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Pepper plants response to metal nanoparticles and chitosan in nutrient media

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Abstract

Neutral nanoparticles (NPs) of iron, zinc, copper or their compositions were introduced into nutrient Murashige-Skoog (MS) medium instead of traditional metal salts with/without chitosan supplements to obtain healthy and crop productive plants. Metal NPs concentrations were: Fe NPs (0.06, 0.3, 3.0); Zn NPs (0.016, 0.08, 0.4); Cu NPs (0.004, 0.0008, 0.00016) (mg L^{-1}). Pepper (*Capsicum annuum*) cultivar LJ-King was grown on modified media in aseptic conditions. After 45 days, root and stem length, root activity, green mass gain and chlorophyll content in leaves were measured. Pepper plants grown on media with metal NPs showed increased root length and activity (for Fe, Zn, Cu NPs and their composition) and chlorophyll content in leaves (for NPs Fe and Cu). However, addition of Chitosan modified the effects. Monitoring of pepper grown on MS with NPs composition (Fe: Zn: Cu as 3.0:1.0:0.004 mg L^{-1}) with electron microscope demonstrated no NPs accumulation in roots, sprouts and leaves.

Keywords: pepper, iron nanoparticles, copper nanoparticles, zinc nanoparticles, root activity, root length, chlorophyll content, chitosan, nutrient medium.
Abbreviations: GM _green mass; LCC_chlorophyll content in leaves; MS -Murashige-Skoog nutrient medium; NPs_nanoparticles; PPC_pepper plants cultivation; RA_root activity; RL _root length; SL_ stem length; with/without Chitosan _+Ch/-Ch.

Introduction

Pepper (*Capsicum annuum*) fruits are one of the expressive crops in the world's agriculture. The increase in yield production can be achieved by improvement of soil fertility, use of genetics advances and breeding of high-performance cultivars. Furthermore, application of active substances (e.g. hormones) or tissue culture employment can be considered. Numerous researchers had shown that nanostructure materials could be used in agriculture and stimulate plant growth in the open and protected environments. Progress in biotechnology includes plants and tissues growing on artificial media with a balanced composition of nutrients essential for proper development. Nanomaterials are different metal oxides, magnetic particles, nanotubes, ceramics, silicates, polymers, dendrimers, emulsions, quantum dots. Nanostructures in practice increase biomass, grain and vegetables yields, and so enhanced crop production. Pest resistance and seed material quality improvement by application of nanoparticles have also been reported (Bhagat et al., 2015; Bindraban et al., 2015; Dasgupta et al., 2015; Parisi et al., 2015). The increase in crops yield and quality due to plant nutrition optimization and pathogen protection are actual tasks (Garcia et al., 2010; Ghormade et al., 2011; Perez-de-Luque and Rubiales, 2009; Ruttkay-Nedecky et al., 2017; Servin et al., 2015). Collection, creation and maintenance of valuable species and rapid reproduction of plant clones and intensive production of the vegetative progeny of plants are hardly reproducible under common conditions. These acts are also closely associated with new biotechnology methods. The use of nanostructures for improvement and cultivation of plants and possibly for clone reproduction is promising. Addition of titan dioxide (nanoanatase) NPs to the MS medium at concentration range of 10-40 mg L^{-1} was used to improve parsley seed germination and seedling quality (Dehkourdi and Mosavi, 2013). These authors reported that 30 mg L^{-1} NPs was the best concentration for seed treatment and led to the seed germination index rise and viability potential increase, stimulation of roots and sprouts growth and green yield mass increase.

Addition of multilayer carbon nanotubes to MS medium at the effective concentrations 0.01 – 0.20 pg ml^{-1} accelerated tomato seeds germination and increased germination percentage. Tomato plants grown on a nutrient medium with carbon nanotubes showed larger biomass yield against controls, but root length did not differ. Biris and Khodakovskaya (2012) studied the effects of nanotubes on water absorption processes in seeds.

Nutrient media, such as Murashige-Skoog, Gamborga, Heller etc., contain the balanced nutritional complex of obligatory components essential for the plants development, including organic substances (vitamins, carbohydrates, amino acids and/or protein hydrolysates) and inorganic salts (nitrogen, phosphorus, boron, potassium, calcium, magnesium, sulfur, iron) and trace elements (manganese, zinc, copper, molybdenum, etc). We used MS medium for experimental metal salts substitution with metal NPs, both with/without chitosan addition. The nanoparticles have been considered reasonable, taking into account the specific features of their effects in biosystems (Glushchenko et al., 2000-2017). Metal NPs are 7-50 times less toxic than metal ions from chemical compounds and demonstrate poly functional and prolonged effects in biotic doses (doses 10-50 times smaller than maximum tolerated doses). NPs stimulate vital processes and can distribute through organs and tissues of plants. The effects depend on particles structure and their physical and chemical characteristics. Copper NPs and natural polysaccharides are synergists (Bogoslovskaja et al., 2014; Glushchenko et al., 2002; Rakhmetova et al., 2010, 2015). Chitosan was selected as an absorbing agent for plants metabolism products (Malerba and Cerana, 2016; Li et al., 2016).

The aim of this study was to understand (A) how neutral metal nanoparticles (Fe, Zn, Cu) or their compositions can be used instead of charged metal ions in the nutrient MS medium and (B) how they may affect the morphometric and physiological growth parameters in plant development under aseptic conditions, and (C) how chitosan supplement can modify the effect of NPs.

Results

Mean values of root length, sprout height, root activity, green mass and chlorophyll content were analysed using ANOVA. Significant treatment effects ($p \leq 0.05$) were verified by Tukey's test.

Modified MS medium prepared with metal NPs and chitosan

We propose a new modified plant nutrient MS medium with iron, zinc, copper NPs instead of traditionally used metal salts. A detailed description of the method for nutrient media with metal nanoparticles preparation has been given in our patent (Hui et al., 2017). We added metal nanoparticles at the concentrations (mg L^{-1}): Fe NPs = 3.0, 0.3, 0.06; Zn NPs= 0.4, 0.08, 0.016; Cu NPs = 0.004, 0.0008, 0.00016 instead of iron, zinc, copper salts, both with/without Chitosan (+Ch/-Ch), into nutrient MS medium (Fig. 1). It should be noted that Fe NPs concentrations (3.0, 0.3, 0.06 mg L^{-1}) were 1.9, 19, 93 times less than standard ferrous ion content in MS medium, respectively. Zn NPs concentrations (0.4, 0.08, 0.016 mg L^{-1}) were 4.9, 24.5, 122.5 times less than zinc from Zn sulphate and Cu NPs (0.004, 0.0008, 0.00016 mg L^{-1}) were 1.6, 8.0, 40 times less than Cu from copper sulphate (in terms of metal).

