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Determination Of The Uptake And Effects Of TiO2 Nanoparticles In Cucumber (Cucumis sativus)

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Dedication

This work is dedicated to my beautiful family; my father, mother, Christian, Oliver and Diego, thank all of you for your never-ending love and support.

DETERMINATION OF THE UPTAKE AND EFFECTS OF TiO_2 NANOPARTICLES IN CUCUMBER (*CUCUMIS SATIVUS*)

by

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DISSERTATION

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Abstract

The profuse use of nanoparticles (NPs) in consumer products has raised concerns about their impacts in environmental and human health and possible transfer into the food chain through plants. Cucumber (*Cucumis sativus* L.) is a widely cultivated garden vegetable that could be in contact with NPs through biosolids. In this dissertation research, the impact of TiO_2 NPs was evaluated in cucumber plants grown in hydroponics and sandy loam soil. Hydroponically grown plants were treated for 15 days with 0-4000 mg/L of TiO_2 NPs, and their vegetative tissues were studied using synchrotron micro X-Ray Fluorescence (micro-XRF) and Micro X-ray Absorption Near Edge Structure (micro-XANES). In soil, the cucumber seeds were germinated and grown to full maturity with 0-750 mg/kg of TiO_2 NPs. At harvest, vegetative tissue and fruits were analyzed using synchrotron micro-XRF, micro-XANES spectroscopic techniques, and biochemical assays. Fourier transform infrared (FTIR) spectroscopy was used to determine possible changes in macromolecules of cucumber fruit. Results from hydroponic experiments showed that TiO_2 significantly increased root length at all concentrations (average >300%). In addition, micro-XRF analysis showed that the Ti was taken up from the hydroponic solution and transported through the xylem from the root to leaf tissue, including to trichomes. The micro-XANES spectra showed that Ti found in the vascular system of cucumber was present as TiO_2, demonstrating that TiO_2 NPs were not biotransformed. Results from soil experiments showed a significant increase in catalase activity at all NP concentrations; while ascorbate peroxidase decreased at 500 mg kg^{-1} in cucumber leaves. In addition, leaves from 750 mg kg^{-1} TiO_2 NPs treatment, showed an increase in total chlorophyll content. FTIR spectra of fruits from TiO_2 NP treated plants showed significant differences ($p \leq 0.05$) in all band areas, suggesting modification in macromolecules of cucumber fruits. Furthermore, micro-XRF and micro-XANES results showed that TiO_2 NPs were translocated from roots to fruit without biotransformation or crystal modification, suggesting that TiO_2 NPs could be introduced

into the food chain with unknown consequences for human health. To our knowledge, this is the first report on the presence and effects of TiO_2 in the edible portion of cucumber plant grown in soil with TiO_2 NPs.

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This dissertation relies on synchrotron analysis, spectroscopic techniques and other biochemical analyses to give insights on the effects, speciation and distribution of TiO_2 nanoparticles (NPs) in cucumber fruit, which could represent a possible pathway of nanomaterials into the food chain. This dissertation is composed of 4 chapters:

a. Chapter 1 contains preliminary concepts of nanotechnology and its applications in the commercial market. Specific information about TiO_2 is highlighted. Background information on cucumber is also included.

b. Chapter 2 includes research approaches to determine the presence of TiO_2 in vegetative tissues by using synchrotron Micro-XRF and Micro-XANES techniques.

c. Chapter 3 contains the report about analyses of cucumber fruit from control and TiO_2 treated plants.

d. Chapter 4 contains general conclusions of the impacts of TiO_2 NPs on cucumber plants.

CHAPTER 1

Introduction

1.1 NANOTECHNOLOGY

Nanotechnology has developed drastically over last two decades, owing to elaborate integrated research among various scientific disciplines. Although its uses are diverse, the world has a lot more to see in various applications in the near future. Nanotechnology has been defined as the manipulation of matter's structure at a molecular level.[1] In addition, it enables the production of molecular systems within the range of one-billionth of a meter, resulting in materials with uncommon electrical, optical, and magnetic properties.[2] These materials are often referred as engineered nanoparticles (NPs), which exhibit non-typical characteristics when compared with their bulk materials. The main reason that these materials show unique characteristics in comparison with their conventional forms is the increased relative surface area, which leads to a stronger chemical reactivity.[3] Engineered NPs are already being used in a broad range of commercial products and processes. They can be found in cosmetics, sunscreen, clothing, tires, paint, electronics, pharmaceuticals, cleaning products and medicine.[4] Table 1.1 displays the estimates of nanomaterial's distribution in different product categories.

The different applications of nanotechnology have generated a high investment from companies and agencies in this field. Previous reports have shown that the investment of agencies in nanotechnology continues growing at a consistent rate. Rocco, et al., reported that the annual investment from private and public companies reached $15 billion in 2008.[5] Additionally, in 2011, reports indicated that the value of products incorporating nanotechnology reached about $200 billion worldwide.[5] On the other hand, this exciting technological advancement has been associated with risks; for example, it has been reported that NPs could enter human cells and might have unknown toxicological consequences

due to their small size and reactivity.[6]

Table 1.1: Nanomaterial product distribution.[7]

Nanomaterial	Product group	% of total use
Nano-TiO_2	Cosmetics (incl. sunscreens)	70-80
	Coatings & cleaning agents	<20
	Plastics	<20
	Paints	10-30
	Cement	1
	Others	<10
Nano-ZnO	Cosmetics (incl. sunscreens)	70
	Paints	30
CeO_x	Chemical mechanical planarization	45-80
	Fuel catalyst	1-50
	UV-coatings, paints	5-10
CNTs	Composites & polymer additives	20
	Materials	80
	Composites	50
	Batteries	50
Fullerenes	R&D	80
Nano-Ag	Paints, coatings & cleaning agents	10-30
	Textiles	30-50
	Consumer electronics & conductivity	10-20
	Cosmetics	20
	Medtech	20
	Anti-microbial coatings	80-100
Quantum dots	Light conversion for LED/OLED	90
	Lab use for imaging	10

1.2 TiO_2 NPs

TiO_2 NPs have been reported to be one of the most produced among the engineered nanomaterials.[7] TiO_2 is a white, highly insoluble, thermally stable oxide that occurs in nature. It is generally found in the form of rutile, anatase and brookite minerals,[8] these differ from each other in shape, size and three-dimensional structure. Rutile has been reported to be the most common and stable form found in nature, while anatase and brookite are both semistable forms of TiO_2.[9] The two major TiO_2 polymorphs commercially manufactured are anatase and rutile. In 2008, was estimated that the

annual worldwide production of TiO_2 was of approximately 10,000 tons.[7] Due to its inert and opaque characteristics, TiO_2 has been used in a broad range of applications and consumer products, and it has been estimated that about 70% of the total use of these nanoparticles is utilized in the cosmetic industry (Table 1.1).[7]

Recently, TiO_2 has been studied due to its potential toxic health effects and the International Agency for Research in Cancer (IARC) classified TiO_2 in 2006 as a possible carcinogenic to humans. However, reports have suggested that TiO_2 toxicity is correlated with its form, size, shape and surface area. Several studies have reported that the TiO_2 anatase form appears to have greater toxic effects in comparison with other forms.[10] For example, studies in A549 epithelial cells reported that TiO_2 anatase exhibited greater toxicity showing cell membrane damage and a reduction in cell viability in comparison with its different forms.[11] It has also been reported that nanofilament-shaped TiO_2 NPs induced a higher cytotoxicity in lungs cells in comparison with nanospheres.[12]

Reports have estimated that due to the use of NPs in a broad range of applications, 60-86% of these materials will end up in landfills.[13] The previously mentioned study reported that the main products that contribute to the release of NPs into soil, water and air are personal care products such as cosmetics, coatings and paints (Figure 1.1).[13] Therefore, due to the fact that TiO_2 NPs are widely used in the aforementioned products, it is of main importance to understand and provide an assessment of possible risks.

Few studies have been focused in the toxicity of NPs in plant systems. For example, recent studies reported that the exposure to TiO_2 NPs caused a decrease in secondary lateral roots of garden peas (*Pisum sativum*) and damaged cell surface of *Rhizobium leguminosarum* bv. viciae affecting plant development and subsequently nodule development, which delayed the nitrogen fixation process.[14] Contrasting results have been reported from studies with plants; in *Lepidium sativum* TiO_2 anatase at 10000 mg kg^{-1} stimulated root growth, while at 1000 mg kg^{-1} showed a toxic effect.[15]

Previous studies have used micro X-ray fluorescence (μ-XRF) and micro X-ray absorption (μ-XAS) spectroscopy analyses to study the accumulation of TiO_2 NPs in plants.[16,17] For example, studies in wheat have demonstrated that TiO_2 anatase and rutile were translocated from root-to-shoot without

crystal phase modification.[16] However, to our knowledge there are no previous reports on the distribution and speciation of TiO_2 anatase/rutile NPs in cucumber fruit. Therefore, in this dissertation we evaluated the uptake and effects of TiO_2 rutile and anatase crystalline forms, in edible plants that could be a possible pathway to the food chain and might have unknown consequences in human health.

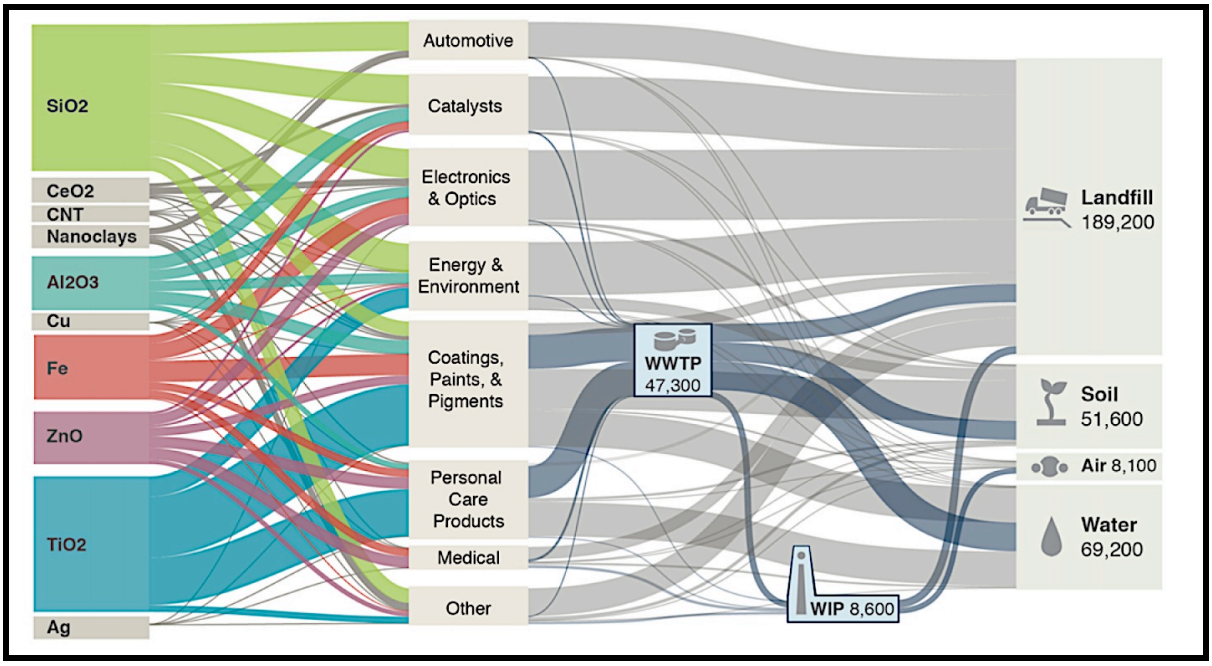

Figure 1.1: Estimated global mass flow of ENMs from production to their release. [13]

1.3 Background on *Cucumis sativus* L.

In order to determine risks and necessary changes to protect the environment from potential toxic substances that may cause harmful ecological effects, the U.S. Environmental Protection Agency recommended several crop plant species for toxicological studies.[18] Among the recommended species are corn, soybeans, tomato, cucumber, lettuce, cabbage, oat, ryegrass, and onion. The studies in the present dissertation research are focused on cucumber plants. Cucumber is a widely commercial and cultivated garden vegetable from the Cucurbitaceae family. Cucumber fruit is mostly consumed as a

fresh vegetable due to its rich content of potassium, phosphorous, magnesium, fiber and folic acid, moderated amounts of Vitamin A and C and as a low calorie food.[19] Even tough cucumber is widely consumed around the world, its largest production is concentrated in China, (62%), Iran, Turkey, Russian Federation, Netherlands and the U.S.A. (3%).[19] In the U.S.A., Florida is the leading producer state, with about 19 percent of cucumber production in the nation. The USDA reported that the consumption of cucumber in the United States has increased during the past four decades, reporting an average of 2.8 pound per capita in 1970's to up 6.8 pound per capita in 2010.[20] Additionally, reports indicate that 60 percent of cucumber is consumed fresh, while the rest is consumed as pickled products (Figure 1.2). Since cucumber is widely produced and consumed freshly, it is possible that it could be in contact with NPs through biosolids and direct agrichemical application; however, there is still lack of information about the possible accumulation of Ti/TiO_2 in aerial structures of plants treated with TiO_2 NPs or if the crystalline form changes after being transported through the xylem of the plants. It is necessary to increase the knowledge concerning TiO_2 NPs transport from roots to the upper parts of the plants and the possible transformation of these NPs within the plants. Thus, the present dissertation describes the translocation, distribution, and potential changes in oxidation state of TiO_2 NPs in cucumber plants.

