Short Scientific Report

doi: 10.25081/jpc.2023.v51.i1.8470
Journal of Plantation Crops, 2023, 51(1): 44-46

Influence of ZnO and TiO_2 nanoparticles in the establishment and growth of *in vitro* callus cultures of coffee

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(Manuscript Received: 22.04.2022, Revised: 29.03.2023, Accepted: 29.03.2023)

Keywords: contamination rate, explants, inoculation media, nanoparticles, recovery

Coffee is an important plantation crop in the tropics, and is produced in more than 80 countries across the world. Coffee ranks second as the most valuable commodity exported by developing countries in international trade. Coffee belongs to genus *Coffea* with over 100 species (Razafinarivo, 2013) among which only two species, *Coffea arabica* L. (Arabica) and *Coffea canephora* Pierre ex A. Froehner (Robusta) are commercially cultivated. Among them, arabica coffee occupies 70 percent of global production and offers beverages with superior cup quality (Coffee Guide, 2014).

Traditionally, coffee is seed propagated. However, it results in the segregation of traits of the parental plant. Vegetative means of propagation is imperative to develop true-to-type plants from elite genotypes. *In vitro* propagation of coffee using leaf explants is a promising technique for large-scale multiplication of elite arabica hybrids and improved robusta cultivars (Sondhal and Sharp, 1977). However, tissue culture technology in tree species has several bottlenecks like recalcitrant nature of plants, genotype specificity, poor *in-vitro* response of explants and the selection of appropriate explants (Devasia *et al.,* 2020). A major obstacle in the adoption of the tissue culture technique in coffee propagation is the high microbial contamination (fungal and bacteria) causing a severe loss of explants and growth media (Sreenath and Muniswamy, 2000). The antimicrobial effect of nanoparticles (NP) of zinc oxide (ZnO) and titanium oxide (TiO_2)has been proven in the *in vitro* cultures of banana, grapes, barley, tobacco and several vegetable crops (Safavi, 2011; Mandeh *et al.*, 2012; Helaly *et al.*, 2014; Alharby *et al.,* 2016; Pal *et al.*, 2018; Aquisman et al., 2020). Recently, the antimicrobial activity of ZnO NP has been reported in coffee tissue culture (Devasia *et al.,* 2020). Accordingly, a study was conducted to test the efficacy of ZnO and TiO_2 NPs in reducing *in vitro* contamination in arabica and robusta coffee explants collected from field-grown plants.

Explant preparation and callus induction

Young, tender second pair of leaves of Chandragiri, S. 5086 (38/12), S. 5085 (18/11) (*Coffea arabica*) and CxR (*Coffea canephora*) were collected as explants from the experimental plots of the Central Coffee Research Institute, Chikmagalur District, Karnataka, during April 2019. The fresh leaves were placed under running water for 15 to 20 minutes; treated with 0.5% bavistin for 10- 12 minutes and rinsed with double distilled water. After 2- 3 rinses the leaves were treated with 70% alcohol for 6-7 minutes. After removing alcohol by rinsing with double distilled water, the leaves were treated with 1% sodium hypochlorite (NaOCl) solution for 10 to 12 minutes. Inside LAF, the leaves were cut into small squares of 0.5 x 0.5 cm and placed in citric ascorbic acid solution (citric and ascorbic acid solutions in 50: 50 ratio) and excess solution on

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the explants were removed using a sterilized blotting paper. The explants were then placed with abaxial side facing the media. The leaf explants were incubated in MS media (Murashige and Skoog, 1962) supplemented with 2, 4-D (1 mg L^{-1}) and kinetin (4 mg L^{-1}) containing synthetic TiO_2 (Titanium IV oxide TiO_2- Anatase- SRL) and ZnO Nanoparticles (Zinc Oxide Nanopowder Type I-SRL) at different concentrations of 0, 15, 30, 45, and 60 mg L^{-1} for callus induction. Agar Agar (0.8%) was added to the media as a gelling agent. The experiments were laid out in three replications under each treatment and 30 explants per replication.

