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Effect of nanoparticles on biological contamination of *in vitro* cultures and organogenic regeneration of banana

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Abstract

This study aimed to identify biological contaminants of banana *in vitro* cultures. We also tested the effect of Zn or ZnO nanoparticles on elimination of some bacterial and fungal contaminants and their influence regeneration. Nine strains of bacterial contaminants (*Cellulomonas uda*, *Cellulomonas flarigena*, *Corynebacterium panrometabolum*, *Bacillus megaterium*, *Staphylococcus* spp., *Klebsiella* spp., *Erwinia cypripedii*, *Pseudomonas* spp. and *Proteus* spp.) and four fungal (*Fusarium* spp., *Aspergillus* spp., *Penicillium* spp. and *Candida* spp.) contaminants were identified in nanoparticles-free media of banana *in vitro* cultures. They eventually led the explants death. The contamination-free cultures of banana *in vitro* cultures were obtained as a result of application of nano Zn and ZnO particles to the culture MS media, with no negative effect on regeneration. The callus growth decreased while total proline as well as SOD, CAT and POX activities were increased significantly, when the nanoparticles doses increased. The highest percent of somatic embryogenesis was observed in MS media supplemented with 100 mg/L nano Zn followed by nano ZnO. Excellent shooting, rooting and regenerated plantlets were observed also in MS+100 mg/L nano Zn and ZnO. Regenerated plantlets were successfully acclimatized with about 98% efficiency for the experimental period (one month). Nanoparticles treated somaclones accumulated more proline, chlorophyll, antioxidant enzymes activity and developed more dry weight accumulation than the control. In conclusion, the microbial contaminants in banana *in vitro* culture can effectively be eliminated by incorporation of nano Zn and nano ZnO particles on growth media at different concentrations. However, 100 mg/L dose was preferable because it showed the best effects on increasing the regeneration of plantlets with well-formed root systems. Further studies are needed to investigate the mechanisms and the side effects of nanoparticles on genetic stability of banana *in vitro* cultures.

Keywords: Banana, Nanoparticles, Phytotoxicity, Microbial contaminates, Regeneration.

Abbreviations: Zn_Zinc; ZnO_Zinc Oxide, SOD_superoxide dismutase; POX_peroxidase and CAT_catalase.

Introduction

Plant cells growing *in vitro* are considered to be under some degree of stress and may be predisposed to direct infection, even by microbes which are not normally pathogenic to them. Thus, microbial contaminants are the major challenges in plant's *in vitro* propagation during different stages of culture processes such as initiation of callus and sub-culturing. It was reported that, sub-culturing process is a major source of contamination (5-15% for each sub-culture) due to insufficient sterilization of explants, growth media, working tools and operators (Omamor et al., 2007). Ödutanyo et al. (2004) found that the major bacterial contaminants are *Pseudomonas syringae*, *Bacillus lichensformic*, *B. subtilis*, *Comebacterium* sp. and *Erwinia* spp. while *Alternaria tenius*, *Aspergillus niger*, *Aspergillus fumigates* and *Fusarium culmorum* have been reported to be the major fungal contaminants frequently occur in plant tissue culture (Msogoya et al., 2012). In banana (*Mousa sapientum*, L.), huge number of explants are destroyed in the cultures due to endogenous microbial contaminants. It was reported that about 40-60% of banana *in vitro* cultures lost in spite of using main aseptic reliable procedures (Msogoya et al., 2012). Moreover, the growth media, in which the plant tissue is cultivated, is also a source of nutrients for microbial growth, both original constituents of the medium and exudates from the plant cells. Thus, pathogens, endophytes, epiphytes and incidental contaminants may all occur and interfere with growth of the plant tissue culture and compete for nutrients. In addition, some of them produce phytotoxins which result in culture mortality, tissue necrosis and reduces shoot proliferation and rooting (Khane 2003). For instance, *Aspergillus niger* and *A. flavus* have reportedly produced oxalate and aflatoxin poison, respectively, that kill plant tissues (Obuekwe and Osagie 1989). To date, production of banana plants through *in vitro* micropropagation has become routine work in commercial planting in many private sectors

of Egypt. Most of these works have been hampered by microbial contaminants. Generally, surface sterilization of the explants eliminates most epiphytic contaminants except endophytic ones (Habiba et al., 2002). An application of systemic fungicides before the collection of plant materials can suppress microbial contaminants in plant *in vitro* cultures (Mngomba et al., 2007).

However, microbial contaminants at the base of the explants and around them are a great problem. Alternatively, an incorporation of antibiotics and antifungal into the growth media of plant cultures has been suggested to eliminate microbial contaminants (Habiba et al., 2002). Moreover, toxicity of nanoparticles was also suggested for their sensitivity, simplicity, low cost and suitability for unstable chemicals or samples (Brunner et al., 2006). Nanotoxicology, an emerging discipline is receiving increasing attraction and has been the research focus of many publications (Wu 2003; Nel et al., 2006). Engineered nanoparticles can be grouped into four types. The metal based materials such as nanogold, nanozinc (nano Zn) and nanoscale metal oxides like ZnO and Al_2O_3 are among common nanoparticles (USEPA, 2005). Some nanoparticles, such as Fullerene and TiO_2 have been widely used as test materials to reveal their nanotoxicity mechanisms. However, available information on the topic is too scarce to reach any consensus on nanotoxicity and its mechanism, particularly for the nano Zn and nano ZnO particles which used in this study. These nanoparticles generally showed negative effects on the tested target organisms or cell despite in different treatment methods used (Yang and Watts 2005 and Wang et al., 2006) and have a dose dependent response (USEPA, 2005). There are many unresolved issues and challenges concerning the biological effects of nanoparticles (<100 nm). Attentions to the appropriate experimental design and interpretation are needed to provide a defensible scientific understanding of the biological effects of nanoparticles (Murachov, 2006). Therefore, the objectives of this study were (1) to provide new information about phytotoxicology of nano Zn and nano ZnO particles incorporated into the growth media; (2) identification of bacterial and fungal contaminants of banana *in vitro* cultures; and (3) to evaluate the efficacy of these nanoparticles on the explants regeneration.

Results and Discussion

Identification of microbial contaminants

Presence of bacterial and fungal contaminants was noticed only in nanoparticles-free media (control) throughout the developmental stages of banana shoot tip explants regeneration (Fig. 1).

Nine bacterial strains were isolated from the contaminated cultures. These contaminants of banana *in vitro* cultures and the essential biochemical tests are presented in Table (1). Four strains were gram positive, whereas the rest were gram negative. The gram positive isolated were *Cellulomonas uda*, *Cellulomonas flarigena*, *Corynebacterium panrometabolum* and *Bacillus megaterium*. The gram negative isolates were *Staphylococcus* spp., *Klebsiella* spp., *Erwinia cypripedii*, *Pseudomonas* spp. and *Proteus* spp. All of the nine bacterial strains were non spore former except *Bacillus megaterium* strain. Moreover, three bacterial strains out of nine isolated were exogenous bacteria (*Proteus* spp., *Erwinia* spp. and *Staphylococcus* spp.) and the rest were endophytic. In banana culture, Msogoya et al. (2012) identified only four exogenous and endophytic bacterial strains from the isolated bacteria contaminants. They isolated *Proteus* spp., *Erwinia* spp., *Klebsiella* spp. and *Staphylococcus* spp. The other isolated strains in this study have been frequently reported in other plant tissue cultures (Habiba et al., 2002 and Odutanyo et al., 2007).

Several workers (Meghwal et al., 2000 and Khane, 2003) indicated that exogenous bacteria are generally easy to eliminate using normal surface sterilization techniques. Being on plant surface, the occurrence of exogenous bacteria in plant tissue cultures in the present investigation may be due to an insufficient surface sterilization of explants, tools and culture jars. Moreover, the growth media, in which the plant tissues are cultivated, is a good source of nutrients for microbial growth. The endophytical strains like *Cellulomonas* and *Klebsiella* has been isolated in internal tissues of banana, maize and wheat (Markins et al., 2003). Khane et al. (2003) reported that endophytic bacteria are beneficial to host plants as they enhance plant defense against diseases but become problematic in tissue cultures when total asepsis is required. In this investigation, the presence of endophytic bacteria was noticed which might be due to the insufficient of surface sterilization of the explants. The elimination of endophytic bacteria may be effective when stronger and systemic sterilants such as mercuric are used. Four fungal genera were isolated from the contaminated culture throughout the experimental period. These genera were *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp. and *Candida* spp.

The isolated fungal contaminants found in this study have been frequently reported in plant tissue cultures (Msogoya et al., 2012). They are exogenously found in soil, water and plant surfaces (Cassels, 1996) and also as endophytes in some plant species (Suryanarayanan, et al., 2000). The later authors, found *Fusarium solani* and *F. oxysporum* as endophytic fungi in banana and pumpkin plants, while *Penicillium* spp. as well as *Aspergillus flavus* and *A. niger* were reported as exogenous fungus in internal tissues of mallow parts (Ödutayo et al., 2007). On the other hand, Hector and Domer (1983) reported that *Candida* is a genus of yeasts that only occurs in animals and humans as a harmless commensally or endosymbiont.