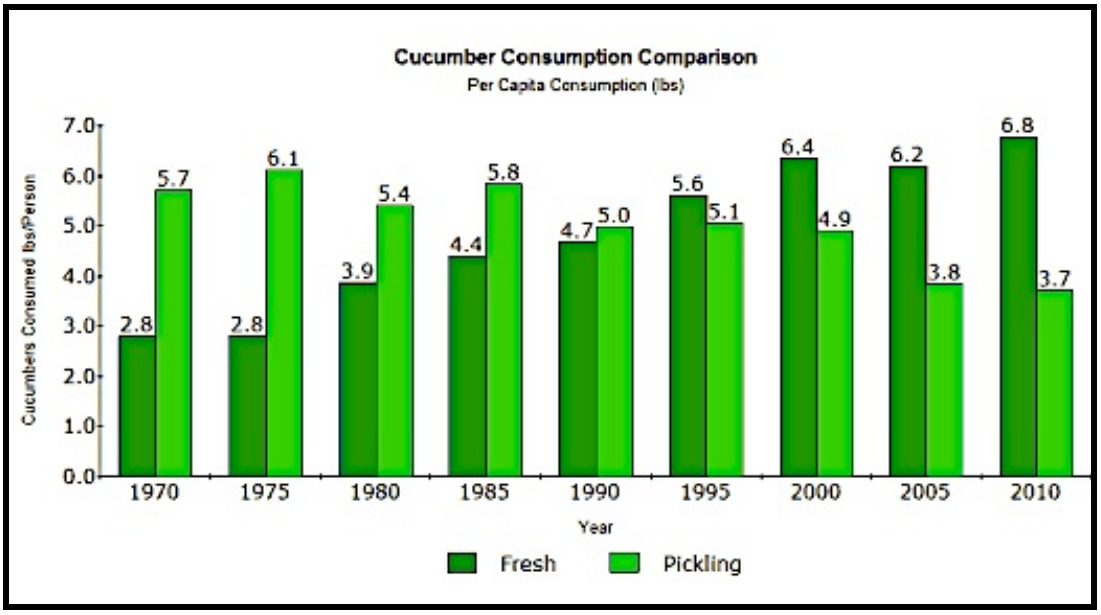

Figure 1.2: U.S. Cucumber Consumption. Courtesy by USDA[17]

1.4 OBJECTIVES

The general objective of this research is to evaluate the potential toxicological/physiological effects, distribution, and accumulation of TiO_2 NPs in cucumber plant.

The specific objectives are to:

1. Determine if cucumber seedlings absorb TiO_2 NPs at an early growth stage.
2. Determine if TiO_2 NPs translocate or biotransform throughout cucumber plant.
3. Determine if TiO_2 NPs remain in the same oxidation state and crystalline phase in cucumber seedlings and mature plants.
4. Determine the growth effects of TiO_2 NPs in cucumber plants.
5. Determine if TiO_2 NPs can be translocated to the cucumber fruit.

1.5 HYPOTHESIS

This investigation was performed under the working hypothesis that:

1. Cucumber plants uptake TiO_2 NPs and translocate them to the aerial parts.
2. TiO_2 NPs interact with the plants and modify the physiological and morphological properties of cucumber plants.
3. TiO_2 is translocated to the cucumber fruit without any biotransformation.

CHAPTER 2

Synchrotron Micro-XRF and Micro-XANES Confirmation of the Uptake and Translocation of TiO_2 Nanoparticles in Cucumber (*Cucumis sativus*) Plants

Abstract

Advances in nanotechnology have raised concerns about possible effects of engineered nanomaterials (ENMs) in the environment, especially in terrestrial plants. In this research, the impacts of TiO_2 nanoparticles (NPs) were evaluated in hydroponically grown cucumber (*Cucumis sativus*) plants. Seven-day-old seedlings were treated with TiO_2 NPs at concentrations varying from 0 to 4000 mg L^{-1}. At harvest, the size of roots and shoots were measured. In addition, micro X- ray fluorescence (micro-XRF) and micro X-ray absorption spectroscopy (micro-XAS), respectively, were used to track the presence and chemical speciation of Ti within plant tissues. Results showed that at all concentrations, TiO_2 significantly increased root length (average >300%). By using micro-XRF, it was found that Ti was transported from the roots to the leaf trichomes, suggesting that trichomes are possible sink or excretory system for the Ti. The micro-XANES spectra showed that the absorbed Ti was present as TiO_2 within the cucumber tissues, demonstrating that the TiO_2 NPs were not biotransformed.

Resulting publication from this research:

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2.1 INTRODUCTION

Nanotechnology includes the fabrication and use of different nanomaterials (NMs), including nanoparticles (NPs). Proper- ties derived from NPs surface area, chemistry, shape, and surface charges, among others, allow their utilization in numerous goods and consumer products.[1] Reports indicate that the number of nanoproducts worldwide increased by 521% since March 2006 to August 2011.[2] TiO_2 NPs are among the most used nanomaterials.[3] These NPs are used in sunscreens, [4] surface antibacterial and antiviral disinfectants,[5] organic pollutant removers,[6] gas sensors,[7] solar cells,[8] food coloring in powdered doughnuts,[9] skim milk as a fat substitute to provide the white color,[10] and in paints.[11] However, this variety of uses and the release of TiO_2 NPs from paints by weather conditions, increase the possibility of environmental dispersion of TiO_2 NPs with unknown consequences.

Previous reports have associated TiO_2 NPs with DNA damage in human lung epithelial cells, inflammatory effects after inhalation in rats,[12-14] and increases in the levels of reactive oxygen species in human and rat alveolar macro- phages.[15] TiO_2 NPs also induce oxidative stress in the brain of rainbow trout (*Oncorhynchus mykiss*)[16] and cause micronuclei formation and apoptosis in Syrian hamster embryo fibroblast.[17] Studies have also shown that TiO_2 NPs can affect plant species in different ways. Du and coworkers[18] reported that TiO_2 NPs reduce wheat (*Triticum*) growth and induce significant changes in soil enzyme activity, showing toxicity for the soil ecosystem. TiO_2 NPs also produce DNA damage and reduce root elongation in onion (*Allium cepa*)[19] and inhibit the apoplastic water flow through nanosized root cell wall pores of maize (*Zea mays*) seedlings, reducing leaf growth and transpiration.[20] Other studies reported a positive correlation between external TiO_2 nano treatments and the inhibitory rate of rapeseed (*Brassica napus*) germination and activation rate of root elongation.[21] However, TiO_2 NPs cause insignificant toxicity in willow (*Salix* sp.) trees[22] and are able to promote light absorption of chloroplast. These NPs also regulate the gene expression of light harvesting complex II and

photosynthesis in *Arabidopsis thaliana*.[23] In *Pinus tabulaeformis*, TiO_2 NPs increase seed germination and seedling growth.[24] In addition, TiO_2 NPs mixed with SiO_2 accelerate soybean germination and seedling growth.[25] Previous studies have shown that TiO_2 can accumulate within plant tissues. For example, in *A. thaliana*, TiO_2 NPs modified with alizarin red S and sucrose, enter into plant cells and accumulate in specific subcellular locations.[26] In maize TiO_2 NPs accumulate in epidermal cells of roots with restricted movement to the cortex and no presence in the vascular system.[27] To our knowledge, there are no previous studies on the effects of TiO_2 NPs in cucumber plants. Cucumber is widely used as garden vegetable and it has been reported that in 2009 the consumption of cucumber was about 6.6 pounds per capita in the U.S, which indicates a rise in cucumber consumption by 15% since 1995.[28]

Synchrotron micro X-ray fluorescence (micro-XRF) combined with micro X-ray absorption near edge structure (micro- XANES) has been used to investigate the speciation of elements in 2D maps of elemental distribution in environ- mental samples.[29] In a previous investigation we reported the use of these techniques to study the As speciation in soil and plant samples of the desert plant *Prosopis juliflora-velutina.*[30] We also used these techniques to study the distribution and speciation of Zn in mesquite plants (*Prosopis juliflora-velutina*) grown with ZnO NPs.[31] Larue et al.,[32] used micro-XRF to map Ti in the vascular system of wheat roots treated with TiO_2 NPs. In addition, by using XANES these researchers found that Ti was in the form of TiO_2 NPs within the roots of wheat plants, and that their crystalline phase did not change. However, there is still lack of information about the possible accumulation of Ti/TiO_2 in aerial structures of plants treated with nano TiO_2, or if the crystalline form would change after transport through the xylem of the plants. The fact that TiO_2 NPs can be absorbed without modification by roots, suggests these NPs can potentially reach edible portions of crop plants (leaves or fruits), posing a threat for human health. It is necessary to increase the knowledge concerning TiO_2 NPs transport from roots to the upper parts of the plants and the possible transformation of these NPs within the plants. Thus, the aims of this research were to determine the translocation, distribution,

and oxidation state of Ti in TiO_2 NP treated cucumber plants. Micro-XRF and micro-XANES were used to study the distribution and oxidation state of Ti within cucumber tissues.

2.1 MATERIALS AND METHODS

2.1.1 Preparation of TiO_2 Suspensions

Semispherical TiO_2 NPs (27 ± 4 nm, Evonik Degussa Corp., Parsippany, NJ) were provided by the University of California Center for Environmental Implications of Nanotechnology (UC-CEIN). Previous characterization showed that the TiO_2 NPs had a surface area of 51.5 m^2 g^{-1} and both anatase (82%) and rutile (18%) crystalline phases were present.[33] The TiO_2 NPs were suspended in a modified Hoagland nutrient solution previously described in literature.[34] Suspensions were prepared to obtain concentrations of 0, 50, 250, 500, 1000, 2000, and 4000 mg TiO_2 NPs L^{-1}. The NP suspensions were stirred for 5 min and sonicated for 30 min in an ice bath (BioLogics, Cary, NC). All suspensions were prepared the same day before each experimental set up and were adjusted to pH 5.8. Because the solubility of TiO_2 NPs at the pH used in this study is extremely low,[35] the effect of ionic Ti was not investigated.

2.1.2 Seed Germination and Plant Growth

Cucumber seeds (Westar Seeds International, El Centro, CA) were stirred for 30 min in a 4% $NaClO_4$ solution, followed by rinsing with sterilized Millipore water (MPW). Subsequently, the seeds were kept for one hour in sterilized MPW, placed in germination paper towels and soaked with an antibiotic- antimycotic solution (A5955, Sigma, St. Louis, MO). After five days in the dark, the seedlings were exposed to light for one more day. Then, similar plants were selected and transferred to 250 mL Mason jars containing the TiO_2 NP suspensions. Control treatment was the Hoagland nutrient

solution, without TiO_2 NPs. Quadruplicate sets of ten plants per jar were set (280 plants per experiment). Aquarium pumps were used to provide oxygen and to maintain suspended the TiO_2 NPs. Control and TiO_2 NP treated plants were grown for 15 days at room temperature on a 16 h light photoperiod and instantaneous light intensity of 54 μmol m^{-2} s^{-1} of photosynthetically active radiation obtained from four 34 W Phillips lamps. Suspension levels were maintained to the same volume by adding MPW every day. At harvest, roots were rinsed with 0.01 M HNO_3 solution followed by three rinses with MPW; then, plants were separated in roots and shoots, and their size was determined.

