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Short Communication

Investigation of ZnO Nanoparticles on *In Vitro* Cultures of Coffee (*Coffea Arabica L.*)

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Abstract

Tissue culture is a promising technique to produce a large number of true to type plants in coffee. One of the major obstacles encountered in in-vitro propagation is the high percentage of contamination of explants which is mainly observed when field grown plants are used as the source of explants. Several research studies were carried out to reduce the percentage of microbial contamination either using disinfectants during explant preparation or antifungal and anti-bacterial chemicals in media. The present paper elucidates the effect of ZnO Nanoparticles (ZnO-NPs) in reducing contamination and enhancing recovery of in vitro cultured leaf explants of arabica coffee (Coffea arabica). MS media containing ZnO-NP at three different concentrations were tested in an improved hybrid line of Coffea arabica (S.4595). Among the various concentrations tested, media containing 25mg/L of ZnO-NPs showed maximum recovery of explants. ZnO-NPs also positively influenced the induction of callus and somatic embryos which was significantly higher than the control.

Keywords: *Nanoparticles, Hybrid line, In vitro culture, Contamination, ZnO-NPs, Enhanced recovery.*

1. INTRODUCTION

Coffee is an important plantation crop of the tropics and is the beverage of choice among consumers across the globe. Coffee is produced in more than 80 countries and ranking second in international trade exchanges. Coffee belongs to genus *Coffea* which comprises of 124 species [1] of which only two species, *Coffea arabica* L (Arabica) and *Coffea canephora* Pierre ex A. Froehner (Robusta) are commercially cultivated.

Traditionally, coffee is propagated through seeds [2]. However, since the seed progenies tend to segregate due to possible recombination during meiosis, vegetative propagation is considered to be an alternative approach for large scale production of elite plants. In coffee, multiplication of elite F_1 Hybrid plant can be achieved through orthotropic sucker cuttings. However, the process is not only labour intensive, but also difficult to be practiced due to the non-availability of suitable suckers in large numbers from the elite plants. In this context, tissue culture provides an alternative to produce true to type elite plants in large numbers and in a short period [3].

Several explants such as young stem, leaf, root, anther, immature zygotic embryos, ovule etc. are suitable for somatic embryogenesis [4, 5, 6]. In coffee however, *in vitro* multiplication using leaf explants is considered to be advantageous due to the year-long availability of leaf material. *In vitro* propagation of coffee plants through somatic embryogenesis using leaf explants have long been optimized in various species [7,8]. Successful plant regeneration using other explants in coffee have also been reported [9,10].

Although the tissue culture protocol using leaf explants was standardised in coffee, establishment of *in vitro* culture using leaf explants from the field grown plants remains a considerable challenge. One of the major obstacles associated with field grown plants is the high percentage of microbial contamination of explants. The maximum loss of primary cultures was due to microbial contamination (both fungus and bacteria) which were present either on the surface or endogenously inside the explants [3, 11].

Although various antifungal and anti-bacterial chemicals are available for use in *in vitro* cultures, they were often detrimental to the growth and differentiation of cultured explants. In recent years various studies have proved the antimicrobial properties of nanoparticles. Nanoparticles have been used for surface disinfection and recovery of explants in tissue culture experiments of potato [12], barley [13], valerian [14], grape vine [15], olive [16], tobacco [17] and banana [18]. Among various nanoparticles, ZnO-NPs, in particular are found to improve callus growth and plant regeneration [19, 20] and in the production of contamination free cultures in Banana [18]. The antibacterial property of ZnO-NP was also proved in the studies of Pal et al., 2017 and Aquisman et al., 2020 wherein ZnO-NP was synthesized using plant extracts and tested for its anti-microbial properties [21, 22].

The present study was designed with an objective to test the efficacy of ZnO-NPs in controlling the growth of microorganisms and its effect on growth and differentiation of coffee leaf explants under *in vitro* conditions.