To obtain an aqueous suspension, a sample of the NPs powder is dispersed in water with an ultrasonic disperser in a controlled manner under cooling. Preparation of NPs compositions need *ex tempore* manner, i.e., MS medium (made without Fe or Zn or Cu salts) will be ready and chilled. For nutrient medium sterility, the NPs suspension blending procedure is carried out under flaming. Planting process in aseptic conditions is illustrated in Fig 1.

Effects of metal nanoparticles on the root length and activity

Iron, zinc, copper NPs and their combinations effects (both with/without chitosan) on the length and activity of *LJ-king* pepper roots are presented in Tables (1-4).

Pepper plants cultivation (PPC) on modified medium containing Fe NPs instead of ionic metals at concentrations 0.06, 0.3 and 3.0 mg L^{-1}, resulted in significant root length increase (by 54%, 118% and 102%, respectively, in comparison with control) (Table 1). The statistically reliable positive effects were observed for Fe NPs concentrations 0.3 (as +Ch and —Ch) and 3.0 mg L^{-1} (—Ch).

Pepper cultivation on nutrient medium with Zn NPs instead of Zn sulphate at concentrations 0.016, 0.08 and 0.4mg L^{-1}, resulted in RL increase by 62%, 80% and 71%, respectively, compared to control, for groups —Ch, and by 54%, 52% and 101% - for groups +Ch (Table 2). Zn NPs concentrations 0.08 (—Ch) and 0.4 mg L^{-1}(+Ch) effects were statistically reliable.

PPC in a nutrient medium with Cu NPs instead of copper ions at concentrations of 0.00016, 0.0008 and 0.004 mg L^{-1}, induced RL increase by 34%, 57% and 54%, respectively, for groups —Ch (Table 3). Statistically reliable positive effects were observed for Cu NPs concentration at 0.0008 mg L^{-1}.

PPC in MS medium containing Fe, Zn, Cu NPs compositions (Fe:Zn:Cu NPs with concentrations 3.0:0.4:0.004) instead of iron, zinc, copper ions, led to RL increase in comparison with control by 58 % for group —Ch and 38% for group +Ch (Table 4). Addition of chitosan to nutrient media with iron, zinc, copper combination Fe: Zn: Cu 0.3: 0.08: 0.0008 mg L^{-1} - increased the RL by 47%, compared to control. The combination of the smallest concentrations of Fe NPs (0.06 mg L^{-1}), Zn NPs (0.016 mg L^{-1}) and Cu NPs (0.00016 mg L^{-1}) led to 19% reduce in RL for group —Ch and 42% increase in RL for +Ch group l.

The pepper plants cultivated on MS medium with Fe NPs at 0.06 and 0.3 mg L^{-1} demonstrated RA increase by 59% and 58% for groups —Ch and by 57%, 77% - for groups +Ch (Table 1).

Replacement of zinc sulfate with Zn NPs at concentrations 0.016, 0.08 and 0.4 mg L^{-1} (—Ch) in MS medium led to RA increase by 31%, 56% and 38%, respectively (Table 2).

Use of Cu NPs instead of copper sulfate at concentrations 0.00016, 0.0008 and 0.004 mg L^{-1} (—Ch) enhanced pepper RA by 18%, 61% and 21%, respectively. Addition of chitosan with NPs modified RA in comparison with —Ch (Table 3).

The PCC on MS medium, containing: 0.06 mg L^{-1} Fe NPs + 0.016 mg L^{-1} Zn NPs + 0.00016 mg L^{-1} Cu NPs, or 0.3 mg L^{-1} Fe NPs + 0.08 mg L^{-1} Zn NPs + 0.0008 mg L^{-1} Cu NPs or 3.0 mg L^{-1} Fe NPs + 0.4 mg L^{-1} Zn NPs + 0.004 mg L^{-1} Cu NPs, instead of ions, stimulated RA by 98%, 51% and 91%, respectively. Supplement of NPs media with chitosan modified the effect, but RA exceeded control parameters (by 70, 88 and 52% respectively) (Table 4).

Table 1. Root length and activity in pepper LJ-king plants grown on MS media with Fe NPs with/without chitosan.

Fe NPs content MS medium, mg L^{-1}	Chitosan content in MS medium, mg L^{-1}	Root length, cm	Root length treated/control, %	Root activity, mg h^{-1}	Root activity, (treated/control), %
0	-	7.54±0.83 a	100	0.535±0.038 ab	100
3.0	-	15.25±1.42 b	202.3	0.498±0.030 a	93.2
0.3	-	16.42±1.25 b	217.8	0.844±0.038 bc	157.8
0.06	-	11.63±1.47 ab	154.2	0.848±0.071 bc	158.6
3.0	100	11.63±1.49 ab	154.2	0.518±0.073 a	96.9
0.3	100	15.21±0.50 b	201.7	0.944±0.091 c	176.5
0.06	100	12.36±0.71 ab	163.9	0.840±0.086 bc	157.1

Experimental data were counted as a ratio of treated values against control ones (%). Control – usual MS medium (without NPs). Test series were repeated n=9 (root length) and n=3 (root activity). Data are presented as a mean ± SE

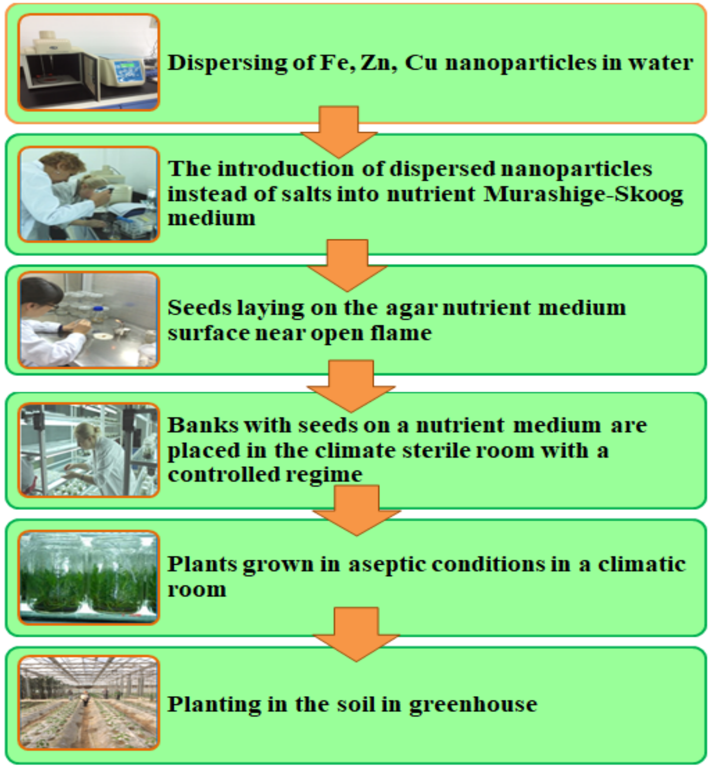

Fig 1. Scheme of experiment using addition of iron, zinc, copper nanoparticles instead of metal salts to nutrient Murashige-Skoog medium.

