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Effect Assessment of TiO2 Nanoparticles Exposure on Medicago by Monitoring Morphophysiology

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EFFECT ASSESSMENT OF TiO_2 NANOPARTICLES EXPOSURE ON MEDICAGO BY MONITORING MORPHOPHYSIOLOGY

A Capstone Project Presented in Partial Fulfillment

of the Requirements for the Degree Bachelor of Biochemistry

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By

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I Dedicate this Thesis to Linnea Neslon, who kept me sane during the semester I wrote it, to Dr. Devesh Shukla, who showed me how to process and analyze my results, to Dr. Sahi and Dr. Sharma, who gave me the opportunity, and my family, for setting me on my current path.

ABSTRACT

In recent years titanium dioxide nanoparticles (TiO_2NPs) have been ingredients in everything from paints to cosmetics, and even in some kinds of food. This growth in use has resulted in a substantial increase in the amount of titanium released into the environment, which could have detrimental effects on nearby plant and animal life. Currently, the number of studies conducted on the effects of TiO_2NPs is quite small, especially when it comes to edible crops.

Because of this lack of research data, this study has been designed to assess the effect of TiO2 NPs exposure on growth and physiology of *Medicago truncatula*. This plant was chosen because each species has a unique reaction to nanoparticles, and it also an important feed crop for the cattle industry. The plants were grown in Turface MVP® soil that had been treated with 250, 500, 1000, and 2000 parts per million of TiO_2NPs for two weeks and then examined for changes in biomass, metal ion concentrations, and gene expression related to antioxidant and photosynthesis.

The results varied between the different experiments, but in general the dry weight showed a decrease in mass from the control to the treated soils. The metal nutrients estimation, which recorded a spike in titanium content in the 500 and 1000 PPM samples, showed a correlation between the titanium and important building blocks such as phosphorus, and a majority of the genes tested showed a spike in shoot expression at 250 PPM relative to control mark followed by a decline with the other samples. Altogether, it appears that TiO2 NPs adversely affect the growth of Medicago at high concentration exposure.

VITA

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INTRODUCTION

Naturally generated nanomaterials, which are particles less than 100 nm across that can be made of both organic and inorganic matter, have existed for as long as the earth itself. Volcanic dust is made of nanoscale shards of volcanic glass, and most organic molecules, including proteins, RNA, and short segments of DNA, count as nanoparticles as well. Plants are used to these naturally occurring nanoparticles, and have pathways for dealing with them (Heiligtag et al, 2013). This is not the case with manufactured nanoparticles, or MNPs.

MNPs are a relatively new addition to the environment. The process of making them is as old as the Roman Empire, but to nature a few thousand years is a mere flicker. The number of surviving artifacts that use nanoparticles is small, but a notable example is the Lycurgus Cup, which appears green when lit from the outside and glows red when lit from within (Heiligtag et al, 2013). This effect was caused by infusing gold nanoparticles into ruby glass. Another example is glazed ceramics, where two layers of silver nanoparticles around 430 nm apart cause a phase shift between blue and green (Heiligtag et al, 2013). Even then, MNPs weren't used or manufactured commonly, and instead were the purview of the elite.

It wasn't until the 20th century that humanity started to mass produce MNPs. Nanoparticles have electrical and chemical effects vastly different from other, larger samples of the same material, thanks to their small size and high surface area to volume ratio. This makes them excellent for a wide variety of uses where macro materials would fall short. For example, titanium dioxide has a high refractive index, making it excellent

for white paint, sunscreen (Fig. 2) and improving the color of white foods such as sour cream (Cox et al, 2016).

In modern industry, zinc, iron, titanium, copper, cerium, gold, silver, nickel, and aluminum have been converted into oxide particles less than 100 nm across, and added to medicine, food, paint, solvents, solder, magnets, and many other products. In food, these particles are mostly for cosmetic effect and preservation, as they are nonreactive and can act as safe dyes (Sheth et al, 2012) and emulsion controllers (Dickinson, 2011), while their industrial uses include enhancing the extraction rate of oil (Onyekonwu et al, 2010) and improving cell morphology in linear polypropylene (Zheng t al, 2010). These products are then consumed and thrown out, resulting in 1.31 million tons (Cox et al, 2017) of MNPs being released into the environment every year as of 2014. Waste treatment plants are not equipped to deal with most MNPs, so this flood is almost entirely unchecked.

The exact influence of nanoparticles on the environment is unknown, but initial studies on their effects on plant growth paint an alarming picture. Plants that uptake nanoparticles have a different reaction based on the size, shape, and composition of the given particle or the species of plant exposed. For example, Fe_2O_3 causes reduced biomass in Mycorrhizal Clover (Tripathi et al, 2017). Copper oxide lowers root growth in Raphanus sativus (Radishes) (Tripathi et al, 2017). Silver NPs decrease the rate of transpiration in Cucurbita pepo (Field Pumpkins) (Tripathi et al, 2017). Zinc oxide reduces chlorophyllous content in Pisum sativum (garden peas) (Tripathi et al, 2017). Bentonite clay inhibits leaf growth in Zea mays (maize) (Tripathi et al, 2017). Silicon also inhibits seed germination in Cucurbita pepo (Field Pumpkins) (Tripathi et al, 2017).

MNPs are small enough to slip between the plant's cells, and tend to get stuck in their pores, resulting in a buildup of toxins, water, nutrients, and other organic matter that needs to travel through blocked pathways. MNPs can also shred a plant's cell walls from the inside out (Tripathi et al, 2017), resulting in severe structural damage that sometimes proves fatal. Plants have no defenses against this kind of nanoparticle, and are adversely affected when MNPs are introduced to the environment on an industrial scale.

However, some MNPs are not as destructive as their fellows. In the right concentrations, certain MNPs have no effect at all on plant health at all, or even a positive one. For example, titanium dioxide is unusually beneficial at low concentrations, with previous studies reporting as much as a 100% biomass increase for tomatoes grown in soil with it (Tiwari et al, 2017). When TiO_2 NPs damage the cell walls in the roots, more secondary and tertiary roots grow from the holes, increasing the roots overall growth rate (Cox et al 2017). For a material that first attracted research as a pollutant, the idea that TiO_2 NPs might be a form of fertilizer instead is not insignificant. Farms are always looking for a way to increase crop yield, and anything that can provide such a massive boost should be investigated.

The catch is that each plant species has a different ideal treatment threshold, and must be tested individually. The exact reason behind this variation is unclear, but it can be assumed that it has something to do with the differences in biochemistry and cell biology between species. While many common food crops have been tested in the past, no one has yet performed any experiments on Medicago truncatula. Medicago truncatula, or the barrel clover, is not a food crop, but a feed crop, primarily fed to cows as a source of protein in the winter. This means that the most immediate effect this research will have

is in the cattle industry. The ability to make more cattle feed for less space, time, and money could help lower the price of beef, allowing for more people to purchase it on a regular basis.

Nanoparticles do have limitations though. Only low concentrations of MNPs have been shown to be beneficial (Cox et al, Tiwari et al, 2017). It's entirely possible that the barrel clover's ideal concentration is too low to achieve reliably, or that the plant doesn't respond favorably to the treatment at all. Nanoparticles do sometimes improve growth, but most of the time they harm more than they help.

The goal of this research is threefold. First, it will study the effects of TiO_2NPs on plant growth, as measured by the overall mass of plants treated with these nanoparticles. Second, the study will measure the accumulation of Titanium in the root and shoot and analyze how that accumulation affects the uptake of macro and micro nutrients such as Iron and Phosphorous, respectively. Third the study will examine the gene expression patterns related to photosynthesis and antioxidant metabolism to determine how an excess of TiO_2 affects these genes's activation and inhibition.

MATERIALS AND METHODS

Medicago truncatula "Jemalong" was the exact species used, as its entire genome has been sequenced, allowing for easy genetic testing. The seeds were acquired from The Western Regional IP Station in the U.S. via the U.S.A. National Plant Germplasm System (PI 442895 SSD). Each came in a packet of 200 seeds, and were planted in sets of 100.

Growth Phase

The seeds were first cleaned in sterile DiH_2O and kept at 4℃ for a week to promote germination. They were then planted in a single tray of MVP soil per group, a special brand primarily used on baseball diamonds that has no nutritional value and a consistency somewhere between sand and gravel, allowing for easy cleaning and transplanting.

The plants were grown in a Percival Intellus Environmental Controller growth chamber at 23℃ and 70% humidity. The growth chamber allowed for a careful, exact control of the plants circadian rhythms and nutrient uptake. The plants were watered between noon and 1:30 P.M. to maximize growth effectiveness.

