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Casein Stabilized Metal and Metal Oxide Nanoparticles for the Efficient *In Vitro* Culturing of *Scoparia dulcis* L.

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Abstract. Unique physicochemical properties of nanoparticles make them a novel candidate in agriculture and related areas. In the present work, we have studied the effect of casein stabilized metal and metal oxide nanoparticles, viz. AgNPs, AuNPs, and CuONPs, on *in vitro* propagation of *Scoparia dulcis* L. We have examined the effect of these nanoparticles on the callus induction, shoot regeneration, and root regeneration capacity. It was observed that Ag, Au, and CuO nanoparticles had interacted with the tissues differently and produced calluses with difference in color, nature, and texture. Nanoparticles also affected shoot regeneration, root regeneration, phenolic compound production, and chlorophyll content. Explants showed a very good response when treated with CuONPs. The formed callus underwent shoot and root regeneration without changing the medium.

Keywords: AgNPs, AuNPs, CuONPs, *Scoparia dulcis*, callus induction.

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Использование стабилизированных казеином наночастиц металлов и оксидов металлов для эффективного культивирования *in vitro Scoparia dulcis* L.

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Аннотация. Уникальные физико-химические свойства наночастиц делают их перспективными кандидатами для использования в сельском хозяйстве и смежных областях. В настоящей работе исследовано влияние стабилизированных казеином наночастиц металлов и оксидов металлов (AgNP, AuNP и CuONP) на рост *in vitro Scoparia dulcis* L. Исследовано влияние этих наночастиц на индукцию каллуса, регенерацию побегов и корней. Показано, что наночастицы Ag, Au и CuO по-разному взаимодействовали с тканями и образовавшиеся каллусы отличались по цвету, природе и текстуре. Наночастицы также оказывали влияние на регенерацию побегов и корней, продукцию фенольных соединений и содержание хлорофилла. Экспланты показали хороший отклик на применение наночастиц CuO. Образовавшиеся каллусы были способны к регенерации побегов и корней без смены среды.

Ключевые слова: наночастицы, Ag, Au, CuO, *Scoparia dulcis*, индукция каллуса.

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Introduction

Scoparia dulcis L. is an important plant, which is used as herbal medicine to treat various diseases like cancer, bronchitis, hypertension, etc. Most of these biological properties are attributed to the phytochemicals present in the plant. Because of the high potential of *S. dulcis* in traditional medicine, its large-scale production through tissue culture is very important (Annie, Jayachandran, 2008).

Establishment of the totipotency of plant tissue, differentiation of callus, and vegetative multiplication under controlled *in vitro* conditions have opened a novel approach in the plant science. Plant tissue culture is a versatile technique for the development of plantlets in laboratory under controlled conditions. The basis of the plant tissue culture is the ability of plant cells to regenerate into a whole plant. Plant tissue culture is mainly used for the large-scale production of rare and endangered

species. It can also be used to produce virus-free plants from the shoot tip culture of virus infected plants (Dagla, 2012).

Metal nanoparticles have their application in various fields including medicine because of their unique physicochemical properties. In agriculture, the nanoparticles are commonly used as fertilizers. Application of nanoparticles in tissue culture is very limited (Sarkar et al., 2012). In our study, we try to investigate the influence of synthesized metal and metal oxide nanoparticles (Ag, Au, and CuO NPs) on the callus formation in the tissue culture of *S. dulcis*.

Previous studies revealed that the plants could produce mineralized nanoparticles naturally and these are required for growth. The study of artificially engineered nanoparticles in plant growth and development is a novel field of research and yet to be explored. Large surface to volume ratio and electron exchange engineering ability help them form favorable interactions with biomolecules present inside the cell (Sharma et al., 2012). Nanoparticles have received tremendous attention as the material for the improvement of crop yield (Jain et al., 2018). Engineered nanoparticles are also used to deliver agrochemicals and nutrients in a controlled manner, which is essential for the growth improvement, facilitating efficient utilization of nutrients and disease resistance (Athanassiou et al., 2018).

Materials and Methods

Materials

S. dulcis, MS tissue culture medium, indole-3-butyric acid (IBA), butyric acid (BA), mercuric chloride, 70 % ethanol, NaOH, agar, distilled water, casein stabilized nanoparticles [silver (AgNPs), gold (AuNPs), and copper oxide (CuONPs)] were used for the study.

