SEMINAR REPORT

Nanoparticles in plant tissue culture

By

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CERTIFICATE

This is to certify that the seminar report entitled **"Nanoparticles in palnt tissue culture"** has been solely prepared by **Rasha Fathima A. A. (2018-11-005)**, under my guidance and has not been copied from seminar reports of any seniors, juniors or fellow students.

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DECLARATION

I, Rasha Fathima A. A. (2018-11-005) declare that the seminar entitled **"Nanoparticles in plant tissue culture"** has been prepared by me, after going through various references cited at the end and has not been copied from any of my fellow students.

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CERTIFICATE

This is to certify that the seminar report entitled **"Nanoparticles in plant tissue culture"** is a record of seminar presented by **Rasha Fathima A. A. (2018-11-005)** on 19th December, 2019 and is submitted for the partial requirement of the course MBB 591.

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1. Introduction

This an era where every aspect of life has a touch of nano, be it the cosmetics we use, the textiles we wear, the appliances we use, the gadgets we employ, the food we eat, or the environment we live in; whether we like it or not, nanomaterials are already in us, on us and around us. In 1959 Richard Feynman, in his classic lecture at the annual meeting of the American Physical Society at Caltech said that “There's Plenty of Room at the Bottom”. With this he laid the conceptual foundations for the field now called nanotechnology and urged scientists to start looking down to smaller invisible materials for bigger solutions and answers. Today nanotechnology has harnessed progess in chemistry, physics, materials science and biotechnology to create novel materials that have unique properties because their structures are determined on the nanometer scale.

Plant tissue culture is the core of plant biology, which is important for mass propagation, conservation, genetic manipulation and bioactive compound production. The success of *in vitro* plant culture depends on several factors such as genotype, the physiological status of the donor plants, the type of explants, surface disinfection methods, the culture medium, plant growth regulators, the size of the culture vessels, spectral quality, light intensity, photoperiod and temperature. In recent years, the application of nanoparticles has successfully led to the elimination of microbial contaminants from explants and demonstrated the positive role of nanoparticles in callus induction, organogenesis, somatic embryogenesis, somaclonal variation and secondary metabolite production (Kim *et al.*, 2017)

2. Nanoparticles

A nanoparticle (NP) is a particle with at least one dimension ranging between 1-100 nm (Strambeanu *et al*., 2015).

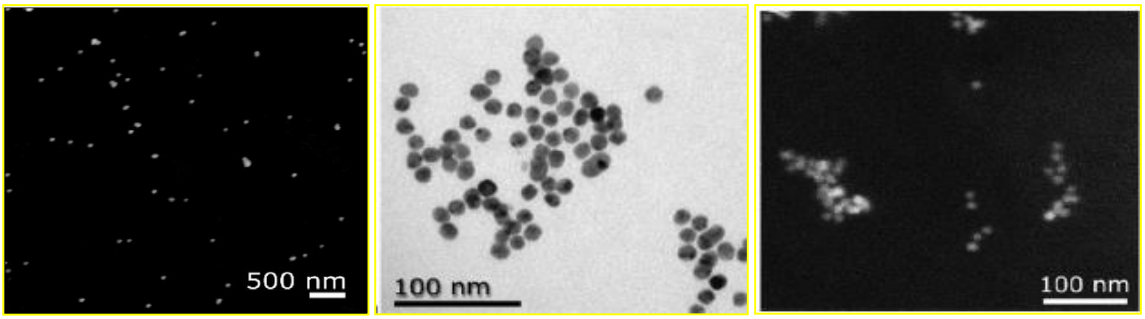

Fig.1: Images of gold nanoparticles

3. Nanoparticles used in plant tissue culture

3.1 Silver nanoparticles

Ag NPs have broad spectrum of antimicrobial activity and reduce various plant diseases caused by fungal pathogens and antibacterial pathogens in plant tissue culture (Abdi *et al*., 2008). Micromolar doses (1–10 μM) of silver ions are sufficient to kill bacteria in water, while silver can be toxic at high doses to mammals and freshwater and marine organisms, probably compromising the growth and shape of animal cells by disrupting a variety of biological functions (Maneerung *et al*., 2008). The application of silver NPs in plant tissue culture has been reported for the elimination of microbial contamination in plant cultures, callus induction, organogenesis, somatic embryogenesis, somaclonal variation, and metabolite production through the introduction of nanomaterials (Sarmast and Salehi 2016). The cost of silver nanoparticle is about Rs. 2041.2 g^{-1}.

3.2 Titanium dioxide nanoparticles

Nanosized titanium dioxide was among the first nanomaterials made readily commercially available to a wide variety of research activities (Reyes-Coronado *et al.*, 2008). The application of TiO2 is highly dependent on its three-dimensional structure, shape, and size. TiO2 displays multiple modes of inhibitory action to microorganisms, in tissue culture. TiO2 caused the antibacterial effect through membrane damage mechanisms in some bacterial species such as *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (Sadiq *et al*., 2010). The cost TiO_2 nanoparticle is about Rs.141.16 g^{-1}.

3.3 Zinc oxide nanoparticles

Zinc oxide is present in the earth crust in zincite form, but mostly they are produced synthetically. It is less toxic and compatible with human skin. Nano-ZnO supplemented with MS media promoted somatic embryogenesis, shooting, and regeneration of plantlets and also induced proline synthesis and activity of superoxide dismutase, catalase, and peroxidase, thereby improving tolerance to biotic stress (Helaly *et al.*, 2014). ZnO NPs inhibited the growth of conidiophores and conidia of *Penicillium expansum*, which finally led to the death of fungal mats (Abd-elsalam, 2013). The cost ZnO nanoparticle is about Rs. 201.7 g^{-1}.

3.4 Other commercially available nanoparticles

Nanoparticles	Purpose	Cost (Rs. g^{-1})
Au	Disinfection, differentiation morphogenesis and somaclonal variation	89161
Cu NP	Differentiation and morphogenesis	5362
Al_2O_3	Disinfection, enhancement of secondary metabolites	102.76
CuO	Disinfection	197.24
Fe_3O_4	Disinfection	286

Table 1: some other nanoparticles used in plant tissue culture

4. Application of nanoparticles in plant tissue culture

4.1 Nanoparticles for decontamination in tissue culture

Microbial contamination is a serious problem faced in plant tissue culture. Major source this contaminaton is explants themselves and the laboratory environment. Therefore, sterilization of laboratory equipments and explants are very important. Several sterilizing agents such as bromine water (BW), calcium hypochlorite (CaOCl), ethanol (EtOH), hydrogen peroxide (H_2O_2), sodium hypochlorite (NaOC1), mercuric chloride ($HgCl_2$), silver nitrate ($AgNO_2$), antibiotics, and fungicides are used for sterization of explants. However, in some cases this may cause phytotoxicity and adversely effects growth and regeneration of explants. Metal and metal oxide NPs have been proven to be useful for the elimination of

various microorganisms. A wide range of NPs such as silver (Ag), aluminum oxide (Al_2O_3), CuO, iron oxide (Fe_3O_4), gold (Au), magnesium oxide (MgO), nickel (Ni), silicon (Si), SiO_2, titanium dioxide (TiO_2), and ZnO have been reported to possess antimicrobial activities against various microorganisms (Wang *et al.*, 2017).