2.1.3 Nitrogen Determination

For nitrogen determination, triplicate sets of four plants per jar were set for each treatment. Control and TiO_2 treated plants were grown for 30 days in hydroponics, in the same conditions as described in previous section. Cucumber plants treated with 0 and 4000 mg TiO_2 NPs L^{-1} were weighed and oven-dried for 48 h. Dry samples were digested in Kjeldahl (TDK) digestion tubes with 10 mL of concentrated H_2SO_4 (97%) and a 1.5 g mixed solution of $CuSO_4 \cdot 5\ H_2O$ (16.6%) with Na_2SO_4 (83.4%). The tubes were placed on the Kjeldahl digestor for 1 h at 450 °C. The digestion residue was cooled down and deionized water added until the samples reached a light blue color. The distillation was performed after the addition of NaOH (50%) using a Labconco 65410 Rapidstill II apparatus. An aliquot of 125 mL solution of 4% (w/v) H_3BO_3 and Shiro Toshiro indicator was made in order to collect the distilled samples. Distilled samples were titrated with HCl and, consequently, Kjeldahl nitrogen was measured by quantifying the HCl volume used for each sample.

2.1.4 Micro XRF and μXANES Data Acquisition

For the micro-XRF and micro-XANES studies, the cucumber plant samples were washed as described above. Carefully cleaned root samples were dissected 0.5 cm up from the root tip, frozen in

liquid nitrogen, and embedded into Tissue Tek resin (Sakura Finetek USA, Torrance, CA). After embedding in the resin, the samples were axially sectioned at 30 μm thick in a Microtome plus cryostat (Triangle Biomedical Sciences, Durham, NC) and mounted onto Ultralene window film. Leaf cucumber samples were dissected along the central veins and prepared following the same method as in roots. Micro-XRF mapping of the distribution of Ti and other elements in the leaves and roots was performed with an incident energy at 5.1 KeV during the 16 bunch mode and continuous mode at beamline ID21 of the European Synchrotron Radiation Facility (ESRF, Grenoble France).[36] The storage ring current during data acquisition ranged between 60 and 90 mA (16 bunch) and 180 and 200 mA (continuous) operating at 6.0 GeV. The beam was focused with the use of a Fresnel zone plate to a size of 0.33 × 0.65 μm (VxH) and the fluorescence signal was detected with a Si drift detector. Two photodiodes were used to measure the incident and transmitted beam intensities. Dwell time and distance of the detector was optimized for each image keeping the detector dead time below 15%. The X-ray fluorescence data was pro- cessed using PyMCA software.[37] For micro-XANES data acqui- sition, the energy of the monochromator (Si111) was scanned from 4.95 to 5.1 KeV and the zone plate was translated in the beam axis in order to maintain the beam focus. The final Ti- Kedge spectra were the sum of 5-60 individual scans with 0.1 s integration time and 0.5 eV resolution step. Each individual spectrum was inspected for beam induced changes and the samples were stable in all cases. XANES data analysis was carried using the Athena software.[38] Spectra were energy-calibrated with respect to a Ti foil (inflection point at 4.9707 KeV) and the pre- edge background was subtracted and normalized using a linear pre-edge. Reference materials analyzed were semispherical TiO_2 NPs (27 ± 4 nm, Evonik Degussa Corp.). Reference spectra from 140 nm anatase TiO_2-NPs, and 50 nm rutile TiO_2-NPs were obtained from the group of Dr. Marie Carriere (CEA, France).

2.1.5 Statistical Analysis

The data reported for root and shoot growths were averages of three replicates ± standard errors (SE). A one-way ANOVA test was performed followed by Tukey-HSD (honestly significant difference) test performed with the statistical package SPSS Version 12.0 (SPSS, Chicago, IL). In all cases the statistical significance was based on a p value < 0.05.

2.2 Results and Discussions

In this section, we discuss the findings from the growth analysis, nitrogen determination and synchrotron analysis. Additionally, we provide micro-XRF images showing the presence of Ti in cucumber plant tissue. Furthermore, we present micro-XANES spectra of TiO_2 within the cucumber tissues.

2.2.1 Growth Analysis

The root and shoot growth of control and TiO_2 NP treated plants is shown in Figure 2.1. As seen in this figure, at all concentrations the TiO_2 NPs significantly increased ($p \leq 0.05$) root elongation. The root sizes presented an increasing trend up to the treatment of 500 mg L^{-1}, showing no further increases at higher TiO_2 NP concentrations. The data suggest that 500 mg L^{-1} is the highest concentration of TiO_2 NPs that cucumber allows for root elongation stimuli. Previous studies have shown that TiO_2 NPs at concentrations below 100 mg L^{-1}, did not produce toxic effect on willow threes,[22] while nanoanatase TiO_2, promoted chlorophyll biosynthesis, photosynthesis, and improved the growth in spinach (*Spinacia oleracea*).[3] Another study showed that, in spinach, TiO_2 NPs increased the activity of RuBisCo activase and promoted the adsorption of nitrate, accelerating the transformation of inorganic nitrogen to organic nitrogen, which produced a positive effect in chlorophyll production and plant growth.[37] In the present

study, total nitrogen determination in TiO_2 NP treated plants and control plants showed 51.16% more nitrogen in TiO_2 NP treated roots, compared to control roots (Table 2.1). However, the nitrogen content in aerial plant parts was statistically similar for both control and TiO_2 NP treated plants (Table 2.1). The present study has shown that at all concentrations tested, TiO_2 NPs increased the size of cucumber roots. We hypothesized that TiO_2 NPs promote plant root growth by stimulating nitrogen accumulation and thus, protein formation.

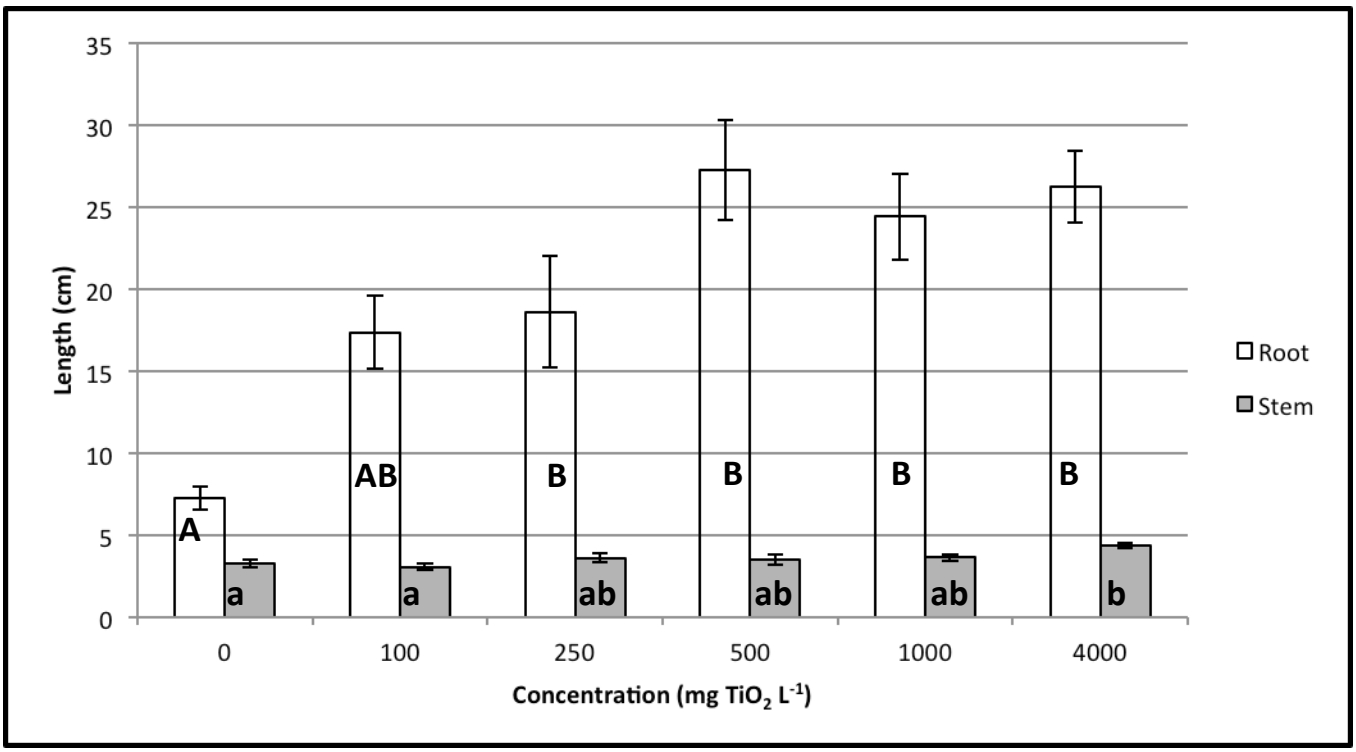

Figure 2.1: Root and shoot length (cm) of hydroponically grown cucumber plants treated for 15 days with TiO_2 NPs up to 4000 mg/kg. Plants were set at room temperature and 16 h light photoperiod given by four 34 W Phillips lamps. Uppercase letters represent differences between treatments. Lowercase letters represent differences between tissues of the same treatment. Error bars stand for standard error ($\alpha \leq 0.05$).

Table 2.1 Nitrogen Determination in control and 4000 mg/kg TiO_2 NPs treated cucumber (*Cucumis sativus*) plants grown in hydroponics for 30 days. [a] From 12 plants combined.[b] From 16 plants combined.

Nitrogen Kjeldahl	Weight (g)	% Nitrogen	% Protein
Control root[a]	0.316	2.103352	13.1460
Control shoot[a]	0.498	2.299584	14.3724
Treated root (4000 mg TiO_2 NPs L^{-1})[b]	0.505	3.179481	19.8718
Treated shoot (4000 mg TiO_2 NPs L^{-1})[b]	0.510	2.137573	13.3598

2.2.2 Micro-XRF and Micro-XANES Analysis

The spatial distribution and speciation of TiO_2 in the roots, leaves, and trichomes of cucumber plants was studied using synchrotron micro-XRF and micro-XANES. Figure 2.2 (A) shows the transversal section of the root central cylinder of a plant treated with TiO_2 NPs at 500 mg L^{-1}; while Figure 2.2 (B) shows the tricolor micro-XRF map of that cross section. In Figure 2.2 (B), the red color stands for Ti, green for Ca, and blue for K. Moreover, as shown in Figure 2.2 (C-D), the Ti/TiO_2 NPs found their way to penetrate the transport system of the cucumber plant. The tricolor micro-XRF map showed Ti at high concentration in the phloem cell walls. Accumulation of toxic elements in cell walls has been reported for heavy metals such as Pb.[34, 39] Cell wall components such as lignin binds heavy metals and the plants use this as a defense mechanism against metal toxicity.[40] Figure 2.2 (F) displays the micro-XANES spectra of selected areas from the cucumber root. The points 1-6 in Figure 2.2 (B) and 7 and 8 in Figure 2.2 (E) indicate the root spots where the micro- XANES spectra were acquired. As

shown in Figure 2.2 (F), Ti in the root epidermis/cortex (spot 7) was present as TiO_2 (81% anatase +19% rutile). Similarly, spots 1-6 from the xylem show Ti was present as 82% anatase and 18% rutile (same composition as the supplied TiO_2 NPs). These results agree with those obtained by Larue et al.[32] in wheat plant root, where they found the presence of TiO_2 within the wheat root tissues in the same chemical form as the supplied TiO_2 NPs. In contrast, the spectrum in phloem (after translocation to leaves), is very similar to the one of pure rutile. These results suggest rutile has preferential translocation in the cucumber plants. However, this needs to be further studied in order to distinguish from preferential translocation and possible anatase dissolution followed by precipitation in the rutile crystalline phase. Figure 2.3 (A) shows the optical image of a transverse sectioned cucumber leaf central vein treated with 500 mg TiO_2 NPs L^{-1}. The temperature map of Ti (Figure 2.3(B)) shows the distribution of Ti and displays the spots used for micro-XANES analysis (white boxes). The micro-XRF image of transversal section of the leaf reveals the presence of Ti (red color) in the mesophyll tissue, epidermis and trichomes (Figure 2.3(C)). The micro- XANES spectra indicated that Ti present in the leaf tissue remained as TiO_2 in the crystalline phase of rutile (Figure 2.3(D)). This could explain why only this phase was observed in the root phloem (Figure 2.2(F)).