Synthesis of nanoparticles

All the three types of nanoparticles were synthesized by casein reduction method (Rakhimol et al., 2020). Casein was used as the reducing agent. 10ml 1M NaOH was prepared and stirred well. 0.1 g of casein and 0.01 g of precursors ($AgNO_3$, $AuCl_3$, and $CuSO_4$) were added, and the stirring was continued for 5 minutes. The solution was then incubated for 12 hours at room temperature. It was then centrifuged, and the supernatant was collected. Then, it was lyophilized and suspended in distilled water.

Characterization of nanoparticles

Nanoparticle synthesis was confirmed using UV–Vis spectroscopy, and morphology of the synthesized nanoparticles was analyzed using transmission electron microscopy.

Source of explants

Fresh, healthy, young twigs of *S. dulcis* were collected from Athirampuzha, Kottayam, Kerala and Kottarakkara, Kollam, Kerala. The plant herbarium was prepared and deposited to Regional Herbarium Kerala, Department of Botany, SB College, Changanassery; it acquired the accession number 7639.

Surface sterilization of the explants

Healthy twigs of *S. dulcis* were washed thoroughly under tap water for three to four times to remove the dust and dirt from the surfaces. Then, they were washed with 1 % soap solution for 1 minute and, finally, with water to remove the soap completely. After that, the twigs were surface sterilized using 0.1 % mercuric chloride for one minute. To remove the mercuric chloride from the surface, they were rinsed with double distilled water several times. All the glassware, forceps, and blade holders were cleaned and kept in hot air oven for preliminary sterilization followed by the sterilization under ultraviolet radiation for 20 minutes in the laminar air flow chamber.

The medium was sterilized in the autoclave and the growth hormones were added to the medium after sterilization. Hands were wiped with 70 % ethanol before the experiments (Ahloowalia et al., 2004).

Inoculation and incubation of the explants

After the surface sterilization, the twigs were transferred to a Petri dish containing filter paper, where the stems were cut into 1 cm long explants and transferred into the tubes with sterile medium containing growth hormones, using sterile forceps. The tubes were then sealed with cotton plugs and labeled. The tubes with explants were then incubated under cool white fluorescent light (1000 lux) (Annie, Jayachandran, 2008).

The phytohormones, auxin and cytokinin, were used separately and in combination for the callus, shoot, and root regeneration. Various combinations of IBA and BA were used to identify the combination of phytohormone that gave the best result. During the experiment, time taken for the callus initiation, percentage of response, and nature, color, and texture of callus were determined. The experiment was carried out with ten replicates. Fresh growth index (FGI), callus frequency, and callus index were also calculated (Kittipongpatana et al., 1998) using the following equations.

$$\text{Fresh Growth Index (FGI)} = \frac{\text{Final fresh wt} - \text{initial fresh wt}}{\text{Initial fresh wt}}$$

Callus index = Fresh growth index ×% of response

Subculture

The calluses were cut in sterile environment, sub cultured in the same medium for callus multiplication, and sub cultured in higher concentrations of IBA and BA (10–15 mg/L) for the shoot and root regenerations through indirect organogenesis.

Treatment of tissue culture medium with metal and metal oxide nanoparticles

Effects of Ag, Au, and CuO NPs in tissue culture of *S. dulcis* were determined by adding different concentrations of nanoparticles (2, 4, 6, 8, and 10 mg/L) into the tissue culture medium containing the optimum concentrations of IBA and BA for callus induction. Time taken for the callus initiation, percentage of response, nature, color and texture of callus, FGI, and callus index were determined, and the values were compared with control (callus in medium without nanoparticles). Endophytic contamination in the tissue culture medium was also detected in the nanoparticle-treated and non-treated medium within two weeks of inoculation (Khodakovskaya et al., 2013).

Statistical analysis

The values expressed are mean ±standard error. ANOVA (analysis of variance) and SPSS (statistical package for the social sciences) 10th edition software were used for the analysis of data.

Results and discussion

Even though the same casein based reduction method was used for the synthesis of AgNPs, AuNPs, and CuONPs, the three types of nanoparticles formed differed in size and shape. AgNPs and AuNPs had a spherical shape with 13.5 and 3.5 nm diameters, respectively. CuONPs were spindle shaped with 25 nm thickness (Fig. 1). Different concentrations of auxins and cytokinins were used for the callus formation of explants. Callus formation showed a good response to IBA and BA concentration of 4 mg/L, which was used in all subsequent experiments (Table 1).