Abdi *et al*. (2008) employed Ag nanoparticles for the first time to control bacterial contamination in *Valeriana officinalis* and obtained 89 per cent contamination free culture. The addition of 60 mg mL^{-1} TiO_2 NPs to the MS medium eliminated bacterial contamination in callus cultures of barley after the 4th subculture (Mandeh *et al.*, 2012). Helaly *et al*. (2014) reported that shoot tips obtained from 3 year-old banana suckers when surface-sterilized with 80% NaOCl, 95% EtOH and finally 0.1% $HgCl_2$ for 15 min. failed to eliminate microbial contamination. However, the incorporation of 100 mg L^{-1} Zn NPs or ZnO NPs into the MS medium resulted in contamination-free cultures. Spinoso-Castillo *et al*. (2017) reported that zero contamination was observed in shoot cultures of *Vanilla planifolia* when MS medium was supplemented with 50–200 mg L^{-1} Ag NPs (Fig. 1).

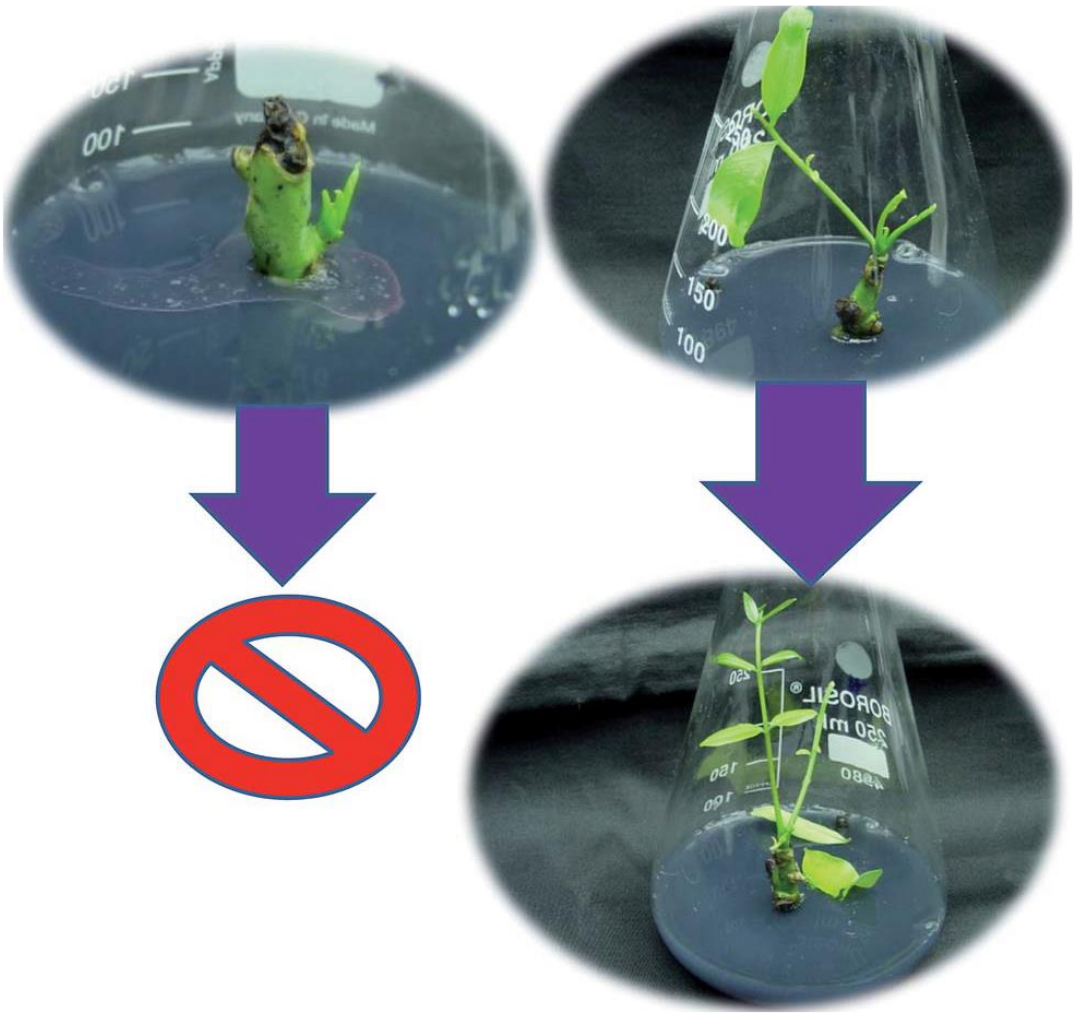

Fig.2: Effect of nanoparticles on microbial contamination.

Various modes of action of silver nanoparticles on bacteria:

Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. There is formation of 'pits' on the cell surface, and there is accumulation of the nanoparticles on the cell surface (Sondi and Solopek-Sondi, 2004). The formation of free radicals by the silver nanoparticles may be considered to be another mechanism by which the cells die (Danilcank *et al*., 2006).

It has also been proposed that there can be release of silver ions by the nanoparticles (Feng *et al*., 2007), and these ions can interact with the thiol groups of many vital enzymes and inactivate them (Matsumura *et al.*, 2003). The bacterial cells in contact with silver take in silver ions, which inhibit several functions in the cell and damage the cells. Then, there is the generation of reactive oxygen species, which are produced possibly through the inhibition of a respiratory enzyme by silver ions and attack the cell itself. Silver is a soft acid, and there is a natural tendency of an acid to react with a base, in this case, a soft acid to react with a soft base (Morones *et al*., 2005). The cells are majorly made up of sulfur and phosphorus which are soft bases. The action of these nanoparticles on the cell can cause the reaction to take place and subsequently lead to cell death. Another fact is that the DNA has sulfur and phosphorus as its major components; the nanoparticles can act on these soft bases and destroy the DNA which would definitely lead to cell death (Hatchett *et al*., 1996).

The interaction of the silver nanoparticles with the sulfur and phosphorus of the DNA can lead to problems in the DNA replication of the bacteria and thus terminate the microbes. It has also been found that the nanoparticles can modulate the signal transduction in bacteria. It is a well established fact that phosphorylation of protein substrates in bacteria influences bacterial signal transduction. Dephosphorylation is noted only in the tyrosine residues of gram-negative bacteria. The phosphotyrosine profile of bacterial peptides is altered by the nanoparticles. It was found that the nanoparticles dephosphorylate the peptide substrates on tyrosine residues, which leads to signal transduction inhibition and thus the stoppage of growth. It is however necessary to understand that further research is required on the topic to thoroughly establish the claims (Shrivastava *et al*., 2007).

