Plant Response on Exposure to Ag Nanoparticles: A Study with *Vigna subterranea*

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**Abstract**

Effects of silver nanoparticles (Ag NPs) exposure on two geographical cultivars of *Vigna subterranea* were investigated. After inoculation in half strength Hoagland medium amended with Ag NPs for 15 days, both physiological and biochemical responses were evaluated. Exposure significantly decreased plant growth by up to 85%. Interestingly, Ag NPs exposure significantly decreased mean shoot biomass in all treatments but increased root mass (34% and 66%) in relation to control. Chlorophyll production was reduced by approximately 46% (in the more tolerant) and 86% (in the more sensitive) for the two cultivars and observed catalase activity was about 50% of the activity in NPs stressed root tissues for the sensitive cultivar. It may be perceived that the inherent stress is associated with observed surge in catalase activity across all cultivars. Also, the observed increase in catalase activity is positively correlated at 99.9% level (r=0.9571, n=10) with decreasing chlorophyll content on exposure. Recorded ascorbate peroxidase activity was higher in leaf tissues. Statistical analysis revealed marked difference between superoxide dismutase activities of *V. subterranea* cultivars and also between treatments. Time trend of transpiration rate revealed a decreasing order throughout growth period.

**Keywords:** Antioxidant enzymes; *Vigna subterranea*; Silver nanoparticles exposure; Nanotoxicity; Transpiration rate

**Abbreviations:** Ag NPs: Silver Nanoparticles; NPs: Nanoparticles; RL: Root Length (cm); PW: Plant Weight (g); NL: Number of Leaves; LW: Leaf Width (cm); LL: Leaf Length (cm); 67 C: Cultivar 67 Spiked With Zero Silver Nanoparticles; 67 Ag NPs: Cultivar 67 Spiked With Silver Nanoparticles; 01 C: Cultivar 01 Spiked With Zero Silver Nanoparticles; 01 Ag NPs: Cultivar 01 Spiked With Silver nanoparticles; S: Shoot Tissues; R: Root Tissues; USDA: Plant Genetic Resources Conservation Unit of USDA Griffin Georgia USA; UI: The Plant Resources Conservation Unit of the University of Ilorin, Nigeria

**Introduction**

Nanoscience and nanotechnology are the study and application respectively of materials at the nanoscale to leverage their amplified properties such as lighter weight, improved strength and reactivity and greater regulation of light spectrum, in comparison to the larger-scale counterparts. Although many new materials and devices with a vast range of applications, such as in medicine, electronics, biomaterials energy production, military operations, agriculture and consumer products are dividends of nanotechnology, toxicity and environmental impacts of nanomaterials [1] like in any other new technology remain a concern. Nano-sized particles exist in nature and can be fashioned from a variety of products, such as carbon or minerals like silver, but nanomaterials as defined by NIEHS [2] must have at least one dimension that is less than approximately 100 nanometres. Gaharwar et al. [3] and Cho et al. [4] chronicled recent application of nanomaterials to include a range of biomedical applications, such as tissue engineering, drug delivery, and biosensors (sensor development for toxic substances detection for example). Also, the catalytic activity of these nanomaterials may imply potential risks in their interaction with biomaterials and the overwhelming number of new manufacturer-identified nanotechnology [5] will only heighten existing safety concerns.

Better understanding of NPs fate and effects on biota has been challenging given the potential for food chain contamination and for an uncharacterized pathway of human exposure [6]. Rico et al. [7] highlighted the unknown interactions of engineered nanomaterials agricultural systems that are recipients of a number of organic chemicals and inorganic amendments. Effort targeted at circumventing any potential threat in the production, use, or disposal of nanoscale products and devices will amount to preparedness in this booming and emerging field.