Table 2. Root length and activity in *LJ-king* pepper plants grown on MS media with Zn NPs with/without chitosan.

Zn NPs content MS medium, mg L^{-1}	Chitosan content in MS medium, mg L^{-1}	Root length, cm	Root length treated /control, %	Root activity,mg h^{-1}	Root activity, treated /control), %
0	-	7.54±0.83 a	100	0.535±0.038 a	100
0.4	-	12.88±1.31 ab	170.9	0.741±0.162 a	138.5
0.08	-	13.57±0.76 b	180.0	0.835±0.100 a	156
0.016	-	12.21±1.06 ab	162.0	0.702±0.080 a	131.3
0.4	100	15.14±1.81 b	200.8	0.948±0.079 a	177.2
0.08	100	11.50±1.31 ab	152.5	0.712±0.104 a	133.0
0.016	100	11.63±1.39 ab	154.2	0.502±0.065 a	93.8

Experimental data were counted as a ratio of treated values against control ones (%). Control – usual MS medium (without NPs). Test series were repeated n=9 (root length) and n=3 (root activity). Data are presented as a mean ± SE

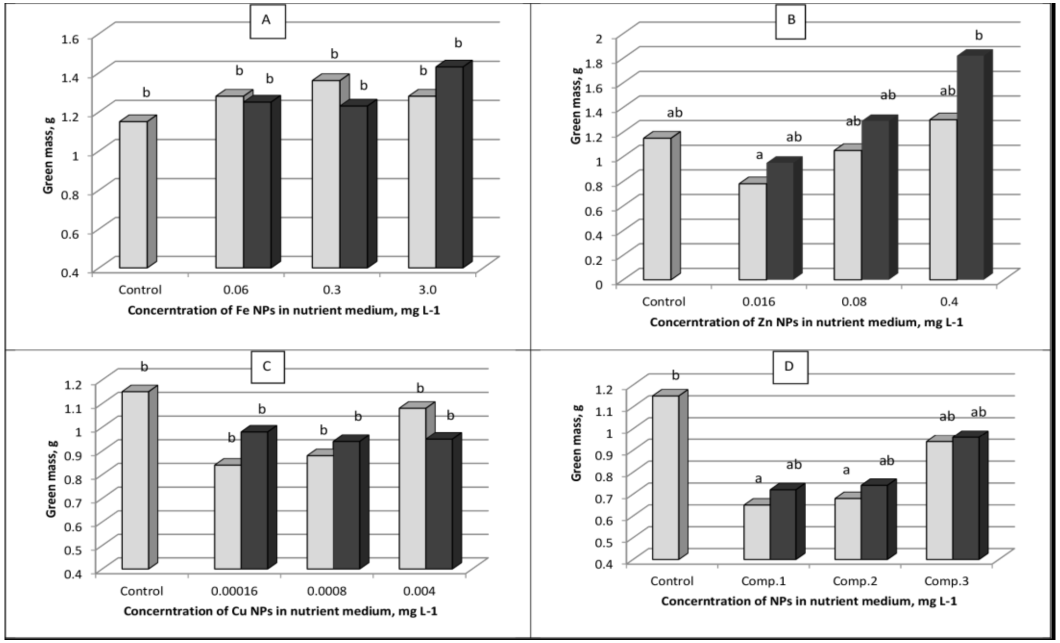

Fig 2. Green mass yield on nutrient medium with iron NPs (A), zinc NPs (B), copper NPs (C) and NPs compositions (D). Light grey columns - without Chitosan, dark grey columns - with Chitosan addition. Control – usual MS medium (without NPs). Fig. D: Composition 1 (Comp.1): Fe 0,06 mg L^{-1} + Zn 0,016 mg L^{-1} + Cu 0,00016 mg L^{-1}; Comp. 2: Fe 0,3 mg L^{-1} + Zn 0,08 mg L^{-1} + Cu 0,0008 mg L^{-1}; Comp. 3: Fe 3,0 mg L^{-1} + Zn 0,4 mg L^{-1} + Cu 0,004 mg L^{-1}

Table 3. Root length and activity in *LJ-king* pepper plants grown on MS media with Cu NPs with/without chitosan

Cu NPs content MS medium, mg L^{-1}	Chitosan content in MS medium, mg L^{-1}	Root length, cm	Root length treated /control, %	Root activity, mg h^{-1}	Root activity, (treated/control), %
0	-	7.54±0.83 a	100	0.535±0.038 a	100
0.004	-	11.64±0.42 ab	154.4	0.645±0.062 a	120.6
0.0008	-	11.86±1.50 b	157.3	0.860±0.065 a	160.7
0.00016	-	10.07±1.13 ab	133.6	0.629±0.047 a	117.6
0.004	100	8.64±0.61 ab	114.6	0.793±0.096 a	148.3
0.0008	100	7.47±0.88 ab	99.1	0.599±0.093 a	111.9
0.00016	100	10.21±1.07 ab	135.4	0.749±0.119 a	140.0

Experimental data were counted as a ratio of treated values against control ones (%). Control – usual MS medium (without NPs). Test series were repeated n=9 (root length) and n=3 (root activity). Data are presented as a mean ± SE