The tissue culture media (MS media) containing optimum concentrations of hormones for callus formation were supplemented with different concentrations, viz. 2 mg/L, 4 mg/L,

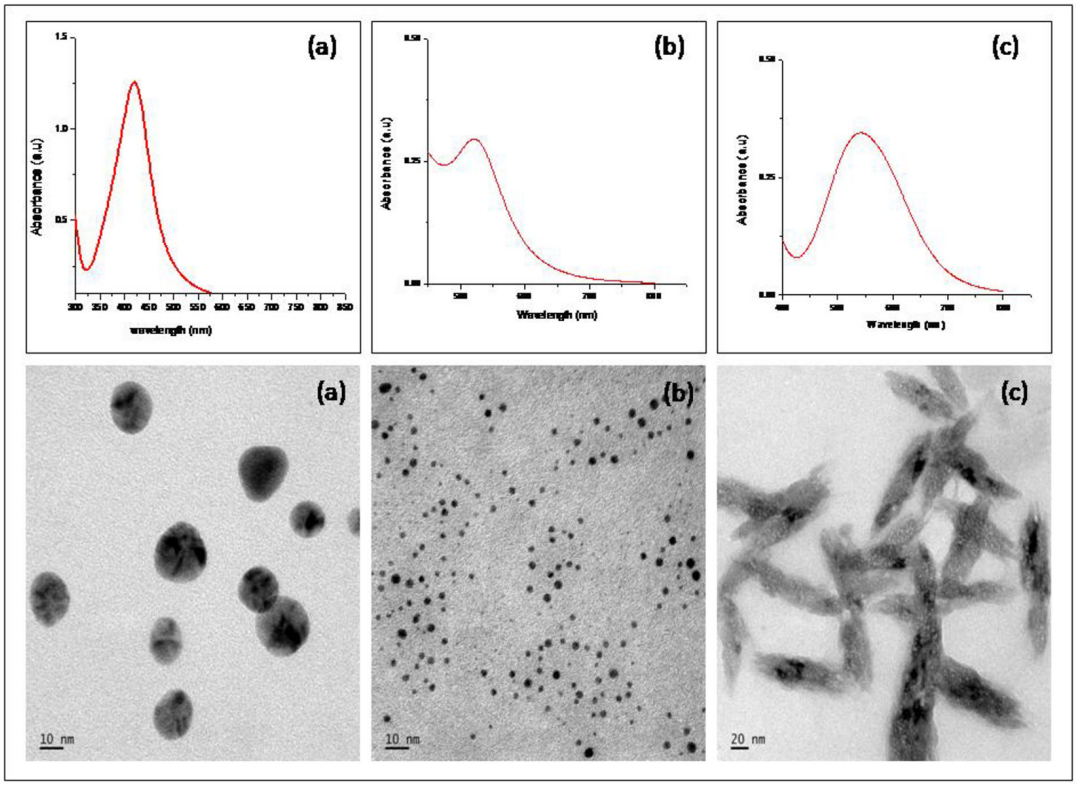

Fig. 1. UV-vis spectroscopy and TEM images of Ag NPs (a), Au NPs (b), and CuO NPs (c)

Table 1. Effect of IBA/BA on callus induction of *Scoparia dulcis* L. (CD value 5, $p<0.01$)

IBA (mg/L)	BA (mg/L)	Minimum no. of days for callus induction	% of response	FGI	Callus index	Nature of callus
1	1	20	50	9.076	453.8	Brownish green, soft, friable
1.5	1.5	20	50	26.111	1305.5	Brownish green, soft, friable
2	2	17	62	21.062	1305.8	Brownish green, soft, friable
2.5	2.5	14	60	31.666	1899.9	Brownish green, soft, friable
3	3	11	76	43.428	3300.5	Brownish green, soft, friable
3.5	3.5	11	93	44.000	4092.0	Brownish green, soft, friable
4	4	7	100	52.529	5252.9	Brownish green, soft, friable
4.5	4.5	9	100	39.309	3930.9	Brownish green, soft, friable
5	5	9	100	25.857	2585.7	Brownish green, soft, friable

and 6 mg/L, of Ag, Au and CuO nanoparticles and were kept for 30 days of incubation. The nanoparticles played a major role in the formation of callus, affecting the nature, color, and texture of callus. Of the three concentrations of nanoparticles, the 4 mg/L concentration was found to give the best results (Table 2).