2. MATERIALS AND METHODS

2.1. Genotype

Three elite plants (14/10, 14/3, 14/8) belonging to the hybrid arabica genotype S. 4595 constitute the material of the present study. S. 4595 is an improved arabica hybrid line derived from the cross between S.2464 and Hibrido-de-Timor (HDT). The fully expanded but tender leaves close to the growing tip were used for explant preparation. Too tender and over matured leaves were avoided. Leaf explants were collected from 18 years old matured plant from the experimental plot of Central Coffee Research Institute (CCRI) during November 2016.

2.2. Explant Preparation

Freshly collected leaves were placed under running water for 15 to 20 minutes for washing and treated with 0.5% Bavistin for 10- 12 minutes and thoroughly rinsed with double distilled water. The leaves were then treated with 70% ethanol for 6-7 minutes and later thoroughly rinsed with distilled water following which the leaves were treated with 1% Sodium Hypo Chlorite (NaOCl) solution for 10 to 12 minutes and thoroughly rinsed with sterile double distilled water for a minimum of east 5-6 times. The leaves were cut into small squares of 0.5 cm x 0.5 cm in CA solution (Citric Acid: Ascorbic acid solution 1:1) in an aseptic condition and excess solution on the explants was blotted out. The explants were then placed with the ventral side facing the media.

2.3. Nanoparticles Preparation

Zinc oxide nanoparticles indicated as Zinc oxide nanopowder (Type I) at a purity of 99.9%. Manufactured by Sisco Research laboratories Pvt., Limited, Maharashtra was used in the study. The properties of the ZnO-NPs is detailed in Table 1.

Table 1. *Properties of ZnO NPs used in the experiment.*

Type	Size (nm)	Purity (%)
ZnO Nano Powder (Type I)	30	99.9

The nanoparticles were suspended directly in deionized water and dispersed by using magnetic stirrer for 30 minutes. The nanoparticles suspensions were filtered (0.7 μm glass filter) and used in

media preparation. Three concentrations of nanoparticles were used in addition to the control 0 (control), 5, 10 and 25 mg/L.

2.4. Media Preparation

MS medium [23] supplemented with 2, 4-D (1mg/L) and kinetin (4mg/L), was used for callus induction and the same media was used as the control. The control media was further supplemented with 5, 10 and 25 mg/L of ZnO NPs and designated as experimental Media I, II and III respectively. The pH of media was adjusted between 5.6 to 5.8 and was sterilised at 15psi, 121º Cfor 20 minutes.

2.5. Experiment Layout

Explants prepared from three plants of S.4595 (14/10; 14/3; 14/8) separately were cultured into four media with two explants in each culture bottle. The culture bottles were incubated in dark for 8 weeks at 23±1ºC with one sub-culturing after 4 weeks. Before the subculture, the explants were examined for bacterial and fungal contamination and recovery of explants were recorded.

2.6. Statistical Analysis

The experimental design followed in the study was Randomised Block Design with three replication for each treatment. Fifteen culture bottles were inoculated with two explants under each treatment. A total of 30 explants per replication per treatment formed the experimental material. The data recorded was subjected to analysis of variance [24] to test the significance of difference in mean values obtained. It was analysed using (LSD) at $P \leq 0.05$ %, means separation using the SAS Software.Duncan's Multiple Range test (DMRT) was carried out to measure specific differences between pairs of means[25].

3. RESULTS AND DISCUSSION

The experimental cultures were observed for microbial contamination and recovery of explants and the details are shown in Table 2. All the three experimental samples showed reduction in contamination percentage and hence increase in recoveries of explants with increased concentration of ZnO-NPs in the media. Maximum recovery of explants was observed in Media III with 25 mg/L ZnO-NPs. In S.4595 14/10 and 14/3, 100 percent recovery of explants was observed in two replications of 25 mg/L ZnO-NPs.