The soil was watered with full strength Hoagland solution every time it dried out, which usually consumed 750-850 ml of solution. This happened once every three days, and resulted in a total of three liters of Hoagland solution over the course of two weeks. After those two weeks had passed, the plants were transplanted to individual pots to prevent root overlap and nutrient interference.

Treatment Phase

Each set of potted plants was split into a cluster of eight for treatment (Fig. 1). The first cluster was a control, and had no titanium added. The second cluster had a titanium soil concentration of 250 Parts Per Million (PPM), the third had a concentration of 500 PPM, the fourth had a concentration of 1000 PPM, and the fifth had a concentration of 2000 PPM. Each of these groups was then watered every day with deionized water (DI-water) at noon for two additional weeks as the treatment was absorbed. MVP soil has poor water retention, and the plants wither and die after as little as two continuous days without watering, so a less frequent schedule was not sufficient. Two trials were conducted in this way, along with an additional control to rectify erroneous readings.

Nanoparticles

The NPs came from a 20% stock sold by US Research Nanomaterials, Inc., product number US7070, with a size of 30-50 nm across, and were mixed directly into the soil by gloved hand. These spheres were chosen because they displayed the most reliable positive results among nanoparticles previously tested.

Harvest

The plants were carefully removed from their planters and washed with DI-water to remove the soil, then separated into the root and shoot sections for the fresh weight. This separation was carried out because plant roots and plant shoots and leaves react differently to stress. As such, their genetic expression and metal ion readings would be significantly different and require individual study.

Half of the samples were then dried in an incubator at 50°C for 3 days to attain the dry weight. The other half was gently crushed and stored at -80°C to be used later for genetic testing.

Biomass

The dry weight, in grams, is a combination of the independent dry root weight, the independent dry shoot weight, and the overall combined dry root and shoot weight. The fresh weight was recorded but not used as the plants were still damp from the harvesting process, which would have muddied the results.

Metal Ions

The metal ion concentration was estimated using an inductively coupled plasma atomic emission spectroscopy, or ICP-OES. Two dried 30mg samples from each trial of root and shoot were digested in 10 ml of concentrated nitric acid, then carefully filtered to remove the remaining plant matter. They were then run through the ICP-OES machine at the WKU South Campus to check for Calcium, Copper, Iron, Potassium, Magnesium, Phosphorous, Sulfur, Titanium, and Zinc content. The ICP-OES did the scan by comparing each sample to a series of controls with set amounts of each compound.

Genetic Expression

The gene expression levels were quantified by RT-qPCR, using the standard method. Ten samples of mRNA were derived from 10 samples exposed to TiO2 NPs. The mRNA was then mixed with a cDNA preparation solution to obtain 10 cDNA samples. Eight distinct primers that corresponded to known enzymes were designed. One of the

primers, Tubulin 1-Beta Chains, was used as an endogenous control. The frozen samples were carefully ground, then processed to isolate the RNA as the protocol dictates detailed below, followed by a dilution to bring it to the proper concentration levels to run the PCR. Each trial, which consisted of two unique markers plus Tubulin Beta-1 Chains (TB), was run through the machine twice, resulting in a total of six tests for each marker in each sample.

RNA Extraction Technique

The manufacturer's protocol was used to extract the RNA from the -80°C fresh samples. The kits used were Qiagen and Sigma-Aldrich. This was done by grinding the frozen samples to a fine powder while submerged in liquid nitrogen, then run through a filtration column with a lysis solution to remove the cellular debris. After the samples were filtered, they were transferred to a binding column and rinsed with wash solutions and DNAse to remove the protein and DNA from the mRNA. 50 µl of RNAse free water were used to remove the mRNA from the column, then a Spectrophotometer Nano Drop was used to determine the resulting mRNA concentration.

RESULTS AND DICUSSION

The purpose of the study was to understand the effects of titanium NPs on Medicago growth. Because of this, the results are split into three subsections: the dry weight measurements recording biomass, the ICP readings measuring nutrient and metal ion uptake, and the RT-PCR tables recording genetic expression.

Biomass

A nanomaterial's most obvious effect will always be on the biomass, or overall size of the plant. This can be measured by drying and weighing the samples after harvest. Fresh measurements were also recorded, but because those fresh weights were still damp from having the soil cleaned away and could not be dried without affecting the genetic expression tests, they are considered unreliable and not shown.

With that in mind, the weight results indicated a preference for the control over any of the concentrations used, although the dry weight (DW) at 250 PPM titanium soil concentration was higher than any other non-control treatment concentration (Fig. 3-5). This preference is primarily visible in the root samples (Fig. 3), while the shoot samples show the control and the 250 PPM concentration had equal growth and mass (Fig. 4). The concentrations of 500 PPM and 1000 PPM were the smallest, causing the most inhibition of the barrel clover's growth, which aligns with previous experiments on tomato that showed a steady decline in growth after peaking at 250 PPM (Tiwari et al. 2017).

Overall, the samples of 500 PPM, 1000 PPM, and 2000 PPM were all shown to have statistical significance when compared to the control, while the 250 PPM sample

was not. This significance measurement was consistent across both root and shoot samples, along with their combined result.

Curiously, both the root and the shoot showed a nonsignificant uptick in mass at 2000 PPM, suggesting that extremely high concentrations of titanium (2000 PPM and higher) might be more beneficial than initially thought. All previous studies used concentrations that were considerably lower than 2000 PPM, with the highest being1000 PPM, which is clearly shown to have a negative influence. It possible that another study into extremely high nanoparticles concentrations along the lines of 2000-5000 PPM might be advisable.

Metal Content

Since part of a nanoparticle's effect on a plant is interfering with its nutrients, waste, and other transportation pathways, another way to measure its influence is to record the metal content. Elements like iron, copper, zinc, calcium, and sulfur are all vital to the ongoing function of plant life, and TiO_2NPs could interfere with their collection and distribution, starving and gorging a plant's cells based on whether a given cell has a deficit or an overabundance of a given metal.

Calcium, copper, iron, potassium, magnesium, phosphorous, sulfur, and titanium were all measured for their concentration in PPM, also known as metal content. Of those elements, potassium (Fig. 12 and 13), magnesium (Fig. 14 and 15), and sulfur (Fig. 18 and 19) showed no significant differences between the control and the other titanium soil concentrations, with magnesium specifically found to be completely unaffected.

Calcium (Fig. 6 and 7) shows an insignificant dip from the control to 250 PPM, followed by a significant spike from 500 PPM to 2000 PPM. It clearly shows that a high concentration of TiO_2NPs is shared by a high concentration of Calcium, and when compared to the weight results (Fig. 3-5) demonstrates a correlation between elevated levels of Calcium and lowered levels of growth.

Copper measurements (Fig. 8 and 9) resulted in a steady, but mostly not significant decline as the concentration increased, save for the 500 PPM and 1000 PPM shoot samples. The shoot also shows a slight uptick at 2000 PPM while the root declines even further, but both differences are just barely not significant. If the tolerance was .075 instead of .05, they would count.

The iron root content (Fig. 10) is much the same, a slow decline in content from the control as the titanium soil concentration increased. The shoot (Fig. 11), however, has a not significant spike at 500 PPM followed by a drop to 1000 PPM and 2000 PPM. The 2000 PPM measurement is significantly different from the control, making it the only measurement of iron to do so.

Phosphorous (Fig. 16 and 17) varies wildly when compared to most of the other metal measurements taken. As a result, most of the recorded Phosphorous concentrations are not significant, with the sole exception of the shoot 250 PPM samples. This sample shows a decline in concentration from the control.

Titanium (Fig. 20 and 21), as would be expected, grows in concentration as the amount of TiO_2NPs added to the soil increased. In the root, the 500 PPM and 2000 PPM were not significant, while the shoot samples of 500 PPM, 1000 PPM, and 2000 PPM

were significant. Between them, they show a steady rise in content levels as the treatment dosage was increased. What was not expected, however, was just how small the concentration was. When compared to elements such as sulfur and magnesium, which both consistently measured around 8000 PPM, titanium ranges from .6 (the smallest control measurement) to 47 (The largest 2000 PPM measurement) PPM, a miniscule fraction of the whole by comparison.

Genetic Expression

While recording the biomass and metal content of the barrel clover can provide an indirect measurement of the treatment's effects, the direct approach is gene expression. The relative frequency of various genes involved in antioxidant and photosynthetic processes will record exactly how the plant is reacting.

Antioxidant genes are a sign of stress. When a plant is stressed, it produces free radicals, negatively charged oxygen atoms and oxygen heavy molecules that will damage any nearby organelles and proteins unless dealt with. A plant has multiple pathways to safely remove the charge, but the most common is turn the free radical into peroxidase and then water. Free radicals are naturally generated by photosynthesis, explaining why the plant has a defense mechanism against them and why measuring them is useful. If antioxidative stress is high, photosynthetic activity is also high, because the latter causes the former. Since a healthy plant performs as much photosynthesis as it possibly can, the higher the expression of those genes the better.