The addition of the AgNPs to the tissue culture medium resulted in the formation of small, creamy, yellow callus around the explants. The callus formed was not very friable or compact. AuNPs lead to the formation of brown friable callus. However, the treatment with CuONPs resulted in the formation of embryogenic callus, which was compact and whitish green (Table 3, Fig. 2).

We found that after 45 days of incubation, the callus treated with 4 mg/L CuONPs started to form micro shoots from the embryogenic callus. It was an advantage, as the callus did not need to be transferred to a shooting medium for regeneration. 4 mg/L concentration of Ag, Au, and CuO NP showed the optimum influence on callus initiation. Callus showed a delayed and weaker response to 2 mg/L concentration of nanoparticles. At that concentration of nanoparticles, the chance of contamination was higher compared to other concentrations. The contamination was reduced as the concentration of nanoparticles was increased. However, higher concentration of nanoparticles reduced callus regeneration capacity.

Incubation of callus in the medium containing 4 mg/L of Ag, Au, and CuONPs showed that the CuONPs accelerated the shoot regeneration process, and multiple shoots were regenerated on

Table 2. Effect of Ag NPs, Au NPs, and CuO NPs on the callus formation

Nanoparticles (mg/L)		IBA/BA (mg/L)	Minimum number of days for callus induction	% of response
AgNPs	2	4/4	7	94
	4	4/4	7	100
	6	4/4	7	96
AuNPs	2	4/4	9	91
	4	4/4	7	100
	6	4/4	7	99
CuONPs	2	4/4	8	100
	4	4/4	7	100
	6	4/4	7	98

Table 3. Difference in color, texture, and nature of the callus after the treatment with nanoparticles

Treatment	Texture of callus	Color of callus	Nature of callus
Control (without nanoparticles)	Friable	Brownish green	Soft
AgNPs	Moderately friable	Creamy yellow	Soft
AuNPs	Friable	Brown	Soft
CuONPs	Compact	Whitish green	Hard

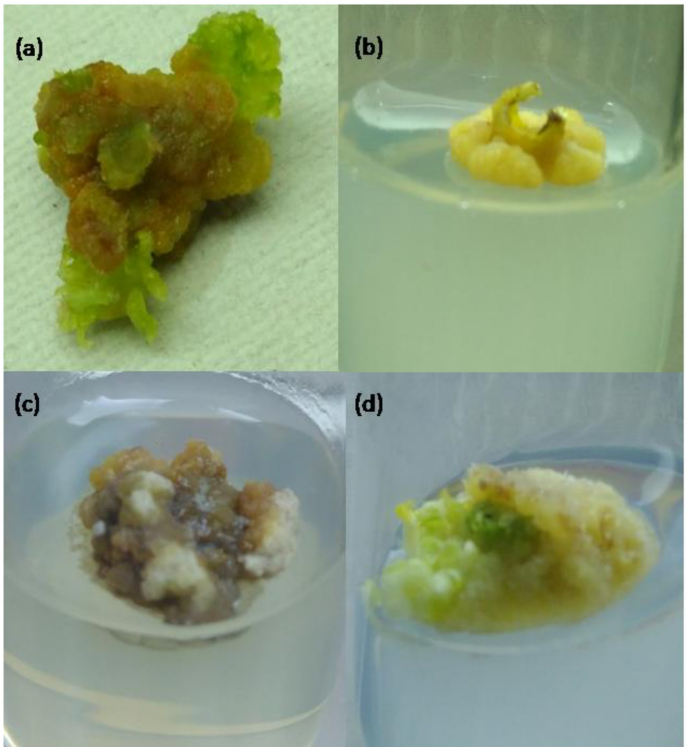

Fig. 2. Effect of Ag, Au, and CuO NPs on callus regeneration of *Scoparia dulcis* L. (a) callus formed in the medium with 4 mg/L IBA and 4 mg/L BA (control). (b) callus formed in the medium treated with AgNPs, (c) callus in the medium treated with AuNPs, (d) callus in the medium treated with CuONPs

the callus after 45 days of incubation. Initiation of root regeneration could be observed after 50 days of incubation on the same medium. 4 mg/L AgNPs also induced regeneration of shoots after 45 days of incubation. But only 2 or 3 shoots were regenerated and the shoots were not healthy. Root regeneration did not occur in these calluses. However, the callus treated with 4 mg/L Au NPs did not show any change even after 60 days of incubation (Fig. 3). It was observed that young leaflets formed from the calluses treated with both AgNPs and CuONPs showed a reduction in green color as compared to the untreated plantlets (Fig. 4). It might be due to the reduction in chlorophyll content of the nanoparticle-treated calluses. But as the plantlets were growing, they turned green. These observations suggested that the nanoparticle treatment could reduce the chlorophyll content of younger leaves only (Fig. 5).

