impose a major threat to quality of life and the agricultural sector (Lion & Olowoyo, 2013).

When heavy metals find their way into the living environment they interact with the living systems of plants and animals, disturbing the normal functioning of the living systems and activating a cellular response (Koeadrith *et al*., 2013). Plants need nutrients in order to survive and these nutrients are accessed by the plants from the soil. Heavy metal ions may also be taken up along with the nutrients and are then found in the plant tissue. Some of the heavy metals include nickel, chromium, zinc, iron, manganese, cobalt, cadmium, aluminium and lead (Ovecka &Takac, 2014).

Heavy metals cause a wide range of adverse effects, such as acute heavy metal toxicity and defects in cells. The chemical similarity of HMs to essential metals means they tend to be substituted with the essential metals necessary for normal cellular function. At high levels, HMs interact with and disrupt vital cellular biomolecules, such nucleic proteins and DNA, causing excessive augmentation and the production of reactive oxygen species (ROS) (Sharma & Dietz, 2009; Yadav 2010; Emamverdian *et al*., 2015). Reactive oxygen species tend to cause a detrimental effect on the cellular reactions in plants, including aerobic respiration and photosynthesis (Karkonen, 2014).

When reactive oxygen species, like hydrogen peroxide, are produced at homeostatically favourable conditions they trigger a defence response that impacts on the proper growth and development of the plant (Klein *et al*., 2013; Qiao *et al*., 2014). High levels of ROS, on the other hand, tend to reduce plant growth, and heavy metal toxicity inhibits the ability of plants to take up water and nutrients, affecting the development of the embryo, flowering and seed formation as a result (Ovecka, 2014). Plants usually scavenge reactive oxygen species in order to avoid oxidation damage, and they do this by using both enzymatic and non-enzymatic mechanisms. Enzymes such as glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase and ascorbate peroxidase (APX) are produced in order to scavenge the deadly effects of reactive oxygen species (Oukarram *et al*, 2015; Dazy *et al*., 2009).

Plants experience stress when they take up heavy metals from the environment and this leads to the damage and disturbance of their ionic homeostasis. In order to minimize the problem of stress resulting from heavy metal uptake, plants have developed a mechanism which detoxifies heavy metals by making use of phytochelatins produced within the plant (Yadav, 2010). Phytochelatins are metal-binding proteins produced by plants to counteract and protect plants from the effects of heavy metals (Ahner *et al*., 1995). Phytochelatins are oligomers of glutathione. Phytochelatins are found in plants, fungi and nematodes and are produced by the actions of the enzyme phytochelatin synthase. Phytochelatins function as chelators, which are important for the detoxification of HMs (Rodrigo *et al*., 2016).

The amount of HMs taken up by plants depends also on the pH value of the soil, clay soil content, the oxide content and also organic carbon content (Xiao *et al*., 2015). OA is the simplest di-carboxylic acid which can be found in plants in the form of soluble salts, free acid and calcium oxalate crystals (Haara *et al.,* 2014). OA is a very important chemical that tends to function as a chelator of metals and it is produced as a by-product in microorganisms that use the TCA and glyoxylate pathway (Keiichi *et al.,* 2014). Basically, chelating agents are ligands. They form complexes with substrates, usually metals, and bonding may be ionic or covalent or through the dissolution of the metals in water (Di Palma *et al*., 2015).

It has been established that OA plays a very important role in fungi and other plant species. They act as an electron acceptor in the manganese peroxidase catalytic cycle by regulating oxygen species through Fenton reaction as an osmotic as well as a pH regulator (Graz & Jarosz-Wilkolazka, 2009). OA has 2 pKa values of 1,23 as well as 4,19 and is mostly found in sediments, forests and soils that are used for agricultural purposes (Tu *et al.,* 2007). The low pKa value of OA is very important as it makes it easy to donate a hydrogen electron whenever needed. Oxalic acids are far more soluble than inorganic compounds, which help them form heavy metal ion complexes easily and reduces its fixation, mobility, availability to and toxic effect on plants (Nigam *et al*., 2007).

Heavy mining of a localised area has been found to be a major cause of heavy metal contamination of soil (Webber 1981; Freedman & Hutchinson 1981; He *et al*., 2005). Mining and refining of metal ores have been reported to cause severe contamination by heavy metals in areas where the mining activities are carried out (Jiang *et al*., 2004). The effect of HMs on plants include the inhibition of growth, structural damage, a general decline in physiological and biochemical activities, and ultimately the general functioning of plants (Cheng, 2003).