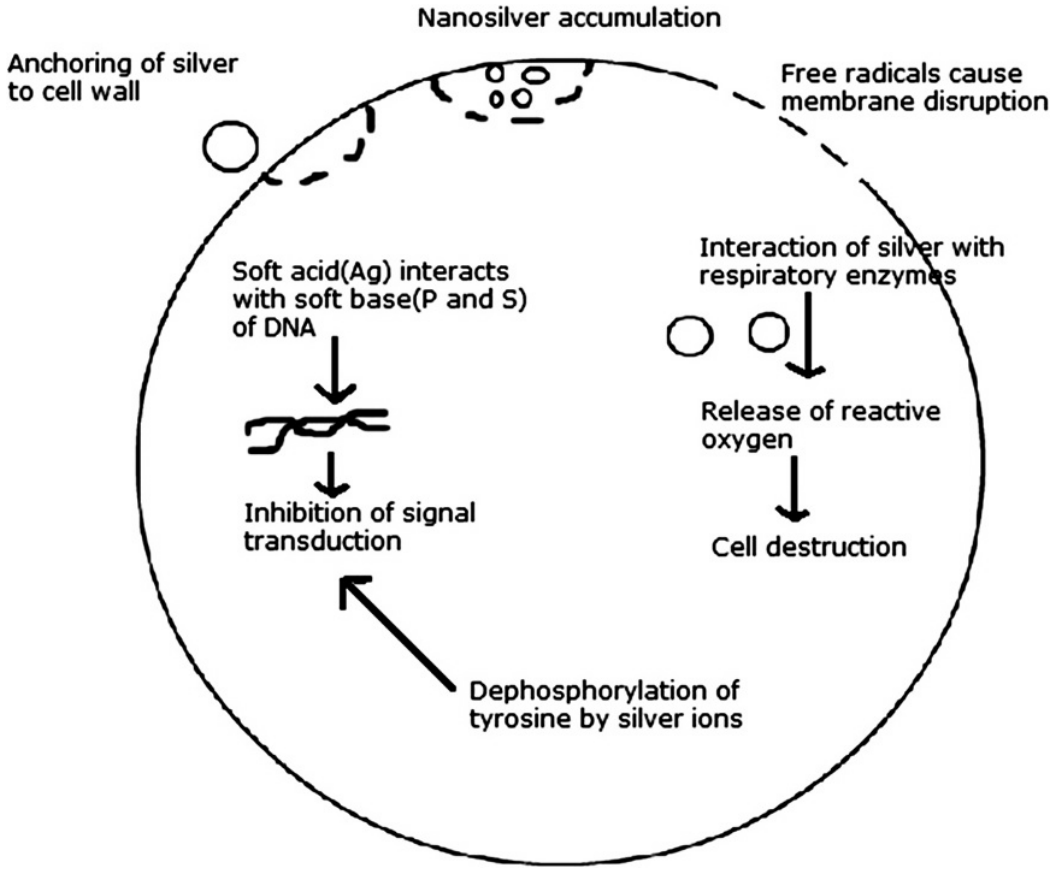

Fig.3: Various modes of action of silver nanoparticles on bacteria (Prabhu and Poulose, 2012)

4.2 Influence of nanoparticles on somaclonal variation

Somaclonal variation can be defined as the genetic changes observed in *in vitro* generated plantlets, cell and organs. This may be caused due to change in chromosome number, chromosome structure, DNA sequence, DNA methylation, mitotic crossing over and activation of transposable elements. It has both advantages as well as disadvantages. This become an advantage when the somaclonal variant is superior to the mother plant. Several studies have demonstrated the phytotoxicity of NPs primarily applied at higher levels. NP treatments affect the mitotic index and DNA integrity, and alter the protein and DNA expression in plants (Atha *et al.,* 2012). However, the exact mechanism behind this is not known.

One the hypothesis is that, the metal nanoparticles produces large amount of Reactive Oxygen Species (ROS) (Tripathy *et al.,* 2017). Increased concentrations of ROS not only affect the cellular viability of exposed plants, but may also affect the integrity of their genetic material and cause nuclear aberrations such as cells with bridges, chromosomal fragments, binucleated cells, and micronuclei (Bello - Bello *et al*., 2018). Further investigations are required in order to determine the effects of a wide range of NPs for enhancing somaclonal variation.

4.3 Nanoparticles for callus induction and organogenesis

Recently several studies have shown that MS media supplemented with NPs show positive effect on callus induction, organogenesis and somatic embryogenesis. Mandeh *et al.* (2017) reported that the number and size of calli increased when barley mature embryos were grown in MS medium augmented with 20 mg L^{-1} 2,4-D and 60 µg mL^{-1} TiO2 NPs. In banana explants, the maximum frequency of somatic embryogenesis was observed in MS medium augmented with 100 mg L^{-1} Zn NPs followed by ZnO NPs. Shoot and root lengths were also increased when adding both NPs to the MS medium (Helay *et al*., 2014). Anwaar *et al*. (2016) reported that supplementation with CuO NPs (15–20 mg L^{-1}) increased organogenesis in rice cultivars. In tomato plants, callus growth and plant regeneration were maximized onmediumcontaining 15mg L^{-1} ZnO NPs and 3.0 g L^{-1} NaCl (Alharby *et al*., 2016).

From all of these reports it can be concluded that the addition of NPs to a plant tissue culture medium affects callus proliferation, shoot multiplication, somatic embryogenesis androoting by altering antioxidant enzyme activities, gene expression, inhibition of ethylene production and production of ROS. However, the actual mechanisms of the promotive or inhibitory effects of NPs on each parameter need to be investigated in detail. The effects of various metal and metal oxide NPs on plants are well documented *in vivo*; such NPs can be used to promote or improve the morphogenetic potential of explants obtained from different plant species. The influence of different concentrations and combinations of NPs on various media (shoot induction, shoot multiplication and rooting media) also deserves evaluation, in order to gain a clear understanding of the underlying mechanisms behind the role of NPs in plant tissue culture (Kim *et al.,* 2017).

4.4 Effect of nanoparticles on secondary metabolites production

Secondary metabolites are bioactive compounds produced by plants. They are produced commercially by by *in vitro* cell or organ culture. The content of secondary compounds in cell and organ cultures was significantly enhanced by optimizing the composition of the culture medium, incorporation of precursors and elicitors and providing appropriate culture conditions. Recent studies has shown that growth medium supplemented with nanoparticles act as a nutrient source as well as elicitor (Hussain *et al*., 2012).

Poborilova *et al.* (2013) reported that the addition of Al_2O_3 NPs (10–100 µgmL^{-1}) to tobacco cell suspension cultures significantly increased the phenolic content. The

accumulation of phenolics in the cells was dose and exposure time dependent. In another study Syu *et al*. (2014) reported that the shape of Ag NPs plays a significant role in the production of anthocyanins in *Arabidopsis.* Treatment with spherical Ag NPs resulted in the highest levels of anthocyanin accumulation in seedlings. In *Aloe vera* suspension culture, cells interacting with 0.625 mg L^{-1} Ag NPs or 120 mg L^{-1} TiO_2 NPs for 48 h exhibited a significantly increased aloin content. The highest content of aloin was obtained subsequent to TiO_2 NP treatment (Raei *et al.*, 2014). Chamani *et al.* in 2018 studied the accumulation of specific bioactive compounds in *Lilium ledebourii* and its dependency on the concentration of ZnO NPs in the MS medium. The highest content of flavonoids, phenolics and anthocyanins was obtained on MS medium supplemented with 25, 75 and 100 mg L^{-1} ZnO NPs, respectively. All of the studies mentioned above confirm the possibility of employing nanoparticles as successful and promising bioactive compound elicitors for secondary metabolite production in plant. Further studies are needed to evaluate the elicitation potential of various other NPs on secondary metabolite production in plant tissue cultures and the corresponding mechanisms.