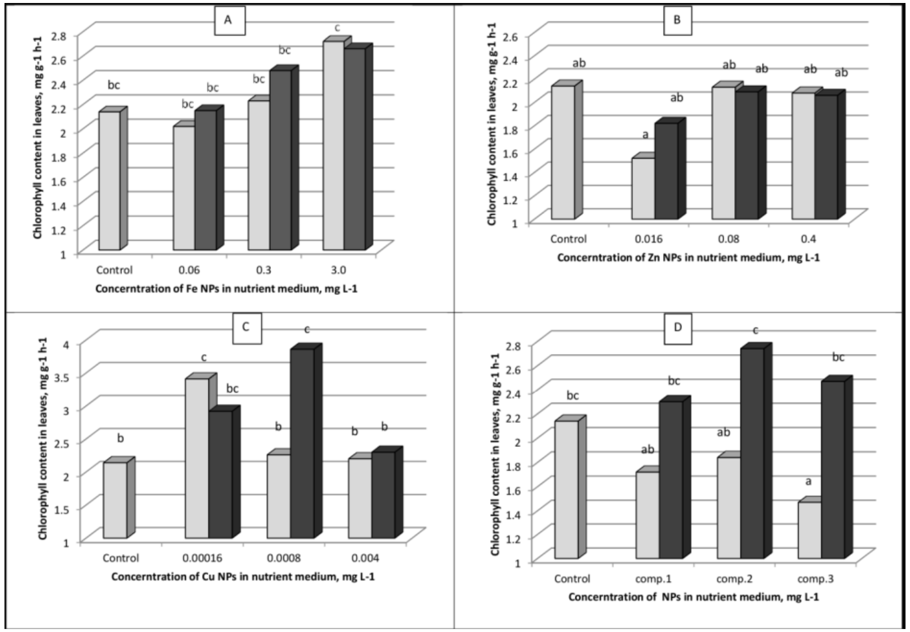

Fig 3. Pepper chlorophyll content in leaves on a nutrient medium with iron NPs (A), zinc NPs (B), copper NPs (C) and NPs compositions (D). Light columns - without Chitosan, dark columns - with Chitosan addition. Control – usual MS medium (without NPs). Fig. D: Composition 1 (Comp.1): Fe 0,06 mg L^{-1} + Zn 0,016 mg L^{-1} + Cu 0,00016 mg L^{-1}; Comp. 2: Fe 0,3 mg L^{-1} + Zn 0,08 mg L^{-1} + Cu 0,0008 mg L^{-1}; Comp. 3: Fe 3,0 mg L^{-1} + Zn 0,4 mg L^{-1} + Cu 0,004 mg L^{-1}.

Table 4. Root length and activity of pepper c.v LJ-king grown on MS media with Fe, Zn and Cu NPs compositions with/without chitosan

Metal nanoparticles content MS medium, mg L^{-1}			Chitosan content in MS medium, mg L^{-1}	Root length		Root activity	
Fe	Zn	Cu	Chitosan	cm	treated /control, %	mg h^{-1}	treated/control, %
0	0	0	-	7.54±0.83 ab	100	0.535±0.038 a	100
3.0	0.4	0.004	-	11.94±1.29 b	158.3	1.024±0.187 b	191.4
0.3	0.08	0.0008	-	8.07±1.13 ab	107.0	0.806±0.040 ab	150.7
0.06	0.016	0.00016	-	6.11±0.76 a	81.1	1.058±0.040 b	197.8
3.0	0.4	0.004	100	10.44±1.16 ab	138.5	0.816±0.059 ab	152.5
0.3	0.08	0.0008	100	11.06±0.69 b	146.7	1.010±0.096 b	188.7
0.06	0.016	0.00016	100	10.71±0.95 ab	142.0	0.911±0.043 ab	170.3

Experimental data were counted as a ratio of treated values against control ones (%). Control – usual MS medium (without NPs). Test series were repeated n=9 (root length) and n=3 (root activity). Data are presented as a mean ± SE

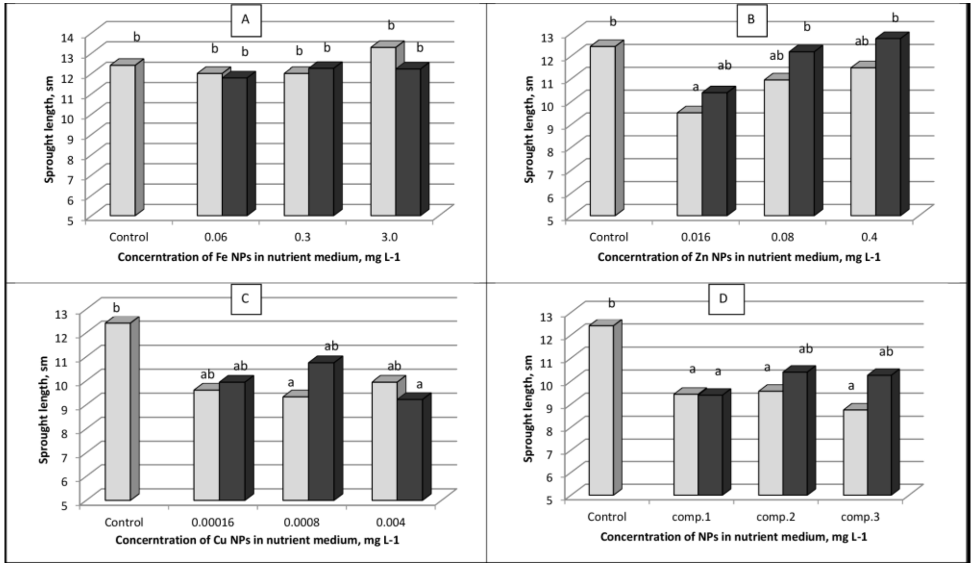

Fig 4. Pepper sprout length on a nutrient medium with iron NPs (A), zinc NPs (B), copper NPs (C) and NPs compositions (D). Light columns - without Chitosan, dark columns - with Chitosan addition. Control – usual MS medium (without NPs). Fig. D: Composition 1(Comp.1): Fe 0,06 mg L^{-1} + Zn 0,016 mg L^{-1} + Cu 0,00016 mg L^{-1}; Comp. 2: Fe 0,3 mg L^{-1} + Zn 0,08 mg L^{-1} + Cu 0,0008 mg L^{-1}; Comp. 3: Fe 3,0 mg L^{-1} + Zn 0,4 mg L^{-1} + Cu 0,004 mg L^{-1}.