Induction of somatic embryos

After incubation for four weeks in dark under normal room temperature, in callus induction medium, the calli were transferred to MS medium supplemented with 2, 4-D (0.5 mg L^{-1}), IAA (0.1 mg L^{-1}) and kinetin (4 mg L^{-1}) with the same concentration of nanoparticles for further growth and somatic embryo induction. The recovery of explants and initiation of callus was recorded one month after inoculation. Growth of callus was recorded three months after incubation.

Explant recovery in media containing TiO_2 and ZnO nanoparticles (Fig. 1 and Fig. 2, respectively), showed a significant improvement in media containing 45 mg L^{-1} ZnO NP in both arabica and robusta coffee. However, the highest recovery of *in vitro* explants was achieved in media containing 30 mg L^{-1} TiO_2 NP in both arabica and robusta coffee. The culture media containing 15 mg L^{-1} TiO_2 nanoparticles showed enhanced callus growth by 48% in arabica whereas 20% enhanced growth was recorded in the media supplemented with 30 mg L^{-1} TiO_2 nanoparticles in robusta genotypes. However, culture media containing TiO_2 nanoparticles above 30 mg L^{-1} reduced the callus growth. Similarly, the effect of ZnO NP on callus growth indicated that the media containing 45 mg L^{-1} ZnO NP enhanced callus growth by 45% in arabica. In robusta genotypes, 37% enhancement in callus formation was recorded when incubated in the media supplemented with 30 mg L^{-1} ZnO NP. In both arabica and robusta genotypes, reduced recovery and growth of explants were observed in media containing higher concentration of ZnO NP. Recent studies on coffee (Devasia *et al.,* 2020) also reported improved growth of callus in 25 mg L^{-1} ZnO NP

To conclude, addition of TiO_2 and ZnO NP in the growth media significantly improved the recovery of *in vitro* explants. Callus growth of 20% and 48% in robusta and arabica coffee was recorded using TiO_2 NP and 37% and 45% in robusta and arabica coffee using ZnO NP. An increase in the concentration of NP in the media hindered the recovery and growth of explants in both arabica and robusta coffee.

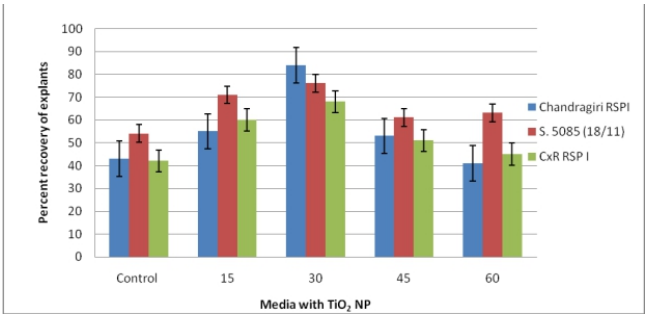

Fig.1. Effect of TiO_2 NP media on the recovery of coffee leaf explants in arabica and robusta.

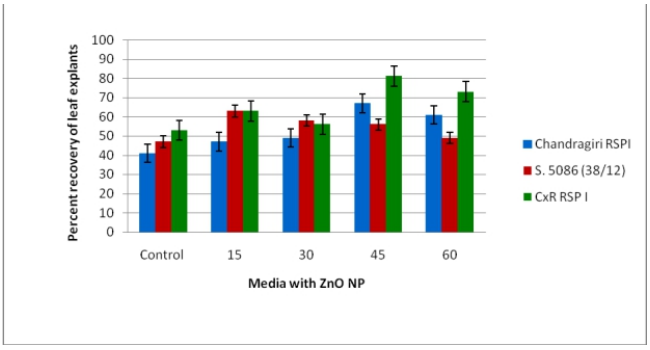

Fig.2. Effect of ZnO NP media on recovery of coffee leaf explants in arabica and robusta.

a. Leaf explants in 0 mg L-1 ZnO NP

b. Leaf explants in 15 mg L-1 ZnO NP

c. Leaf explants in 45 mg L-1 ZnO NP

Fig.3. Remission of fungal contamination of coffee leaf explants grown in media containing different concentration of ZnO Nanoparticles.

Acknowledgements

Sincere thanks to Dr. Surya Prakash Rao Director of Research, Coffee Board for all support and encouragement and Dr. V. B. Suresh Kumar, Divisional Head, Plant Breeding & Genetics, CCRI for providing the plant material for the study.

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