The mean recovery percentage of explants recovered is shown in Table 3. The data indicated 88.33 to 97.67 percent recovery in media containing 25 mg/L ZnO-NP while control media showed a recovery of 57.33 to 73.33 percent(Table 3). The statistical analysis of the data indicated significant variation among the three media tested along with control (Table 3), indicating a significant effect of the media with ZnO-NPs. Media supplemented with 25mg/L ZnO- NPs. showed a maximum recovery (Average 94.56%) which was significantly higher than the rest.

Table 2. *Percentage of explant recovered in three experiments using ZnO NP.*

Batch/ Media	Media I			Media II			Media III			Media IV(Control)		
Expt 1	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
S.4595 14/10	53	73	73	53	60	60	100	100	93	56	56	60
S.4595 14/3	80	80	86	67	73	73	100	93	100	73	80	67
S.4595 14/8	73	73	80	80	80	80	86	86	93	60	63	60
Media 1- 5 mg/L ZnO NP ; Media 2- 10 mg/L ZnO NP; Media 3- 25 mg/L ZnO-NPs												

Table 3. *Table of means for observations on percentage recovery of explants of three plants of S.4595 tested in four media.*

Media/Batch	S.4595 (14/10)	S.4595 (14/3)	S.4595(14/8)
Control	57.33 [b]	73.33[b]	61.00 [c]
Media I	66.33 [b]	82.00[b]	75.33 [ab]
Media II	57.67[b]	71.00[b]	80.00[ab]
Media III	97.6[a]	97.67 [a]	88.33[a]

Media 1- 5 mg/L ZnO NP; Media 2- 10 mg/L ZnO NP; Media 3- 25 mg/L ZnO-NPs
Means with similar superscripts indicate on par recovery, while the means with different superscript shows significance in recovery.

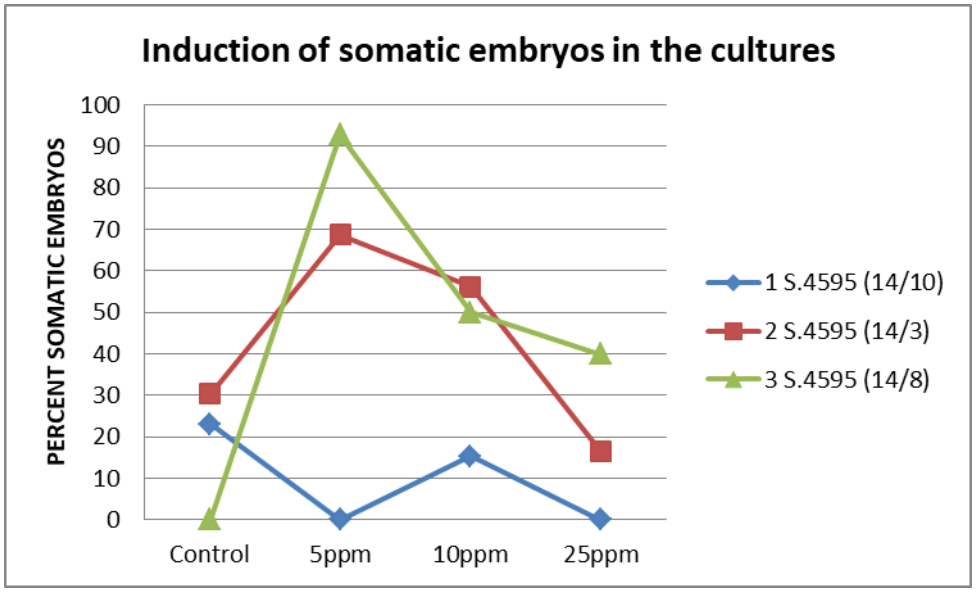

Figure 1*. The percent induction of Somatic embryos in S.4595 14/10; S.4595 14/3; S.4595 14/8 in Control, Media I (5* mg/L*),Media II (10* mg/L*) and Media III (25* mg/L *)and ZnO-NPs.*

Further, the mean recovery of explants in media supplemented with 5 and 10mg/L ZnO-NPs was found to partially improve the recovery. Hence the addition of ZnO-NPs in the media was found to significantly improve the recovery of explants at concentration of 25mg/L by effectively suppressing fungal and bacterial contamination.