Therefore, it is expected that the incidence of *Cassells* spp. in banana *in vitro* cultures in nanoparticles-free medium of this study might be due to an insufficient asepsis among workers during one or more stage of the developmental stage of the explants micropropagation. Similar conclusion was reported by Mosogoya et al. (2012).

Similarly, the occurrence of exogenous fungal contaminants in banana *in vitro* cultures was possibly due to an inadequate surface sterilization during tissue culture operations. Several studies have also reported the incidence of exogenous fungal contaminants in plant *in vitro* cultures due to insufficient sterilization (Khane, 2003; Mosogoya et al., 2012).

Culture susceptibility test of isolated microbial contaminants

Data in Tables (3 a, b, c and d) of susceptibility test indicated that nanoparticles of Zn and ZnO were effective in the suppression of bacterial and fungal contaminants at all concentrations. The Nano Zn and ZnO are broad-spectrum anti-bacterial and fungal agent that suppresses their growth. They are usually effective against a wide range of gram negative and gram positive bacteria as well as fungal contaminants. It could be mentioned that the effectiveness of Zn and ZnO against identified bacteria and fungi in this study was accompanied without having any advance effects on explants regeneration and differentiation to plantlets

Table 1. Morphological and biochemical characterization as well as identification of the selected bacterial contaminants isolates of banana in vitro cultures (+= Positive result and -= Negative result).

Name of isolate	*No. of isolates*	*Vegetative cells*	*Gram Reaction*	*Spores*	*Methyl Red Test*	*Arginine hydrloase test*	*Kovacs oxidase test*	*Starch hydrolysis test*	*Gelatin hydrolysis test*	*Casein hydrolysis test*	*Fluorescent pigment test*	*Lactose utilization test*	*Citrate test*	*Catalase test*	*Voges proslauer test*
Cellulomonas spp.	*1*	*Rods (Short)*	+	*Non*	-	+	-	-	-	-	-	-	-	+	-
Cellulomonas spp.	*2*	*Rods (Short)*	+	*Non*	-	+	-	+	-	-	-	-	-	+	-
Corynebacterium spp.	*3*	*Rods (Long)*	+	*Non*	-	+	+	-	-	-	-	-	-	+	+
Bacillus megaterium	*4*	*Rods (Long)*	+	*Former*	-	-	-	+	+	+	-	-	-	+	-
Staphylococcus spp.	*5*	*Cocci*	+	*Non*	-	-	-	+	-	+	-	+	+	+	-
Klebsiella spp.	*6*	*Rods*	-	*Non*	-	-	-	-	-	+	-	+	+	+	+
Erwinia cypripedii	*7*	*Rods (Long)*	-	*Non*	+	+	-	-	-	-	-	+	+	+	+
Pseudomonas sp.	*8*	*Rods (Short)*	-	*Non*	-	-	-	+	-	+	+	-	-	+	+
Proteus spp.	*9*	*Rods*	-	*Non*	+	-	-	-	-	-	-	-	-	+	+

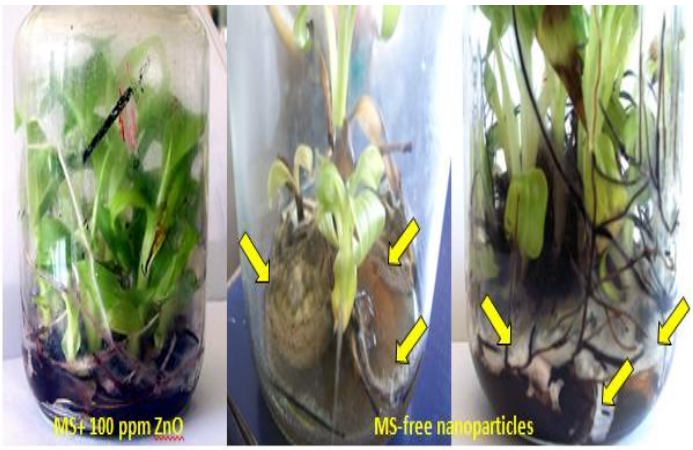

Fig 1. Effect of ZnO nanoparticles and MS-free nanoparticles on microbial contamination in *in vitro* culture of banana during shooting stage.

formation. The *Fusarium* wilt of banana, caused by *Fusarium oxysporum* f. sp. *cubense* is an important disease in many parts of the world. This fungus has the ability to establish itself systemically in the xylem vessels of banana plants, causing vascular discoloration, yellowing and wilting symptoms. The *Fusarium* wilt of banana has been threatening for many years to the banana industry. The *Fusarium* wilt, which is also known as Panama disease, is the most important lethal disease of banana (Ploetz, 2006). Rare available information reported about the influence of nanoparticles on microbial suppression. In addition limited studies reported both positive and negative effects of nanoparticles on higher plants. It has been reported that only 2000 mg/L (among the concentrations of 20, 200 and 2000 mg/L) of the nano Al_2O_3 suspensions has significant inhibitory effects on root elongation of five plant species (Yang and Watts 2005).

However, there are increasing amount of research on the toxicology of nanomaterials. Some researchers have shown the toxicity of nanoparticles such as fullerene, carbon nanotubes and metal oxides to human cells, bacteria and rodents (Brunner et al., 2006; Lam et al., 2006; Soto et al., 2006). But how nanomaterials affect living organisms remains unknown. Though, reactive oxygen species generation (ROS) and oxidative stress are proposed to explain the toxicity of inhaled nanoparticles (Nel et al., 2006). A pioneering study of Oberdörster et al. (2005) to assess potential toxicity of nanomaterials on ecological terrestrial test species (plants, wildlife, soil invertebrates or soil microorganisms) showed that uncoated fullerenes exerted oxidative stress and caused several lipid peroxidation in fish brain tissue, a possible negative impact of nanomaterials on the health of aquatic organisms. There are still many unresolved issues and challenges concerning the biological effects of nanoparticles. Attentions to appropriate experimental design and interpretation are needed to provide a defensible scientific understanding of the biological effects of nanoparticles (Murashov, 2006). Therefore, further studies are needed to study the effects of nanoparticles as a microbial anti-agent on synthesis of cell protein and nucleic acid as well as on the uptake of solutes.

In vitro regeneration

Establishment of callus cultures

Callus initiation was observed at the cut ends and wounded regions of the shoot tip explants after two weeks of culture. The whole surface of the explants was covered with the callus within 4 weeks of incubation. Fig. 1 shows that nanoparticles had a significant effect on the percentage of explants showing callus formation in shoot tip explants while the control without nanoparticles showed lowest response due to the microbial contamination of the media. The responded explants were died in control medium without subculturing on a fresh medium and had no ability to respond due to the contamination. The MS medium supplemented with nano Zn or ZnO particles at 100 mg/L resulted in the highest callus induction followed by nanoparticles at 200 mg/L. The best medium for callus induction from shoot tip explants was MS medium supplemented with 100 mg/L nano Zn which caused 92% callus induction followed by treatment consisting of MS medium supplemented with 200 mg/L and 50 mg/L as well as nano ZnO inducing 79.5% and 63.1% callus, respectively. However, there was no significant difference between the two applied nanoparticles, comparing them with the corresponding level. The results showed that the callus obtained from shoot tip explants on MS medium supplemented with nanoparticles of Zn and ZnO at 100 and 150 mg/L was compact, modular yellowish green in colour and showed good callus growth and had an ability to be developed by subculturing in the following steps. Similarly, callus obtained at 50 mg/L nano Zn and ZnO particles was compact, creamy yellow in color and showed good growth and had proliferation ability.

Callus differentiation, shoot bud induction and shoot regeneration

Callus derived from all experiments were cultured on callus differentiation and shoot regeneration media containing various concentration of nano Zn and ZnO particles. Data presented in Fig. 2 revealed that the regeneration potential, represented by number of shoots/callus piece, was increased with an increase in nanoparticles levels up to 100 mg/L. Less increase was noticed at 200 mg/L. Therefore, the highest number of shoots/callus piece were formed on MS basal medium supplemented with 100 mg/L nano particles followed by MS + 150 mg/L and MS + 50 mg/L. The lowest value of the regeneration value was detected in nanoparticles-free media due to the microbial contaminants. The average number of shoots/callus piece reached to 2.5 shoots. In this study, increase of regeneration potential with nano Zn and nano ZnO may be due to the effect of Zn on plant growth. the Zn is an essential element for plants, animals and humans, but it is toxic at high levels with the effective concentrations (EC_{50}- substrate Zn concentration resulting in 50% biomass reduction) from 43 to 996 mg/L among various plant species (Paschke et al., 2006). However, mechanism of nanotoxicity remained unknown. It would be closely related to the chemical composition, chemical structure, particle size and surface area of the nanoparticles. In this study, toxicity of nanoparticles on microbial contaminants may be due to two

Table 2. Isolates fungi from banana *in vitro* cultures.