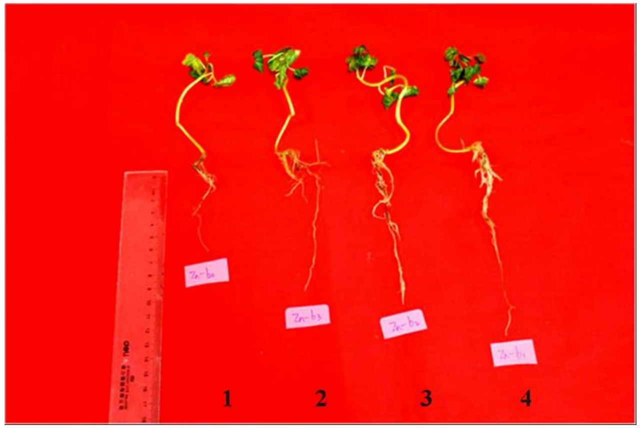

Fig 5. The photo of pepper c.v LJ-king plants grown on a nutrient medium containing zinc nanoparticles and chitosan: (1) –control plant; (2) – grown with zinc nanoparticles at a concentration 0.016 mg L^{-1}; (3) – with zinc nanoparticles at a concentration 0.08 mg L^{-1}; (4) – with zinc nanoparticles at a concentration 0.4 mg L^{-1}.

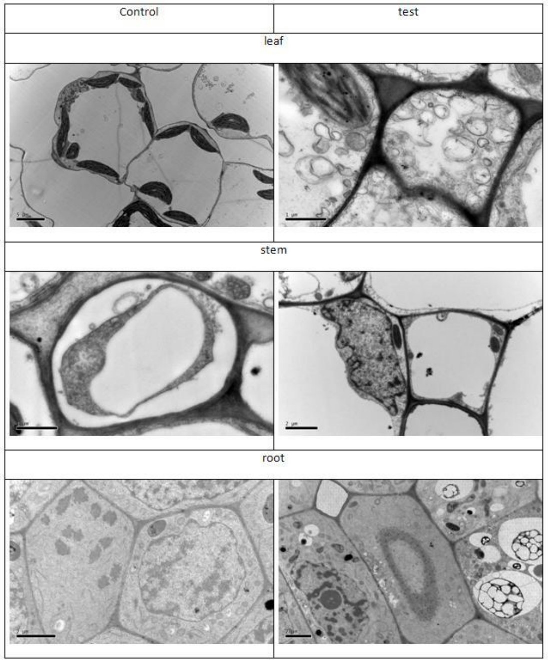

Fig 6. Electron microscope photos of leaf, stem and root slices of pepper, grown on nutrient medium with NPs (Fe:Zn:Cu) in ratio (3.0 : 1.0 : 0.004) mg L^{-1}.

Pepper green mass (GM)

Metal salts substitution with neutral NPs induced changes in pepper GM. It increased slightly (about 10%-20%) under Fe NPs at concentrations 0.06, 0.3 mg L^{-1}(as for —Ch so for +Ch) (Fig. 2)

GM was decreased under Zn NPs at concentrations 0.016 and 0.08 mg L^{-1}. Chitosan addition enhanced GM yield. Pepper GM under copper NPs was smaller than in untreated groups. Pepper GM was also reduced in the case of metal NPs compositions (both +Ch/—Ch) .

Leaves chlorophyll content (LCC)

Addition of Fe NPs (0.3 mg L^{-1} and 3.0 mg L^{-1}) increased LCC (Fig. 3). Pepper grown on MS media with zinc NPs (0.016 mg L^{-1}) showed reduced LCC. Increased chlorophyll in leaves was observed for Cu NPs at 0.00016 mg L^{-1}(—Ch), by 59%. Chitosan additive induced extra growth of LCC up to 80% after application of 0.0008 mg L^{-1} Cu NPs. The observed changes were statistically significant.

Under NPs compositions, LCC was reduced in —Ch groups, but after addition of supplementary chitosan it exceeded the control values by 10 - 30%.

Pepper sprouts length

Introduction of Fe NPs did not influence SL (Fig. 4). In other experements metal NPs and their compositions lowered SL. Chitosan addition to metal NPs preparations re-increased SL, some of them to control level.

Pepper seedlings grown on a nutrient media with nanoparticulated vital elements and chitosan looked as compact healthy plants with a well-developed root system (Fig. 5).

Electron microscope data

Slices of plant's leaves, stems and roots were prepared and electron microscopy processed to study metal NPs impregnation in pepper organs from nutrient medium with NPs composition Fe: Zn: Cu as 3.0 :1.0 : 0.004 mg L^{-1} (Fig. 6).

Only native structures appeared to be visualized. Metal NPs were not observed in plant tissues.

Discussion

Modern methods for production of high-quality planting material include plants grow in controlled aseptic conditions. Nutrient substrates, as a rule, are balanced compositions with optimal contents of macro- and microelements. This study was carried out to evaluate the effect of substitution of traditional trace elements with neutral NPs (Fe, Zn, Cu and their compositions) on growth and development of pepper plants under aseptic conditions. We developed an improved nutrient medium with neutral metal NPs instead of appropriate ions. In order to modify nutrient medium, we used as a base MS medium formulation, in which instead of common trace element additives such as iron, zinc, copper as salts or chelates, we blended Fe, Zn, Cu NPs or their combinations as ingredients with the original nutrient medium formulation staying unaltered.

Results on metal NPs biological effects have already been presented in our works (Glushchenko et al., 2002; Rakhmetova et al., 2010, 2015, 2017; Bogoslovskaja et al., 2014). NPs were less toxic than salts and they had prolonged and poly functional action, showing synergistic affects with low-molecular chitosan derivatives. It has been reported that replacement of iron, zinc, copper salts with nanoparticles in broilers forage premixes led to an increase in live weight of chick's, reduced the forage consumption and guaranteed 100% preservation of livestock (Egorov et al., 1987).

A significant problem with nano sized materials is choosing of effective concentrations. Metal NPs are known to produce stimulation of metabolic processes at concentrations at doses smaller than maximum tolerated dose (Glushchenko et al., 2002). So, we used Fe NPs in concentration ranges about 1.9-93, Zn NPs- 4.9-122.5, Cu NPs - 1.6-40 times less than ions concentrations from sulfates in the MS medium (Hui et al., 2017).

Chitosan has been reported as plant growth stimulating factor, possessing high sorption activity for metabolic products of growing plants and bacteria, protecting plants from infections and promoting crop productivity (Malerba and Cerana, 2016; Li et al., 2016; Solís-Cruz, 2017). We have used chitosan Taynshi (China), known in Chinese medicine as detoxifier. After chitosan addition to the nutrient media, bacteria, fungi or mold contaminations did not occur.