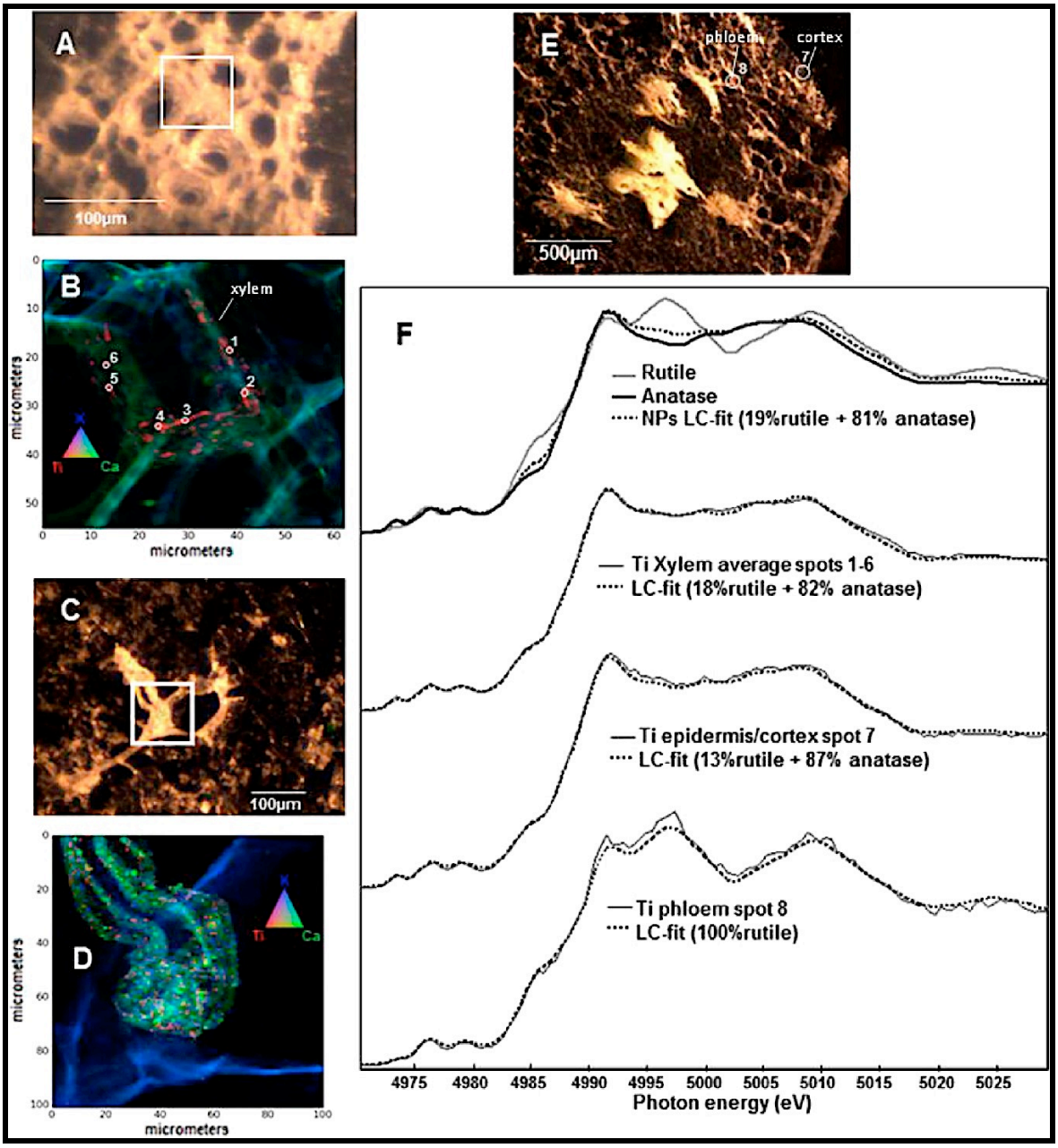

Figure 2.2. Images of root cross sections of cucumber plants treated for 15 days with 500 mg L^{-1} TiO_2 NPs. Video microscope image of cucumber root vascular cylinder (A) and a phloem cylinder (C). Tricolor micro-XRF images of cucumber root xylem (indicated in A) (B) and phloem cylinder (indicated in C) (D). Red color stands for titanium, green for calcium, and blue for potassium. E) Video microscope image of cucumber root cross section where the white circles (7 and 8) indicate areas where micro-XANES was acquired. Map acquired at 5.1 KeV with 200 ms dwell time, and 0.3 μm^2 pixel. (F) Shows the micro-XANES spectra of reference materials (TiO_2 NPs anatase and rutile) and linear combination fitting of the marked spots in (B) and (E). Spot 1-6 where acquired in focused mode (beam size 0.33 × 0.65 μm^2) and spots 7 and 8 with the use of a 50 µm pinhole.

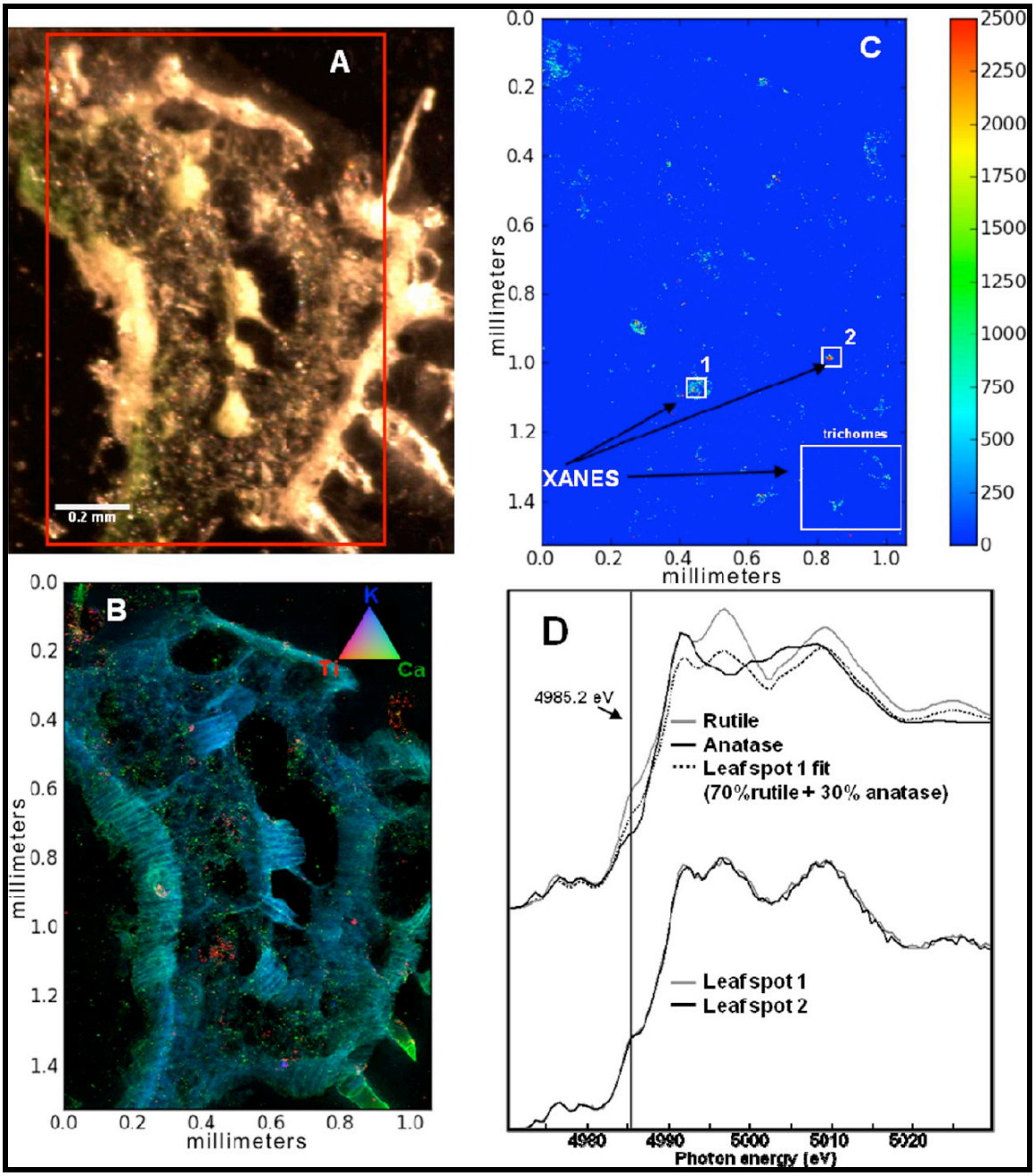

Figure 2.3. Images of transverse sections of cucumber leaf treated for 15 days with 500 mg L^{-1} TiO_2 NPs. (A) Video microscope image of the cucumber leaf transverse section. (B) Tricolor micro-XRF map. Red color stands for titanium, green for calcium, and blue for potassium. The XRF map was acquired at 5.1 KeV, 200 ms dwell time, 3 μm^2 pixel size and 0.33 × 0.65 μm^2 beam size. (C) Ti temperature map, color scale units are raw intensity. The scale was adjusted to 2500 in order to enhance the medium range intensity regions; the max in some of the red spots was ~8000. White box marked areas indicate where micro-XANES was acquired with the use of a 50 µm pinhole. (D) Micro-XANES spectra of reference materials and spot 1 and 2 from Figure 2.3(C).

Thrichomes (leaf hairs) are glandular and nonglandular outgrowth of the epidermis of most of the Cucurbita species. These structures protect the plant from heat, sunlight, herbivores, and water loss.[41] Plants also release several substances to the surface through trichomes. Previous studies have suggested that trichomes are involved in the protection of the plant against diverse environmental challenges such as heavy metal detoxification.[42] In the present study, trichomes of the cucumber leaf were analyzed by micro-XRF and micro-XANES to investigate their possible participation as sink for TiO_2 NPs. Figure 2.4(A) shows the optical image of longitudinally sectioned trichomes in the cucumber leaf. Figure 2.4(B and C) shows the Ti temperature maps of the base and stem of the trichomes as well as the spots chosen to obtain the micro-XANES spectra (circled). The circle represents the illuminated area for micro- XANES acquisition in unfocused mode with a 50 μm pinhole. The micro-XRF images of the leaf trichomes (Figure 2.4(D)) revealed that the cucumber is possibly using the trichomes as possible sink or excretory system for the TiO_2 NPs. It has been reported that in hydroponically grown cucumber plants, supplemented with silicon at 100 mg L^{-1}, silica accumulate in cucumber trichomes possible due to the different permeability of trichomes membranes.[43]