Photosynthetic genes are much more direct measurements. They record how often proteins required to perform the necessary reactions are created, and a higher

frequency means more photosynthesis being performed more quickly, causing the proteins already created to wear out and need replacement.

Just recording the gene expression directly doesn't provide context and isn't helpful, so the gene expression described is relative to two points of reference. The first is Tubulin Beta-1 Chains, referred to as TB, a protein that is used to build microtubules and not affected by the treatment. It serves as a baseline to ground the expression rates. The second point of reference is the control sample. After all the samples have been compared to the TB, the control is set as the standard and the other samples are compared against it. When a sample is shown to have a relative expression of two, it means that sample had twice as many copies of that gene as the control did. This is much more useful when measuring whether a treatment makes a certain gene more active or less active.

Antioxidant Relative Genetic Expression

The first gene measured was L-ascorbate peroxidase, or APX (Fig. 22), a protein that turns H_2O_2 into water, completing the last step in the neutralization of free radicals (Caverzan et al, 2012). While the gene shows an increase beyond the control at 250 PPM in both root and shoot as well as an increase in the shoot only at 500 PPM and 2000 PPM, the only significant results are the 1000 PPM and the root's 2000 PPM measurements. This shows a reduction in antioxidative strength, which suggests fewer free radicals and less active photosynthesis at 1000 PPM, while other treatment levels resulted in more of both.

Glutathione reductase, or GR, a protein that splits glutathione disulfide into two copies of glutathione, which is a molecule used to fight oxidative stress (Chang et al,

1978), was consistently down regulated in all samples regardless of treatment concentration (Fig. 23). While only the 250 PPM and 2000 PPM samples could be considered significant, having the gene downregulated in all samples strongly suggests that this result is reliable. An interesting note is the split between root and shoot. The shoot samples of GR follow the pattern of the biomass results, while the root mirrors that of the patterns found in magnesium, phosphorous, and sulfur. It's possible that this gene directly affected those metal contents, although the content of sulfur might be responsible for this gene's expression rather than the reverse.

Monodehydroascorbate reductase (MDHAR), which uses NADH and an H^+ ion to turn to two copies of monodehydroascorbate into two copies of ascorbate (Park et al, 2016), was the first gene measured that showed a splitting trend between the root and the shoot. The root samples were down regulated across all concentrations (Fig. 24), with only the 500 PPM sample not being significantly so, while the shoot samples were upregulated at 250 PPM and unchanged at 500 PPM and 2000 PPM, though only the slight down regulation at 1000 PPM was significant. This suggests more stress in the leaves where photosynthesis can occur, and less in the roots where it can't, implying a more efficient use of the gene than in the control.

This trend is continued with phospholipid hydroperoxide glutathione peroxidase (PGHP), which combines two glutathione molecules into a glutathione disulfide to remove the hydroperoxide part of a lipid hydroperoxide (Imai et al, 2003). All the down regulated root samples are significant, as well as the up regulated 250 PPM samples, which is a full 50% more active than the control (Fig. 25). The 500 PPM shoot sample is

also up regulated, but not significantly, and the 1000 PPM and 2000 PPM samples show no change from the control.

The third gene to follow this trend is homoglutathione synthetase (GS). The gene codes for a protein that uses ATP to synthesize glutathione (Cruz de Carvalho et al, 2009), an important antioxidant molecule. All root samples are significantly down regulated by between 50% and 75%, while the shoot samples are unaffected except for 250 PPM, which is both significant and an increase of over 250% (Fig. 26). Of all the genes measured, this one provided the strongest results.

Cofilin/actin-depolymerizing factor-like protein, or CAD, is the final antioxidant gene measured. CAD, which is responsible for binding actin and severing filaments (Maciver et al, 1998), also follows the above trend (Fig. 27), with a significant 86% increase over the control in the shoot but no significant difference in the root at 250 PPM, followed by no change, significant or otherwise in the 500 PPM samples, and a significant downregulation in 1000 PPM and 2000 PPM. The upregulation at 250 PPM has a significance value of 0.007, or 99.3%, which is considerably higher than the standard 0.05 (95%).

Photosynthetic Relative Genetic Expression

Ribulose bisphosphate carboxylase small chain, a.k.a. RuBisCO or RBC, catalyzes the first step in the Calvin cycle (Tabita, 2007), is the first photosynthetic gene measured and follows the trend set by the antioxidants very strongly. The roots samples are all significantly downregulated (Fig. 28), with the highest down regulation being the 250 PPM samples, while the shoot is strongly upregulated at 250 PPM (130%) and 500

PPM (43%). The only shoot sample that did not have significant results was the 1000 PPM sample, which was within 4% of the control and as such essentially unchanged.

Thylakoid lumenal 15.0 kDa protein, or TL, is a membrane protein imbedded in the thylakoid membrane that participates in the light reactions of photosynthesis (Kieselbach et al, 1998). This gene is unusual in that the root sample is more upregulated than the shoot, especially at 250 PPM (Fig. 29). That upregulation is also the root's only significant result, while the shoot's only significant result is the upregulation at 500 PPM.

While LHC, or Light Harvesting Complex I chlorophyll A/B binding protein (one of the key components of photosynthesis) has an upregulation in the shoot of 75% at 250 PPM, the unusually large error margin prevents the data from being significantly different from the control (Fig. 30). The only significant results are the 1000 PPM root and shoot and the 2000 PPM shoot samples, with the other concentrations not significantly affected.

CONCLUSION

In conclusion, the results seem to show an increase in the presence of nutrients and photosynthetic gene expression at the 250 PPM content level over the control, followed by a decline as the concentration increases, but a decrease in overall plant mass when compared to the control. Ultimately, this suggests an error on the part of the researcher when selecting which plants received which treatment. Because of the nutrient free properties of the MVP soil, most plant growth was completed before the treatment process began, and if the largest plants got placed in the control because they drew the eye and as such were selected first, then it would have skewed the entire experiment. This has a few possible fixes: the researcher could be more careful when selecting which plants to place in the control, or the plants could be given Hoagland solution in addition to the treatment, allowing for continued growth while under the treatment's influence and increasing the effect it would have on the final biomass levels.

This error does not appear to carry over to the metal content or PCR experiments, both of which suggested an improvement at the 250 PPM treatment level. Despite APX (Fig. 22), GR (Fig. 23), and MDHAR (Fig. 24) being linked, each making up a different section of the same antioxidative process, only MDHAR provided significant results, while the other two either were down regulated or didn't have a sufficiently pronounced change between any sample and the control.

Meanwhile, MDHAR (Fig. 24), PGHP (Fig. 25), GS (Fig. 26), CAD (Fig. 27), and RBC (Fig. 28), all showed strong, significant upregulation at 250 PPM in the shoot but down regulation or no effect on the root. Since this result was not expected under the

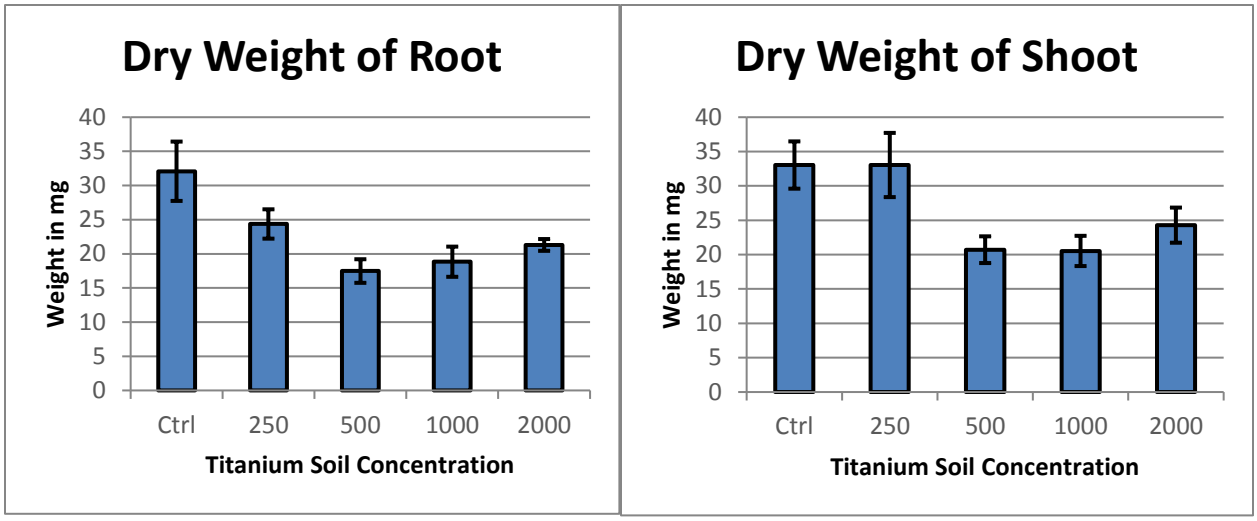
null hypothesis, much less to such a degree, it suggests that 250 PPM, or a concentration close to that amount, does in fact provide some benefit, and a study with more plants and stricter growth standardization would show a biomass increase at that concentration.