The major positive effect for RL and RA was found at 0.3 mg L^{-1} concentration of Fe NPs. Root growth stimulation after Fe NPs treatment for some plants was noted by many authors (Li et al., 2015; Alidoust et al., 2013; Ghafariyan et al., 2013; Jeyasubramanian et al., 2016). The Zn and Cu NPs, along with some other metal NP compositions increased pepper RL, although to a less extent than Fe NPs.

Chitosan addition usually decreased RL under Fe, Zn, Cu NPs, but values still remained higher than in control groups. Probably it was due to the high affinity of the natural polysaccharide chitosan, reducing availability of elements to the growing plants (Monier et al., 2010).

However, when chitosan was added to MS with Fe:Zn:Cu NP preparations at 0.3:0.08:0.0008 mg L^{-1} or 0.06:0.016: 0.00016 mg L^{-1}, the RL was larger in plants grown on (—Ch) media.

Not only root system dimensions provided plant growth but also RA. Enhanced RA was observed for Fe, Zn, Cu NPs, except for Fe NPs at 3.0 mg L^{-1}.

Zn NPs (0.016 and 0.08 mg L^{-1}), Cu NPs and NPs compositions led to SL decrease up to 20%. The results correlate with other researche data (Ruttkay-Nedecky et al., 2017; Azamal et al., 2014). Chlorophyll is the main component of photosynthesis. The maximal LCC in pepper leaves was under Cu NPs (0.00016 mg L^{-1}). Fe NPs (3.0 mg L^{-1}) increased LCC by 30%; Fe: Zn: Cu (0.3: 0.08: 0.0008 mg L^{-1}) composition (+Ch) increased chlorophyll content by 36 %.

Zinc NPs and NPs compositions (—Ch) reduced LCC. The LCC reduction was reported for ZnO NPs (Wang et al., 2016).

The accumulation of nanoparticles in the root tip zone, an active transport in the plants vascular and NPs accumulation in the outer layers of root tissues have been reported (Davis et al., 2017; Krajcarová et al., 2017). But our electron microscopic data on roots, leaves, sprouts slices, even 45 days after cultivation on a nutrient medium with metal NPs, did not reveal any accumulation of nanoparticles in plant tissues.

Our data show that nutrient media with metal NPs in aseptic conditions induce plants responses associated with the nature of the metal NPs, concentrations, ratios in NPs compositions and the chitosan supplements. Pepper plants cultivated on nutrient media with metal NPs instead of salts both with/without Chitosan had well-developed active root systems, compact stems and chlorophyll content in leaves, depending on metal, concentrations and ratios of nanoparticles in the nutrient media. We studied metal NPs at concentrations significantly lower than usually tested by researchers, so metal NPs toxicity problem (Ruttkay-Nedecky et al., 2017; Azamal et al., 2014) was not observed and discussed.

Materials and Methods

Plant materials

Our experimental plant material was pepper (*Capsicum annuum*) LJ-King cultivar.

Experimental location

The study was conducted at the Shenzhou Space Biotechnology Group, CAST, located in Beijing, China and Institute for Energy Problems of Chemical Physics of Russian Academy of Sciences, located in Moscow, Russian Federation.

Experimental design

The experiment was performed in a randomized design, with 25 treatments in 8-9 replications. Groups of treatment:

I) Control group – original MS medium.

II) MS medium without ionic Fe, but with Fe NPs, added instead of $FeSO_4$, in concentration: 2a: 3.0 mg L^{-1}; 2b: 0.3 mg L^{-1}; 2c: 0.06 mg L^{-1}.

III) Fe NPs supplements instead of ionic Fe in MS medium in concentration: 3a: 3.0 mg L^{-1}; 3b: 0.3 mg L^{-1}; 3c: 0.06 mg L^{-1}; with Chitosan 100.0 mg L^{-1} added to each group.

IV) MS medium without ionic Zn, but with Zn NPs, added instead $ZnSO_4$, in concentration: 4a: 0.4 mg L^{-1}; 4b: 0.08 mg L^{-1}; 4c: 0.016 mg L^{-1}.

V) Zn NPs supplements instead of ionic Zn in MS medium in concentration: 5a: 0.4 mg L^{-1}; 5b: 0.08 mg L^{-1}; 5c: 0.016 mg L^{-1}; with Chitosan 100.0 mg L^{-1} added to each group.

VI) MS medium without ionic Cu, but with Cu NPs, added instead of $CuSO_4$, in concentration: 6a: 0.004 mg L^{-1}; 6b: 0.0008 mg L^{-1}; 6c: 0.00016 mg L^{-1}.

VII) Cu NPs supplements instead of ionic Cu in MS medium in concentration: 7a: 0.004 mg L^{-1}; 7b: 0.0008 mg L^{-1}; 7c: 0.00016 mg L^{-1}, with Chitosan 100.0 mg L^{-1} was added to each group.

VIII) MS medium without ionic Fe, Zn, Cu, but with composition, containing Fe NPs, Zn NPs, Cu NPs, added instead of $FeSO_4$, $ZnSO_4$, $CuSO_4$, in concentration: 3.0, 0.4, 0.004 mg L^{-1}, respectively.

IX) Composition, containing Fe NPs, Zn NPs, Cu NPs, was added instead of ionic Fe, Zn, Cu in MS medium in concentrations: 3.0, 0.4, 0.004 mg L^{-1}, respectively, along with Chitosan 100.0 mg L^{-1}.

X) MS medium without ionic Fe, Zn, Cu, but with composition, containing Fe NPs, Zn NPs, Cu NPs, added instead of $FeSO_4$, $ZnSO_4$, $CuSO_4$, in concentrations: 0.3, 0.08, 0.0008 mg L^{-1}, respectively.

XI) Composition, containing Fe NPs, Zn NPs, Cu NPs, added instead of ionic Fe, Zn, Cu, in MS medium in concentrations: 0.3, 0.08, 0.0008 mg L^{-1}, respectively, with Chitosan 100.0 mg L^{-1}.

XII) MS medium without ionic Fe, Zn, Cu, but with composition, containing Fe NPs, Zn NPs, Cu NPs, added instead of $FeSO_4$, $ZnSO_4$, $CuSO_4$, in concentrations: 0.06, 0.016, 0.00016 mg L^{-1}, respectively.

XIII) Composition, containing Fe NPs, Zn NPs, Cu NPs, was added instead of ionic Fe, Zn, Cu in MS medium in concentrations: 0.06, 0.016, 0.00016 mg L^{-1}, respectively with Chitosan 100.0 mg L^{-1}.