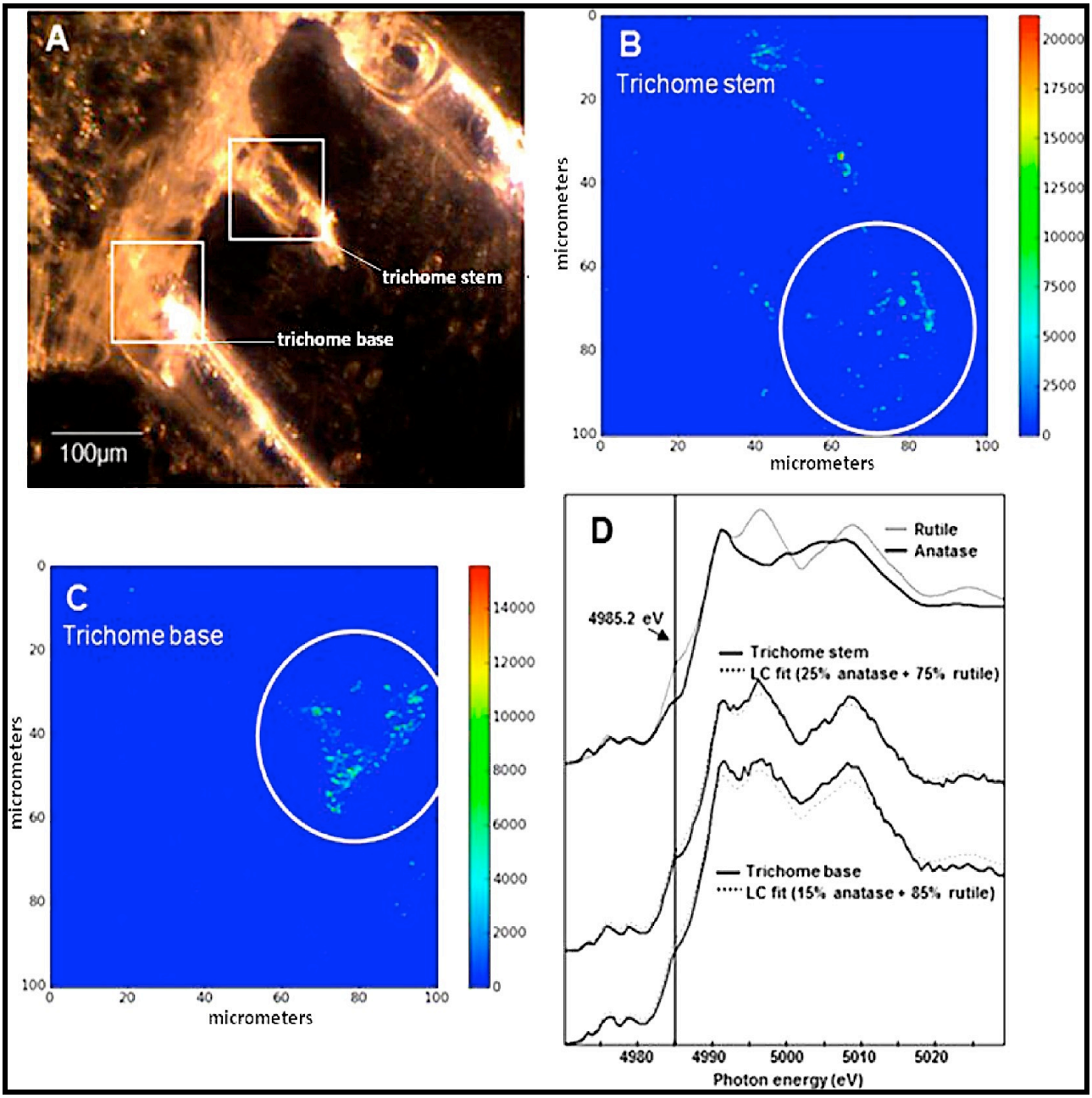

Figure 2.4: Images of cucumber leaf trichomes from plants treated for 15 days with 500 mg L^{-1} TiO_2 NPs. (A) Video microscope image of trichomes from the cucumber leaf. (B) Ti temperature map of the trichome stem from Figure 2.4(A). (C) Ti temperature map of the trichome base from Figure 2.4(A). Color scale units are raw intensity. Circles represent the illuminated area for micro-XANES acquisition in unfocused mode with a 50 µm pinhole. Maps acquired at 5.1 KeV with 300 ms dwell time, 0.5 μm^2 pixel and 0.33 × 0.65 μm^2 beam size. (D) micro-XANES spectra of reference materials (TiO_2 anatase and rutile) and trichomes from Figure 2.4(B) and (C).

2.3 CONCLUSION

In summary, the micro-XRF and micro-XANES analyses of transversal sections of root and leaf of cucumber have proven that TiO_2 NPs are absorbed by roots and transported to the aboveground plants parts. The Ti was detected mainly in the root dermis and cortex, but it was also found in structures from the main nutrient transport systems (phloem and xylem). In the leaf, Ti was found in the dermis, mesophyll, vascular system, and trichomes. The plants were supplied with anatase/rutile mixed TiO_2 NPs and the rutile phase was mainly found in the aerial tissues of the plants whereas anatase remained preferentially in the root tissues. Phloem translocation plays an important role in fruit formation; [44] thus, it is possible that the TiO_2 NPs present in the phloem can be stored in cucumber fruits. According to Menard et al., [3] in the United States the modeled concentrations of TiO_2 NPs in sewage sludge, which is commonly used as soil amendment, vary from 107 to 523 mg kg^{-1}. This means that cucumber grown in sewage sludge amended soil could represent a possible pathway for the entrance of TiO_2 NPs into the food chain.

CHAPTER 3

Synchrotron verification of TiO_2 accumulation in cucumber fruit: A possible pathway of TiO_2 nanoparticle transfer from soil into the food chain

Abstract

The transfer of nanoparticles (NPs) into the food chain through edible plants is of great concern. *Cucumis sativus* L. is a freshly consumed garden vegetable that could be in contact with NPs through biosolids and direct agrichemical application. In this research, cucumber plants were cultivated for 150 days in sandy loam soil treated with 0 to 750 mg TiO_2 NPs kg^{-1}. Fruits were analyzed using synchrotron μ-XRF and μ-XANES, ICP-OES, and biochemical assays. Results showed that catalase in leaves increased (U mg^{-1} protein) from 58.8 in control to 78.8 in the 750 mg kg^{-1} treatment; while ascorbate peroxidase decreased from 21.9 to 14.1 in the 500 mg kg^{-1} treatment. Moreover, total chlorophyll content in leaves increased in the 750 mg kg^{-1} treatment. Compared to control, FTIR spectra of fruit from TiO_2 NP treated plants showed significant differences ($p \leq 0.05$) in band areas of amide, lignin, and carbohydrates suggesting macromolecule modification of cucumber fruit. In addition, compared with control, plants treated with 500 mg kg^{-1} had 35% more potassium and 34% more phosphorous. For the first time, μ-XRF and μ-XANES showed root-to-fruit translocation of TiO_2 in cucumber grown in soil without biotransformation. This suggests TiO_2 could be introduced into the food chain with unknown consequences.

Resulting publication from this research:

Servin, A.D., Morales, M.I., Castillo-Michel, H., Hernandez-Viezcas, J.A., Munoz, B., Zhao, L., Nunez, J.E., Peralta-Videa, J.R., Gardea-Torresdey, J.L. 2013. Synchrotron Verification of TiO_2 Accumulation in Cucumber Fruit: A Possible Pathway of TiO_2 Nanoparticle Transfer From Soil Into the Food Chain. *Environmental Science and Technology* 47, 11592-11598.

3.1 INTRODUCTION

Multiple applications of nanotechnology have generated a high demand for engineered nanoparticles (NPs). It is estimated that by 2020, the worldwide value of products for nanotechnologies will reach US $3 trillion.[1] Recent reports indicate that the most widely used metal oxide nanoparticle is titanium dioxide (TiO_2) with up to 10,000 t per year of global production.[2] Approximately 50-80% of the total production of TiO_2 NPs is used in the cosmetic and sunscreen industries.[3,4] Among other uses, TiO_2 NPs have been used in coatings, plastics, paints, cement,[4] and as a photo-catalyst.[5] As a result of the high production and uses of TiO_2 NPs, environmental release is inevitable.

Previous reports have shown that TiO_2 NPs can be correlated with major toxicological problems.[6] For example, *In vitro* experiments conducted on human lung cells, have shown that TiO_2 NPs in their anatase crystalline phase inhibited cell growth, induced oxidative stress,[7] increased intracellular cytokine contents,[8] and caused cell death by an intrinsic apoptotic pathway.[9] *In vitro* studies on nervous cells reported induction of apoptosis in embryonic striatum cells, as well as the internalization of TiO_2 in BV2 and N27 cytoplasm cells;[10] while necrotic and apoptotic cells were observed in studies with white human blood cells.[11] Other studies have shown that TiO_2 NPs also can affect bacteria and plant species. For example, studies in *Escherichia coli* showed that TiO_2 NPs induced significant oxidative stress, resulting in DNA damage and cell death.[12] In addition, TiO_2 NPs increased the level of malondialdehyde, an indicator of lipid peroxidation in *Allium cepa* and *Nicotiana tabacum,* suggesting DNA damage could ocurr.[13] In contrast, other reports have indicated minimal toxicity of TiO_2 NPs in plants. For example, studies in *Arabidopsis thaliana* roots showed relatively weak impact on genes involved in responses to TiO_2 NPs exposure.[14]

Previous studies have used micro X- ray fluorescence (μ-XRF) and micro X-ray absorption spectroscopy (μ-XAS) analyses to study the accumulation and translocation of NPs in plants. Larue et al.,[15] reported that TiO_2 NPs were accumulated in roots and shoots in hydroponically grown wheat

(*Triticum aestivum*). Similarly, in a previous study, we demonstrated that cucumber (*Cucumis sativus*) plants grown in hydroponics conditions take up and transport TiO_2 NPs from the roots to leaf trichomes.[16] However, there are no previous studies on the translocation of TiO_2 NPs into the cucumber fruit and the effects of these NPs on the cucumber quality. Cucumber consumption in the United States has increased since the 1960's, reaching 10.3 pounds per capita; 60% consumed as fresh produce and 40% as pickled products.[17] If these NPs reach the cucumber fruit, they could be introduced into the food chain with unknown consequences.

In the present study, cucumber plants were grown until fruit production in soil treated with TiO_2 NPs. The total chlorophyll content and activity of antioxidant enzymes catalase (CAT) and ascorbate peroxidase (APX) were measured in mature plants. At harvest, the fruit was analyzed by synchrotron μ-XRF and FTIR techniques in order to determine the possible presence of TiO_2 NPs, as well as induced changes in key macromolecules, respectively. In addition, changes in nutrient accumulation in the fruit were determined by using inductively coupled plasma-optical emission spectroscopy (ICP-OES).

3.2 Materials and Methods

In this section, we describe the methodology used in the preparation of TiO_2 NPs suspensions, soil preparation and cucumber growth. Also, in this section we provide information about the sample preparation for the chlorophyll content experiment, CAT/APOX assays, ICP-OES, FTIR and synchrotron analysis.

3.2.1 TiO_2 NP suspensions

Semispherical TiO_2 NPs (27 ± 4 nm, Evonik Degussa, Nippon Aerosil) were provided by the University of California Center for Environmental Implications of Nanotechnology (UC-CEIN). These NPs were previously characterized by Keller at al.[18] and were found to have a surface area of 51.5 $m^2 g^{-1}$ and both anatase (82%) and rutile (18%) crystalline phases were present. For the present study, the NPs

were suspended in Millipore water (MPW) and diluted to have 0, 250, 500 and 750 mg TiO_2 NPs kg^{-1} soil. In order to avoid precipitation, the suspensions were stirred for 5 min, and sonicated in a water bath before mixing with the soil (Crest Ultrasonics, Trenton, NJ) at 10°C for 30 min (120 V, 3 Amps, 50/60 Hz).

3.2.2 Soil Preparation

The soil for this experiment was collected from the top 50 cm of irrigated cotton field near Fabens, TX and previously characterized as sandy loam soil.[19] The soil was sieved through a 2 mm mesh prior to experimental use. After sieving, portions of 4000 g were put in plastic trays and mixed with the TiO_2 NP suspensions. The soil with NPs was placed into pots of 24 cm diameter x 23 cm high. Each treatment (suspension) was replicated three times.

3.2.3 Cucumber Growth

Cucumber seeds (Westar Seeds International, El Centro, CA) were stirred for 15 min in a 4% NaClO solution, followed by a rinsing with sterilized MPW. Then, seeds were stirred for one hour in sterilized MPW. Subsequently, triplicate sets of ten cucumber seeds per pot (120 plants per experiment) were planted at 2cm depth from the surface and placed into the growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) at a controlled temperature of 25/20°C day/night, 65% relative humidity, light intensity of 340 μmole $m^{-2}s^{-1}$, and a 14 h photoperiod. After one month of growth under the TiO_2 NP treatments, plants were thinned to have only five plants per pot. Control and TiO_2 NP treated plants were watered with MPW every day and no fertilizer was added. The experiment lasted 150 days. Hand-pollination was accomplished by transfer pollen with an artist brush.

3.2.4 Chlorophyll Content

To investigate the impact of TiO_2 NP exposure on chlorophyll production in 120-day old plants, five leaves per replicate were analyzed using the Minolta Chlorophyll Meter SPAD- 502 (Minolta, Japan) as previously described in literature. [20]

3.2.5 CAT/APX assays

To determine cucumber stress response to TiO_2 NP exposure, the activity of catalase and ascorbate-peroxidase were determined. Control and TiO_2 NP treated plants were sampled prior to fruit formation (120 days after exposure to TiO_2 NP treatments). Fresh true leaves were washed with 0.01 M HNO_3 solution followed by a rinse with MPW in order to remove leaf surface adhering NPs. Extracts of leaves of three plants per pot were used to determine the activity of CAT and APX, as it has been previously described in literature, [21] with minimal modifications.[22] A ratio of 10% w/v of cucumber leaves was homogenized with 25 mM monopotassium phosphate buffer (KH_2PO_4) at a pH of 7.4. The extracts were centrifuged for 5 min at a temperature of - 4 °C at 10,000 rpm (Eppendorf AG bench centrifuge 5417 R, Hamburg, Germany).

For the CAT assay, 50 µL of the sample were mixed with 950 µL of 10 mM H_2O_2 in a quartz cuvette, having a final volume of 1 mL. The absorbance at 240 nm was recorded for 3 min in a Perkin Elmer Lambda 14 UV/Vis Spectrometer (single-beam mode, Perkin-Elmer, Uberlinger, Germany). For the APX assay, the activity was evaluated according to Murguia et al.[23] with minor modifications.[22] A volume of 100 µL of the sample was placed in a quartz cuvette and mixed with 886 µL of 0.1 M KH_2PO_4 buffer at pH 7.4, 4 µL of a 25 mM ascorbate solution, and 10 µL of 17 mM H_2O_2. The absorbance was recorded at 265 nm for 3 min in a Perkin Elmer Lambda 14 UV/Vis Spectrometer.