Name of genus	Name of species
Fusarium spp.	*Fusarium solani* *Fusarium oxysporum*
Aspergillus spp.	*Aspergillus flavus* *Aspergillus niger*
Penicillium spp.	
Candida spp.	

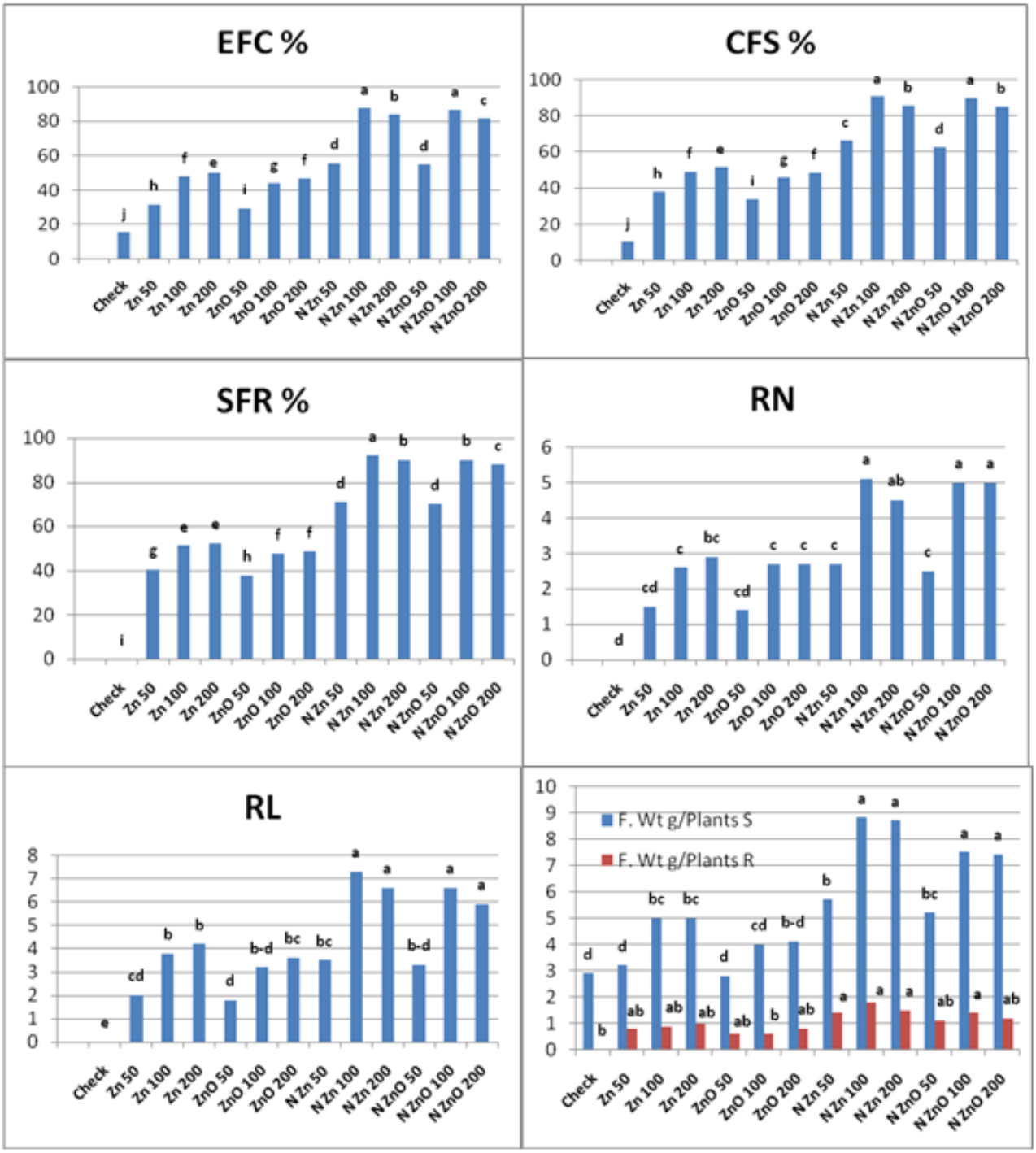

Fig 2. Mean percentage values of the explant forming callus (EFC), callus forming shoots (CFS) (Shooting %), shoot forming roots (SFR), number of roots RN)/plantlets, root length (RL) as well as shoots and roots fresh weight/plant as affected by Zn and ZnO particles.

different actions: (1) the chemical toxicity based on chemical composition, e.g. release of toxic ion; and (2) stress or stimuli caused by the surface, size and/or share of the particles. It was confirmed that solubility of oxide nanoparticles greatly affect the cell culture response (Brunner et al., 2006).

Rooting and plantlets hardening

Fig. 2 shows that the nanoparticles-free medium (control) did not cause rooting. The maximum rooting (89.0%), average number of roots per shoot (6.57%) and maximum average root length of 2.93 cm was achieved on medium containing nano Zn at 100 mg/L followed by 150 mg/L and nano ZnO at 100 mg/L and 150 mg/L, respectively. The minimum rooting of 57%, average number of root per shoot 3.10 and lowest average root length of 1.61 was achieved on medium containing nano ZnO at 50 mg/L followed by nano Zn at the same level. Roots could contact nanoparticles directly. Therefore, rooting (number, length and weight) was affected by Zn and ZnO nanoparticles more than shooting. Moreover, rooting would have a dose-dependent response, whereas shoots could grow to a certain degree even though rooting was halted in the presence of nano Zn and nano ZnO in the cultures. Thus S/R ratio was increased.

The same table also shows that the survival percent of plantlets was about 90% after 6 weeks of transfer to earthen pots containing soil and sand and remained constant thereafter, indicating treatment with Zn and ZnO nanoparticles caused successful establishment of tissue culture, raised number of plantlets accompanied with suppuration of microbial contaminants.

On the other hand, the results show that none of the explants responded well to a nanoparticles of Zn and ZnO free medium. Additional Zn and ZnO nanoparticles to the medium suppressed the microbial contaminants without affecting on shoot tip explants *in vitro* regeneration. Furthermore, nanoparticles increased the regeneration potential of banana. Callus formation (%) was much higher compared to nanoparticles-free medium. Zn and ZnO nanoparticles at 100 mg/L were found to be satisfactory on prevention of microbial contaminants and produced healthy plants. In the case of nanoparticles-free medium, the explants did not produce healthy shoots and plantlets became yellow. Falkiner (1990) mentioned that the agents who act specifically on bacterial cell walls would be more suitable to control infection in plant tissue cultures. A possible explanation may be that Zn and ZnO nanoparticles inhibit bacterial cell wall synthesis preventing contamination.

Consequently, the explants will be physiologically active greatly. Available information on nanotoxcity is scarce to reach any consensus on its mechanism, particularly for the nanoparticles used in this study (Zn and ZnO). Previous research data on the nanoparticles generally showed negative effects on the tested organisms such as cells, despite different test methods used (Wang et al., 2006). However, nanoparticles are preferable than the bactericide or fungicide due to their sensitivity, simplicity, low cost and suitability over unstable chemicals (Wang et al., 2001).

Organogenesis is an outcome of the process of dedifferentiation followed by re-differentiation of cells. Dedifferentiation favors unorganized cell growth and the resultant callus has randomly divided meristems (Deepike and Komwar 2010).

Oberdörster et al. (2005) indicated that dose-response curve of nano Zn and nano ZnO are "T" shaped. There was a threshold, below which no obvious symptom appeared. However, root length decreased with increasing dose after the threshold. Paschke et al. (2006) showed the phytotoxicity of nano Zn and nano ZnO on growth of radish, rape and ryegrass (less than 50mg/L). This was lower than the phytotoxic effect of Zn^{2+} on some plant species (65-156 mg/L).

Although, reports on phytotoxic effects of nanoparticles are scanty, the antifungal agent has been reported to suppress larval development in mussel *in vitro* culture (Owen et al., 2010). However, both positive and negative effects of nanoparticles on higher plants were reported. It was pointed out that a mixture of nano scale SiO_2 (nano SiO_2) and TiO_2 (nano - TiO_2) could increase nitrate reductase in soybean (*Glycine max* L.), enhance its abilities of absorbing and utilizing water and fertilizer, stimulate its antioxidant system and apparently hasten its germination and growth (Lu et al., 2002). Nano-TiO_2 was reported to promote photosynthesis and nitrogen metabolism and then greatly improve growth of spinach at a proper concentration (Hong et al., 2005 a, b; Zheng et al., 2005; Yang et al., 2006 b). After investigating the phytotoxicity of nano Zn and nano ZnO on microbial contaminants of *in vitro* banana tissue, with no impact on explants regeneration, it could be concluded that such materials can generate adverse biological effects in living cells. Particles in such a size (<100 nm) fall in the transitional zone between individual atoms or molecules and the corresponding bulk material, which can modify the physiochemical properties of the material e.g. performing exceptional feats of conductivity, reactivity and optical sensitivity. The side effects of nanoparticles are hardly known for it has not yet been used to suppress fungal contaminants in plant tissue culture.