Seeds were placed in sterile bottles with 50 ml of modified MS. Each test version included 5 glass bottles with three pepper seeds in each. Data (RL, RA) were counted as experimental groups mean values ratios against control ones (%).

Nanoparticle production and characteristics

The electro neutral iron, zinc and copper nanoparticles were produced by flow-levitation method as described in (Guen and Miller, 1983; Jigatch et al., 2000). Physicochemical characteristics of NPs were obtained from TEM-microscopy and X-ray diffraction meter, as already described (Hui et al., 2017). Metal nanoparticles had the average diameter: the iron particles: 27.0 ± 0.51 nm, zinc: 54.0 ± 2.8 nm, and copper: 79.0 ± 1.24 nm. In iron nanoparticles, the crystalline metallic phase was 53.6%, the iron oxide phase of Fe_3O_4 was 46.4%, and the oxide layer thickness was 3.5 nm. Copper and zinc nanoparticles consisted of a crystalline metallic phase with an oxide film thickness of 0.5-1.0 nm.

Preparation of the nutrient MS medium with metals nanoparticles and chitosan

MS nutrient medium was prepared as described (Murashige and Skoog, 1962) and sterilized at 120 °C under 0.1 MPa for 20 min in an autoclave "Hirayama, HVE-50" (Japan).

NPs aqueous suspensions were prepared in flasks with distilled sterile water. Samples were dispersed for 30 sec thrice by "Scientz JY 92-IIN" Dispergator (China) in an ice bath. Stock NPs suspension was diluted by sterile distilled water to the required concentrations with the subsequent dispersing and cooling. Metal NP suspensions (or there appropriate dilutions) were added to sterile, cooled to 45 °C nutrient medium under flaming. The original MS nutrient medium was used as a control.

100 mg of Tyanshi Chitosan were put in 1 L of sterile cooled to 45 °C base MS nutrient medium under flaming and kept stirring on a magnetic stirrer for 2 minutes for preparation of a nutrient medium with Chitosan additive.

50 ml of the cooked nutrient medium was immediately dispensed under flaming into 200 ml sterile bottles, sealed with Parafilm or polypropylene caps, and left in sterile room. Nutrient medium prepared with such procedure was used for seeds germinating and plants growth.

Plant cultivation

Seeds were placed in a sterile vessel with breeding substrate. Banks with seeds were put on racks in a sterile room with a controlled permanent regime: temperature 22-25° C, humidity 36%, lighting 3500-3000 Lux 12/12 hours day/night. We checked seed germination after 15 days. After 45 days, we evaluated morphometric indicators such as root and stem height and green mass gain. Root activity as a physiological parameter was measured with triphenyl tetrazolium chloride method according to Adebusoye et al. (2012). The chlorophyll content in leaves was evaluated as in (Lichtenthaler and Buschmann, 2001).

Transmission electron microscopy

Specimens from pepper plants were sampled after 45 days of cultivation and cut into pieces with size of 1 mm^2. They were pre-fixed for two hours in 2.5% gluteraldehyde in 0.1 M phosphate buffer (pH 7.4). Samples were finally fixed in 1% osmium acid solution. After dehydration in acetone, the samples were transited in epoxypropane and embedded on epoxy resin 812. Ultrathin sections were cut with Leica EM UC7 ultramicrotome (Germany), and stained with 3% uranyl acetate and 3% lead citrate. Specimens were photographed with a Hitachi H-7500 Transmission electron microscope (Japan).

Statistical analysis

All experiments included 8-9 multiples of test series, and the data were presented as a mean ± SE. Experimental data for RL and RA were counted as a ratio of test values against control ones (%). Data analysis was carried out with Statistica 20.0. Significant treatment effects ($p \le 0.05$) were verified by Tukey's test.

Conclusion

In our study we substituted the traditional for trace elements (Fe, Zn, Cu) supplementation ionic forms in nutrient medium with neutral NPs at the biotic doses as individually or in compositions, both with/without chitosan. Some preparations showed positive effects on plant growth (root height and activity, green mass, sprout height, chlorophyll content). Effects depended on NPs metal species and concentrations. The method ensures generation of healthy planting materials with the better morphometric and physiological parameters. Electron microscopic control did not find any nanoparticles accumulation in roots, leaves and sprout tissues. Therefore, metal NPs at efficient concentrations used in plant nutrient substrates insures an efficient and safety route for plants cultivation.

References

Adebusoye O, Onanuga AO, Jiang P, Sina A (2012) Effect of phytohormones, phosphorus and potassium on cotton varieties (*Gossypium hirsutum*) root growth and root activity grown in hydroponic nutrient solution. J Agr Sci. 4(3):93-110.

Alidoust D and Isoda A (2013) Effect of gamma Fe_2O_3 nanoparticles on photosynthetic characteristic of soybean (*Glycine max* (L.) merr.): foliar spray versus soil amendment. Acta Physiol Plant. 35:3365–3375.

Azamal H and Khwaja SS (2014) Phytosynthesis of nanoparticles: concept, controversy and application. Nanoscale Res Lett. 229(1):1-11.

Bhagat Y, Gangadhara K, Rabinal C, Chaudhari G, Ugale P (2015) Nanotechnology in agriculture: a review. J Pure Appl Microbiol. 9:737–747.

Bindraban PS, Dimkpa C, Nagarajan L, Roy A, Rabbinge R (2015) Revisiting fertilisers and fertilisation strategies for improved nutrient uptake by plants. Biol Fert Soils. 51(8):897–911.

Biris AS and Khodakovskaya MV (2012) Method of using carbon nanotubes to affect seed germination and plant growth. EP № 2499107, London, GB. Williams Powell Staple Court.

Bogoslovskaja OA, Rakhmetova AA, Ovsyannikova MN, Olkhovskaya IP, Gluschenko NN (2014) Antibacterial effect of copper nanoparticles with differing dispersion and phase composition. Nanotech Rus. 9(1-2): 82-86.

Dasgupta N, Ranjan S, Mundekkad D, Ramalingam C, Shanker R, Kumar A (2015) Nanotechnology in agro-food: from field to plate. Food Res Int. 69:381–400.

Davis RA, Rippner DA, Hausner SH, Parikh SJ, McElrone AJ, Sutcliffe JL (2017) In vivo tracking of copper-64 radiolabeled nanoparticles in lactuca sativa. Environ Sci Technol. 51(21):12537-12546.