Studies in Banana using Zn and ZnO-NPs also reported reduction of bacterial and fungal contaminants at concentrations of 50, 100 and 200 mg/L [18].

Statistical analysis also indicated the significant variation in recovery percentage between the three different plant samples tested (Table 3). Among the three plants tested, all the plants showed a significant recovery in media containing 25 mg/L ZnO NP (Table 3) followed by increased recovery in 10 mg/L and 5mg/L ZnO NP in genotype S.4595 (14/8). However the other genotypes (S.4595 (14/3) and

(14/10)) showed increased recovery in media with 5mg/L ZnO NP followed by 10 mg/L ZnO NP which were however higher recovery compared to their respective controls. This highlights the genotypic differences observed in coffee. Earlier studies [26] also reported similar responses in somatic embryogenesis obtained from cultured embryos in coffee.

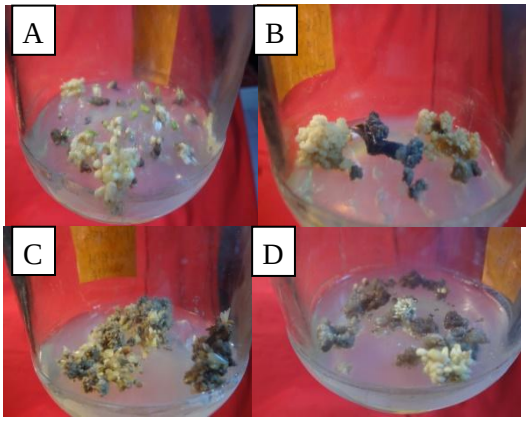

Figure 2 a, b, c, d. *Initiation of somatic embryos in S.4595 (a) 14/3 in 25* mg/L *of ZnO NP; (b)14/8 in 10* mg/L*; (c) 14/3 in 5* mg/L*; (d) 14/3 in Control.*

Observations were also recorded on the initiation of somatic embryos in the experimental cultures. [Figure 1 and Figure 2 a, b, c, d]. High percentage of induction of somatic embryos of 69% and 93% respectively was observed in cultures of S.4595 (14/3 and 14/8) at media concentration of 5mg/L ZnO NP compared to control in S.4595 (14/3) (30%) and S.4595 (14/8)(0%). The percentage of somatic embryogenesis reduced as concentration of ZnO NP increased. In Banana also, the use of Nano Zn and Nano ZnO increased in regeneration potential, which was reported to be due to the beneficial effect of Zn on plant growth [18].

Zinc is an essential element for plants, animals and humans, but is toxic at very high levels among various plant species [27].

Hence, studies are being carried out to understand the long term effect of ZnO NP on callus differentiation, induction of somatic embryos and plantlet regeneration.

4. CONCLUSION

Identification of new techniques for the reduction on contamination of explants in tissue culture experiments is an immediate requirement. For the first time, the effect of NP has been tested in Coffee tissue culture. The study resulted in the identification of antimicrobial properties of ZnO NP and its effect in reduction of contamination in tissue culture of coffee at a concentration of 25 mg/L. MS media with ZnO NP at 25 mg/L concentration showed maximum recovery of explants in all the genotypes up to 94.56 percent compared to 63.89 percent recovery in control. The media containing ZnO NP also showed an improvement in the induction of somatic embryos. Further, no inhibitory effect on the growth of explants was observed at this concentration. This is the first report in coffee for the use of ZnO Nanoparticles for culture recovery and also for promoting growth of callus cultures and somatic embryogenesis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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