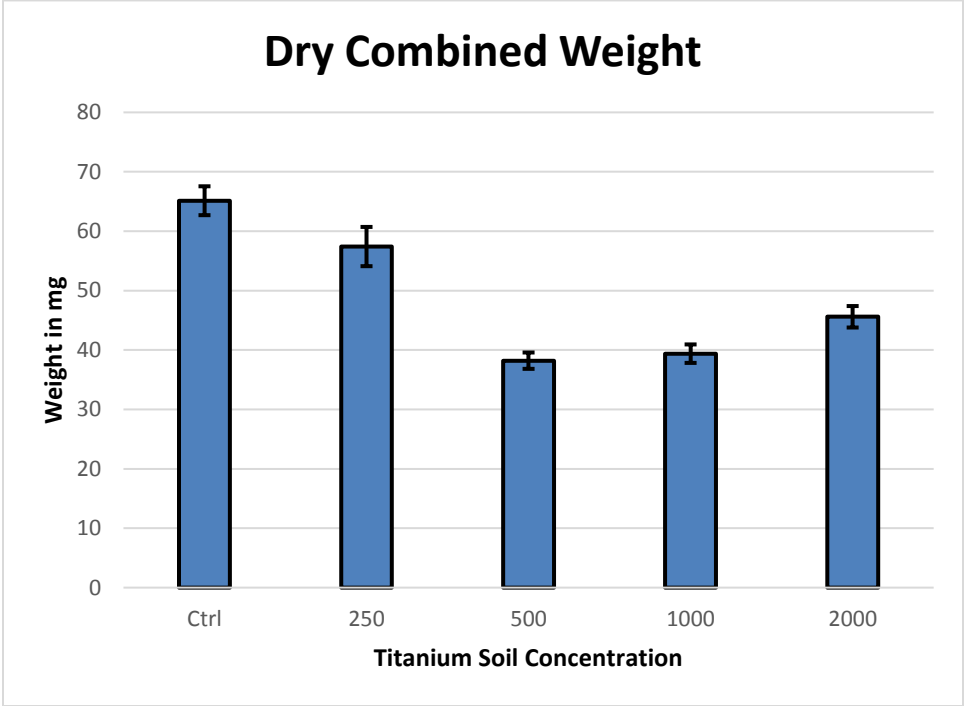
By far the most definitive result of the research is that 1000 PPM is the worst possible titanium soil concentration, resulting in the smallest plants, the lowest genetic expression, and the worst metal content, even more so than the 2000 PPM samples. This concentration must be avoided at all reasonable costs.

The other important results are the conclusion that 250 PPM is not the ideal concentration for Medicago, but is closer than the other tested. A follow up experiment would be to measure 100-500 PPM treatments along with a control and see which of those had the best results. The final important result was the slight uptick in growth and gene expression at 2000 PPM. This suggested that extremely high concentrations might not be as detrimental as initially thought, and a second follow up experiment could focus on testing treatments in the 2000-10000 PPM range.

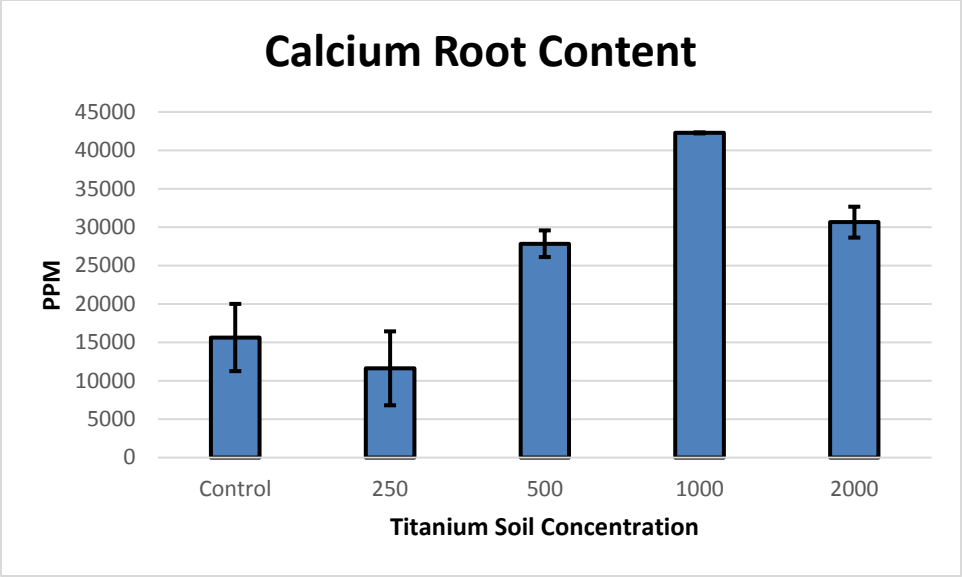
Figure 1 | Picture of Medicago Control Samples at the start of the treatment stage.

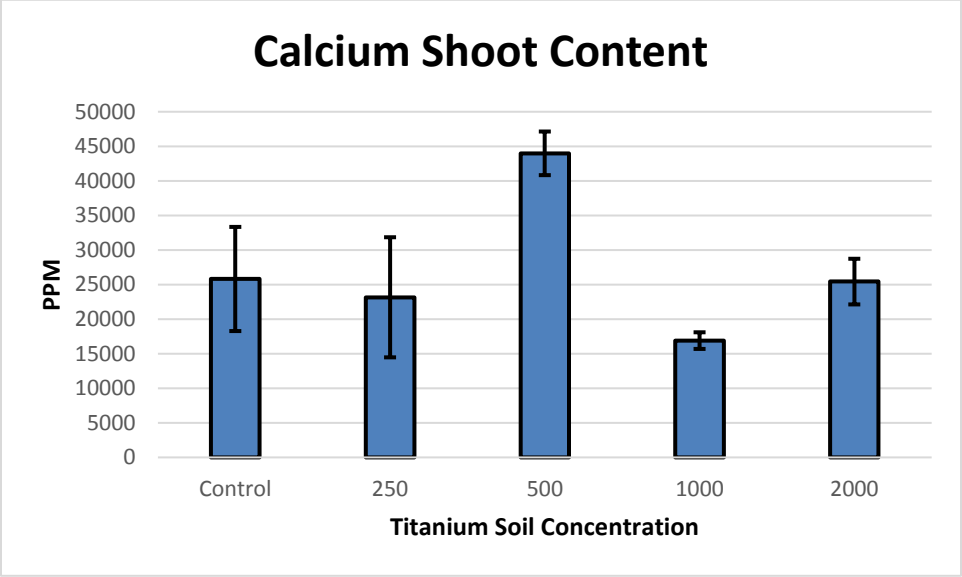
Figure 2 | Example of Product (Sunscreen) advertising the use of titanium NPs.