5. Case Studies

5.1 Case study -1

Effect of zinc oxide (ZnO) nanoparticles on physiology and steviol glycosides production in micropropagated shoots of *Stevia rebaudiana* Bertoni. (Javed *et al*., 2016)

5.1.1 Aim

This study aims to address the effects of different concentrations (0, 0.1, 1.0, 10, 100 or 1000 mgL^{-1}) of engineered zinc oxide (ZnO) nanoparticles (34 nm in size) on growth parameters, steviol glycosides (rebaudioside A and stevioside) production and antioxidant activities in the tissue culture grown shoots of *Stevia rebaudiana* Bertoni.

5.1.2 Preparation of medium containing ZnO nanoparticles

The nodal segments of *Stevia rebaudiana* were placed into 25 mL of Murashige and Skoog (MS) medium containing 3% (w/v) sucrose without any plant growth regulators. Total six media treatments in Petri plates (90 ×15 mm) were prepared containing 0, 0.1, 1, 10, 100 or 1000 mgL^{-1} of ZnO nanoparticles. The pH of different media was adjusted to 5.7-5.8 and afterwards, 0.8% (w/v) plant agar was added for the solidification of media. Later on, the culture media were autoclaved for 15 min at 121 ˚C temperature and 1.06 kg cm^{-2} pressure.

5.1.3 Growth conditions of shoot organogenesis

The stock plants of *S. rebaudiana* were obtained, and their continuous multiplication in a growth room having 16 h photoperiod provided by cool-white fluorescent light of 35 µmol $m^{-1}s^{-1}$ irradiance. The temperature of the growth room was set to 24 ± 1 ˚C with an average relative humidity (55-60%).

The shoot nodes excised from four-week-old stock plants were incubated in different types of solid MS medium, i.e., provided with various concentrations of ZnO nanoparticles. This experiment was conducted in triplicate. Total 15 nodal explants (replicates) were used per treatment. After four weeks, various physiological parameters were recorded including mean percentage of shoot formation, mean length of shoots, mean number of leaves, and fresh weight of the shoots were determined.

5.1.4 Result and Discussion

5.1.4.1 Determination of growth parameters

Fig. 3 shows that the increasing concentration of ZnO nanoparticles in the growth of *S. rebaudiana* up to a certain threshold level, i.e., 1 mgL^{-1}, but once this level is reached, further increase of ZnO nanoparticlescause toxicity in *S. rebaudiana*.

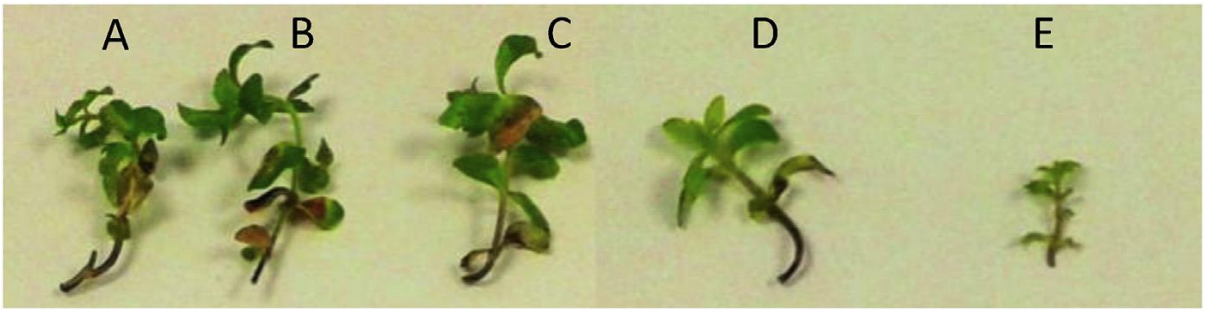

Fig.4: Effect of various concentrations of ZnO nanoparticles on the shoot formation in *Stevis rebaudiana*. Length of shoots, number of nodes and number of leaves formed in control medium (A), at a concentration of 0.1 ppm (B), concentration of 1.0 ppm (C), 10 ppm concentration (D), and at 1000 ppm concentration of ZnO nanoparticles (E).

Table 1 clearly indicates that the highest amount of shoot formation (~90%) occurred in MS medium supplemented with 1.0 mgL^{-1} of ZnO nanoparticles. However, the least shoot formation frequency (~31%) was found in MS medium containing 1000 mg L^{-1} ZnO nanoparticles. Maximum shoot length (4.6 cm) was obtained with MS medium augmented with 1.0 mgL^{-1} ZnO nanoparticles, followed by MS medium containing 0.1 or 10 mgL^{-1} ZnO nanoparticles (4.5 cm) and MS control medium (4.1 cm). Based on our data, the highest

number of nodes (5.4) and leaves (14.2) were produced by 1.0 mgL^{-1} ZnO nanoparticles treatment, whereas the lowest number of nodes (2.0) and leaves (5.45) developed in the treatments provided with 1000 mgL^{-1} ZnO nanoparticles. Fresh weight of shoots was also found to be highest in MS medium fortified with 1.0 mgL^{-1} ZnO nanoparticles (0.59 g) and lowest fresh weight (0.07 g) was observed in 1000 mgL^{-1} ZnO nanoparticles treatment

ZnO NP (mgL^{-1})	Per cent shoot formation	Mean shoot length (cm)	Mean no. of nodes	Mean no. of leaves	Fresh weight of shoot (g)
Control	85.7	4.1	4.2	10	0.16
0.1	85.4	4.5	4.4	13	0.44
1.0	89.4	4.6	5.4	14	0.59
10	85.5	4.5	3.6	13.8	0.36
100	50.5	3.6	3.3	12.3	0.27
1000	30.8	1.5	2.0	5.4	0.07

Table 2: Comparison of physiological parameters in *stevia rebaudiana* at different concentrations of ZnO nanoparticles.

5.1.4.2 Steviol glycosides estimation

Fig 3. shows that *S. rebaudiana* produces more steviol glycosides (rebaudioside A and stevioside) in the presence of ZnO nanoparticles up to 1 mgL^{-1}, thereafter, increasing concentrations gradually decreased the steviol glycosides. The amount of rebaudioside A enhanced from 2.07% in control MS treatment to 2.80% in MS treatment supplemented with 0.1 mgL^{-1} ZnO nanoparticles. Furthermore, rebaudioside A quantity obtained from shoots grown in MS medium containing 1.0 and 10 mgL ZnO nanoparticles was found to be 3.65 and 3.11%, respectively. At 100 mg L^{-1} ZnO, a drastic decrease in rebaudioside A amount (1.02%) was clearly observed. The decline in rebaudioside A quantity was more evident (0.14%) when shoots were allowed to grow in MS medium containing 1000 mgL^{-1} ZnO nanoparticles. Similarly, stevioside content was 0.73% in the control group, while it was 1.12% and 1.17% in treatments having 0.1 and 1.0 mgL^{-1} ZnO nanoparticles, respectively. A sudden decline (from 0.99 to 0.35%) occurred by increasing ZnO nanoparticles concentration from 10 to 100 mgL^{-1}. In addition, at the highest concentration (1000 mgL^{-1}), stevioside was not detected at all.