In this research, we investigated the use of OA as an ameliorating agent of HM in *Zea mays*. This is with the aim of improving plant defence against HM and boost the yield and production of maize, which is stable in HM infected soil.

Materials and Methods

Plant growth and preparation: Sixty (60) maize seeds were surface sterilized in 0.35% sodium hypochlorite for 10 minutes and washed five times using distilled water. The seeds were then soaked in distilled water five times. Each soaking lasted for about an hour. They were then ready to be planted. The maize seeds were planted at the University of Zululand, Department of Botany inside the greenhouse to control watering. The groups were subdivided as follows:

Procedure for planting the maize seeds: The seeds were planted in a 2L, 20cm plastic pot filled with river sand at room temperature in a greenhouse. Each seed was pre-soaked in distilled water, and planted at 10 mm deep in a plastic pot. The seeds were watered at 2-day intervals with distilled water until germination. At the V2 stage of plant development, the plants were separated into 6 groups of treatment. Each group contained 10 plants per group and each plant was treated for a period of 29 days.

Preparation of nutrient solution: The nutrient solution was prepared using the method described by Hoagland & Arnon (1950) Table 1.

Table 1. Reagents used to prepare a nutrient solution.

Components	Stock solutions	mL stock solution /L
Macronutirents		2,5 ml
KNO_3	202g/L	2,5 ml
$Ca(NO_3)_2$. $4H_2O$	236g/0,5L	1,5 ml
Iron (sprint 138 iron chelate)	12g/L	1 ml
$MgSO_4.7H_2O$	493g/L	1 ml
NH_4NO_3	80g/L	1 ml
Micronutrients		
H_3BO_3	2.86g/L	1 ml
$MnCl_2.4H_2O$	1.81g/L	1 ml
$ZnSO_4.7H_2O$	0,22g/L	1 ml
$CuSO_4.5\ H_2O$	0,051g/L	1 ml
$H_3MoO_4.2\ H_2O$	0,09g/L	1 ml
KH_2PO_4 (pH to 6.0)	136g/L	0,5 ml

Stock solution was made and stored in bottles. Each aliquot was added to 800 mL of double distilled water and made up to 1L. The resulting nutrient solution was then ready to be used on the plants.

The maize plants were harvested after the 29-day treatment Table 2. The roots were properly washed to remove the soil and allowed to dry. The plants were divided into 2 groups of 30 plants, 5 from each treatment. The first group was used for growth parameters and the second group was used for enzyme assay.

Measurement of growth parameters: Growth parameters, like root length and dry matter content for all plants, were recorded following standard procedure. For root length, a tape measure was used to measure the length of the plants as per concentration from the first brace roots to the tip of the longest root. For dry weight measurements, the harvested plants were carefully blotted to remove any surface moisture and dried at 80ºC for 72 hours (Klein *et al*., 2013).

Biochemical enzyme assay: The roots were harvested, washed and ground in a phosphate saline buffer (pH 6.8) at a ratio of 0.5:3 (g:ml). Next, the mixture was homogenized for 10 minutes and centrifuged for about 15 minutes at 3200 rpm. Supernatant was collected and stored at -80º C for enzyme assay.

Table 2. Subdivision of maize groups based on the treatments they received for the 29-day treatment.

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Control-Nutrient solution	OA 1mM	200µM HM + 1mM	400µM HM + 1mM OA	200µM HM	400µM HM

Table 3. Growth parameters of the maize plant.

	RFW	RDW	SL	SFW	RL
Control	8,432 ± 0,436	6,532 ± 0,028	74 ± 1,854	317 ± 18,32	317 ± 18,321
Oxalic Acid	11,358 ± 0,370***	8,68 ± 0,3931***	81,8 ± 3,548***	377 ± 6,505***	377 ± 6,505***
200mM Ni + OA	5,996 ± 0,119***	4,24 ± 0,161***	67,8 ± 0,769*	291,6 ± 13,085	291,6 ± 13,085
400mM Ni + OA	2,984 ± 0,325***	3,268 ± 0,122***	57,8 ± 2,55***	281 ± 17,458*	281 ± 15,937**
200mM Ni	1,854 ± 0,404***	1,32 ± 0,111***	52,2 ± 2,142***	144,4 ± 13,003***	144,4 ± 13,003***
400mM Ni	0,976 ± 0,091***	0,682 ± 0,096***	47,8 ± 2,613***	91,2 ± 3,933***	91,2 ± 3,933***