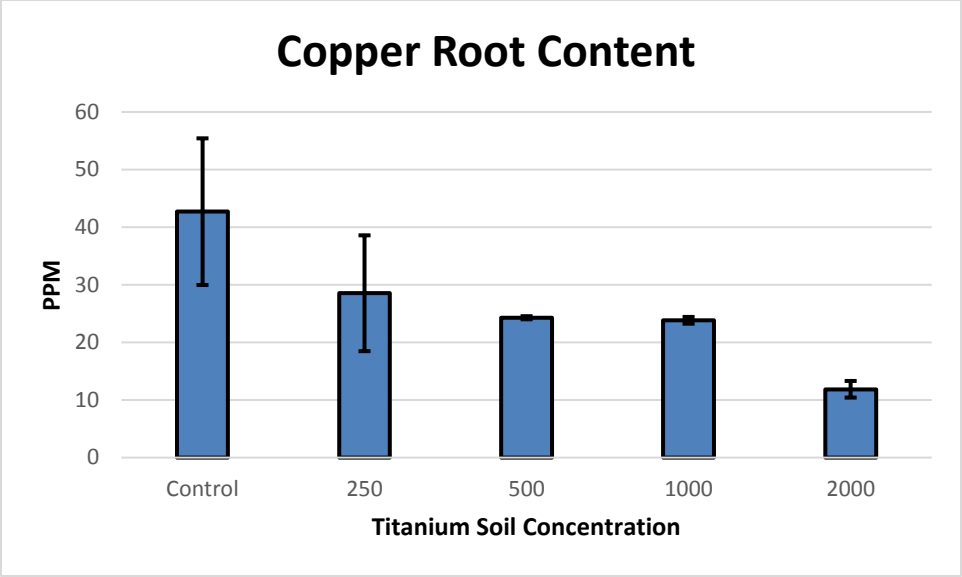


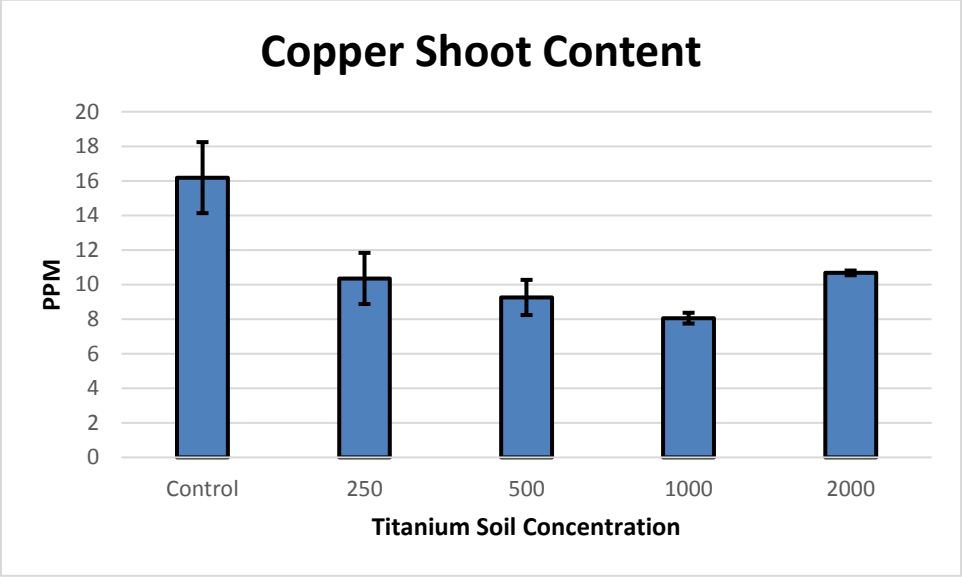
Figures 3, 4, and 5 | Effect of 0-2000 PPM TiO_2 NPs on the separate root and shoot weight and overall combined weight of *Medicago truncatula*. The bars represent standard error.



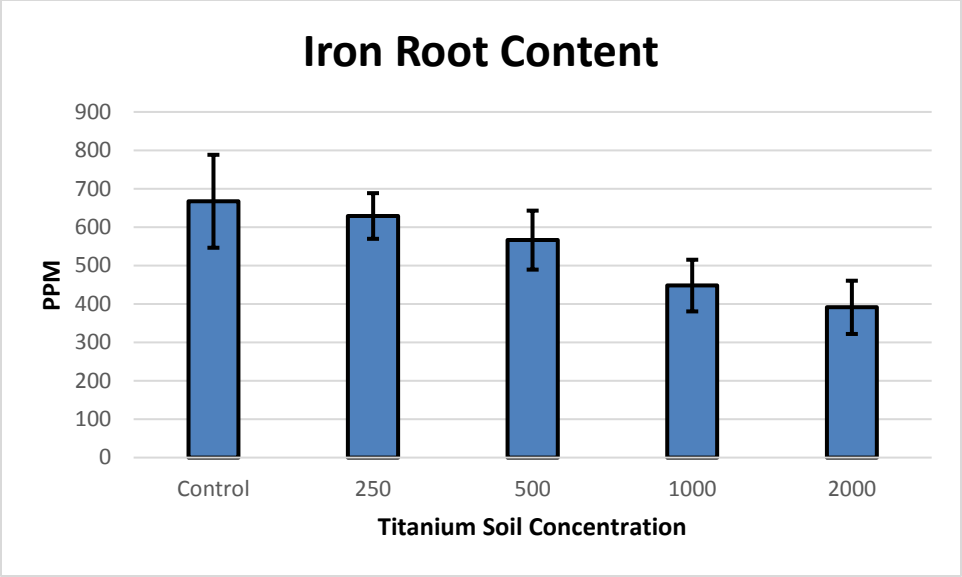


Figures 6 and 7 | Effect of 0-2000 PPM TiO_2NPs on the calcium concentration of *Medicago truncatula*. The bars represent standard error.





Figures 8 and 9 | Effect of 0-2000 PPM TiO_2NPs on the copper concentration of *Medicago truncatula*. The bars represent standard error.



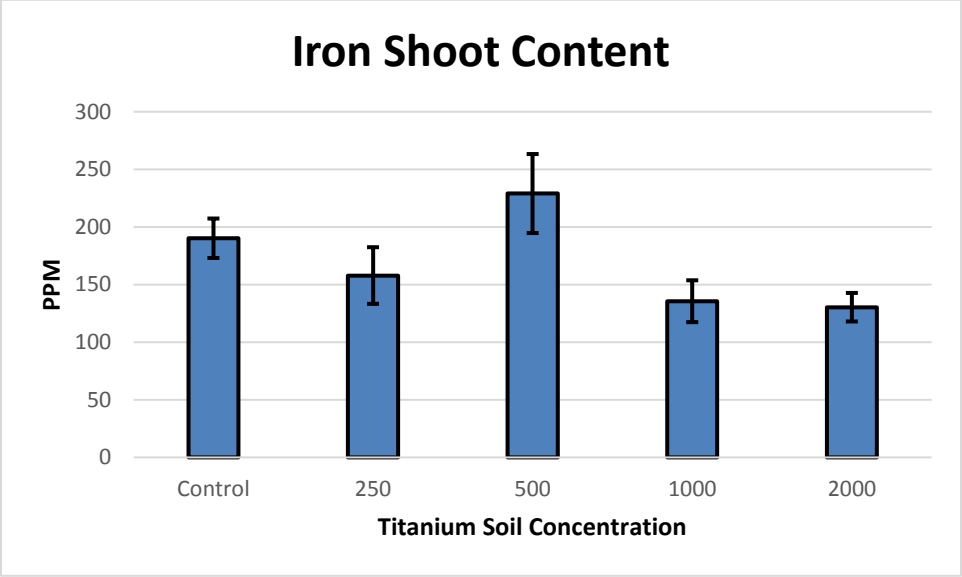
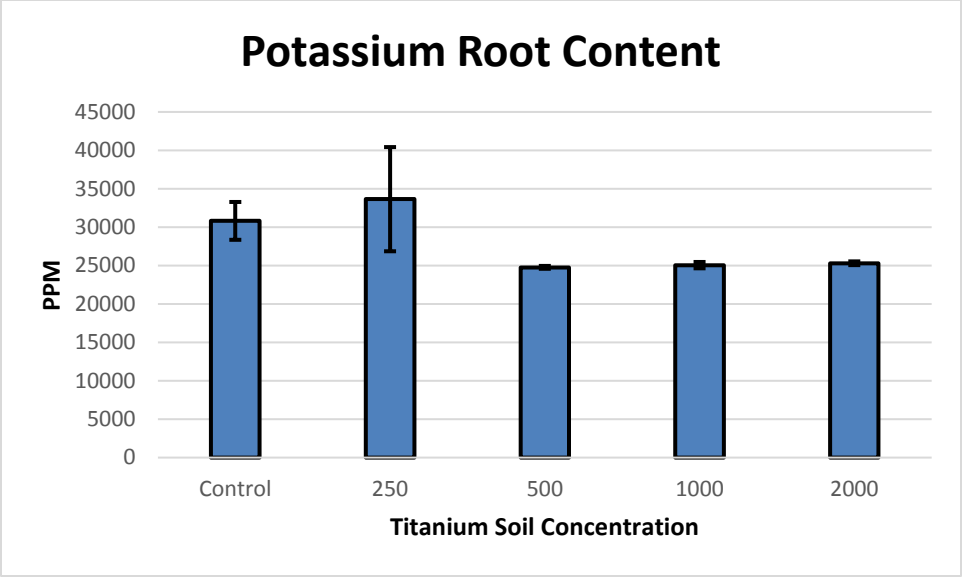
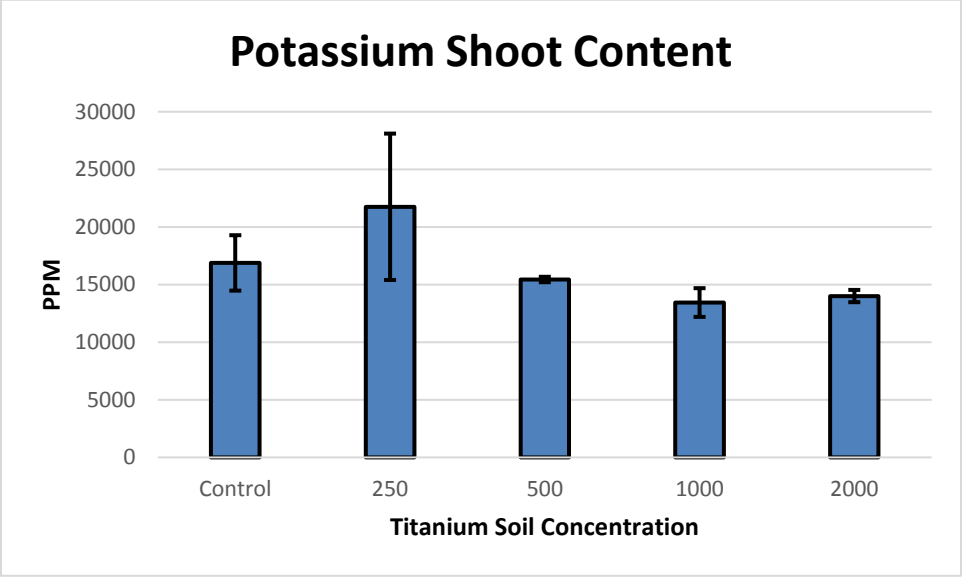
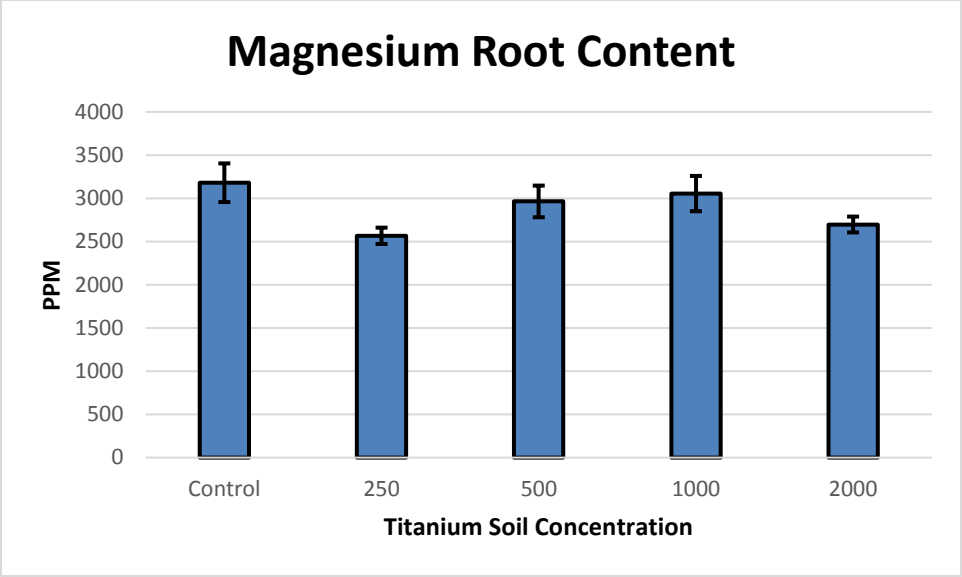