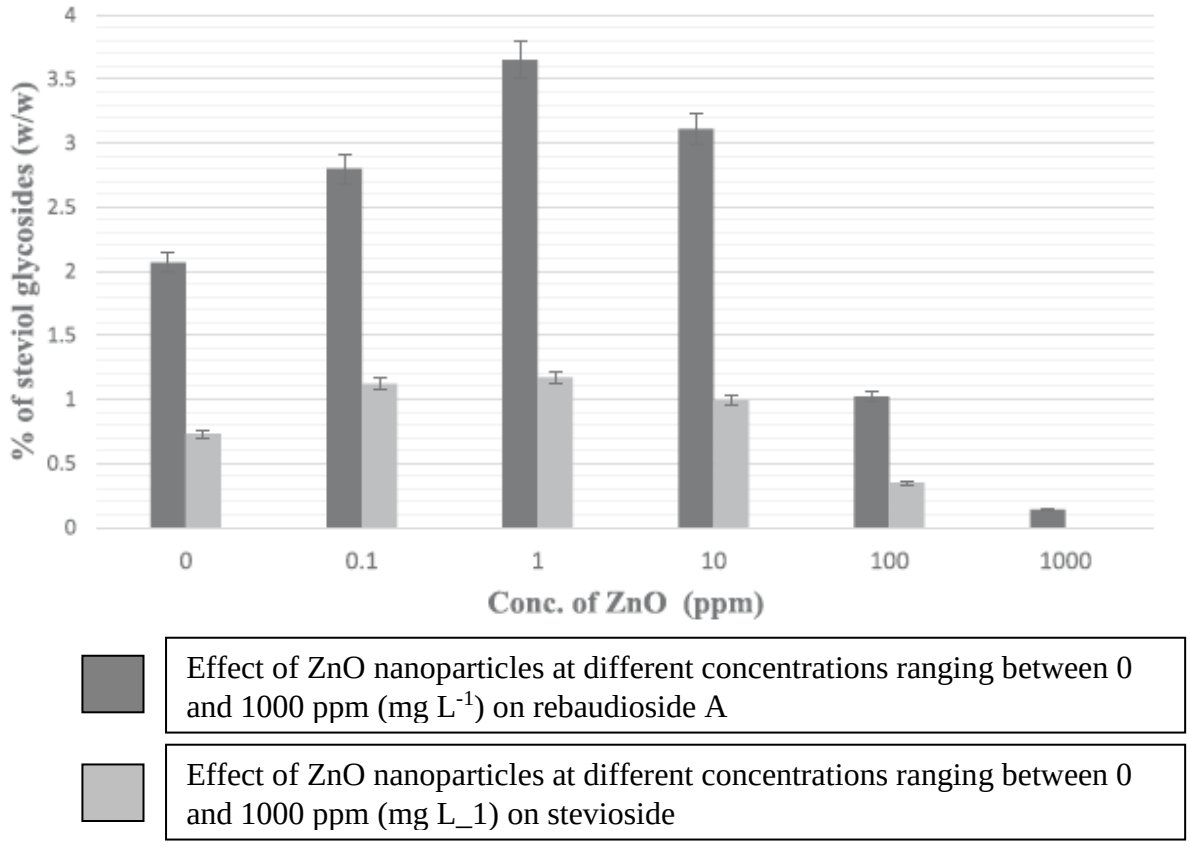

Fig. 5: Effect of ZnO nanoparticles on steviol glycoside production

5.1.4.3 Anti-oxidative activity:

In Table 2, MS medium containing 1 mgL^{-1} ZnO nanoparticles was the most productive in terms of total antioxidant (11.7 mg AAE mg_1) in 4 weeks old *S. rebaudiana* leaves. The lowest antioxidant activities were obtained from extracts containing 1000 mgL^{-1} ZnO employed in MS medium. Both MS media containing 100 and 1000 mgL^{-1} ZnO had lower total flavonoid and total phenolic contents, total antioxidant capacity as well as total reducing power than the control group.

ZnO NP (mgL^{-1})	Total antioxidant capacity (μg AAE mg^{-1})
Control	9.84
0.1	10.8
1.0	11.7
10	10.9
100	7.36
1000	4.62

Table 3: Comparison of total antioxidant capacity at different concentration of ZnO nanoparticles.

5.2 Case study - 2

Nano silver: a novel nanomaterial for removal of bacterial contaminants in valerian (*Valeriana officinalis* L.) tissue culture (Abdi *et al*., 2008)

5.2.1 Aim:

To evaluate the potential of nano silver (Ag) to remove bacterial contamination in *Valeriana officinalis* nodal explants.

5.2.2 Plant materials and culture conditions

Greenhouse grown valerian (*Valeriana officinalis* L.) mother plants were used in this study. These plants were tested by culturing their stem explants in potato dextrose agar (PDA) medium for internal contamination assay. The explants were first surface sterilized with 70% ethanol for 1 min and 10% Clorox (containing 5.25% sodium hypochlorite) for 1 min and then rinsed four times with sterilized distilled water. The cause of internal contamination was identified with special laboratory methods, in the Department of Plant Pathology, Shiraz University, as Xanthomonas genus. After testing, the mother plants were divided into two groups: with internal contamination (group 1) and without internal contamination (group 2). For group 1, a number of 20–25 cm stems were cut and transferred to the laboratory immediately. They were cut to the length of about 0.5–1 cm and prewashed in water supplemented with 10 drops of a weak household detergent solution for 10 min and then placed under running tap water for at least 30 min. Nano silver solution (NS) at different concentrations (25, 50 and 100 mgL^{-1}) and exposure times (30, 60, 180, 300, 600 and 1,200 min) was used at two stages; before and after surface sterilization along with the control.

Initial experiment showed that in high exposure times (300, 600 and 1,200 min) explants turned to bleach. Therefore, high exposure time's results were omitted.

For the treatment without surface sterilization, after prewashing in water and keeping under running tap water, nodal segments were dipped at appropriate times and concentrations of NS solution. After this treatment, the explants were rinsed four times with sterilized distilled water. For the treatment before surface sterilization, after dipping explants in NS solution, the explants were surface sterilized (as mentioned above).

For the treatment after surface sterilization, the explants were rinsed with sterilized distilled water and dipped in NS solution with appropriate concentrations at different times. After recut, the sterilized explants were dipped in NS solution before being transferred to the culture vessels. After sterilization, about 1 cm single node explants were cultured on a modified MS medium containing salts, organic constituents, 30 g l-1 sucrose, 8 gL^{-1} agar and 5 mgL^{-1} Kin and 0.1 mgL^{-1} NA. The pH of media was adjusted to 5.8 by 0.1 N HCl before autoclaving for 15 min at 121˚C and 1.5 kg cm^{-2} pressure. Cultures were kept under a 16 h

photoperiod of 30 mm $m^{-2}s^{-1}$ light intensity emitted by two cool white fluorescent lamps at 25 ± 3_C. Explants in group 2 were just prewashed, surface sterilized and cultured without NS treatment. All the culturalconditions in this group were similar to those in group 1.

5.2.3 Results

Cultures subjected to NS solution treatment before surface sterilization showed low percentage of disinfected valerian explants (Table 4). In all the treatments, the percentage of fungal contamination was zero. Among the treatments, highest percentages of disinfection (32%) were observed when the explants were dipped in 100 mgL^{-1} NS solution for 180 min. Using NS solution after surface sterilization was successful. Treatment with 100 mgL^{-1} of NS for 180 min after rinsing the explants in sterilized distilled water was the most successful disinfection treatment. This treatment had significant differences with other treatments. The 11% contamination left after this treatment was bacterial contamination (data not shown). Also, this treatment did not have any negative impact on measured characters in micropropagation of valerian in four subsequent subcultures.