Biochemical characters

Fig. 3 shows that proline concentration in all *in vitro* regeneration stages of banana explants increased gradually, in response to increasing nano Zn and nano ZnO. Low dose of nanoparticles (50 mg/L) caused about 4-fold increase in proline concentration. However, significant highest increase in proline concentration (about 8 fold) was observed at 200 mg/L nanoparticles. The increase was highest under nano ZnO and the lowest under nano Zn. Moreover, proline concentration was increased gradually with advancement of the explants regeneration up to fully developed *in vitro* regenerated somaclones and thereafter tended to decrease in the acclimated one-month-old plantlets. The low proline concentration was found in induced callus stage and the highest in fully developed plantlets. Again, it could be mentioned that addition of nanoparticles used to the cultures media prevented bacterial and fungal contaminants and favored growth of *in vitro* regenerated somaclones of banana. Similar results were reported by Baskaran and Jayabalan (2009) who found that BVN fungicide prevented fungal contamination of the moist soil mixture. The use of biochemical markers, such as proline can be used for improving crop resistance to stress condition. Nanoparticles play an important role as stress agent (USEPA, 2005).

Table 3. Percentage of detected microorganism contaminants of banana *in vitro* cultures throughout the various regeneration stages in response to Zn and ZnO particles. (a) Callus initiation (b) Shooting (c) Rooting (d) Formed plantlets.

a)

Particles	Level mg/L	*F. solani*		*F. oxysporum*		*A. niger*		*A. flavus*		*Penicillium* spp.		*Candida* spp.		Bacteria	
Check		22.80	a	27.93	a	14.25	a	12.75	a	7.81	a	3.28	a	26.05	a
Zn	50	21.20	b	25.97	b	13.25	ab	11.86	ab	7.26	ab	3.05	a	24.23	b
	100	19.61	c	24.02	c	12.26	bc	10.97	bc	6.72	a-c	2.82	a	22.40	c
	200	17.33	de	21.23	d	10.83	cd	9.69	d	5.93	b-d	2.49	a	19.80	d
ZnO	50	18.70	cd	22.9	c	11.69	cd	10.46	cd	6.40	bc	2.69	a	21.36	c
	100	16.87	e	20.67	d	10.55	d	9.44	d	5.78	cd	2.43	a	19.28	d
	200	14.14	f	17.32	e	8.84	e	7.91	e	4.84	d	2.03	ab	16.15	e
Nano Zn	50	5.24	g	6.42	f	3.28	f	2.93	f	1.80	e	0.75	bc	5.99	f
	100	0.00	h	0.00	g	0.00	g	0.00	g	0.00	f	0.00	c	0.00	g
	200	0.00	h	0.00	g	0.00	g	0.00	g	0.00	f	0.00	c	0.00	g
Nano ZnO	50	4.79	g	5.87	f	2.99	f	2.68	f	1.64	e	0.69	bc	5.47	f
	100	0.00	h	0.00	g	0.00	g	0.00	g	0.00	f	0.00	c	0.00	g
	200	0.00	h	0.00	g	0.00	g	0.00	g	0.00	f	0.00	c	0.00	g

b) Shooting

Particles	Level mg/L	*F. solani*		*F. oxysporum*		*A. niger*		*A. flavus*		*Penicillium* spp.		*Candida* spp.		Bacteria	
Check		24.80	a	30.38	a	15.50	a	13.87	a	8.49	a	3.57	a	28.33	a
Zn	50	23.06	b	28.25	b	14.42	ab	12.90	ab	7.90	ab	3.32	ab	26.35	b
	100	21.33	c	26.13	c	13.33	bc	11.93	bc	7.30	a-c	3.07	ab	24.37	c
	200	18.85	d	23.09	d	11.78	cd	10.54	d	6.46	cd	2.71	ab	21.53	d
ZnO	50	20.34	c	24.91	c	12.71	cd	11.38	cd	6.97	bc	2.93	ab	23.23	c
	100	18.35	d	22.48	d	11.47	d	10.27	d	6.29	cd	2.64	ab	20.97	d
	200	15.38	e	18.84	e	9.61	e	8.60	e	5.27	d	2.21	b	17.57	e
Nano Zn	50	5.70	f	6.99	f	3.57	f	3.19	f	1.95	e	0.00	c	6.52	f
	100	0.00	g	0.00	g	0.00	g	0.00	g	0.00	f	0.00	c	0.00	g
	200	0.00	g	0.00	g	0.00	g	0.00	g	0.00	f	0.00	c	0.00	g
Nano ZnO	50	5.21	f	6.38	f	3.26	f	2.91	f	1.78	e	0.00	c	5.95	f
	100	0.00	g	0.00	g	0.00	g	0.00	g	0.00	f	0.00	c	0.00	g
	200	0.00	g	0.00	g	0.00	g	0.00	g	0.00	f	0.00	c	0.00	g

c) Rooting

Particles	Level mg/L	*F. solani*		*F. oxysporum*		*A. niger*		*A. flavus*		*Penicillium* spp.		*Candida* spp.		Bacteria	
Check		24.00	a	29.40	a	15.00	a	13.43	a	8.22	a	3.45	a	27.42	a
Zn	50	22.32	b	27.34	b	13.95	ab	12.49	ab	7.64	ab	3.21	ab	25.50	b
	100	20.64	c	25.28	c	12.90	bc	11.55	bc	7.07	a-c	2.97	ab	23.58	c
	200	18.24	d	22.34	d	11.40	cd	10.20	d	6.25	cd	2.62	ab	20.84	d
ZnO	50	19.68	c	24.11	c	12.30	cd	11.01	cd	6.74	bc	2.83	ab	22.48	c
	100	17.76	d	21.76	d	11.10	d	9.93	d	6.08	cd	2.55	ab	20.29	d
	200	14.88	e	18.23	e	9.30	e	8.32	e	5.10	d	2.14	b	17.00	e
Nano Zn	50	5.52	f	6.76	f	3.45	f	3.09	f	0.00	e	0.79	c	6.31	f
	100	0.00	g	0.00	g	0.00	g	0.00	g	0.00	e	0.00	c	0.00	g
	200	0.00	g	0.00	g	0.00	g	0.00	g	0.00	e	0.00	c	0.00	g
Nano ZnO	50	0.00	g	6.17	f	3.15	f	2.82	f	0.00	e	0.73	c	5.76	f
	100	0.00	g	0.00	g	0.00	g	0.00	g	0.00	e	0.00	c	0.00	g
	200	0.00	g	0.00	g	0.00	g	0.00	g	0.00	e	0.00	c	0.00	g

d) Formed plantlets

Particles	Level mg/L	*F. solani*		*F. oxysporum*		*A. niger*		*A. flavus*		*Penicillium* spp.		*Candida* spp.		Bacteria	
Check		22.36	a	27.39	a	13.98	a	12.51	a	7.66	a	3.22	a	25.55	a
Zn	50	20.79	b	25.47	b	13.00	ab	11.63	ab	7.12	ab	2.99	a	23.76	b
	100	19.23	c	23.56	c	12.02	bc	10.76	bc	6.59	a-c	2.77	a	21.97	c
	200	16.99	de	20.82	d	10.62	cd	9.51	d	5.82	b-d	2.44	a	19.42	d
ZnO	50	18.34	cd	22.46	c	11.46	b-d	10.26	cd	6.28	a-c	2.64	a	20.95	c
	100	16.55	e	20.27	d	10.34	d	9.26	d	5.67	cd	2.38	a	18.90	d
	200	13.86	f	16.98	e	8.66	e	7.75	e	4.75	d	1.99	a	15.84	e
Nano Zn	50	5.14	g	6.30	f	3.21	f	2.88	f	1.76	e	0.74	b	5.88	f
	100	0.00	h	0.00	g	0.00	g	0.00	g	0.00	f	0.00	b	0.00	g
	200	0.00	h	0.00	g	0.00	g	0.00	g	0.00	f	0.00	b	0.00	g
Nano ZnO	50	4.70	g	0.00	g	2.93	f	2.63	f	1.61	e	0.68	b	5.36	f
	100	0.00	h	0.00	g	0.00	g	0.00	g	0.00	f	0.00	b	0.00	g
	200	0.00	h	0.00	g	0.00	g	0.00	g	0.00	f	0.00	b	0.00	g