Widespread presence of silver nanoparticles in consumer products such as antimicrobial coatings, and many textiles, keyboards, Silver Nanowires, wound dressings, biomedical devices, photonic devices, conductive inks, pastes and fillers, has attracted many researchers in a quest to understand how they interact with biological systems and the environment. In their product description, Oldenburg [8] described the unique and desirable properties of Ag NPs to include: diagnostic applications as they are used in biosensors and innumerable assays where the silver nanoparticle materials can be used as biological tags for quantitative detection; antibacterial applications in their incorporation in apparel, footwear, paints, wound dressings, appliances, cosmetics, and plastics for their bactericidal property; conductive applications as they are used in conductive inks and integrated into composites to enhance thermal and electrical conductivity; and optical applications in their ability to efficiently harvest light and for amplified optical spectroscopies including metal-enhanced fluorescence and surface-enhanced Raman scattering.

Nwaichi et al. [9] reported the socioeconomic role played by an indigenous African legume, *V. subterranea* in the semi-arid regions of Africa. They also submitted that the crops can grow in marginal, low-input environments, and occur in the local environment. Also,
the seeds make a complete food, as it contains sufficient quantities of proteins, carbohydrates and lipid, and these may predispose this crop to anticipated implication with increasing use and disposal of nano products; hence the rationale for choice in this study.

Scarce data, however, are available on the response in important crops subjected abiotic stress due to nanoparticles, as well as on the involvement of antioxidant enzymes in this type of stress in anticipation to the rising patronage of nanoparticles.

This study aims to evaluate nanotoxicity of Ag NPs exposure on selected cultivars of *V. subterranea* at the physiological level and investigate the activity of antioxidant defence system there-of.

**Methods**

**Materials and conditions of growth**

Silver nanoparticles (99.99% purity; <20 nm) was obtained from US Research Nanomaterials, Inc. (Houston, TX US). Using an ultrasonic probe, prepared 250 mg L\(^{-1}\) and 500 mg L\(^{-1}\) Ag NPs solutions were agitated for 10 mins and left overnight thereafter, to determine particle characteristics in water. Afterwards, the solutions were centrifuged (3000 rpm, 10 mins) and an aliquot of the supernatant taken for particle size and ζ-potential determination (Malvern, Zetasizer Nano ZS90). Respective average particle size and ζ-potential values were 98 nm and −16.7 mV 68 nm at 250 mg L\(^{-1}\) and 90 nm and −20.8 mV at 500 mg L\(^{-1}\). Seeds of seven cultivars (Table 1) of Bambara beans (*Vigna subterranea*) were sourced from ARS Plant Genetic Resources Conservation Unit of USDA Griffin Georgia US and Plant resources conservation unit of the University of Ilorin Nigeria. Seeds were scarified and sown in racks containing Vermiculite to raise seedlings (rapidly ground in liquid N\(_2\)) were homogenized using 1.5 mL of phosphate buffer (25 mM L – Methionine, 57 µM Nitrobluetetrazolium, [10] was adopted as follows: 2.2 mL of the assay mixture included 50 mM phosphate buffer pH 7.0, 0.1% (w/v) Bovine Serum Albumin, 50 mM Phosphate buffer at pH 7.0, 2% (w/v) β – Mercaptoethanol and 0.1% (w/v) Ascorbate) and was centrifuged at 4°C for 20 min at 4,000 rpm in a refrigerated centrifuge (Eppendorf Centrifuge 5810R). The clear supernatant was used as the enzyme source. A modified method of Chance and Maehly (1995) was adopted as follows: One millilitre of extraction buffer (mixture of 0.1% (w/v) Bovine Serum Albumin, 50 mM phosphate buffer pH 7.0, 0.1% (w/v) Ascorbate and 10 mM H\(_2\)O\(_2\) and 0.2 mL of enzyme extract. Absorbance was taken at 290 nm and 1 min interval for 30 mins. The specific activity of catalase was expressed as units mg\(^{-1}\) protein min\(^{-1}\).