The linear slope at 3 minutes recording was used to calculate the specific activity for the H_2O_2 decomposition, by using an extinction coefficient of H_2O_2 and corrected by protein content determined

by Bradford assay.

3.2.6 Elemental Analysis

For macro and microelements quantitation, triplicates samples per treatment of cucumber fruit were washed with 0.01 M HNO_3 and MPW to remove surface adhering NPs. Subsequently, cucumber fruit were dried at 60 °C for 48 h. The dried samples were microwave-assisted acid digested using a CEM microwave (CEM $Mars_X$, Mathews, NC) following the US-EPA 3051 method (1200 W, 5 min ramping to 175 for 10 min at a pressure of 350 PSI). Samples were digested with 3 mL plasma pure HNO_3 (SCP Science, New York) and the digest were adjusted to a volume of 30 mL using MPW. Concentrations of macro and micronutrients (Ca, Mg, K, P, Fe, Mn, Na, Cu, Zn, B, and Ni) in cucumber fruit were determined using ICP-OES (Perkin-Elmer Optima 4300 DV). Ti concentrations were not determined in plant samples due to difficulties to fulfill the requirements for the digestion of TiO_2 NPs. A certified standard reference material (NIST-SRF 1570A, Metuchen, NJ) was used for calibration and quality assurance/quality control. In addition, an external certified standard of each element was used after every 10 samples to monitor the matrix effect on the analytes.

3.2.7 FTIR Analysis

Three week-old cucumber fruit harvested from plants cultivated for 150 days in soil treated with the TiO_2 NPs were lyophilized for 48 h, as it has been previously reported in literature.[24] The dried cucumber fruits were grinded and placed on a sample holder plate. Then, samples were analyzed in a FT-IR Spectroscopy (Perkin Elmer, Spectrum 100, Universal ATR Sampling Accessory) with a range of 650-3950 cm^{-1}.

3.2.8 Micro-XRF and Micro-XANES Data Acquisition

μ-XRF and μ-XANES were used to analyze three week-old cucumber fruit harvested from plants cultivated for 150 days in soil treated with TiO_2 NPs. The fruits were frozen in liquid nitrogen and stored at -80 ºC until analysis. Fruit of cucumber plants exposed to 750 mg TiO_2 kg^{-1} and control were sent to the European Synchrotron Radiation Facility (ESRF, Grenoble, France) under cryogenic conditions. Once at the beamline, portions of fruit were immersed in Tissue Tek resin and plunged freeze in liquid nitrogen. Samples were axially sectioned at 40 μm thickness using a cryomicrotome. Micro-XRF mapping of Ti K-edge was performed under cryogenic conditions with a 5.1 KeV incident beam at beamline ID21 of the ESRF. The beam was focused with the use of KB mirrors to a size of 0.300 x 0.700 μm^2 (VxH). The fluorescence signal was detected by a Si drift detector. Two photodiodes were used to measure the incident and transmitted beam intensities. Dwell time and distance of the detector were optimized for each XRF map keeping the dead time below 15%. The XRF data was processed using the PyMCA software.[25] For micro-XANES data acquisition, the energy was selected using a Si111 monochromator and scanned from 4950 to 5100 eV. The final Ti K-edge spectra were the sum of 10-30 individual scans with 0.1 s integration time and 0.5 eV resolution step. Reference materials were TiO_2 (100% anatase) spherical NPs of 4 nm nominal diameter and TiO_2 (100% rutile) spherical/elongated 137 nm particles analysed as powder pellets in transmission and fluorescence mode.[16]

3.2.9 Statistical Analysis

The data reported for chlorophyll content was an average of five replicates; the rest of the data were averages of three replicates. A one-way ANOVA, and Duncan test was used to determine statistical significance for enzyme assays, while Tukey's HSD test was used for the remainder analysis. The significance was determined with $p \leq 0.05$.

3.3 RESULTS AND DISCUSSION

In this section we provide insights on the effects, speciation and distribution of TiO_2 NPs on mature cucumber plant as well as cucumber fruit grown in sandy loam soil. Also, we present micro-XRF images showing the presence of Ti within cucumber fruit tissue. Additionally, we show Micro-XANES spectra of TiO_2 in cucumber fruit. The results on this section show the root-to-fruit translocation of TiO_2 in cucumber without biotransformation.

3.3.1 Chlorophyll Content.

The chlorophyll content in leaves of 120 day-old cucumber plants was determined using a SPAD- 502 chlorophyll meter (Figure 3.1). In the TiO_2 NP treated plants, the chlorophyll content in leaves increased as the external concentration of NPs increased. The highest chlorophyll content was recorded in leaves of plants treated with 750 mg TiO_2 kg^{-1}. This concentration was significantly higher compared with control leaves ($p \leq 0.05$). Chen et al.[26] reported that TiO_2 anatase-rutile NPs (21 nm particle size) increased the contents of chlorophyll *b* on a unicellular green alga *Chlamydomonas reinhardtii.*[26] Other reports indicate that TiO_2 NPs can affect chlorophyll content of diverse plant species. For example, Sadiq et al.[27] reported the reduction of total chlorophyll content in algal species as the concentration of TiO_2-anatase (25 nm particle size) NPs increased. Song et al. [28] reported no differences in chlorophyll content in tomato upon exposure to 1000 and 5000 mg TiO_2 NPs/L (anatase: rutile 80:20, 27 nm particle size). The effect of TiO_2 NPs on pigments is still an open question.

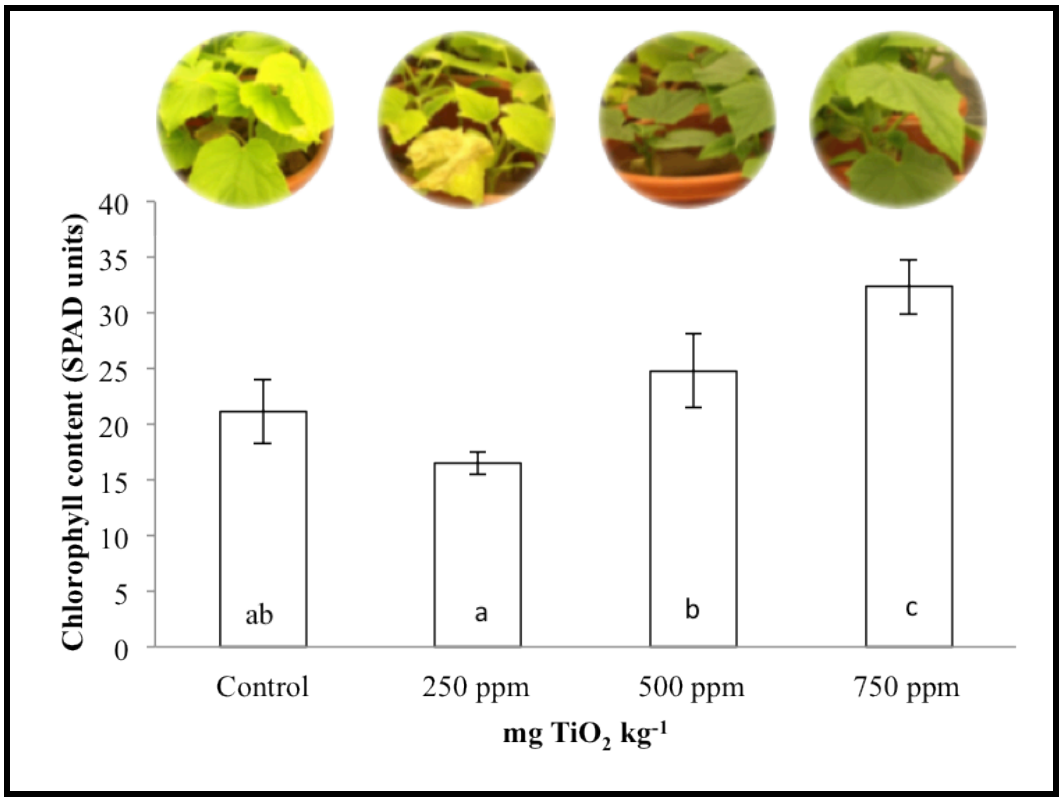

Figure 3.1: Total chlorophyll content of cucumber leaves treated for 120 days in inorganic soil with TiO_2 NPs up to 750 mg kg^{-1}. Plants were set in the growth chamber at a temperature of 25/20°C day/night, 65% relative humidity, 14 hour photoperiod and 340 µmole $m^{-2}s^{-1}$. Lowercase letters represent statistically significant differences between treatments. Error bars stand for standard error ($\alpha \leq 0.05$).

3.3.2 Catalase (CAT) and Ascorbate Peroxidase (APX) Activity

The effects of TiO_2 NPs on CAT and APX specific activities are shown in Figure 3.2. As seen in this figure, compared to control, all TiO_2 NP treatments increased CAT activity in leaves (Figure 3.2(A)). This suggests that TiO_2 exposure increased generation of H_2O_2 and, consequently, a higher activity of CAT was observed. Previous studies have suggested that TiO_2 NPs (rutile) increase the activity of CAT in plants; a response to protect chloroplast membranes from ROS.[29] Recent studies have also reported an increase in CAT activity in spinach chloroplasts treated with TiO_2-anatase NPs.[30] As it can be seen in Figure 3.2(B), compared to control, APX activity in leaves decreased at 500 and 750 mg

TiO_2 NPs kg^{-1}. These results suggest that TiO_2 NPs produce stress in cucumber plants; since CAT was increased at all TiO_2 NP concentrations and APX decreased at high TiO_2 NP concentrations.

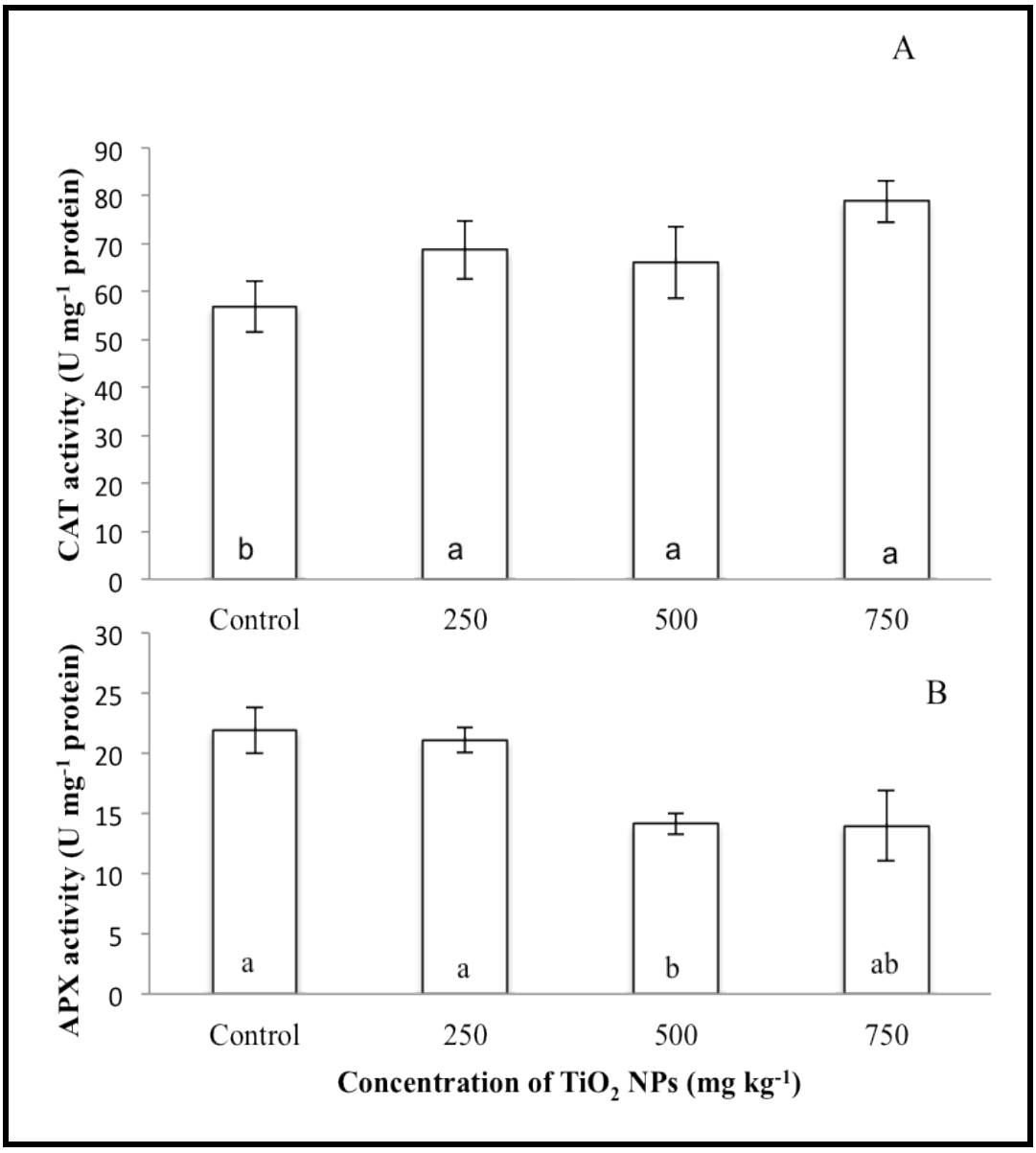

Figure 3.2: Activity of catalase (A) and ascorbate peroxidase (B) in leaves from cucumber plants treated for 120 days in inorganic soil with 0-750 mg TiO_2 NPs kg^{-1}. Data are means of five replicates ±SE (standard error). Lowercase letters among columns indicate statistically significant differences between TiO_2 NPs treatments. Error bars stand for standard error ($\alpha \leq 0.05$).