Root fresh weight (RFW), Root dry weight (RDW), Shoot length (SL), Shoot fresh weight (SFW), Root length (RL). Plant parameters in maize was measured after the period of 29 days treatment that was initiated at V2 stage of vegetative growth. Table represent the means ± SD of the 6 independent groups with 5 separate plants used for each treatment ($p<0,05$)

Etermination of catalase, SOD and GSH activities in *Zea mays*: Catalase, SOD and GSH were determined using a Catalase, SOD and GSH kit according to the manufacturer's instructions (Sigma –Aldrich). The method of Halliwell & Gutteridge (1990) was used to determine malondialdehyde (MDA)—TBARS. 1.5 ml of TCA (10 %) was added to 100 µl of the homogenized root and incubated for 10 minutes. The mixture was centrifuged for 20 minutes at 3500 rpm . Then, the supernatant was collected and mixed with 1.5 ml of 1% TBA. The collected mixture was boiled for a period of 30 minutes in a water bath and allowed to cool. 2 ml of n-butanol was added. The presence of a butanol layer was measured at an absorbance of 532 nm. The value of non-specific absorption at 600 nm was then subtracted from this value.

Statistical analyses: The results are presented as a ± standard error of the mean (SEM). Data was analysed using a one-way analysis of variance (ANOVA).

Results and Discussion

The enzymes ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) are antioxidant enzymes that help to prevent formation of the highly toxic ROS hydroxyl radical (Mittler, 2002). ROS in plants tend to be very reactive and toxic and damage DNA, proteins, carbohydrates and lipids which invariably causes oxidative stress, and lipid peroxidation in cells (Mittler, 2002).

Exposure to extreme environmental conditions including extreme temperatures, heavy metals, drought, air pollutants, nutrient deficiency as well as salt stress tends to increase the formation of intracellular ROS in plants (Gill and Tuteja, 2010). Plant cells employ antioxidant defence mechanisms to protect their cellular integrity against the toxic oxygen intermediates which are formed as a result of environmental stressors (Gill & Tuteja, 2010).

HMs inhibit growth, cause structural damage, as well as a decline in physiological and biochemical activities and the general function of plants (Cheng, 2003). At high concentrations, some HMs tend to decrease the yield of crops (Liu *et al*., 2003). The effects of OA on HM induced stress on growth parameters in *Zea mays* were investigated in this study.

Citric and OAs are organic acids. They form complexes with metals and are relatively stable, with a greater ability to move and form complexes with metals from the soil compared to acetic and formic acid which do not form stable complexes (De Wit *et al*., 1993; Ravichandran, 2004 and Kim *et al*., 2013).

The results clearly indicate that Nickel (HM) has a severe adverse effect and impact on plant growth parameters (such as shoots, roots and fresh weight). The effects shown in Table 3 suggest that the growth parameters of maize are severely affected by Ni, but growth parameters are improved when OA is added to heavy metal induced plants. Approximately 67% increase in root fresh weight was observed in plants treated with OA and Ni when compared with those treated with HM alone (Table 3). Table 1 shows a 79% increase in the root dry weight of plants treated with OA and Ni compared to those treated with Ni alone. A 68% increase in the root length of plants was observed with the addition of OA compared to plants induced with Ni (Table 3). Plants treated with OA and Ni showed a 17% and 68% increase in shoot length and shoot fresh weight compared to those treated with Ni alone (Table 3). It is clear that the application of OA reduced the effects of Ni on the plants. The plants treated with OA alone had the highest shoot length, root length, root fresh weight, shoot fresh weight and root dry weight. The physiological and biochemical impacts of Ni showed structural damage including inhibition in growth and in the general functioning of plants.

The results of the study were similar to those of Athar & Ahmad (2002); Cheng (2003); Oancea *et al*., (2005) Chibuike & Obiora (2014). The results were also similar to other findings where different stress factors were introduced, like salinity which induced oxidative stress in plants (Misra *et al*., 1997; Memon *et al*., 2010; Amira & Qados, 2010 and Klien *et al*., 2015).

Transition metals are essential for plants to maintain growth. However, when concentrations increase, they become toxic, induce stress and reduce growth parameters such as shoots and roots (Zhou *et al.,* 2013).