**Ascorbate peroxidase assay:** Plant tissues weighing 300 mg (rapidly ground in liquid N\(_2\)) were homogenized using 1.5 mL of extraction buffer (mixture of 0.1 mM EDTA, 100 mM Phosphate buffer at pH 7.0, 2% (w/v) β – Mercaptoethanol and 0.1 mM Ascorbate) and was centrifuged at 4°C for 20 min at 4,000 rpm in a refrigerated centrifuge (Eppendorf Centrifuge 5810R). The clear supernatant was used as the enzyme source. A modified method of Chance and Maehly [10] was adopted as follows: 2.2 mL of the assay mixture included 50 mM phosphate buffer pH 7.0, 0.1 mM EDTA, 0.6 mM Ascorbate and 10 mM H\(_2\)O\(_2\) and 0.2 mL of enzyme extract. Absorbance was taken at 290 nm and 1 min interval for 30 mins. The specific activity of APx was expressed as units mg\(^{-1}\) protein min\(^{-1}\).

**Superoxide dismutase assay:** Plant tissues weighing 100 mg (rapidly ground in liquid N\(_2\)) were homogenized using 1.0 mL of extraction buffer (mixture of 0.1% (w/v) Bovine Serum Albumin, 50 mM Phosphate buffer at pH 7.8, 8.05% (w/v) β – Mercaptoethanol and 0.1% (w/v) Ascorbate) and was centrifuged at 4°C for 20 min at 4,000 rpm in a refrigerated centrifuge (Eppendorf Centrifuge 5810R). The clear supernatant was used as the enzyme source. In a foil wrapped beaker, 3.0 mL of the assay mixture containing 50 mM phosphate buffer pH 7.8, 9.9 mM L – Methionine, 57 µM Nitroblue tetrazolium, 0.025% (w/v) Triton X – 100 and 0.0044% (w/v) Riboflavin.

**Chlorophyll content assay**

50 mg of fresh plant leaves were soaked in 15 mL tubes containing 10 mL 95% ethanol each. Absorbance values were measured spectrophotometrically at 645 nm and 663 nm for chlorophyll a (chl a) and chlorophyll b (chl b) respectively. The tubes were wrapped with foil and put in a dark room at temperature of 25 ± 3°C for 3 days after which absorbance values were taken.

**Enzyme extraction and assay**

**Catalase assay:** Plant tissues weighing 100 mg (rapidly ground in liquid N\(_2\)) were homogenized using 1 mL of phosphate buffer (25 mM KH\(_2\)PO\(_4\) at pH 7.4) and was centrifuged at 4°C for 20 min at 4,000 rpm in a refrigerated centrifuge (Eppendorf Centrifuge 5810R). The clear supernatant was used as the enzyme source. A modified method of Chance and Maehly (1995) was adopted as follows: One millilitre of the assay mixture included 950 µL of 10 mM H\(_2\)O\(_2\) and 50 µL of enzyme extract. A blank (buffer) was run in same way. A unit of catalase activity is defined as the amount of enzyme that degrades 1 µmole of H\(_2\)O\(_2\) min\(^{-1}\) under the assay conditions prescribed. ThermoScientific Evolution 605 UV – Visible Spectrophotometer was employed for reading at 240 nm and 30 secs interval for 2.5 mins. The specific activity of catalase was expressed as units mg\(^{-1}\) protein min\(^{-1}\).

**Ascorbate peroxidase assay:** Plant tissues weighing 300 mg (rapidly ground in liquid N\(_2\)) were homogenized using 1.5 mL of extraction buffer (mixture of 0.1 mM EDTA, 100 mM Phosphate buffer at pH 7.0, 2% (w/v) β – Mercaptoethanol and 0.1 mM Ascorbate) and was centrifuged at 4°C for 20 min at 4,000 rpm in a refrigerated centrifuge (Eppendorf Centrifuge 5810R). The clear supernatant was used as the enzyme source. A modified method of Chance and Maehly [10] was adopted as follows: 2.2 mL of the assay mixture included 50 mM phosphate buffer pH 7.0, 0.1 mM EDTA, 0.6 mM Ascorbate and 10 mM H\(_2\)O\(_2\) and 0.2 mL of enzyme extract. Absorbance was taken at 290 nm and 1 min interval for 30 mins. The specific activity of APx was expressed as units mg\(^{-1}\) protein min\(^{-1}\).