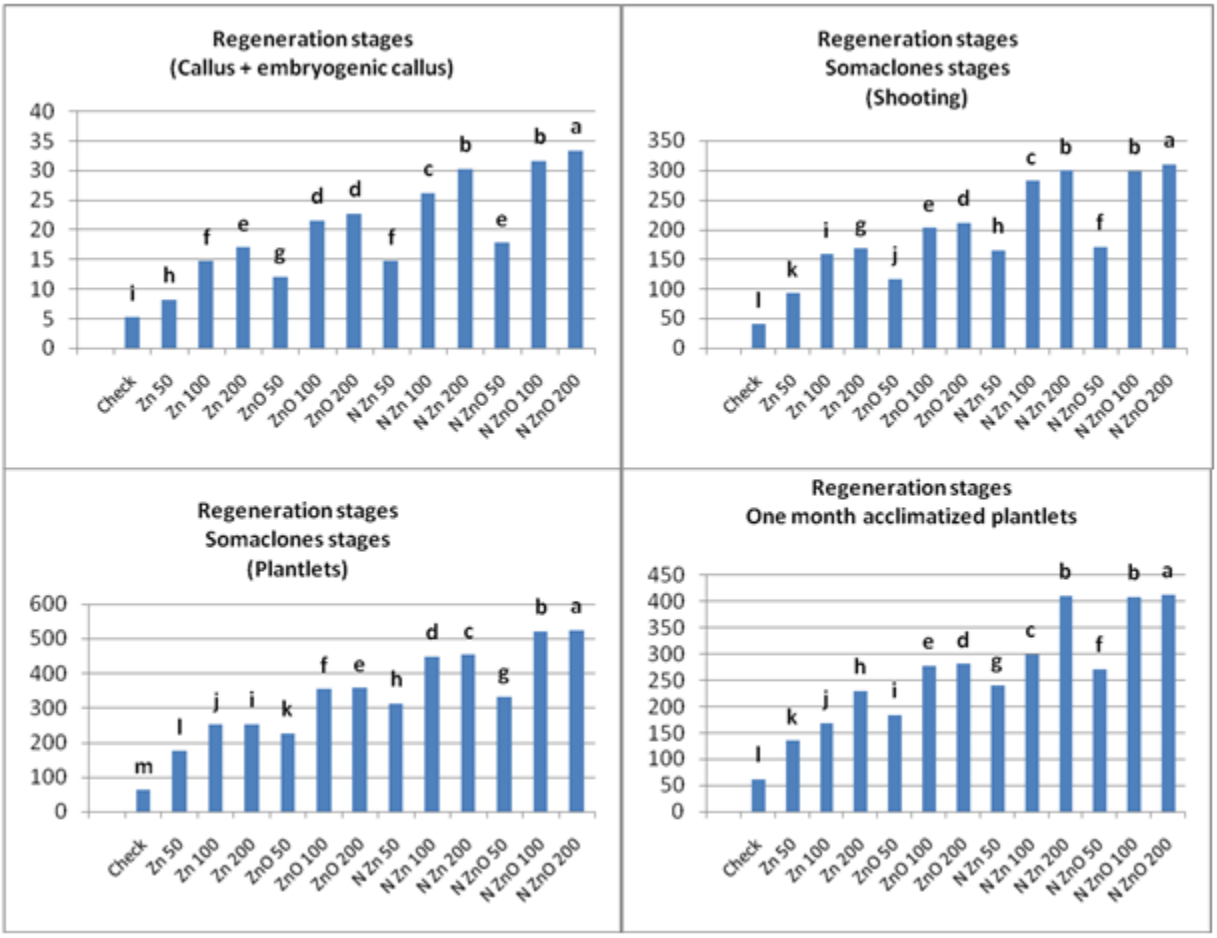

Fig 3. Proline concentrations (µg/g fresh weight) of callus developed *in vitro* regenerated somaclones (RS) and one-month-acclimatized plantlets of banana in response to Zn and ZnO particles.

Accumulation of proline in call and callus cultures exposed to stress condition has been reported by Alkayri and Al-Bahrany, (2002) and Joshi et al. (2011). Concentration of proline gives better protection against biological unfavorable consequences. Proline may interact with enzymes to preserve protein structure and activities. The presence of high contents of proline gives better protection against biological unfavorable of dehydration-induced thermodynamic perturbation (Hamilton and Heckathom, 2001). Nanoparticles-treated somaclones showed higher amount of total chlorophyll and chlorophyll a/b ratio than the control. Total chlorophyll and chlorophyll a/b ratio were found to be highest in fully developed *in vitro* regenerated somaclones under control and nanoparticles treated somaclones. However, percentage increase was greater in acclimatized plants (Fig. 4). In Fig. (4), increased chlorophyll a/b ratio (under increasing total chlorophyll) during nanoparticles stress (treatment) signifies greater chl. a rather than chl. b. The chlorophyll a/b ratio is an indicator of the antenna sizes of PS I and PS II (Parida and Das, 2005). They added that, the core antenna contains only chl. a, whereas the outer antenna contains both chlorophyll a and chl, b. Therefore, higher chlorophyll a/b ratio in acclimatized plantlets than regenerated somaclones suggests greater efficiency of PS I and PS II antenna complex leading to better capacity of photosynthesis even under stress condition. In addition, the results indicated that nanoparticles may induce the somaclones to tolerate stress conditions. Our data indicated that the proline content of the regenerated somaclones was increased gradually in response to increased nanoparticles. Maximum proline content was observed in fully developed *in vitro* regenerated somaclones and the acclimatized plantlets and the lowest in callus and the embryogenic callus stages (very sensitive to nanoparticles stress). Under stress conditions, membrane integrity must be maintained to prevent protein denaturation (Joshi et al., 2011). The POX enzyme activities; however, significantly increase under 200 mg/L dose of either nano Zn or nano ZnO particles. The increase was the highest in the full developed *in vitro* somaclones (shooting) and the lowest in acclimatized plants, one- month- age. This trend may be due to the increase in dry weight accumulation with advancement in plant age. The data also indicated that there are no significant differences between the effects of nano Zn and nano ZnO in this respect. Similar results have also been reported by Lu et al. (2002) who indicated that superoxide dismutase (SOD), Catalase (CAT) and peroxidase (POX) activity of the developed plantlets of soybean with a mixture of nano SiO and nano TiO_2 treatment could be significantly increased, leading to better seed germination and seedling growth. Few studies

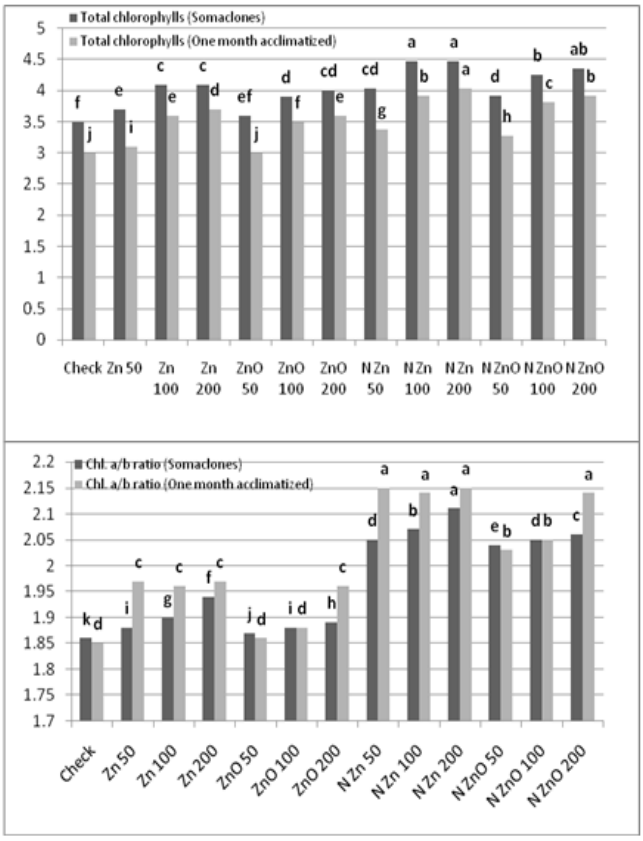

Fig 4. Total chlorophyll concentrations (mg/g fresh weight) and Chl. a/b ratio of fully developed *in vitro* regenerated somaclones and one-month-acclimatized plantlets of banana in response to Zn and ZnO particles.