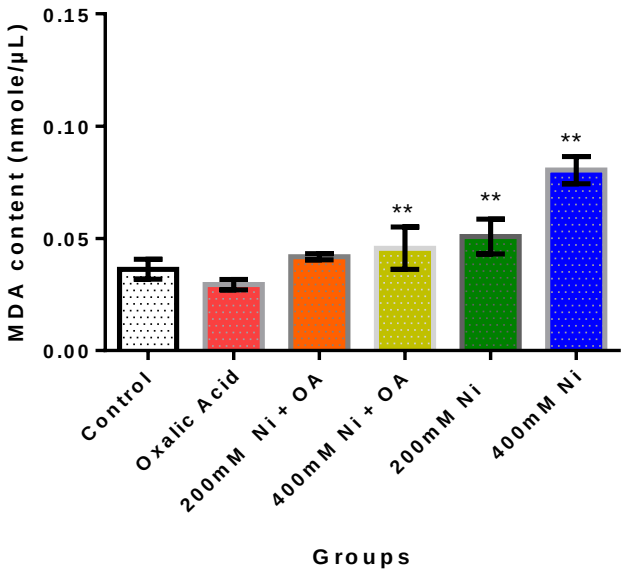

Fig. 1. MDA content. MDA content in response to exogenously applied OA and Ni stress. MDA content was measured spectrophotometrically in maize plant roots. Assays were done at stage V2 of the vegetative growth for a period of 29 days. Bars represent the mean± SEM of 6 independent experiments on 5 separate plants for each treatment ($p<0,05$).

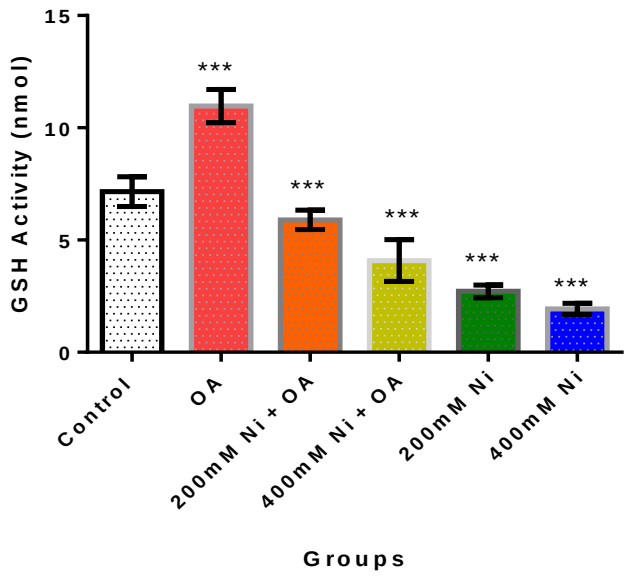

Fig. 2. GSH activity. GSH activity in response to exogenously applied OA and Ni stress. Enzymatic activity was measured spectrophotometrically in maize plant roots. Assays were done at stage V2 of the vegetative growth for a period of 29 days. Bars represent the mean± SEM of 6 independent experiments on 5 separate plants for each treatment ($p<0,05$).

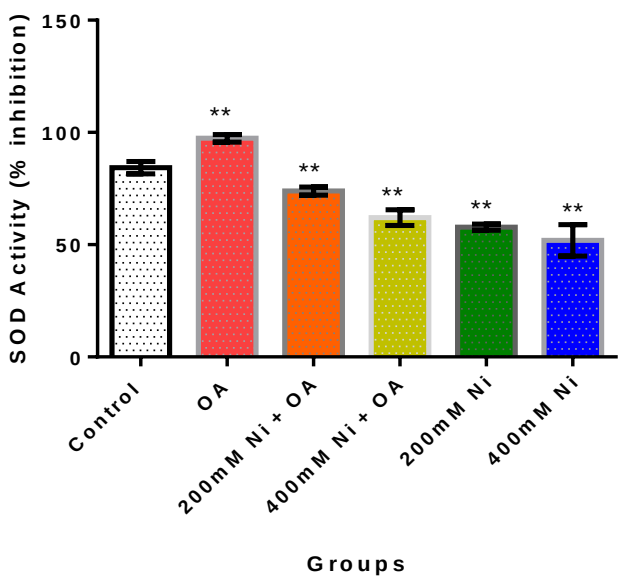

Fig. 3. SOD activity. SOD activity (% inhibition) in response to exogenously applied OA and Ni stress. Enzymatic activity was measured spectrophotometrically in maize plant roots. Assays were done at stage V2 of the vegetative growth for a period of 29 days. Bars represent the mean± SEM of 6 independent experiments on 5 separate plants for each treatment ($p<0,05$).