Treatment (time and concentration)	Before surface sterilization contamination per cent	After surface sterilization contamination per cent
control	98	99
30 min.		
25 mgL^{-1}	97	90
50 mgL^{-1}	97	91
100 mgL^{-1}	96	82
60 min.		
25 mgL^{-1}	95	80
50 mgL^{-1}	94	71
100 mgL^{-1}	88	63
180 min.		
25 mgL^{-1}	78	32
50 mgL^{-1}	73	28
100 mgL^{-1}	68	11

Table 4: Comparisons among different treatments used for disinfection of *Valeriana officinalis* L. single node explants 3 weeks after culturing on the medium

5.3 Case study - 3

Cytotoxic, Genotoxic, and Polymorphism Effects on *Vanilla planifolia* Jacks ex Andrews after Long-Term Exposure to Argovit® Silver Nanoparticles (Bello-Bello *et al.,* 2018)

5.3.1 Aim: To study the cytotoxic and genotoxic effect of AgNPs in *Vanilla planifolia* plantlets after very long exposure time of six weeks.

5.3.2 *In Vitro* Establishment and culture conditions

Stems of 20 cm length were cut from young *V. planifolia* plants kept under greenhouse conditions. The leaves were removed, and 2 cm length nodal segments were cut off for use as explants.The were surface sterilized and cultured in 2.2 - 15 cm test tubes with 15 mL MS medium supplemented with 3 gL^{-1} of sucrose without growth regulators. Culture medium pH was adjusted with 0.1 N sodium hydroxide until pH = 5.8. 0.25% (w/v), phytagel was added as a gelling agent, and then it was autoclaved for 15 min at 120 ˚C. The explants were incubated at 24 - 2 ˚C, 16 h light photoperiod with 40 µmol m^{-2} s^{-1}. After two subcultures of four weeks each, 2 cm length shoots were used for different treatments with AgNPs (0, 25, 50, 100 and 200 mgL^{-1}).

5.3.3 Effects of AgNPs on *Vanilla planifolia* physiological parameters

Administration of silver nanoparticles to *Vanilla planifolia in vitro* culture show a dose-dependent effect in different growth parameters such as shoot length and the number and length of roots. The shoot length, the number of leaves, and the number and length of the roots of plants exposed to 25 and 50 mgL^{-1} of AgNPs (1.5 and 3.0 mgL^{-1} of metallic silver) show no significant differences compared with the untreated plants used as a negative control group. On the other hand, the plants exposed to AgNPs concentrations of 100 and 200 mgL^{-1} (6 and 12 mgL^{-1} of metallic silver) showed an important decrease in number and length of roots, clearly appreciable by the naked eye as shown in Fig. 4.

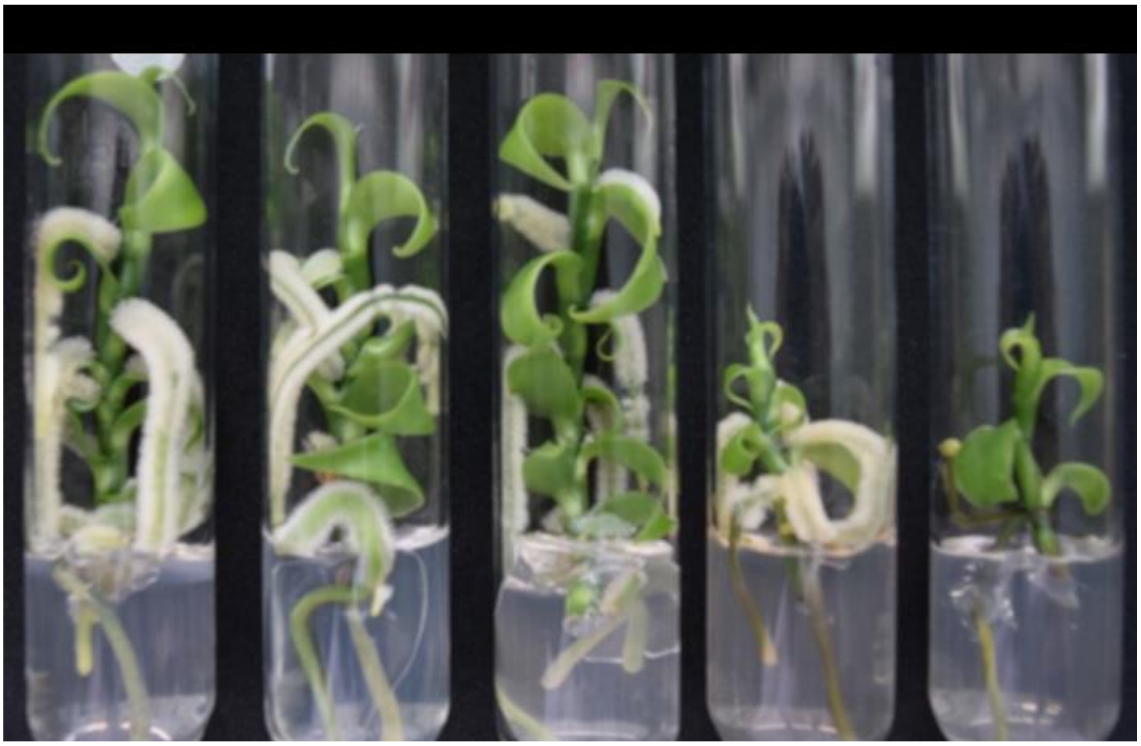

Fig.6: Effect of AgNPs on *in vitro* elongation and rooting of *V. planifolia* after six weeks of *in vitro* culture. From left to right 0, 25, 50, 100, and 200 mgL^{-1} of AgNPs (0, 1.5, 3, 6, and 12 mgL^{-1} of metallic silver).

5.3.4 Cytotoxic and genotoxic effectsof AgNPs

The lower concentrations of silver nanoparticles (25 and 50 mgL^{-1}) do not generate significant damage in the genetic material neither as cells with bridges (CB), chromosomal fragments (CF), binucleated cells (BN) or micronuclei (MN) compared with the control group, but an increase in the frequency of CB, CF, and BN were observed with the increase of silver nanoparticles concentrations without the presence of MN.

On the other hand, the higher concentrations of AgNPs, 100 and 200 mgL^{-1} (6 and 12 mg of metallic silver), administered to *Vanilla planifolia* continue with the observed tendency in the lower concentrations, increase aberrations in nuclear material as the concentration of AgNPs increase. The frequency of micronuclei registered was 1.5 and 3 with the exposure to 100 mgL^{-1} (6 mgL^{-1} of metallic silver) and 200 mgL^{-1} (12 mgL^{-1} of metallic silver), respectively. This study that reports the genotoxicity on *Vanilla planifolia* Jacks. using the micronuclei test. Figure 5 shows the chromosomal aberrations found in root tip cells of *Vanilla planifolia* exposed to AgNPs 200 mgL^{-1} (12 mgL^{-1} of metallic silver).