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### Table 1: Collected cultivars of *V. subterranea* for the study.

<table>
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<tr>
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<tr>
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<td>UIH 001</td>
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<td>Nigeria</td>
<td>UI</td>
</tr>
</tbody>
</table>

USDA denotes Plant Genetic Resources Conservation Unit of USDA Griffin Georgia USA; UI denotes the Plant resources conservation unit of the University of Ilorin Nigeria.
In the dark at room temperature (25 ± 3°C), 0.05 mL of enzyme extract was added to 1.45 mL of assay mixture. Absorbance (440 nm) values for test and blank (assay mixture only), were taken at the 20th minute. The specific activity of SOD was expressed as units mg⁻¹ protein min⁻¹.

**Protein content assay:** One microliter of supernatant was read off at 280 nm using Thermo Scientific NANODROP 2000 Spectrophotometer connected to VOSTRO DELL Computer. Buffer and Hygromycin B were used as blank and reference protein respectively. Hygromycin B from Streptomyces hygroscopicus (potency of 1210 U mg⁻¹) was purchased from Phytotechnology Laboratories. Manufacturer’s protocol was followed.

**Statistical analysis**

Data collected from ten replications per treatment were presented as mean ± S.E. and subjected to statistical test of significance using the Student’s t-test. Correlation co-efficient was used to determine the association among cultivars under study at 95% confidence level. Each treatment was compared to its control experiment.

**Results and Discussion**

**Effect of silver nanomaterials on plant mass and other growth indices**

Over 70% of the cultivars exposed to 500 mg L⁻¹ Ag NPs dried up between days 6 and 9 and hence were completely excluded in the final assays. The cultivars with concentration, 250 mg L⁻¹ Ag NPs were therefore reserved for analysis. The observed drying may be due to loss of leaves’ cuticle leading to widening of the stomatal pores and poor desiccation tolerance. At the end of the 15 d experiment, a marked decrease in shoot and root biomass (Figure 1) was recorded. However, an inverse order between the shoot and root tissues were the case when percentage of total mass contributions were calculated. Shoot tissues decreased in percentage of total mass from 84% and 82% (control) to 79% and 70% (Ag NPs exposed) for cultivars 01 and 67 respectively. Shoots for G. Max and C. pepo decreased in their percentage total biomass contribution from 77% and 84% to 77% and 82% in the exposure (500 mg mL⁻¹ Ag NPs) study by De La Torre-Roche et al. [11]. On the other hand, root tissues increased in their% total biomass contribution from 16% and 18% (control) to 26% and 30% (Ag NPs exposed) for cultivars 01 and 67 respectively. Similarly, a decrease from 23% and 16% to 41% and 19% respectively for G. Max and C. pepo, were reported by De La Torre-Roche et al. [11]. Besides reduced biomass, De La Torre-Roche et al. [11] observed 47% and 25% increment in root contribution to total plant biomass for G. Max and C. pepo respectively in relation to their respective controls. These calculations gave 26% for the sensitive cultivar 01 and 67% for the more tolerant cultivar 67 from our results.