We examined the effect of higher concentrations (8 & 10 mg/L) of synthesized nanoparticles on callus formation of *S. dulcis* explants. We found that the callus formation was low compared to 4 and 6 mg/L concentrations of nanoparticles. After 25 days of incubation, regeneration of callus stopped and blackening of callus occurred (Fig. 6). It might be because of the effect of nanoparticles on cellular metabolism of the callus.

In our study, we observed that casein stabilized metal and metal oxide nanoparticles could tune the color, texture, and nature of callus. There were many studies addressing the effect of Ag and Au NPs on seed germination (El-Temsah, Joner, 2012; Arora et al., 2012; Kumar et al., 2013) but no study was devoted to the effect of these nanoparticles on the regeneration of calluses and their morphology. The mechanism behind

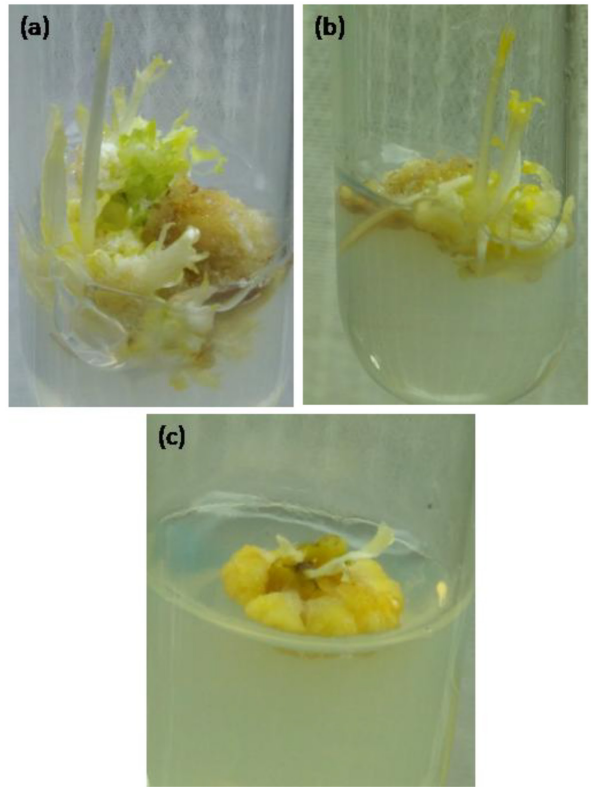

Fig. 3. Shoot and root regeneration of callus treated with nanoparticles. (a) multiple shoot regeneration of the callus treated with 4 mg/L CuONPs after 45 days of incubation, (b) root initiation of the shoots grown in the medium containing 4 mg/L CuONPs after 50 days of incubation, (c) shoot regeneration on the callus grown in medium containing 4 mg/L of AgNPs after 45 days of incubation