Dehkourdi EH and Mosavi M (2013) Effect of anatase nanoparticles (TiO_2) on parsley seed germination (Petroselinum crispum) in vitro. Biol Trace Elem Res. 155(2):283-286.

Egorov IA, Kureneva VP, Glushchenko NN, Fatkullina LD, Fedorov YuI (1987) Ru Patent. №1346114, 22.07.1987.

Garcia M, Forbe T, Gonzalez E (2010) Potential applications of nanotechnology in the agro-food sector. Ciênc Tecnol Aliment. 30(3):573–581.

Ghafariyan MH, Malakouti MJ, Dadpour MR, Stroeve P, Mahmoudi M (2013) Effects of magnetite nanoparticles on soybean chlorophyll. Environ Sci Technol. 47:10645–10652.

Ghormade V, Deshpande MV, Paknikar KM (2011) Perspectives for nano-biotechnology enabled protection and nutrition of plants. Biotechnol Adv. 29:792–803.

Glushchenko NN, Bogoslovskaya OA, Olhovskaya IP (2002) Physical and chemical regularities of biological effect of high-disperse powders of metals. Chem Phys. 21(4):79-85.

Glushchenko NN, Bogoslovskaya OA, Olhovskaya IP et al. (2000-2017). Web Site: http//www. nanobiology.narod.ru, http//www. nanobiologyblog.wordpress.com.

Guen MY and Miller AV (1983) Flow-levitation method for synthesis of ultrafine metal powders. Phys Chem Mech Surf. 2:150-154.

Hui Z, Min L, Yu C, Jinying L, Qiao S, Tsyao S, Nechitaylo GS, Jigatch AN, Leipunskii IO, Bogoslovskaya OA, Glushchenko NN. (2017) Method of growing the plants with using metal nanoparticles and substratum for its implementation. RU Patent № 2,612,319, Moscow, RF. Fed Serv Intel Prop.

Jeyasubramanian K, Thoppey UUG, Hikku GS, Selvakumar N, Subramania A, Krishnamoorthy K (2016) .Enhancement in growth rate and productivity of spinach grown in hydroponics with iron oxide nanoparticles. Roy Soc Ch Adv. 6:15451–15459.

Jigatch AN, Leipunskii IO, Kuskov ML, Stoenko NI, Storozhev VB (2000). An apparatus for the production and study of metal nanoparticles. Instr Exp Tech. 43(6):839-845.

Krajcarová L, Novotný K, Kummerová M, Dubová J, Gloser V, Kaiser J (2017) Mapping of the spatial distribution of silver nanoparticles in root tissues of *vicia faba* by laser-induced breakdown spectroscopy (LIBS). Talanta. 173:28-35.

Li A, Lin R, Lin C, He B, Zheng T, Lu L, Cao Y (2016) An environment-friendly and multi-functional absorbent from chitosan for organic pollutants and heavy metal ion. Carbohydr Polym. 148:272-280.

Li X, Yang Y, Gao B, Zhang M (2015) Stimulation of peanut seedling development and growth by zero-valent nanoparticles at low concentrations. PLOS One. Apr 22; 10(4):e0122884. doi: 10.1371

Lichtenthaler HK and Buschmann C (2001) Chlorophylls and carotenoids: measurement and characterization by UV – VIS spectroscopy. In: Wrolstad RE, Acree TE, An H, Decker EA, Penner MH, Reid DS, Schwartz SJ, Shoemaker CF(eds): current protocols in food analytical chemistry. Wiley, New York, F4.3.1-F4.3.8.

Malerba M and Cerana R (2016) Chitosan effects on plant systems. Int J Mol Sci. 17(7): 996.

Monier M, Ayad DM, Wei Y, Sarhan AA (2010) Adsorption of Cu(II), Co(II), and Ni(II) ions by modified magnetic chitosan chelating resin. J Hazard Mater. 177(1-3):962-970.

Murashing T and Skoog F (1962) A received medium for rapid growth and bio-assays with tobacco tissue culture. Physiol Plant. 15:473-497.

Parisi C, Vigani M, Rodriguez-Cerezo E (2015) Agricultural nanotechnologies: what are the current possibilities? Nano Today. 10:124–127.

Perez-de-Luque A and Rubiales D (2009) Nanotechnology for parasitic plant control. Pest Manag Sci. 65: 540–545.

Rakhmetova AA, Alekseeva TP, Bogoslovskaya OA, Leipunskii IO, Ol'khovskaya IP, Zhigach AN, Glushchenko NN (2010) Wound-healing properties of copper nanoparticles as a function of physicochemical parameters. Nanotech Rus. 5(3-4):271-276.

Rakhmetova AA, Bogoslovskaja OA, Olkhovskaya IP, Zhigach AN, Ilyina AV, Varlamov VP, Gluschenko NN (2015) Concomitant action of organic and inorganic nanoparticles in wound healing and antibacterial resistance: chitosan and copper nanoparticles in an ointment as an example. Nanotech Rus. 10(1-2):149-156.

Rakhmetova AA, Bogoslovskaya OA, Olkhovskaya IP, Zhigach AN, Iljina AV, Varlamov VP, Gluschenko NN (2017) Wound healing activity of ointments containing copper nanoparticles assembled with chitosan nanoparticles and N-sulfosuktsinoil-N-carboxyimethyl chitosan. Rus J Biopharm. 9(1): 22-25.

Ruttkay-Nedecky B, Krystofova O, Nejdl L, Vojtech A (2017) Nanoparticles based on essential metals and their phytotoxicity. J Nanobiotech. 15:33-38.

Servin A, Elmer W, Mukherjee A, De la Torre-Roche R, Hamdi H, White JC, Bindraban P, Dimkpa C (2015) A review of the use of engineered nanomaterials to suppress plant disease and enhance crop yield. J Nanopart Res. 17: 1–21.

Solís-Cruz B, Hernández-Patlán D, Beyssac E, Latorre JD, Hernandez-Velasco X, Merino-Guzman R, Tellez G, López-Arellano R (2017) Evaluation of chitosan and cellulosic polymers as binding adsorbent materials to prevent aflatoxin B1, fumonisin B1, ochratoxin, trichothecene, deoxynivalenol, and zearalenone mycotoxicoses through an in vitro gastrointestinal model for poultry. Polymers. 9: 529-538. .

Wang X, Yang X, Chen S, Li Q, Wang W, Hou C, Gao X, Wang L, Wang S (2016) Zinc oxide nanoparticles effect biomass accumulation and photo synthesis in *arabidopsis*. Front Plant Sci. 6:1243.