have focused on the effects and mechanisms of nanoparticles on plant growth and its relation with oxidative enzymes, having a photo-catalyzed (Crabtree 1998) nano TiO_2 under light which could cause an oxidation-reduction reaction and produce superoxide ion radical and hydroxide. These reactive oxygen species (ROS) can go after organic substance and be effective antimicrobial agents (Wang et al., 2001). Similar results were reported by Helaly and Hanan El-Hoseiny (2011) on stressed *in vitro* cultures of sweet orange. They achieved higher enzyme activity of superoxide dismutase (SOD), peroxidase (POX), ascorbic peroxidase (Apox), Catalase (CAT) and glutathione reductase (GR). Wang et al. (2001) confirmed that nano SiO_2 treatment could be the cause of increased strength, resistance to disease and; thus, increased yield of rice (Tang and Cao, 2003). Zheng et al. (2005) reported that nano TiO_2 treatment could markedly promote the vigor of aged seeds and chlorophyll formation of spinach, by which the ribulose biphosphate carboxylase activity and photosynthesis is obviously increased and the growth and development of spinach promoted. Similarly, our results indicated that addition of nano Zn and ZnO particles in the *in vitro* media prevents microbial contaminants and favored regenerating and plantlets growth. Moreover, the regenerated plantlets were successfully established (98%) in pots under field conditions. It is well known that photosynthesis is consistent with photo-catalyzed chemical reaction and enzyme catalyzed chemical reaction. As a photo-catalyzed reaction, Hong et al. (2005 a and b) speculated that photosynthetic rate enhancement by nano TiO_2; catalysis might be closely related to photochemical reaction activity such as the absorption of light energy, transforming light energy into electron energy, electron transport rate, oxygen evolution rate and photophosphorylation efficiency and so forth. In this study, it was observed that somaclones developed from nanoparticles Zn and ZnO treated calli attained greater growth and higher amount of proline, chl(s) and a/b ratio, compared to control. Proline may interact with enzymes to preserve protein structure (Joshi et al., 2011). A higher proline content confers better protection against biologically unfavorable of dehydration-induced thermodynamic perturbation (Hamilton and Heckathom, 2001).

Materials and Methods

This study was carried out at the Plant Tissue Culture Laboratory, Horticultural Research Institute, Ministry of Agriculture, Agricultural Research Center (A. R. C). and the Laboratories of Agric. Bot. Dept. and Plant Pathology Dept., Faculty of Agric., Mansoura Univ., Egypt during the period from Jan. 2012 to March 2013.

Isolation, identification and characterization of microbial contaminants

At the end of each culturing period, the developed bacterial and fungal contaminants were isolated from banana cultures and transferred to specific medium. Bacterial isolates were aseptically streaked onto sterile nutrient agar medium and the cultures were incubated at 25±1^{o}C for 24 hr(s). Pure bacterial isolates were obtained by repeated subculturing using a serial dilution technique (Collins and Lyne, 1984).

Fungi were prepared for examinations under a stereoscopic binocular microscope (6-50 X) to study their characters. When necessary the compound microscope was used to confirm the identification after having examined the morphology of conidia and conidiophores. Fungi presented on infested jars were identified by means of comparison with the description sheets of Commonwealth Mycological Institute, Kew, Surrey, England (CMI), Danish Government Institute of Seed Pathology (DGISP) publications as well as publications of Raper and Fennel, 1965; Ellis, 1971; Moubasher, 1977; Booth, 1985; Burrges et al., 1988; and Singh et al., 1991.

The purified bacterial isolates were observed under microscope after staining for morphological characterization based on vegetative cell chap, gram reaction and presence or absence of spores. In addition, essential biochemical tests were conducted per standard methods namely; methyl red, arginine hydrolase, starch hydrolysis, casein hydrolysis, fluorescent pigments, lactose, citrate and catalase productions (Collins and Lyne, 1984; Krieg and Holt, 1984 and Sneath et al., 1986).

For identification, characterized bacterial strains were compared with the standard strains of Bergey's Manual (Krieg and Holt, 1984 and Sneath et al., 1986). The key proposed by Bradbury (1988) was also followed. Fungal isolates were aseptically transferred onto Petri-dishes containing potato dextrose agar (PDA) growth medium. The cultures were incubated at 25±1^{o}C for 5-15 days. The fungal isolates were purified by repeated subcultures onto fresh growth medium (PDA). Slides of pure fungal isolates were prepared and stained with lactophenol cotton blue for identification of the isolates based on microscopic morphological appearance of conidiophores and conidia (Barnett and Hunter, 1972).

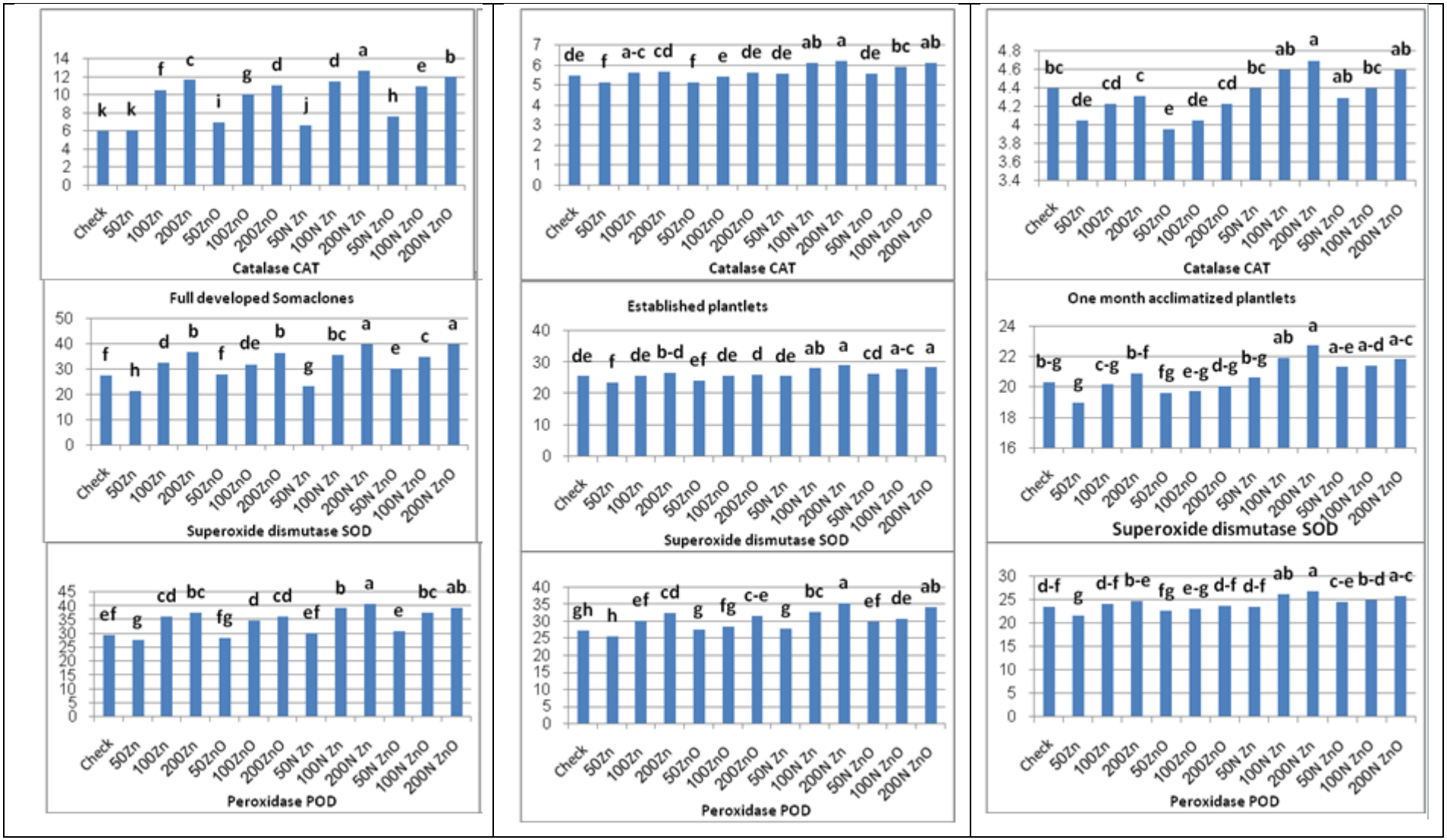

Fig 5. Enzymes concentrations (units/g fresh weight) of developed *in vitro* regenerated explants throughout the various regenerated stages of banana in response to Zn and ZnO particles.

Mother plant sources and explant material

Mother plants, 3-years-old banana; *Musa paradisiacal* L. (*M. acuminate* × *M. balbisiana*) cv. Grand Nain were used as an experimental material. Small suckers of about 60-70 cm in length were carefully removed from the mother plants grown in Agricultural Research and taken immediately to the labs. Shoot tips (explants) were prepared by removing the outer layer tissues from the suckers. The pale white tissue blocks containing the shoot tips and rhizomatous bases were surface sterilized with three disinfectants; Sodium Hypochlorite (NaOCl 80%), Ethanol 95% and Mercuric Chloride ($HgCl_2$ 0.1%) for 15 mins, respectively.