3.3.3 Effect of TiO_2 in macro and micro-elements accumulation in cucumber fruit

In the present study, macro and micronutrients (Ca, Mg, K, P, Fe, Mn, Na, Cu, Zn, B, and Ni) were determined in three week-old cucumber fruit harvested from plants treated with 0- 750 mg TiO_2 NPs kg^{-1} (Table 3.1). As seen in this table, relative to control, fruit from plants treated with 500 mg TiO_2 kg^{-1} had significantly more K and P, compared to the other treatments. At this time we cannot explain why only the uptake of these two elements was increased. However, previous studies have shown that TiO_2 NPs have similar effects than some plant hormones like cytokinins and gibberellins [31] and cytokinins have been found to affect P and K uptake.[32] Phosphorus is essential in plant's development processes which require energy transfer, it is also found in large quantities in fruit and seeds.[33] Previous studies have shown that in *Capsicum annuum* L., there was an increase in P uptake associated with the presence of Ti. The authors also reported an increase in photosynthetic activity associated with the Ti.[34] Therefore, changes in nutrient content can impact the nutritional quality and taste of the crop.[35] The role of K in plants is essential for many biochemical and physiological processes. For example, in controlling the opening/closing of stomata, in the activation of enzymes such as the starch synthetase, and for water/nutrient transport.[36] Therefore, high concentration of K can improve the physical quality and nutritional value of fruit.[36]

Table 3.1. Phosphorus and potassium concentration in cucumber fruit of plants treated for 150 days in sandy loam soil treated with 0 to 750 mg TiO_2 NPs kg^{-1}. Data represent means of three replicates ± SE (standard error). Different letters indicate statistically significant difference between treatments at $p \leq 0.05$.

TiO_2 NPs in soil (mg kg^{-1})	**mg kg^{-1} d wt biomass**	
	Phosphorus	**Potassium**
0	6.0 ± 0.3 *b*	22.4 ± 3.6 *b*
250	5.4 ± 0.3 *b*	21.0 ± 1.0 *b*
500	9.1 ± 0.4 *a*	34.6 ± 1.8 *a*
750	4.9 ± 0.1 *b*	18.4 ± 1.9 *b*

3.3.4 FTIR Analysis

FTIR spectra of macromolecules from the cucumber fruit are displayed in Figure 3.3, and the FTIR band areas of plants are shown in Table 3.2. As seen in this figure, the band area of the amide I group (U) of the cucumber fruit treated with 500 and 750 mg TiO_2 NPs kg^{-1} decreased compared with the control. Furthermore, the lignin I band area (V) of the cucumber fruit increased in plants treated with 250 and 500 mg TiO_2 NPs kg^{-1}, compared with the control. A previous report indicates that the dissolution of TiO_2 is very low; thus, it is assumed that the changes observed in cucumber plant were produced by the TiO_2 NPs.[37] Previous studies with mangosteen fruit (*Garcinia mangostana* L.) have shown correlation of an increase in lignin with impact damage.[38] As it can be observed in Figure 3.3, there is a reduction in the carbohydrate band areas (X) of fruit from plants treated with TiO_2. The peak height of the carbohydrate group decreased as the concentration of TiO_2 NPs increased. The decrease in area of the affected groups is likely due to partial decomposition of these groups, which in turn causes environmental changes in the area for the amide I (U), lignin I (V), and carbohydrate groups (X), which

are not chemically affected. The apparent increase and decrease of the area of these groups (U, V, and X) upon changes in NP concentrations suggests an environmental change around these groups, as a result of chemical changes within the affected functionalities (S, T, W, Y, and Z). Changes of the area corresponding to the aromatic region of the lignins (V) and those of the amide groups (U) may also be a result of coordination interactions of the amide's carbonyl and nitrogen groups with the TiO_2 nanoparticles, which induce a change in the chemical environment of the plant. These types of metal-lignin complexes have been previously reported, and are well known to change the chemical environment of the plant. Morales et al.[21] reported that at 125 and 500 mg kg^{-1} CeO_2 NPs changed the chemical environment of carbohydrates in cilantro (*Coriandrum sativum* L.) shoots, suggesting a change in the nutritional properties of cilantro. The results of the present study indicate that TiO_2 NPs have an effect in the chemical environment of macromolecules in cucumber fruit, which could alter nutritional value and quality.

Table 3.2: FTIR absorption band frequencies of functional group components in plants.[39]

Band	Frequency range (cm^{-1})	Assignment	Type
S	2840-2960	Lipids I	C-H Symmetric/asymmetric stretch
T	1720-1740	Lipids II	C=O stretching of carboxylic/phenolic ester
U	1650	Amide I	Amide: C=O and C-N stretch
V	1635	Lignin I	Aromatic C=C stretch
W	1550	Amide II	N-H deformation and C-N stretch
X	900-1200	Carbohydrate	Carbohydrate fingerprint region
Y	1515	Lignin II	C=C phenolic stretch
Z	845	Lignin III	Aromatic C-H wag of aromatic ring associated with lignin

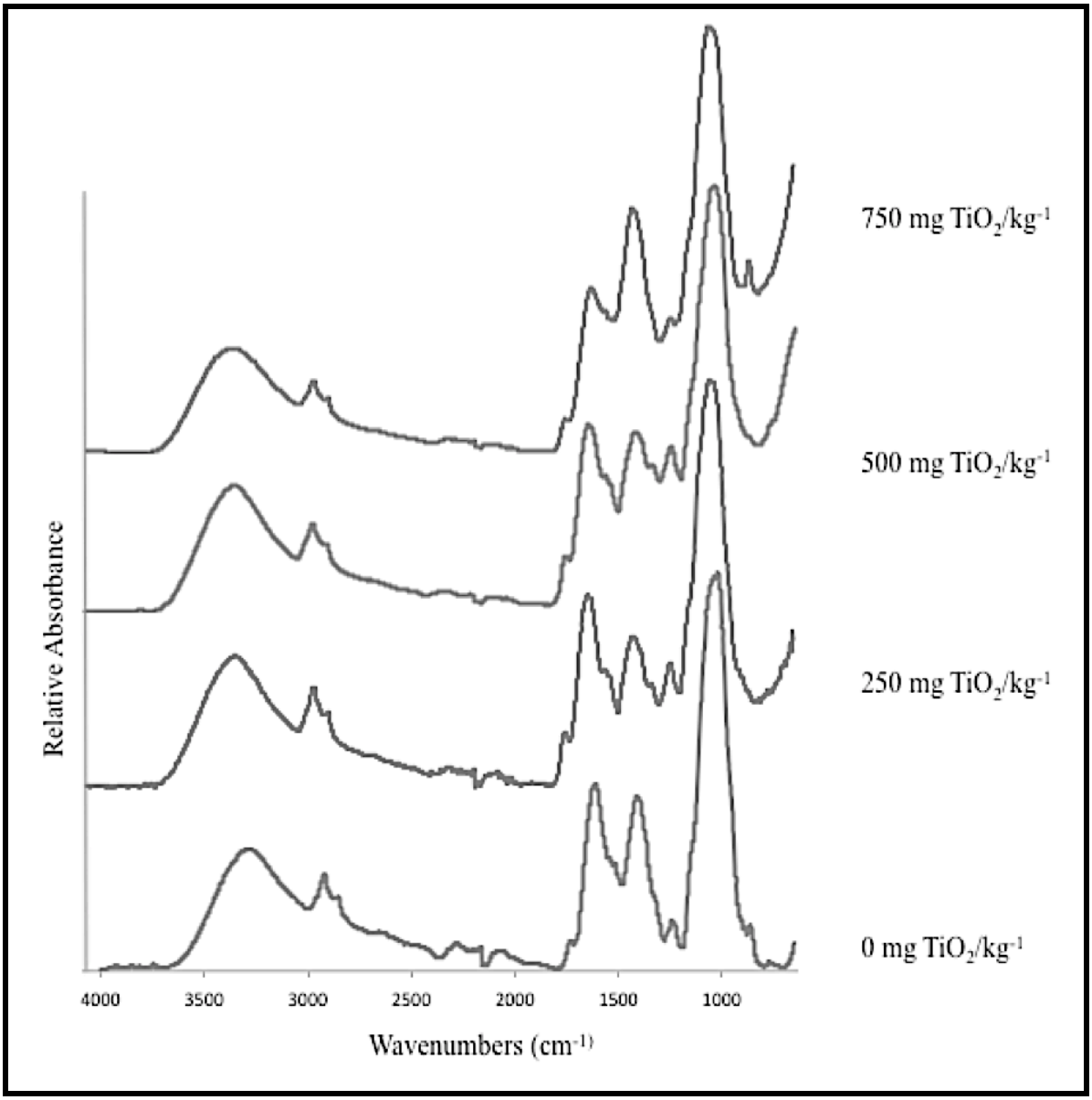

Figure 3.3: FTIR spectra of cucumber fruit treated with 0-750 mg TiO_2 NPs kg^{-1}. Data are means of three replicates. Uppercase letters above spectra indicate the band area from each frequency range, S and T represent lipids, U and W represent amide, V, Y, and Z represent lignin band area.

3.3.5 Micro-XRF and micro-XANES Analysis

Figure 3.4(A) shows the tricolor micro-XRF map of the cucumber fruit cross-section treated with 750 mg TiO_2 NPs kg^{-1} soil. The red color denotes titanium (Ti), blue calcium (Ca), and green potassium (K). The micro-XRF image corroborates the presence of Ti inside the cucumber fruit. Moreover, the temperature map in Figure 3.4(B) shows the distribution of Ti within the cucumber fruit, in which the color scale units represent the Ti intensity regions. This indicates that the TiO_2 NP treated plants absorbed the Ti from soil through the roots and translocated them to the edible portion. The micro-XANES technique was used to determine the chemical form of Ti within the fruit. Figure 3.5 displays the micro-XANES spectra of reference materials (1 and 2) and selected spots (3 and 4) where Ti was located in the cucumber fruit. TiO_2 anatase and rutile were used as reference materials for the present study. As one can see in Figure 3.5, spectrum three acquired from Ti in cucumber fruit resembles the reference material TiO_2 rutile (spectrum two). Moreover, spectrum four resembles the reference material anatase (spectrum one). This indicates the cucumber plants take up both anatase and rutile crystalline phases from the soil and transport them through the tissues without crystal phase modification. In previous investigation, we reported the translocation of TiO_2 NPs from root to the cucumber shoot.[16] We reported that TiO_2 rutile was mainly found in the central vein and trichomes of cucumber leaf, whereas TiO_2 anatase crystalline phase was mainly observed in the root of cucumber plants. The linear combination of spectra obtained from cucumber leaf and trichomes, showed a higher percentage resembling the rutile crystalline phase than the anatase form (70:30, rutile: anatase in leaf; 85:15, rutile: anatase in trichomes).[16] The results of the present study corroborate that both crystalline phases are transported from the roots to the cucumber fruit. These results agree with those reported by Larue et al.[15] who found TiO_2 NPs in the roots and other vegetative parts of wheat plants without any biotransformation or crystal phase modification.