A reduction in leaf length, leaf width, and number of leaves formed were noted with progressive leaf necrosis (Figure 2) and inward curling with time. These changes however affected the older leaves more. Naranjo et al. [12] concluded that necrotic spots are typical for plant–pathogen interactions especially when they are incompatible (avirulent). Although NPs – induced toxicity reduced root length as an inverse order between the shoot and root tissues were the case when percentage of total mass contributions were calculated. Shoot tissues decreased in percentage of total mass from 84% and 82% (control) to 79% and 70% (Ag NPs exposed) for cultivars 01 and 67 respectively. Shoots for G. Max and C. pepo decreased in their percentage total biomass contribution from 77% and 84% to 77% and 82% in the exposure (500 mg mL⁻¹ Ag NPs) study by De La Torre-Roche et al. [11]. On the other hand, root tissues increased in their% total biomass contribution from 16% and 18% (control) to 26% and 30% (Ag NPs exposed) for cultivars 01 and 67 respectively. Similarly, a decrease from 23% and 16% to 41% and 19% respectively for G. Max and C. pepo, were reported by De La Torre-Roche et al. [11]. Besides reduced biomass, De La Torre-Roche et al. [11] observed 47% and 25% increment in root contribution to total plant biomass for G. Max and C. pepo respectively in relation to their respective controls. These calculations gave 26% for the sensitive cultivar 01 and 67% for the more tolerant cultivar 67 from our results.

**Screening for the expression of the H₂O₂ scavenging enzymes**

Catalases and peroxidases are two major systems for the enzymatic removal of H₂O₂ in plants [13]. Table 2 represents the changes of catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) activities. Alterations in studied antioxidant enzymes were noted.
cell from oxidative damage by reactive oxygen species, gave heightened longevity agent and growth regulators [16], it could be inferred that P. vulgaris [14]. In their role having various metabolic functions within organisms, decreased posed nanotoxicity in cultivar 01 but were not marked. Spermidine, 3% and 10% respectively for shoot and root tissues in response to for the more sensitive cultivar 01. APx activity slightly decreased by activation of peroxidase during pH 2.2 treatment was found in two protective role of the enzyme in the system. The increment (Table 2) increased activity of peroxidase in acid rain treated plants suggests the protective role of the enzyme in the system. Chlorophyll content in V. subterranea is due to both an increase in SOD specific activity and a drop in APX specific activity (Table 4). Gupta et al. [18] concluded that other factors like maturation of other quenching systems that may include antenna pigments and pools of antioxidant compounds such as ascorbate, GSH, and tocopherols, could have contributed to resistance to oxidative stress given a stable SOD activity as SOD: APX value than the total activity of each enzyme could be an important factor in determining the level of oxidative stress protection [18]. Observed ratio averaged 1.444 and 2.373 in shoot tissues of cultivars 67 and 01 but decreased to 0.328 and 0.544 respectively. It averaged 0.426 and 0.945 and decreased to 0.325 and 0.346 in the same order for root tissues. The higher SOD: APX ratio in control plants, compared with Ag NPs – exposed plants, is due to both an increase in SOD specific activity and a drop in APX specific activity (Table 4). Gupta et al. [18] concluded that other factors like maturation of other quenching systems that may include antenna pigments and pools of antioxidant compounds such as ascorbate, GSH, and tocopherols, could have contributed to resistance to oxidative stress given a stable SOD activity as SOD: APX value increased from 0.45 at rosette stage to 0.75 at bolting stage in their study. Given higher biomass, root length and leaf width (Figure 2) observe in cultivar 67 under stress, they may have attained higher maturity and may have influenced protection. The ratio of shoot SOD activity for cultivar 67 to 01 was higher in spiked samples but the reverse was the case in root tissues and may be related to compartmentalization.

**Chlorophyll content in V. subterranea under Ag NPs stress**

There was accompanying loss of protein content with chlorophyll content. A positive correlation at 99.9% level (r=0.9429, n=10) between protein content and Chlorophyll content during phytotoxicity was observed. Similarly, a positive correlation (r=0.8571, n=10) was observed between increase in catalase activity at the 99.9% level with the decrease of the chlorophyll content during exposure. A significant decrease in the content of chlorophyll a and b was observed for both studied cultivars, and the content of photosynthetic pigments varied between 46% and 86% for the more tolerant South African and it Nigerian sensitive counterpart respectively.