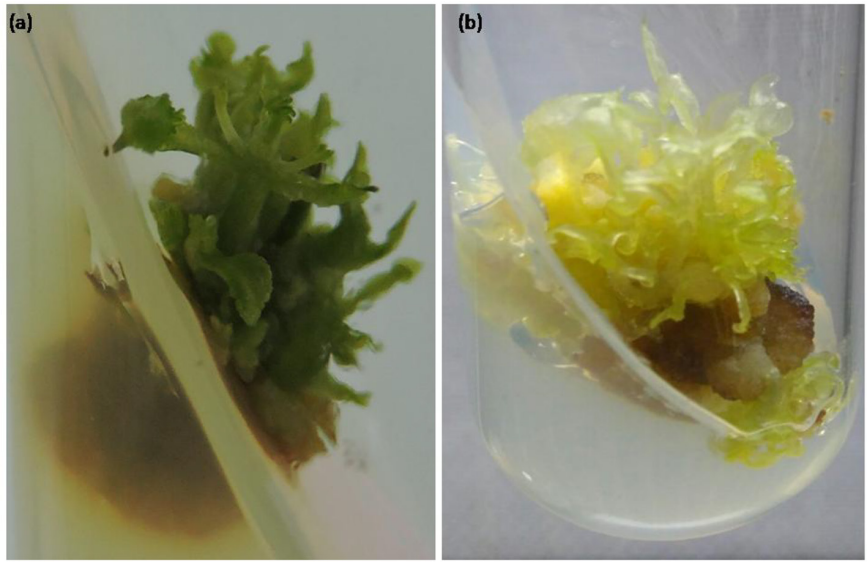

Fig. 4. Difference in the color of the shoots developed from (a) callus grown on medium without nanoparticles; (b) callus grown on medium with CuONPs

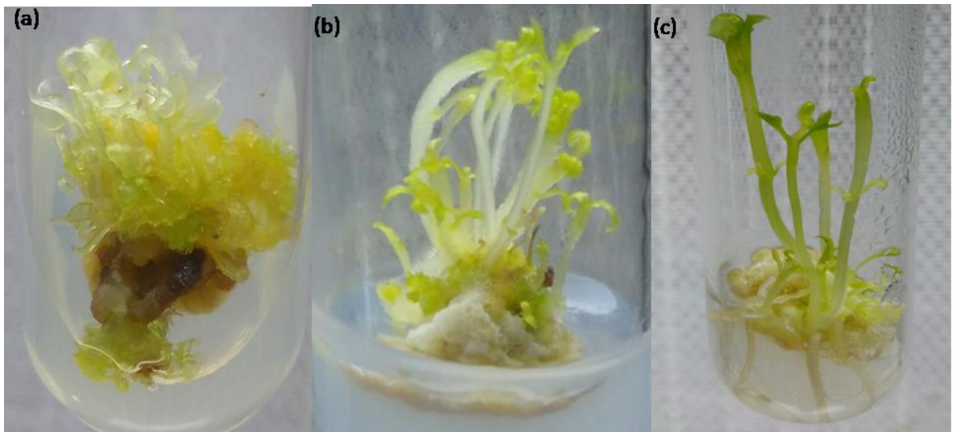

Fig. 5. (a) Reduction in the green color of plantlets grown on medium containing CuONPs (after 40 days); (b, c) Regaining of green color of the leaflet as it gets older (after 45 and 50 days, respectively)

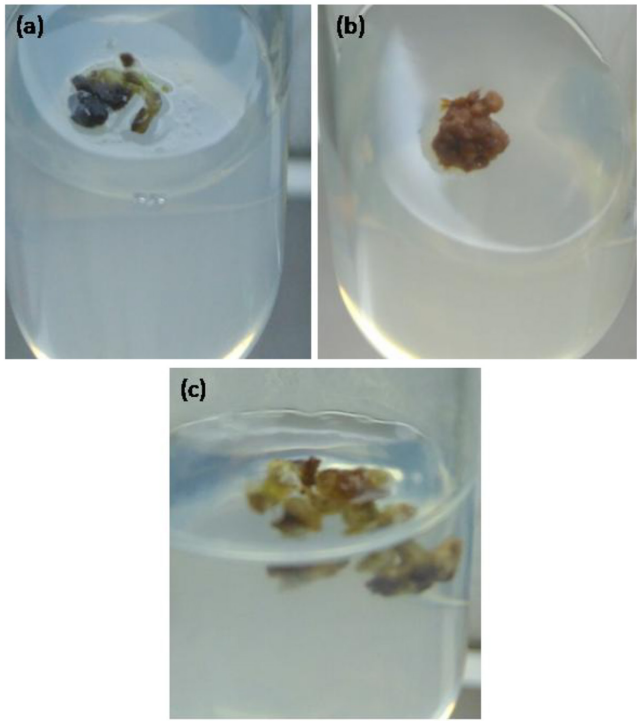

Fig. 6. Blackening of the callus grown on the medium containing 8 mg/L AgNPs (a), AuNPs (b), and CuONPs (c) after 15 days of incubation

the interaction of these metal nanoparticles with the plant tissue is unknown. Of these metal nanoparticles, CuONPs showed the greatest positive effect on the callus, shoot, and root regeneration.