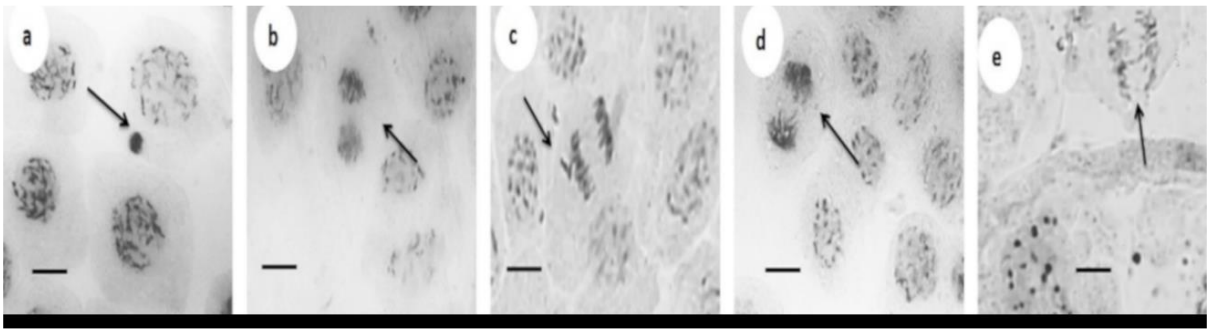

Fig.7: Chromosomal aberration and nuclear aberrations observed in root tips cells of *V. planifolia* at 200 mgL^{-1} of AgNPs after six weeks of *in vitro* culture. (**a**) Cell with micronucleus; (**b**) binucleated cell, (**c**) cell in anaphase with a chromosomal fragment, (**d**) cell in telophase with laggard, and (**e**) cell in anaphase with a bridge. Arrows indicate the produced damage in each case.

5.3.5 Effect of AgNPs on somaclonal variation.

To know the capability of AgNPs formulation studied in the present work to induce somaclonal variation on *Vanilla planifolia* plantlets, an inter-simple sequence repeat (ISSR) analysis was performed. Figure 6 shows the band pattern amplified with UBC (University of British Columbia) primer UBC-825. Exposure to AgNPs increases the polymorphism of *Vanilla planifolia* plantlets. Somaclonal variation was found in all treatments, showing a dose-dependent behavior. Plantlets without exposure to AgNPs showed a polymorphism of 15.28%. The polymorphism increases as AgNPs concentration does. The percentage of polymorphism observed was 18.06, 20.83, 23.61, and 25% for plantlets exposed to 25, 50, 100, and 200 mgL^{-1} of AgNPs for six weeks, respectively.

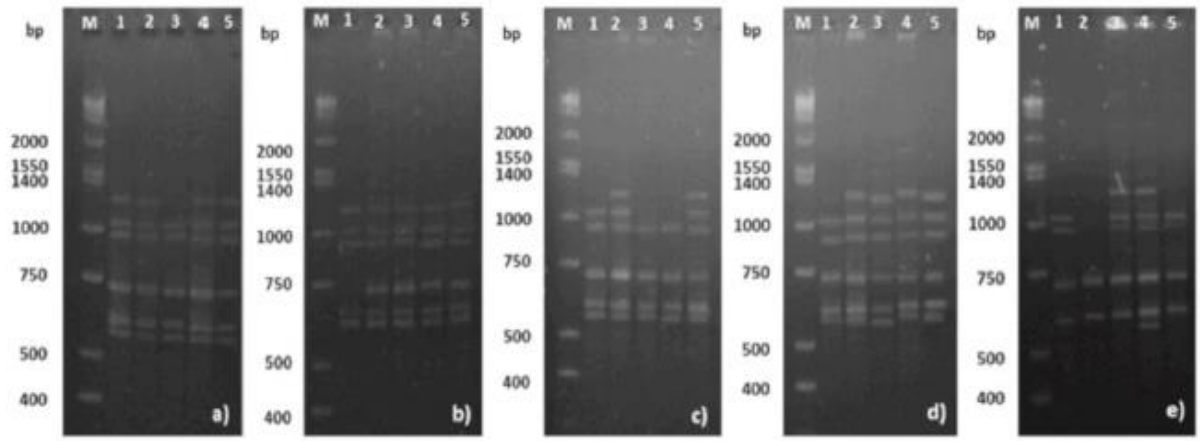

Fig.8: Electrophoresis pattern of ISSR banding profiles of five plants (1–5) of *V. planifolia* exposed to AgNPs for six weeks on *in vitro* culture. The amplification for UBC 825 primer corresponding to (**a**–**e**) 0, 25, 50, 100, and 200 mgL^{-1} of AgNPs, respectively. M = molecular mass marker 1 kbp plus DNA ladder; bp = base pairs.

6. Studies to reduce toxicity of Nanoparticles

More stringent dose-dependent studies are needed to identify the ideally safe NP doses that stay within the positive impacts on plant growth with lesser negative impacts on the environment as well as plants (Kim et al., 2017). Thus, a series of safety evaluation and toxicological risk assessment standards should be formulated, including exposure route and safe exposure doses (Ruttykay-Nedecky et al., 2017) and meticulously applied for plant nanotechnology. The whole life cycle of the NPs should be evaluated. Bioaccumulation, penetration, and translocation of NPs in plants should be evaluated in detail. This being an inter-disciplinary area of research, appropriate awareness of the positive and negative aspects of nanoparticles should be provided to the user.

7. Nanotechnological regulations in India

Realising the emerging importance of safe handling of nanomaterials, the Nano Mission of Govt. of India constituted a Nanoregulatory Task Force consisting of eminent experts to bring out a comprehensive document on “Guidelines and Best Practices for Safe Handling of Nanomaterials in Research Laboratories and Industries”. Under the able guidance of Nano Mission and Nanoregulatory Task Force, Centre For Knowledge Management of Nanoscience and Technology (CKMNT) has brought out this comprehensive document based on published reports by regulatory bodies like Internation Organisation for

Standardization (ISO), Occupational Safety and Health Administration (OSHA), National Institute for Occuppational Safety and Health (NIOSH) and other reports available in public domain.

8. Overveiw of primary regulatory bodies in India (Maclurcan and Radywyl, 2011)

The Ministry of Health is involved in the regulation of nanotechnology applications through its Directorate General of Health Services, under which the Central Drugs Standard Control Organisation (CDSCO) is situated. The CDSCO is responsible for the approval of various drugs and establishing standards, but the implementation takes place at the levels of states and union territories. There are thirty-five state drug controllers (SDCs), which have the primary responsibility of overseeing the regulation, manufacture, sale, and distribution (including licensing) of drugs.

Another authority, although statutory, is administratively based under Director General of Health Services, operating under the MoHFW. It is the Food Safety and Standards Authority, which was recently established to lay down science-based standards for its manufacture, storage, distribution, sale, and importation to ensure the availability of safe and wholesome food for human consumption.

The Ministry of Environment and Forests (MoEF) deals with environmental impacts or hazards that may emanate from new applications of existing or new chemicals and substances. The Central Pollution Control Board (CPCB) discharges most of the functions relating to the prevention and control of pollution, including through hazardous materials. The State Pollution Control Boards (SPCBs) are the state-level authorities under the 1986

Environment Protection Act (EPA). The SPCBs do not look at nanotechnology applications or health applications, but any commercial establishment or manufacturing process will have to adhere to standards laid down by the EPA and Hazardous Materials

Rules, thereby bringing them under supervision of SPCBs.