Nanoparticles examined and preparation

Two types of nanoparticles were examined denoted as nano Zn and nano ZnO (MN6Z) at purity of 99.9%. Zn nanoscale metal (diameter 35 nm) was purchased from Zunye (Nanomaterials Co. Ltd. Shenzhea, China). Nanoscale ZnO was from Hongchen (Material Sci. and Tech. Co. Ltd. Zhejiang, China). The surface area provided by the producers was 40±10 and 50±10 m^2/g for nano Zn and nano ZnO, respectively.

The nanoparticles used were suspended directly onto deionized water and dispersed by ultrasonic vibration (100 W, 40 KHz) for 30 min. Small magnetic bars were placed in the suspensions for stirring before use to avoid aggregation of the particles. The nanoparticles suspensions after centrifugation (3000 g for 1h) and filtration (0.7 µm glass filter) were used for the treatments. Three levels of each nanoparticled were used in addition to the control denoted 0 (control), 50, 100 and 200 mg/L.

Explants regeneration

Calls induction

To study the callogenic response and microbial contaminants in the presence and absence of Zn and ZnO nanoparticles, sterilized shoot tips explants were cultures on MS (Murashige and Skoog, 1962) basal media supplemented with sucrose (30 g/L), 2, 4 D (2 mg/L) and one of each nanoparticles graded levels. The final pH was adjusted to 5.8. Each treatment was replicated 5 times (5 Jars), each jar with 3 explants and the experiment was replicated three times. The media were solidified with 7 g/L agar (Defco, India). Cultures containing specific media were disperses in jars (325 ml) at the rate of 50 ml/jar, capped and autoclaved (at 121^o C at 15 Ibs/insh^2) for 15 min and incubated at 25±1^o C for 6 weeks with two sub-culturing. At the end of callus induction period, bacterial and fungal contaminants were characterized and identified and the infection percentage calculated. Moreover, callus induction frequency estimated as follows as the percentage of explants that produce callus (Lee et al., 2009). Callus colors and types proliferation were also recorded.

Callus proliferation and shooting

Calli obtained from each treatment were cut into small pieces (about 0.8 cm^3) and subsequently transformed on to free auxin MS specific media (contained one of each nanoparticles graded levels) supplemented with BA (3mg/L). Similarity, each treatment consisted of five replicates (culture jars) and the experimental unit cultured at the rate of five callus pieces/jar. All cultures were maintained at the same conditions used for callus proliferation. After shoot but differentiation (4 weeks), the embryogenic calli pieces along with the shoot buds were separated and transformed to the subculture on same corresponding specific freshly media prepared for shooting regeneration. Incubation was taken place for 12 weeks with 3 subculturing. At the end of shooting stage, the observations were taken for microbial contaminants, percentage emberyoginic callus pieces showing shoot bud induction and average number of shoots/callus piece.

Rooting and plantlets acclimatization

Regenerated and developed shoots (3-4 cm in length) were excised from the emberyogenic callus, transformed and cultured on half strength MS media supplemented with one of the different levels of nano Zn and nano ZnO examined and IBA (3 mg/L), activated charcoal (0.5 g/L) and pH adjusted to 5.8. The cultures were incubated at the growth room and maintained under the same conditions previously mentioned. Each treatment consisted of 5 replicates (experimental unit), each with 3 shoots/jar. The experiment was replicated thrice and the observation were recorded after two weeks of incubation for microbial contaminants, percentage of rooting based on number of shoots forming roots, number of roots per shoot and length of roots. For acclimatization, the obtained plantlets were removed from the media, gently washed with tap water and selected based on their size. Plantlets showed well-developed root system (4-5 cm in length) and planted individually in plastic pots (5.5 x 6.5 cm; tyrpido) containing sterilized peat moss + sand + perlite (1: 1: 1 v/v) and irrigated with one tenth MS solution devoid of sucrose in a humid chamber at 26±1^{o}C under 16/8 day/night cycle. One month later, the potted plantlets were grown under greenhouse conditions, covered with transparent plastic bags to maintain the humidity. Acclimatization to the external environmental was done by removing the transparent plastic bags gradually to reduce humidity. After 4 weeks, the observation for microbial contaminants, percentages of the survival plants, fresh and dry weights of the plantlets were recorded. In addition, total chlorophyll was isolated and determined as given by Arnon (1949). Total proline was detected as described by Bates et al. (1973). Antioxidant enzyme activities were also determined.

Antioxidant enzymes activity

Antioxidant enzymes activity (unit/g F. Wt.) were extracted from the plant material in a phosphate buffer (Inskeep and Bloom, 1985) and assayed using the methods of Beauchamp and Pridovich (1971), Herzog and Fahimi (1973) and Bergmeyer (1970) for superoxide dismutase (SOD) (EC 1.15.1.1), peroxidase (POX) (EC 1.11.1.x) and catalase (CAT) (EC 1.11.1.6) respectively.

Statistical analysis

The experimental design for each treatment was randomized complete blocks system as each was replicated thrice and all data recorded on different parameters were subjected to analysis of variance (Gomez and Gomez, 1984). Each of the experimental mean values was compared to its corresponding control. Differences among treatment means were statistically analyzed using the least significant differences test (LSD) at $P \leq 0.05$ %, means separation using the CoStat program.

Conclusion

In this study, we tested phototoxicity of nano Zn and nano ZnO on microbial contaminants in banana *in vitro* cultures and examined the explants regeneration rate. It was found that nano Zn and nano ZnO particles have significant inhibitions on bacterial as well as fungal contaminants and significantly prevent their growth. No marked negative effects on explants regeneration was noticed even at 200 mg/L nanoparticles in the growth media. Nanoparticles concentrations used were most likely lower than the threshold values and did not show any negative effect on banana regeneration. Further investigation should be directed to phototoxicity mechanisms in microorganisms and higher plant species. In addition, further studies are required to investigate the adverse side-effects of nanoparticles on genetic stability of banana *in vitro* cultures.

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References

Al-Khayri JM, Al-Bahrany AM (2002) Callus growth and proline accumulation in response to sorbitol and sucrose-induced osmoptic stress in rice (*Oryza sativa*, L.). Biol Plant. 45:609-611

Arnon DI (1949) Copper enzymes in isolated chloroplasts phenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1-15

Barnett HL, Hunter BB (1972) Illustrated genera of imperfect fungi. Burgress Publishing Company, Minneapolis MN, USA, pp. 241

Baskaran P, Jayabalan N (2009) *In vitro* propagation of *Psoralea carylifolia*, L. by somatic embryogenesis in cell suspention culture. Acta Physiol Plant. 31: 1119-1127.

Bates LS, Waldern RP, Teara ID (1973) Rapid determination of free proline for water stress studies. Plant Soil. 39:205-207

Beauchamp C, Pridovich I (1971) Superoxide dismutase. Impressed assay and assay applicable to acrylamide gels. Anal Biochem. 44:276-284

Bergmeyer HV (1970) Methods of enzymatic analysis. Verlag Chemie. Weinheim, Bergstr, German.

Booth C (1985) The genus *Fusarium.* Commonwealth Mycological Institute, Kew. Surrey, England 237 pp

Bradbury JF (1988) Identification of cultivable bacteria from plants and plant tissue cultures by use of simple classical methods. Acta Hort. 225: 27-37

Brunner TJ, Wick P, Manser P, Spohn P, Grass RN, Limbach LK, Bruinink A, Stark WJ (2006) *In vitro* cyto-toxicity of oxide nanoparticles comparison to asbestos, silica and the effect of particle solubility. Environ Sci Technol. 40: 4374-4381

Burrges LW, Liddell CM, and Summerell BA (1988) Laboratory manual for *Fusarium* research. Incorporating a Key and descriptions of common species found in Australia (second edition). *Fusarium* research Laboratory Department of Plant Pathology and Agricultural Entomology the University of Sydney, 156 pp

Cassells AC (1996) Production of healthy plants. In Proceeding of the Institute of Horticultural Symposium. Micropropagation in Culture. Alerson PG. Dullforce. WM (edition). Nothingham. University of Nothingham Trent Print pp. 53-71

Collins CH, Lyne PM (1984) Microbiological methods (5th ed.). Butterworths Co. Ltd. London, pp. 56-113

Crabtree RH (1998). A new type of hydrogen bond. Science. 282: 2000-2001

Danso KE, Azu E, Elegba W, Asumeng A, Amoatey HM, Klu GYP (2011) Effective decontamination and subsequent plantlet regeneration of sugarcane (*Sacharum officinarum*, L.) *in vitro*. Inter J Integrative Biol. 11(2): 90-96.

Deepike R, Kamwar K (2010) *In vitro* regeneration of *Punica granatum* L. plants from different juvenile expalnts. J Fruit Ornamental Plant Res. 18(1):5-22

Ellis MB (1971) Dematiaceous Hyphomycetes. CMI, Kew, Surrey, England, 608 pp

Falkiner FR (1990) The criteria for choosing an antibiotic for control of bacteria in plant tissue culture. IAPTC Newslet. 60:13-22

Gomez KA, Gomez AA (1984) Statistical procedure for Agricultural Research. 2nd ed. John Wiley and sons Inc. New York, pp. 328-332.