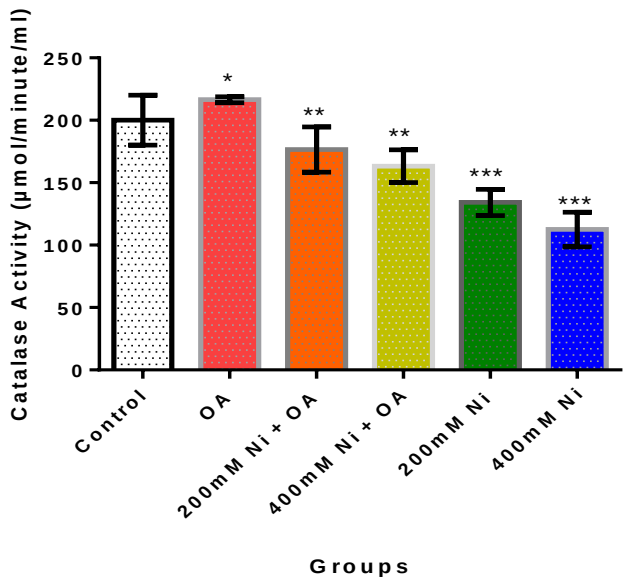

Fig. 4. Catalase activity. Catalase activity in response to exogenously applied OA and Ni stress. Enzymatic activity was measured spectrophotometrically in maize plant roots. Assays were done at stage V2 of the vegetative growth for a period of 29 days. Bar graphs represent the mean± SEM of 6 independent experiments on 5 separate plants for each treatment ($p<0,05$).

It was apparent that when the Ni accumulated above their normal required concentration they became toxic and inhibited the growth of the plants and roots. However, when OA was applied to the plants, the growth parameters were seen to improve in comparison to those treated with heavy metals alone, and were also seen to be higher than the untreated normal control. This could be attributed to OA being involved in the mobilization and dissolution of soluble nutrients and in the detoxification of heavy metals causing nutrients to be available for better plant growth (Jones *et al.*, 2003).

An antioxidant system regulates the effects of oxidative stress levels in plants. These defence systems are metabolites like ascorbate, glutathione, tocopherol and several others. They are also composed of an enzymatic scavenging system which scavenges free radicals such as ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) (Schutzendubel & Polle, 2001; Asada, 1999; Noctor & Foyer, 1998). The exposure of plants to HM disturbs the homeostasis of cells, resulting in the generation of ROS.

We went further by evaluating the effects of OA on the intracellular enzymes involved as antioxidants when the plants were exposed to Ni. ROS, which were produced due to oxidative stress, activated lipid peroxidation. It was clear that the application of OA assisted in the prevention of lipid peroxidation. It was observed that lipid peroxidation was decreased when OA was applied (Fig. 1). Levels of lipid peroxidation were high in treatments with a high levels of Ni (Fig. 1). A 24% increase in the MDA was observed in plants treated with Ni as compared with those treated with a combination of Ni and OA. These results correspond with the findings of Nigam *et al*., (2007); Sadak & Orabi (2015) which reported that OA functions as a chelating agent, mopping up heavy metals, causing less stress to the plant and result in a reduction of any lipid peroxidation that may occur. The levels of GSH tend to increase by 53% in plants treated with combinations of Ni and OA when compared to those induced with Ni alone (Fig. 2). Plants treated with OA alone showed 82% increase in GSH as compared to those with Ni and OA. There was also a 53% increase in plants treated with OA when compared to the untreated plants. Figs. 2-4 show the increase in enzymatic activities in the plants stressed with Ni and OA. The enzymatic activity tends to increase with the treatment of OA. An increase of approximately 22% and 31% SOD and catalase were observed respectively. Plants treated with OA alone showed higher enzymatic activities which were even 15% for SOD and 8% higher than the untreated control. These results correlate with the findings of Sadak & Orabi (2015) where it was demonstrated that exogenously applied OA tends to improve activity by assisting enzymes in fighting against free radicals. The results obtained indicate that the use of OA or other chelating compounds are effective when applied to plants undergoing stress, and even in normal conditions. OA induces an ameliorative effect on plants stressed with Ni. OA improves the physiological and biochemical structure of *Zea mays* and in turn improves yield.

Conclusion

In conclusion, this study demonstrated that when maize plants were stressed by nickel they tended to be affected biochemically and physical appearance. However, the exogenous application of OA tends to detoxify or chelate Ni which is a heavy metal. The activity of OA not only detoxifies Ni, but also stimulates better plant growth and increases the activity of enzymes. These results seem to suggest that plants can be planted in areas where there are defects in the soil, whether it is acidic, stressed by heavy metals, or even salt. The exogenous application of OA will counteract these effects and even promote better plant growth than that found normal soil.

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