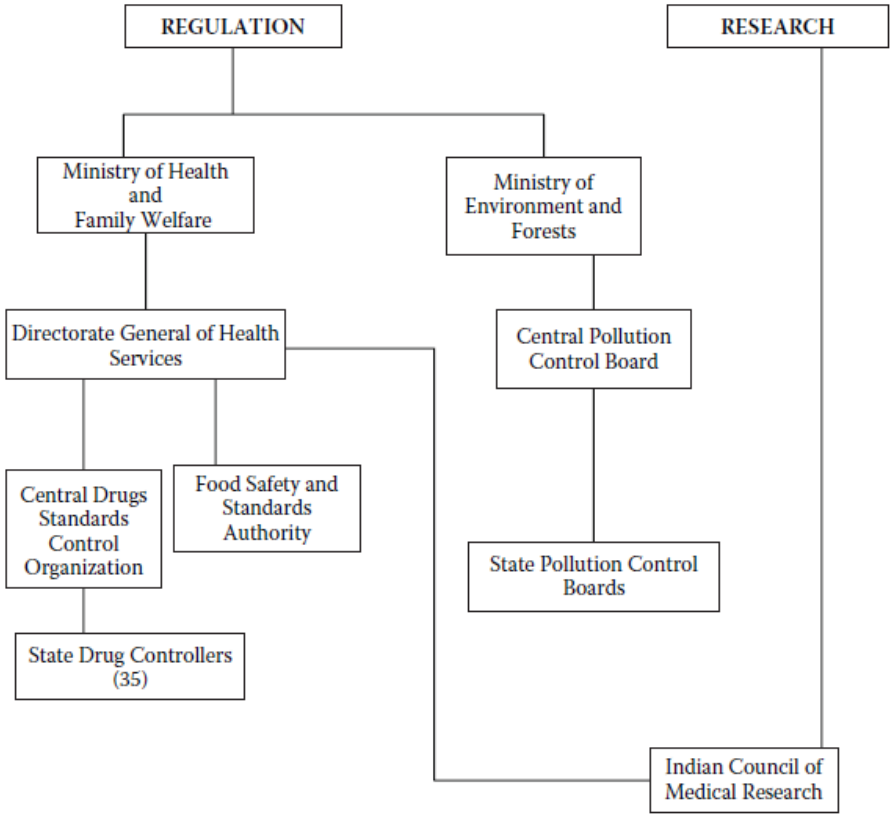

Fig.9: Overview of primary regulatory actors in India.

9. Summary

Nanoparticle is a particle with at least one external dimension ranging between 1 - 100 nm. A wide range of nanoparticles such as silver (Ag), aluminum oxide (Al_2O_3), cupric oxide (CuO), iron oxide (Fe_3O_4), gold (Au), magnesium oxide (MgO), nickel (Ni), silicon (Si), silicon dioxide (SiO_2), titanium dioxide (TiO_2) and zinc oxide (ZnO) have been reported to possess antimicrobial activities against various microorganisms. The application of NPs in plant tissue culture has been reported for the elimination of microbial contamination in plant cultures, callus induction, organogenesis, somatic embryogenesis, somaclonal variation and secondary metabolite production. Studies to be done to reduce nanotoxicity and nanotechnological regulation in India are also covered.

10. Future thrust

- Use of new generation NPs like graphene, quantum dots, dendrimer *etc.*
- More focused research use of nanoparticles in different aspects of plant tissue culture.

11. Discussion

1) Nanoparticles have antimicrobial activity and also have the ability to cause somaclonal variation. So will it affect the genetic makeup of an *in vitro* generated plants, when it is used for decontamination?

Ans: Nanoparticles will not affect the genetic makeup plant when the exposure time is low. To cause somaclonal variation plants are exposed to nanoparticles at high concentration for long time mostly few weeks while for decontamination exposure time is only few hours.

2) Is use of nanoparticles in plant tissue culture cost effective?

Ans: The cost effectiveness of use nanoparticles in plant tissue culture depends on the type and amount of nanoparticles used. In most of the studies the amount nanoparticle used is very less so, it can be used cost effectively.

3) Any work related to use of nanoparticles in plant tissue culture is done in India?

Ans: Yes, many paper related to the use nanoparticles in plant tissue culture was seen published from India.

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MBB 591: Master's Seminar

Name : Rasha Fathima A. A. Venue : Seminar Hall

Admission No. : 2018-11-005 Date : 20-12-2019

Major Advisor : Dr. M. R. Shylaja Time : 10.00 am

Nanoparticles in plant tissue culture

Abstract

Plant tissue culture is the core of plant biology, which is important for mass propagation, conservation, genetic manipulation and bioactive compound production. The success of *in vitro* plant culture depends on several factors such as genotype, the physiological status of the donor plants, the type of explants, surface disinfection methods, the culture medium, plant growth regulators, the size of the culture vessels, spectral quality, light intensity, photoperiod and temperature. In recent years, the application of nanoparticles has successfully led to the elimination of microbial contaminants from explants and demonstrated the positive role of nanoparticles in callus induction, organogenesis, somatic embryogenesis, somaclonal variation and secondary metabolite production (Kim *et al.*,2017)

Nanoparticle is a particle with at least one external dimension ranging between 1 - 100 nm. A wide range of nanoparticles such as silver (Ag), aluminum oxide (Al_2O_3), cupric oxide (CuO), iron oxide (Fe_3O_4), gold (Au), magnesium oxide (MgO), nickel (Ni), silicon (Si), silicon dioxide (SiO_2), titanium dioxide (TiO_2) and zinc oxide (ZnO) have been reported to possess antimicrobial activities against various microorganisms (Wang *et al*., 2017). Abdi *et al*. (2008) employed Ag nanoparticles for the first time to control bacterial contamination in *Valeriana officinalis* and obtained 89 per cent contamination free culture.

Bello-Bello *et al.* (2018) studied the cytotoxic and genotoxic effects of nanoparticles on *Vanilla planifolia* and identified safe concentration to induce variation. At higher concentrations nanoparticles release Reactive Oxygen Species (ROS), which affect the genetic integrity. The addition of nanoparticles to a plant tissue culture media affects callus proliferation, shoot multiplication, somatic embryogenesis and rooting by altering antioxidant enzyme activities, gene expression, inhibition of ethylene production and production of ROS (Kim *et al*., 2017). Nanoparticles added to the plant tissue culture medium act as a nutrient source as well as an elicitor. Raei *et al*. (2014) reported that *Aloe vera* suspension cells interacting with Ag or TiO_2 nanoparticles for 48 h. exhibited a significantly increased aloin

content. Javed *et al.* (2016) reported that when the *in vitro* culture of *stevia rebaudiana* was supplemented with 1 mgL^{-1} of Zno nanoparticles, it showed increased shoot formation and steviol glycoside production.

Realising the importance of safe handling of nanomaterials, the Nano Mission, Government of India constituted a Nanoregulatory Task Force consisting of eminent experts to bring out a comprehensive document on "Guidelines and Best Practices for Safe Handling of Nanomaterials in Research Laboratories and Industries".

Application of nanoparticles in plant tissue culture is emerging as a prominent and promising field with excellent potential towards plant improvement. However, more targeted research is required to clarify and streamline the use of nanoparticles to harness the beneficial effects.

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