Habiba U, Reza S, Saha ML, Khan R, Hadiuzzaman S (2002) Endogenous bacterial contamination during *in vitro* culture of table banana: Identification and prevention. Plant Tiss Cult. 12(2):117-122

Hamilton EW, Heckathom SA (2001) Mitochondrial adaptation to NaCl, complex I is protected by antioxidants and small heat shock proteins, whereas complex II is protected by proline and betaine. Plant Physiol. 126: 1266-1274

Hecror RF, Domer JE (1983) Control of systemic spread of *Candida albicans* with ketoconazole in the stomachs of mice treated with cytarabine. J Infec Dis. 147(5):946-950

Helaly MN, El-Hoseiny H (2011) Combined effects between genotypes and salinity on sweet orange during the developments stages of its micropropagation. Res J Bot. 6(2): 38-67

Herzog V, Fahimi H (1973) Determination of the activity of peroxidase. Anal Biochem. 55:554-562

Hong FS, Yang F, Liu C, Gao Q, Wan ZG, Gu FG, Wa C, Ma Z, Zhou Z, Yang P (2005a). Influences of nano-TiO_2 on the chloroplast aging of spinash under light. Biol Trace Elem Res. 104: 249-260

Hong FS, Zhou J, Liu C, Yang F, Wa C, Zheng L, Yang P (2005b) Effect of nano-TiO_2 on photochemical reaction of chloroplast of spinash. Biol Trace Elem Res. 105: 269-279

Inskeep WP, Bloom PR (1985) Extinction coefficients of chlorophyll a and b in N,N-dimethylformamide and 80% acetone. Plant Physiol. 77:483-485

Joshi RA, Shukla E, Sairam RK (2011) *In vitro* screening of rice genotypes for drought tolerance using polyethylene glycol. Acta Physiol Plant. 33(6): 2209-2217

Khane M (2003) Bacterial and fungal indexing of tissue cultures.http/www.hos.ufl.edu.moreweb/tissueculture/class 1/bacterial%20and%20fungal%20indexing%20of20tissue% 20cultures.doc

Krieg RN, Holt IG (1984) Bergey's annual of systematic bacteriology Vol. 1. Williams and Wikins company, Baltimore, USA pp. 308-429

Lam CW, James JT, Mccluskey R, Arepalli S, Hunter RI (2006) A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks. Crit Rev Toxicol. 30: 189-217

Lee KW, Choi GJ, Kim KY, Hc L, Park HS, Yoon SH, Lee H (2009) High frequency plant regeneration from mature seed derived callus of Italian ryegrass (*Lolium muliforum*) cultivars. Afr J Biotech. 8: 6828-6833

Lu CM, Zhang CY, Wen JQ, Wa GR, Tao MX (2002) Research of the effect of nanometer materials on germination and growth enhancement of *Glycine max* and its mechanism. Soybean Sci. 21: 168-172

Markins M, Enreiro RT and Oliveira T (2003) Genetic refatedness of *Portuguese almond* cultivars assessed by RAPD and SSR markers. Plant Cell Rep. 22:71-78

Meghwal PR, Sharma HC, Singh SK (2000) Effect of surface sterilizing agents on *in vitro* culture establishment of guava (*Psidium guajava,* L.). J Applied Hort. 2(2): 94-95.

Mngomba SA, Toil D, Akinnifesi FK (2007) Effective precondikoning methods for *in vitro* propagation of *Uapaca kirkiana* Muell Arg. Tree species. Afr J Biotech. 6(14):1670

Moubasher AH, El-Kady IA, Farghally SM (1977) The mycoflora of some Egyptian seeds and their potentialities for production of aflatoxins**.** Zeszyty Problemowe Postepow Nauk Rolniczych. 189: 141-147

Msogoya T, Kayagha H, Mutigitu J, Kulebelwa M, Mamiro M (2012) Identification and management of microbial contaminants of banana *in vitro* cultures. J Appl Biol. 55:3987-3994.

Murashige TA, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 15: 473-497

Murashov V (2006) Comments on "Particle surface characteristic may play an important role in phytotoxicity of alumina nanoparticles" by Yang, L.; Watts, D.J. Toxicology letters, 2005, 185: 122-132. Toxicol Lett. 164: 185-187.

Nel A, Xio T, Mädler L, Li N (2006) Toxic potential of materials at the nano level. Science. 311: 622-627

Oberdörster G, Oberdörster E, Oberdörster I (2005) Nanotechnology: an emerging discipline evolving from studies of ultrafine particles. Environ Healt Pers. 113: 823-839

Obuckwe CO, Osagie II (1989) Morphological changes in infected wilt-resistance and wilt susceptible oil palm progenies and hydrolytic enzyme activities associated with *Fusarium oxysporum* pathogens. Oeagureux. 44(11):8-9

Ödutanyo O I, Amusa NA, Okutade OO, Ogunsan YR (2007) Sources of microbial contamination in tissue culture laboratories in southern Nigeria. Afr J Agric Res. 2(3):67-72

Ödutanyo OI, Oso RT, Akinyemi BO, Amusa NA (2004) Microbial contaminants of cultured *Hibiscus carnabinus* and *Telfaria occidentalis* tissues. Afr J of Biotech. 3(9):472-476

Omamor IB, Asemota AO, Eke CR, Eziashi EI (2007) Fungal contaminants of the oil palm tissue culture in Nigerian Institute for oil palm Research (NIFOR). Afr J Agric Res. 2(10):534-537

Owen CT, Alexander JE, McGregor M (2010) Control of microbial contamination during *in vitro* culture of larval anionid mussels. Invert Reprod Develop. 54(4):187-193

Parida A, Das B (2005) Salt tolerance and salinity effect on plants; a review. Ecotoxicol Environ Safety. 60:324-349

Paschke MW, Perry LG, Redente EF (2006) Zinc toxicity threshold for reclamation for species. Water Air Soil Polut. 170:317-330

Ploetz RC (2006) *Fusarium*-induced diseases of tropical, perennial crops. Phytopath. 96:648–652

Raper KE, Fennel DI (1965) The genus *Aspergillus.* The Williams and Wilkins Co., Baltimore 686 pp.

Sarkhosh A, Zamani Z, Fatahi R, Ebadi A (2006) RAPD markers reveal polymorphism among some Iranian pomegranate (*Punica granatum* L.) genotypes. Sci Hort. 111:24-29

Singh K, Frisvad JC, Thrance U, Mathur SB (1991) An illustrated manual on identification of some seed-borne *Aspergilli*, *Fusaria*, *Penicillia* and their Mycotoxins. Danich Government Institute of Seed Pathology for developing countries, Hellerup, Copenhagen, Denmark.

Sneath PH, Mair MS, Sharpe ME, Holt JG (1986) Bergey's manual of systematic bacteriology. Vol.2. Williams and Wilkins company, Baltimore, USA, pp. 1220.

Soto KF, Carrasco A, Powell TG, Murr LE, Garza KM (2006) Biological effects of nano-particulate materials. Mater Sci Eng. 26:1421-1427.

Suryanarayanan TS, Senthilarasu G, Muruganandam M (2000) Endophytic fungi from *Cuscuta reflexa* and its host plants. Fungal Diversity. 4:117-123

Tang SY, Cao YP (2003) Effects of spraying different forms of silicon (Si) on growth as stress resistance of rice plants. Sci Fertil. 2:16-22

USPEA (U.S. Environmental Protection Agency) (2005) Nanotechnology white paper external review. Draft Available from http://www.epa.gov/osa/pdfs.EPA_ nanotechnology_white_paper_external_review_draft_12-02-2005

Wang BWY, Feng WY, Wang C, Jia M, Wang C, Shi W, Zhang F, Zhao L, Chai F (2006) Acute toxicity of nano-and micro scale zinc powder on healthy adult mice. Toxicol Lett. 161:115-123

Wang XD, Sun C, Gao SX, Wang LS, Han K (2001) Validation of germination rate and root elongation as indicator to assess phytotoxicity with *Cucmis sativus*. Chemosphere. 44:1711-1721

Wu WK (2003) Plant Physiology Science Press. Beijing pp. 117-143.

Yang F, Hong FS, You WJ, Liu C, Gao FQ, Wu C, Yang P (2006) Influences of nanoanatase Ti O_2 on the nitrogen metabolism of growing spinach. Biol Trace Elem Res. 110:179-190

Yang L, Watts DJ (2005) Particle surface characteristics may play an important role in phytotoxicity of alumina nano-particles. Toxicol Leu. 158:122-132

Zheng L, Hong FS, Lu SP, Liu C (2005) Effects of nano Ti O_2 on strength of naturally aged seeds and growth of spinach. Biol. Trace Elem Res. 104:83-91