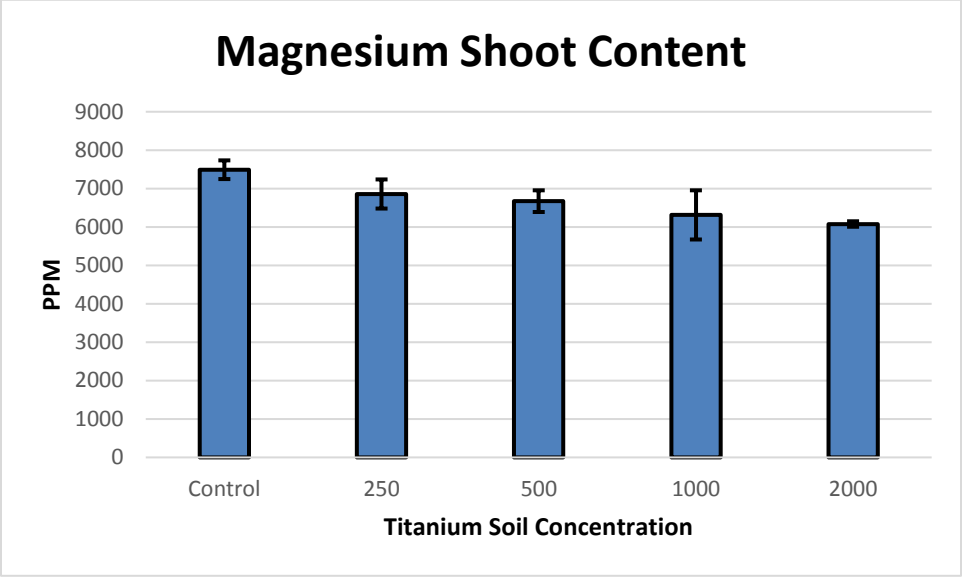
Figure 10 and 11 | Effect of 0-2000 PPM TiO_2 NPs on the iron concentration of *Medicago truncatula*. The bars represent standard error.



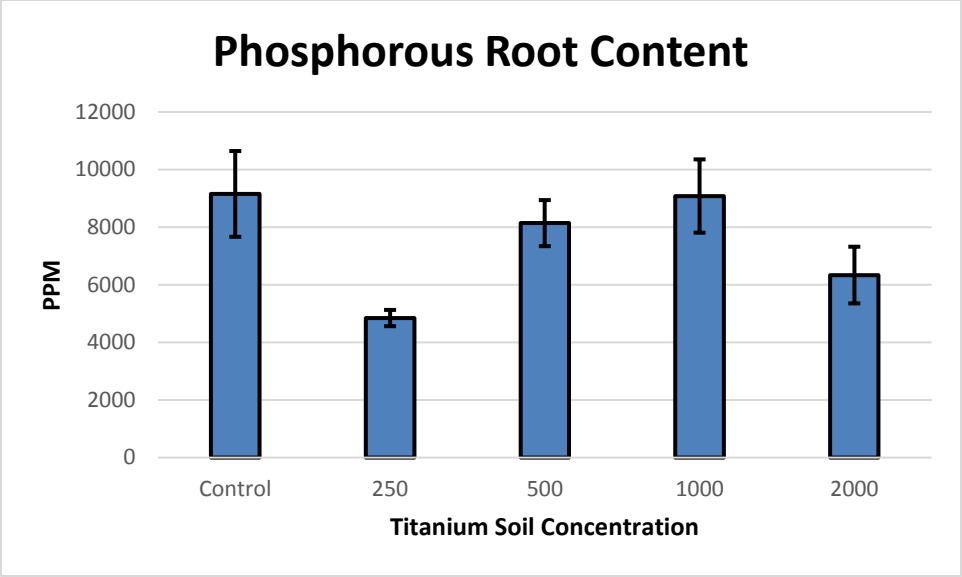


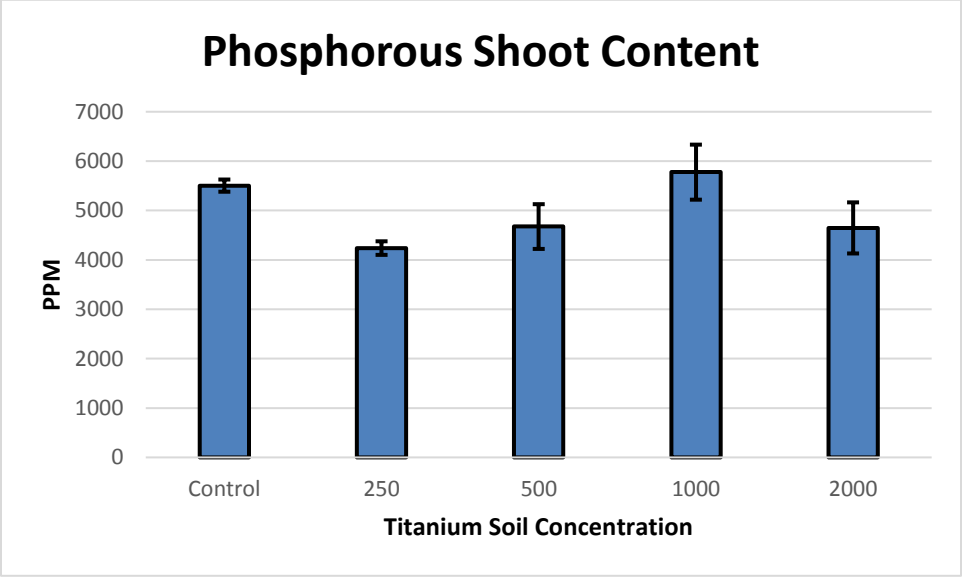
Figures 12 and 13 | Effect of 0-2000 PPM TiO_2NPs on the potassium concentration of *Medicago truncatula*. The bars represent standard error.