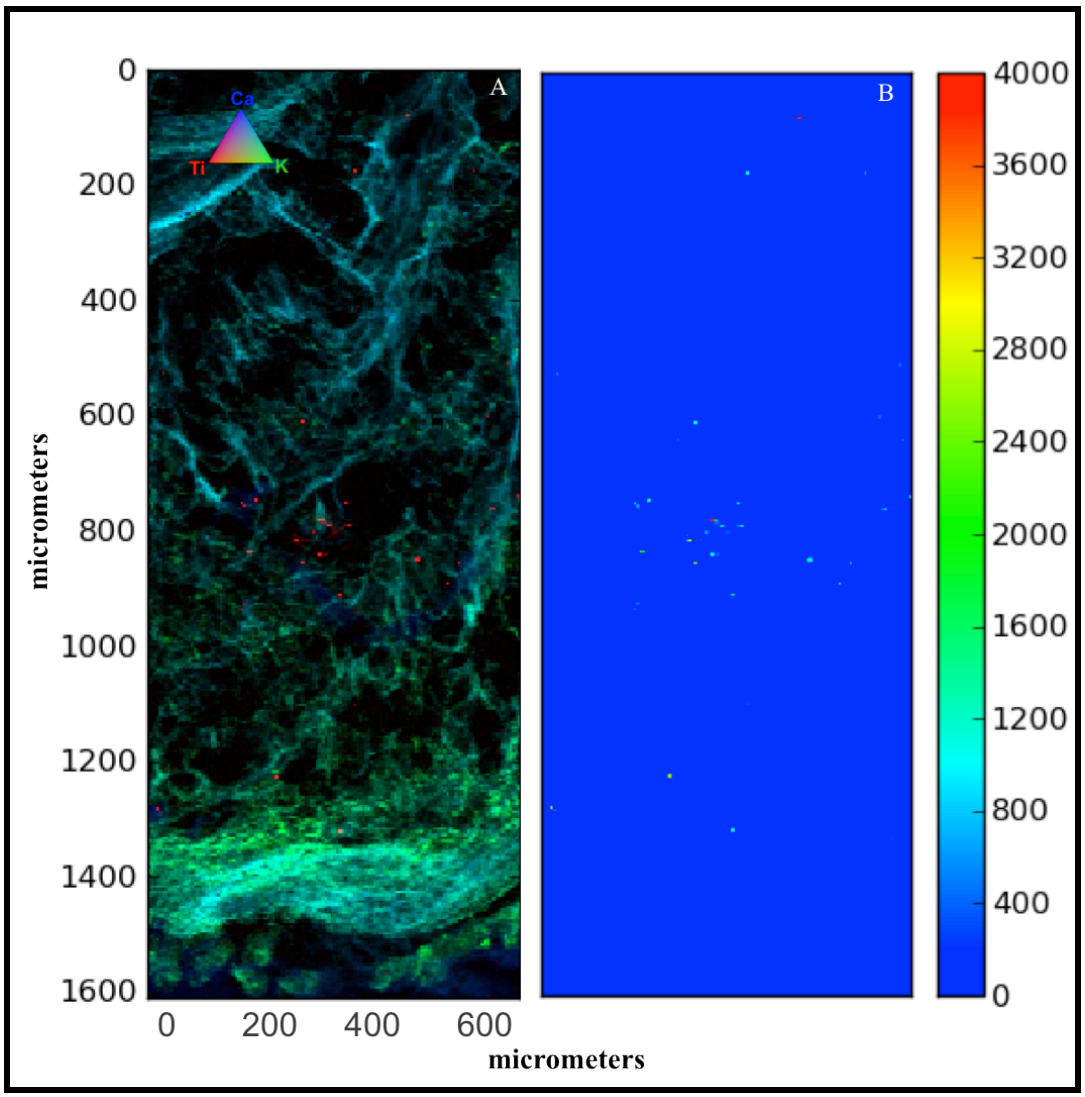

Figure 3.4: (A) Tricolor micro-XRF images of the cross sections of cucumber fruit treated with 750 mg TiO_2 NPs kg^{-1}, red color stands for titanium, green for potassium, and blue for calcium. (B) Ti temperature map, color scale units are counts per second.

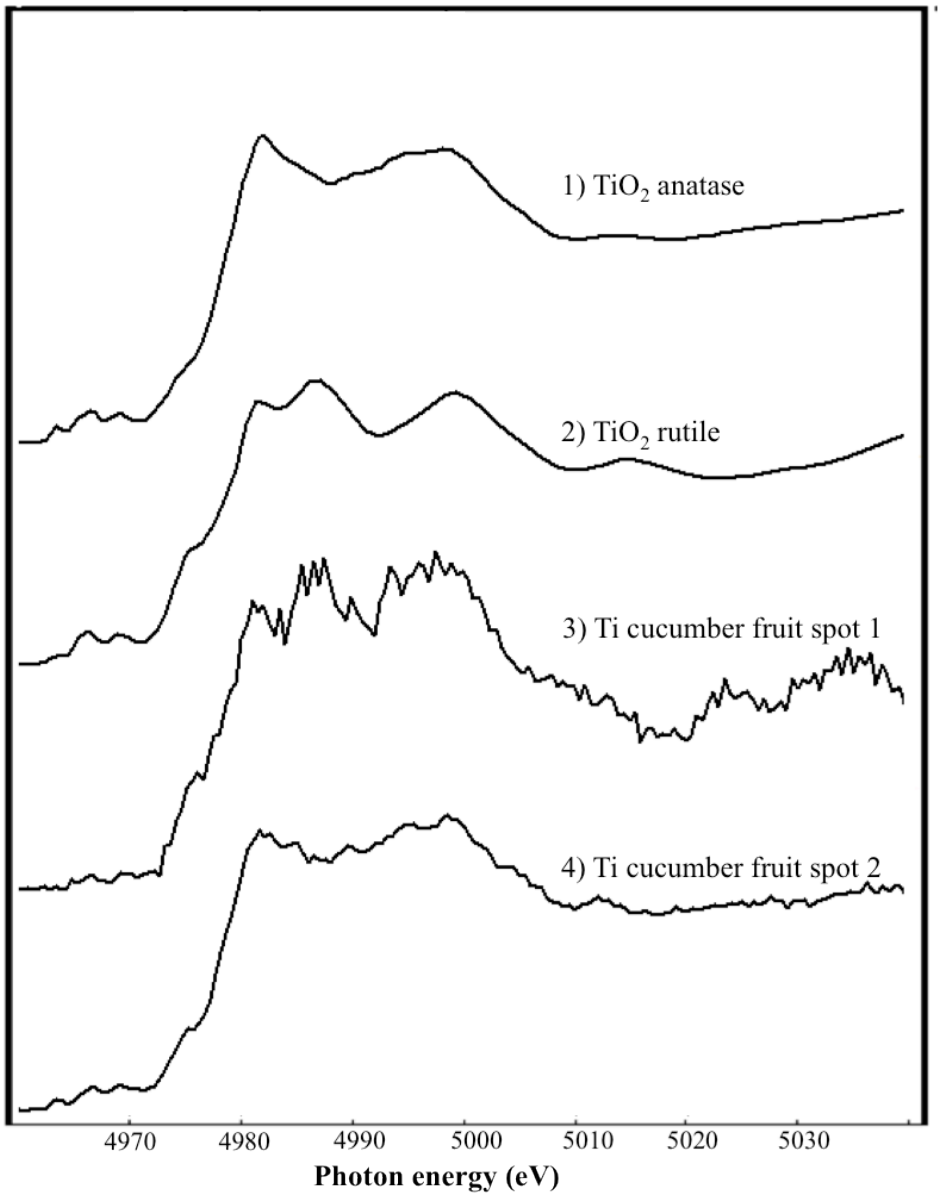

Figure 3.5: Micro-XANES spectra of reference materials (1-2) and spots of interest (3-4) from Figure 3.4 (A).

3.4 CONCLUSION

In summary, the ICP-OES analyses showed that cucumber fruit from plants treated with 500 mg TiO_2 kg^{-1} NPs have a higher content of primary macronutrients P and K. Moreover, FTIR analysis demonstrated that at all concentrations (250-750 mg kg^{-1}) TiO_2 NPs could modify the chemical environment of macromolecules in cucumber fruit. The FTIR analyses suggest significant chemical changes in lipids, amide, lignin and carbohydrates. However, a decrease in nutritional value would be directly correlated to the extent of decomposition of the macromolecules, which is not quantified by these IR experiments. Furthermore, the present study suggests that at 750 mg TiO_2 NPs kg^{-1} exposure results in an increase of chlorophyll content. In addition, all TiO_2 NP concentrations increased catalase activity in cucumber leaves, suggesting that TiO_2 exposure increased the generation of H_2O_2, resulting in a higher catalase activity. Results from synchrotron studies have shown that TiO_2 was translocated without biotransformation or crystalline phase modification to the edible part of cucumber plants, suggesting that TiO_2 NPs could be introduced into the food chain with unknown consequences.

Chapter 4

General Conclusions

The presence of nanomaterials in the environment has raised concerns about their possible transfer to the food chain through plants. The results of this dissertation work have shown the impacts of TiO_2 NPs in cucumber (*Cucumis sativus* L.) plants grown in hydroponics and soil. Our hydroponic studies demonstrated that TiO_2 significantly increased plant root length at all concentrations (average >300%). In addition, our results from the hydroponic experiments showed the presence of TiO_2, mostly in its anatase form, in the root dermis, cortex and nutrient transport system of the root. While, TiO_2 rutile was mostly found in the aboveground part of the plant. The aforementioned suggests that TiO_2 was translocated from the root to the leaf without biotransformation. The soil study was focused on the analysis of fruits from plants exposed to TiO_2 NPs grown in soil. Analyses of the effects of TiO_2 in the physiology of cucumber showed an increase of chlorophyll content at 750 mg TiO_2 NPs kg^{-1}. In addition, all TiO_2 NP concentrations increased catalase activity in cucumber leaves in contrast with the control. Our results showed that catalase in leaves increased when exposed to TiO_2 NPs, (U mg^1 protein) from 58.8 in control to 78.8 in the 750 mg kg^{-1} treatment; while ascorbate peroxidase decreased from 21.9 to 14.1 in the 500 mg kg^{-1} treatment. The μ-XRF and μ-XANES analyses showed that that TiO_2 was translocated without biotransformation or crystalline phase modification to the edible part of cucumber plants. ICP-OES analyses showed that cucumber fruit from plants treated with 500 mg TiO_2 kg^{-1} NPs have a higher content of phosphorous and potassium, primary macronutrients, suggesting that high concentration of potassium and phosphorous can improve the physical quality and nutritional value of the fruit. FTIR analyses demonstrated that at all concentrations (250-750 mg kg^{-1}) TiO_2 NPs could modify the chemical environment of macromolecules in cucumber fruit. The FTIR analyses suggest significant chemical changes in lipids, amide, lignin and carbohydrates. However, changes in nutritional value would be directly correlated to the extent of macromolecules decomposition, which is not quantified by these IR experiments. This research has shown that in the eventual exposure of TiO_2 NPs to cucumber, at similar concentrations like those tested in this study, cucumber fruit might store these NPs and be introduced into the food chain with unknown consequences.

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Vita

Alia D. Servin obtained her bachelor in science in chemistry at the University of Texas at El Paso. In 2009 she joined the Ph.D program in Chemistry under the supervision of Dr. Jorge Gardea-Torresdey, performing research in the area of nanoparticle toxicity in plants. During her time at UTEP she has been recipient of awards including the Outstanding Undergraduate Chemistry Student in Research at UTEP, and the National Science Foundation GK-12 Fellowship. Also, she has been recipient of fellowships from NSF/EPA funded "UC Center for Environmental Implications of Nanotechnology (UCCEIN)," and the USDA funded "Center for Education and Training in Agricultural and Related Sciences (CETARS)". The different research projects that she has been involved at the University of Texas at El Paso have resulted in 5 peer-reviewed publications in various high impact journals, including two manuscripts in *Environmental Science and Technology* (2012, 46, 7637-7643) (2013, 47, 11592-11598), *Journal of Agricultural and Food Chemistry* (accepted for publication), *Applied Spectroscopy* (2012, 66, 719-727), and *Chemical Engineering Journal* (2011, 170, 346-352). In 2012, the European Synchrotron Research Facility (ESRF) at Grenoble France highlighted her article titled "Synchrotron Micro-XRF and Micro-XANES confirmation of the uptake and translocation of TiO_2 Nanoparticles in cucumber plants". In addition, the results of her work have been presented at several national and international scientific conferences. Alia D. Servin will join a USDA funded post-doctoral position at the Connecticut Agricultural Experimental research Station in New Haven, CT.

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