**Formation of reactive oxygen species due to foreign toxic substance** [19] and possibly nutrient depletion may have led to observed leaf dryness from day 5 in the more sensitive cultivar 01 and day 11 in the more tolerant cultivar 67. Formed superoxide may have shut down energy harvest, in addition to formation of precursors of certain amino acids as well as the reducing agent NADH that is used in numerous other biochemical reactions by inactivating citric acid cycle enzyme aconitase, hence poisoning energy metabolism, with attendant release of potentially toxic iron. There was complete dryness of leaves in most replicates for cultivar 01 under Ag NPs stress.

**Observed transpiration pattern in the face of Ag NPs stress**

*V. subterranea* plants were each irrigated with an average of 26.2 mL.
day was least (21 mL d⁻¹ plant⁻¹) for more sensitive cultivar 01 which
study with more tolerant cultivar 67. Transpiration rate on the 8th
stress treatment over control. Similar pattern was observed in this
agriculture. Such findings demonstrate the complex interactions of Ag NPs
exposure; Cultivar 67 under no exposure and Cultivar 67
01 C and 01 AgNp represent cultivar 67 spiked with no Silver nanoparticles,
cultivar 67 spiked with Silver nanoparticles, cultivar 01 spiked with no Silver
nanoparticles, and cultivar 01 spiked with Silver nanoparticles respectively. F.
test compared samples across parameter types and same alphabets indicate no
significant difference at α=0.05. Vertical bars represent mean ± S. E. for n=10.

Such generated data may trigger operators, farmers and households
to apply measures to prevent and reduce nanocontamination as low as
reasonably achievable in order to protect public health. This may include
good agronomic management practice to alleviate contaminant uptake
and biomagnification [22]. It also calls for nanotoxicological acceptable
limits to be set following rigorous experiments and validations by
relevant regulatory bodies.

Techniques used in this work offer unique insights into changes to
some physiological and biochemical processes inside of a crop plant
under Ag NPs stress. An important consideration when studying the
crop plants and interpreting results is that one species does not represent
the high variability and diversity of phylia present in the plant kingdom.
However, this work demonstrated that even same plant species can
behave in dramatically different ways under the same experimental
conditions. This may proffer explanations to some observed variations
within the same conditions.

Conclusion

Our study highlights the relevance of studying physiological and
biochemical responses of crop plant under Ag NPs exposure given
available alterations. The rising use of nanomaterials in commercially
available products like consumer products and agrichemicals such as pesticides and fertilizers has resulted to environmental
nanocontamination, including agricultural systems. Nevertheless,
attendant consequence is not inevitable given heightened interest in
exposure and risk assessment, fate and footprints of NPs in food and
environment. Here, significant changes in anti-oxidant enzymes and
physiological parameters demonstrated that the cell integrity of studied
cultivars have been injured to a varied extent due to Ag NPs stress. It
was also observed that Ag NPs hampered the photosynthetic ability of
tested cultivars and may hence hamper food production. Also, the time
trend of transpiration rate revealed a decreasing trend throughout the
growth period of the crop under Ag NPs stress. A used and presented
approach here offer a novel combination of indicators for identifying
NP-induced perturbances within the crop plant and has contributed to
data pool in the field.

The current study demonstrates that the response of markers of
oxidative stress varies greatly between cultivars and sensitivity of
interest plant. The potential implications of findings on fate of Ag NPs
are a topic currently under keen investigation.

Ethics Approval and Consent to Participate

Not applicable

Consent for Publication

Not applicable

Availability of Data and Materials

Authors will make available any other raw data that may be
requested by researchers through email to the corresponding author.

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Competing Interest

The authors declare that they have no competing interests.
Disclosure Statement

The authors acknowledge that there is no conflict of interest or benefit arising from the direct applications of their research.

Author’s Contribution

EO1 carried out growth experiment and laboratory analyses and drafted the manuscript. EO2 conceived of the study and participated in its design. EO1 and EO2 jointly performed the statistical analysis. All authors read and approved the final manuscript.

References