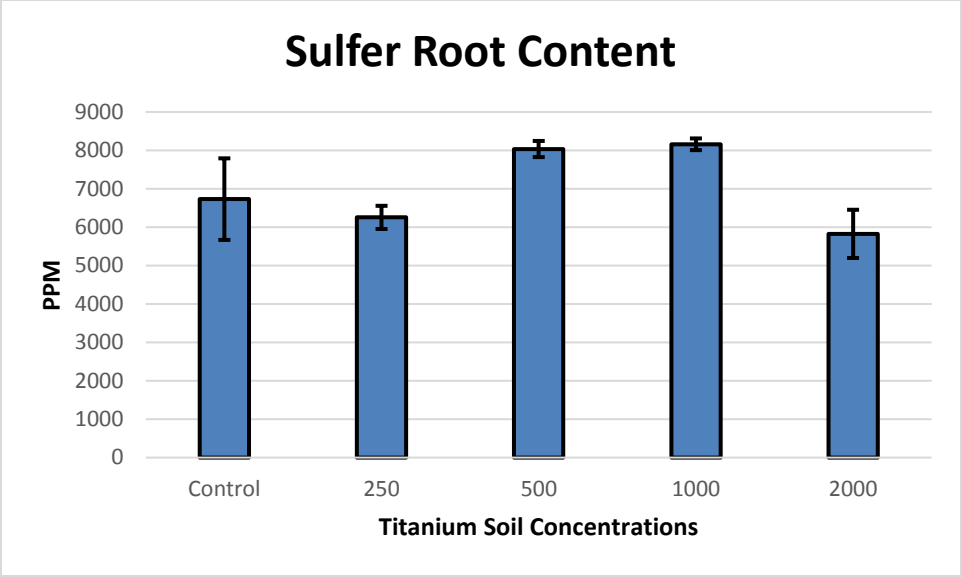


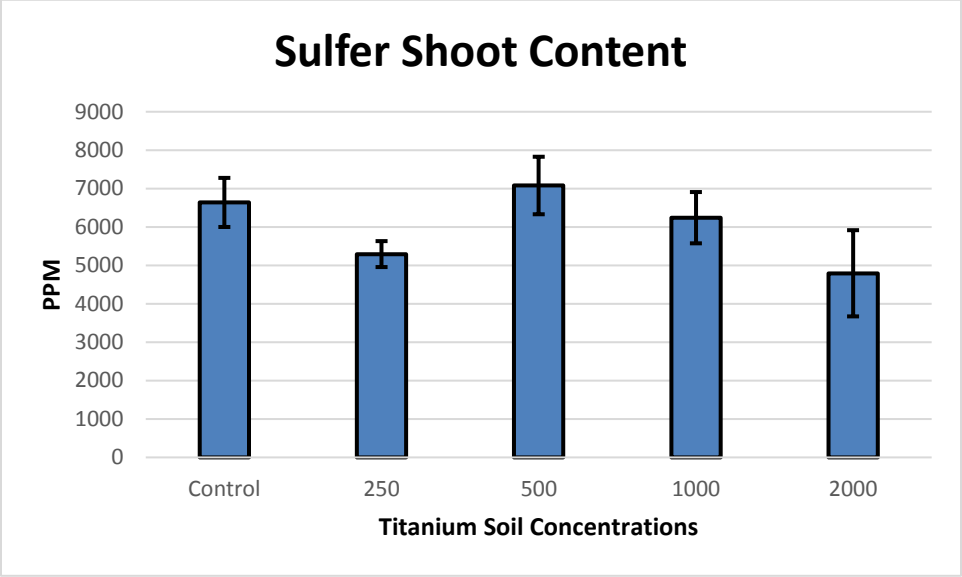
Figures 14 and 15 | Effect of 0-2000 PPM TiO_2NPs on the magnesium concentration of *Medicago truncatula*. The bars represent standard error.



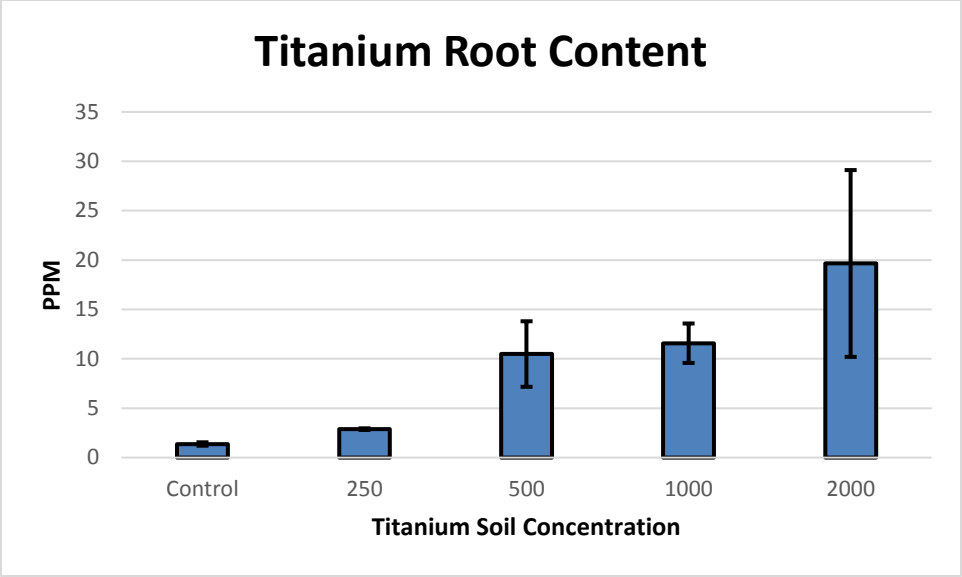


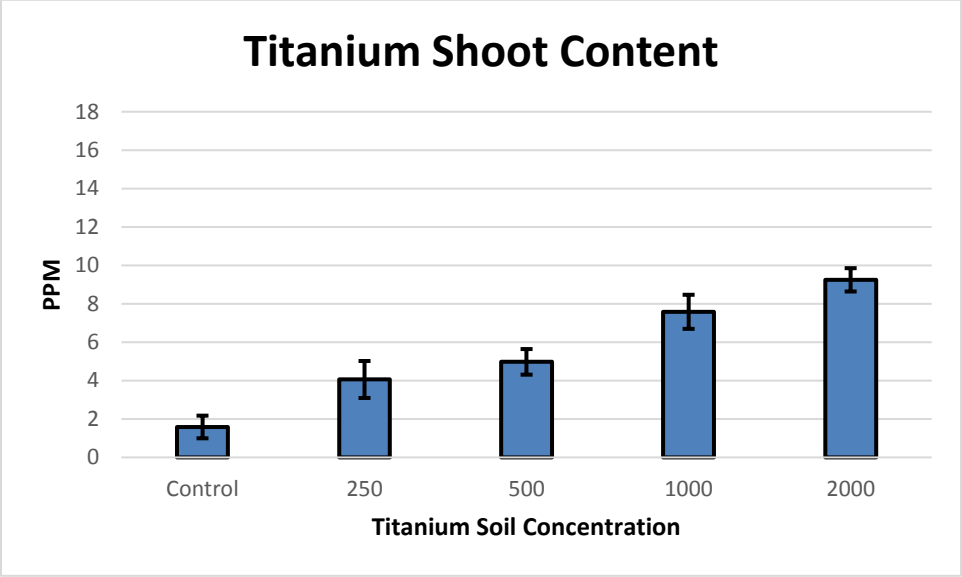
Figures 16 and 17 | Effect of 0-2000 PPM TiO_2NPs on the phosphorous concentration of *Medicago truncatula*. The bars represent standard error.





Figures 18 and 19 | Effect of 0-2000 PPM TiO_2NPs on the sulfur concentration of *Medicago truncatula*. The bars represent standard error.





Figures 20 and 21 | Effect of 0-2000 PPM TiO_2NPs on the titanium concentration of *Medicago truncatula*. The bars represent standard error.

Figure 22 | Effect of 0-2000 PPM TiO_2NPs on the Relative Expression vs. Control of Ascorbate Peroxidase. The bars represent standard error.