Interaction of copper with plants was studied by many research teams, showing that lower concentration of copper was beneficial or essential for the growth of plants. But higher concentration of copper inhibited the growth and

metabolism of plants by the production of a large amount of oxygen free radicals, which interfered in the important cellular metabolic pathways. There was only a small difference between the minimum, optimum, and lethal concentrations of copper in the plants (Mocquot et al., 1996). Our results were also comparable with this. At 2 mg/L CuONPs, the callus formation was slow and less responsive. However, the treatment with 4 mg/L of CuONPs showed fast and productive results. At this concentration, we observed the formation of embryogenic callus within 30 days of incubation, shoot regeneration within 45 days of incubation, and root initiation within 50 days of incubation in the same medium. However, at a concentration above 4 mg/L, the response was delayed and blackening of the callus occurred at a concentration of CuONPs above 8 mg/L. Thus, the minimum concentration of the CuONPs for the response in the tissue culture medium was 2 mg/L, and the optimum and lethal concentrations were 4 mg/L and 8 mg/L, respectively. When compared with the shoots that developed in the standard tissue culture medium, the shoots grown on the medium containing CuONPs were less green. Earlier studies revealed that the chlorophyll pigment of the younger leaves may be affected by copper (Ouzounidou, 1994; Caspi et al., 1999; Zengin, Kirbag, 2007; Shakya et al., 2008; Ke et al., 2017). That might be the reason for reduction in green color in younger leaves. However, the increase in green color of the leaflets indicated that they could regain the chlorophyll content as they were growing. Higher concentration of copper had an inhibitory effect on the root elongation (Nair, Chung, 2015) but most of the studies revealed that lower concentration of copper had no effect on the rooting of shoots (Yuan et al., 2013). However, our observation clearly demonstrated that CuONPs had an important role in the rooting of the shoots by allowing the root formation without changing the auxin and cytokinin concentrations in the tissue culture medium. That was due to the unique physical and chemical properties of the CuONPs. Recent studies on Cu and CuO NPs showed an increase in the callus regeneration of the plants after the treatment with optimum concentrations (Abdel-Wahab et al., 2019; Ibrahim et al., 2019). Our findings also showed good agreement with these results. The size and shape of nanoparticles play a great role in their biological properties (Albanese et al., 2012). Spindle shape of the synthesized CuONPs may be also a reason for their excellent plantlet regeneration capacity.

The color of the callus grown on the nanoparticles changed because of the effect of nanoparticles on phenolic secretion of the callus. Studies revealed that at the higher phenolic secretion, the tissue will be browner, and the callus growth is inversely proportional to the phenolic secretion (Ozyigit, 2008; Reis et al., 2008). Our results showed a good agreement with these findings. Callus formed on the medium containing AuNPs was brownish in color with reduction in the regeneration capacity. Callus treated with CuONPs showed a very good regeneration capacity and it was whitish green in color.

All the three nanoparticles showed a dose and time dependent effect. A concentration of 4 mg/L was found to be the optimum concentration of the nanoparticles for their maximum effect. Above a particular concentration, all the nanoparticles showed a dose and time dependent toxicity on the callus. The higher concentration of nanoparticles allowed the explants to regenerate the callus for a limited period of time, but after that, they inhibited the growth and caused the blackening of the callus. Nanoparticles, along with their effect on callus formation, decreased the extent of contamination in the tissue culture medium. Explants on the tissue culture medium without nanoparticles were prone to fungal and bacterial contamination to a greater extent. Contamination

occurred after 10–15 days of inoculation of explants, confirming the presence of endophytic fungi and bacteria. However, treatment with all the three types of nanoparticles (AgNPs, AuNPs, and CuONPs) tremendously reduced the bacterial contamination, proving that the metal and metal oxide nanoparticles are also promising candidates to prevent the endophytic contamination.

Conclusion

The effect of nanoparticles on *in vitro* micro propagation of plants is a novel area of research. To the best of our knowledge, this is the first attempt to study the effect of nanoparticles on callus regeneration. Our study confirmed that the metal nanoparticles could affect the size, texture, and nature of callus. All the metal nanoparticles lead to the formation of calluses with different color, nature, and texture. Treatment of the tissue culture medium with CuONPs resulted in the shoot and root regeneration in the callus inducing medium. It will be also beneficial in the tissue culture of other plants. The action of nanoparticles depends on the size and shape of the nanoparticles used as well as the type of the plant. So, the mechanism of action of nanoparticles will vary with all these parameters. A systematic study is necessary to find out the mechanism of action of these nanoparticles on callus in the tissue culture medium.

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