Figure 23 | Effect of 0-2000 PPM TiO_2NPs on the Relative Expression vs. Control of Glutathione Reductase. The bars represent standard error.

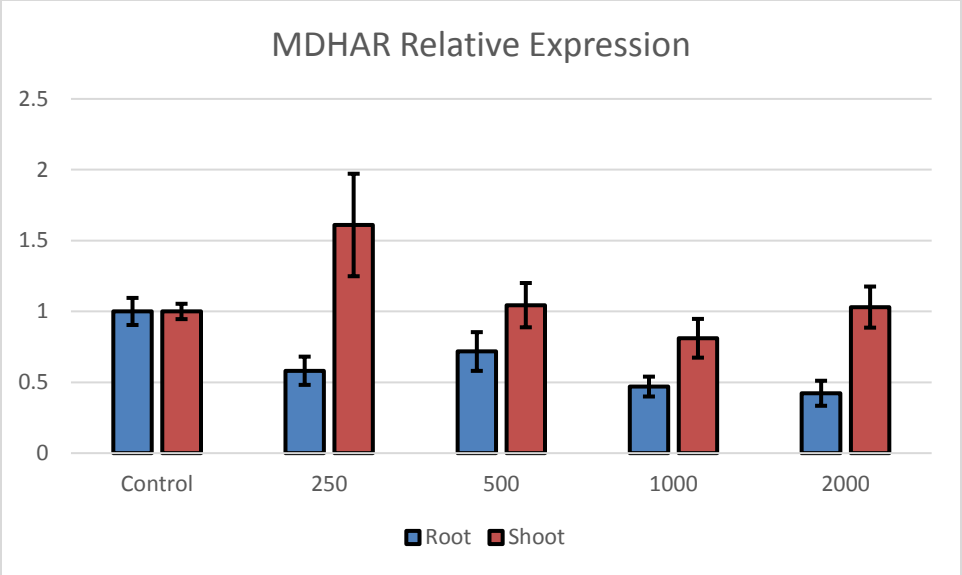


Figure 24 | Effect of 0-2000 PPM TiO_2NPs on the Relative Expression vs. Control of Monodehydroascorbate Reductase. The bars represent standard error.

Figure 25 | Effect of 0-2000 PPM TiO_2NPs on the Relative Expression vs. Control of Phospholipid Hydroperoxide Glutathione Peroxidase. The bars represent standard error.

Figure 26 | Effect of 0-2000 PPM TiO_2NPs on the Relative Expression vs. Control of Homoglutathione Synthetase. The bars represent standard error.

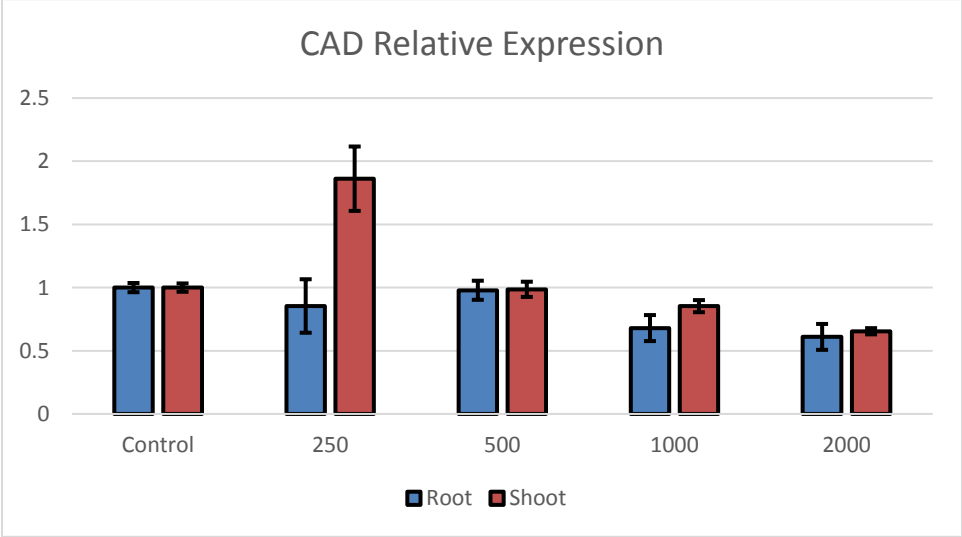


Figure 27 | Effect of 0-2000 PPM TiO_2NPs on the Relative Expression vs. Control of Cofilin/Actin-Depolymerizing Factor-Like Protein. The bars represent standard error.

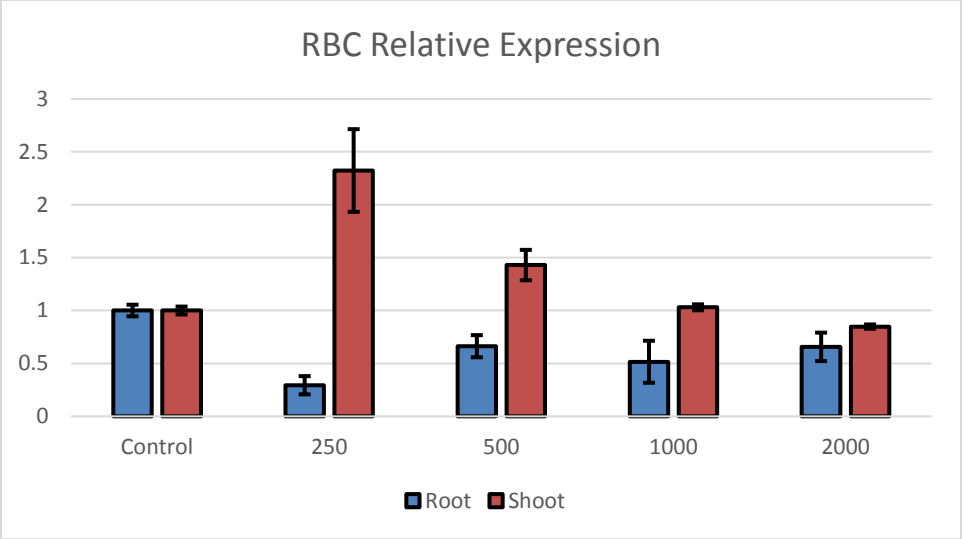


Figure 28 | Effect of 0-2000 PPM TiO_2NPs on the Relative Expression vs. Control of Ribulose Bisphosphate Carboxylase Small Chain (RuBisCO). The bars represent standard error.

Figure 29 | Effect of 0-2000 PPM TiO_2NPs on the Relative Expression vs. Control of Thylakoid Lumenal 15.0 kDa Protein. The bars represent standard error.

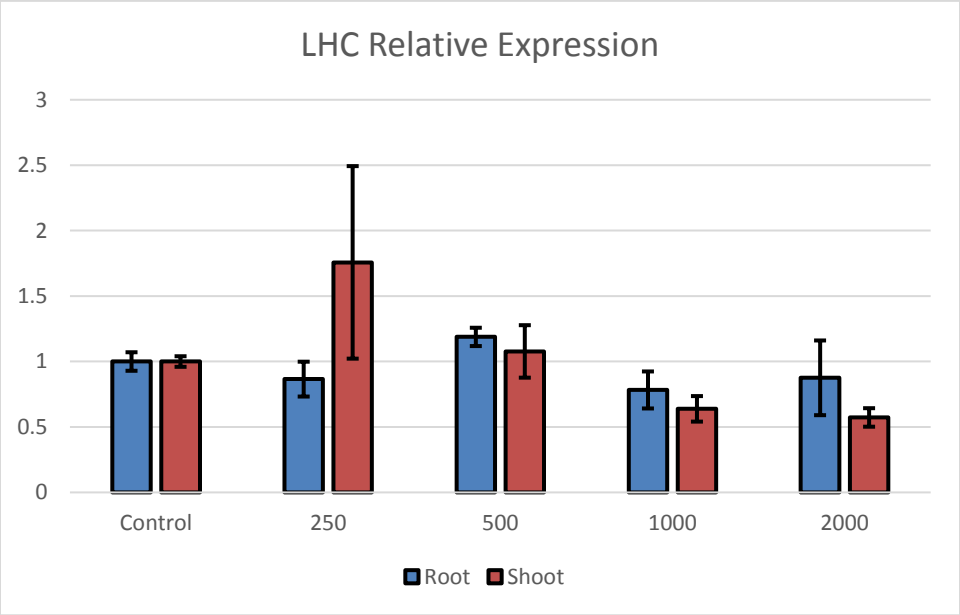


Figure 30 | Effect of 0-2000 PPM TiO_2NPs on the Relative Expression vs. Control of Light Harvesting Complex I chlorophyll A/B binding protein. The bars represent standard error.

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