NANOPARTICLE DRIVEN ELICITATION FOR ENHANCED PRODUCTION OF CURCUMINOIDS FROM SHOOT CULTURES OF *CURCUMA LONGA* L.

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Abstract:
Curcuma longa L. belonging to family Zingiberaceae is a valuable medicinal plant containing pharmaceutically important curcuminoids (Bisdemethoxy curcumin, Demethoxy curcumin and Curcumin). Plant tissue culture is a leading technique utilized for secondary metabolite production. Nanoparticles find their applications as elicitors for enhancing the production of secondary metabolites from *in vitro* plant cell organ cultures. This study was conducted toinvestigate the effect of ZnO, CuO, and Ag nanoparticles on *in vitro* grown plants of C. *longa*. The effect of ZnO (30, 60, 120mg/L) CuO (1, 2 and 5mg/L) and Ag (0.5 and 3mg/L) was examined on growth and curcuminoid production. The *in vitro* shoot cultures were evaluated for biomass increase and secondary metabolite production by augmenting nanoparticles for a period of 40 days. ZnO 60mg/L with MS basal media gave best response for biomass and curcuminoid production after 30 days of inoculation. There was an increase of 10 fold curcuminoid production on 60mg/L ZnO containing media in comparison to control cultures. Agnanoparticles proved to be better than CuO nanoparticles for growth and curcuminoid production.

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Introduction:

Turmeric, also known as *Curcuma longa* L., is a nutraceutical plant from the Zingiberaceae family. It is cultivated throughout Asia's southern and western areas. The primary source of medicinally effective curcuminoids (volatile) and mono- and sesquiterpenes in volatile oil is the rhizomes of the *C. longa* plant. The rhizome has been used for ages in Chinese and Ayurvedic medicine as well as for cooking uses, as a food color, and as a component of many other medicinal treatments (Sharifi et al. 2020). This prominent herbal medicine has long been used to cure a variety of illnesses. Anti-inflammatory, anticancer, antioxidant, anti microbial, antimutagenic, anti-obesity, hypolipide- mic, cardio protective, and neuroprotective benefits are a few of the pharmacological properties of turmeric. Due to its purported pharmacokinetic properties, turmeric is a crucial candidate for additional clinical studies (Sharifi et al. 2020).

Research on nutritional supplementation and multifunctional diets for various disorders has drawn more attention in current years (Mohammadi et al 2018). Curcumin, also known as diferuloyl methane or 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1E, 6E-heptadiene-3, 5-dione, is a polyphenol produced from the turmeric plant (Ahmad et al 2020). The volatile oils and diferuloylmethane are two more of turmeric's primary active components. The key secondary metabolites of *C. longa* are curcumin and two of its related compounds, demethoxy curcumin and bisdemethoxy curcumin. The yellow colored polyphenolic curcumin has been revealed to have pleiotropic properties and has the capacity to affect a variety of signaling molecules. In pastfew years, the importance of curcuminoids and their pharmaceutical effects has increased many Fold (Purpura et al. 2018).Curiously, there is emerging indication that a novel technology or method is being used to increase the production of curcuminoid for societal benefits (Kanubaddi et al. 2018).

Elicitors:

Secondary metabolites are bio - active substances that plant produce in order to protect themselves.

Due to their extensive usage in the pharma sector, these intermediate metabolites are extremely important. They are used widely in the fields of medical science and medication development. The rate of production of these substances in plants is constrained, and the rising demand for them in medicine necessitates increased synthesis of secondary metabolites (Zaeem et al. 2022). Elicitation uses a variety of stimuli, including biotic, abiotic, and signaling molecules, to increase the synthesis of secondary metabolites in plant cell tissue and organ culture (Bharath et al. 2008; Owolabi et al. 2018). Organ cultures are chosen because they overcome the primary limitations of genomic variability and relatively low productivity inherent with unorganized cell suspension cultures. Organ cultures also offer the benefit of tissue specific aggregation with high yield (Sandhya et al. 2021).

Nanoparticles as elicitors:

Regarding the nature of plants, nanoparticles (NPs) produce both harmful and stimulating impacts on them (Ma et al. 2010). There are several ways to prepare NPs. Nanoparticles are synthesized in laboratories using both biological and chemical synthesis. Due to its eco-friendly attributes, biological synthesis—also known as "green synthesis"—is more popular (Sengani et al. 2017). Depending on the dosage of the Nanoparticles and the kind of plant, the elicitation impact varies (Khan 2016; Nadeem 2018; Thuesombat et al. 2014). Now, more and more nanoparticles are being used to elicit secondary metabolites from plants. According to published reports, NPs can infiltrate plant cells, modify how reactive oxygen species (ROS) are metabolized, and trigger signaling processes that are involved in both principal and subsequent metabolism (Marslin et al. 2017; Sun et al. 2020). Though some of the phytopharmaceuticals have displayed significant enhancement in therapeutic herbs owing to the potential of Nanoparticles to control plant secondary metabolism. (Kruszka et al. 2022). The ZnO Nanoparticles have increased the production of anticholinergic chemicals scopolamine and hyoscyamine in the bushy root system of *Hyoscyamus reticulatus L*. (Hosseini et al. 2019). In *Glycyrrhiza glabra L*. seedlings, CuONPs promoted the accumulation of the anti-inflammatory compound glycyrrhizin (Oloumi 2015).

Material and Methods:

***In vitro* plant establishment**:

Rhizomes of C. *longa* were procured from CIMAP, Hyderabad for the *in vitro* plant establishment .The aseptic cultures were established employing rhizome buds as explants. The explants were initially cleaned with running tap water for 30 minutes, and then washed with gentle cleanser. The buds were then surface sterilized with 0.1 percent (w/v) mercuric chloride for three minutes after being treated with 0.2 percent (w/v) bavistine for five minutes. The explants were then rinsed thoroughly with sterile double distilled water (8–10 times) and Inoculated in MS liquid media reinforced with different combinations and concentration levels of phytohormones (BAP, TDZ, KN, NAA, IAA, IBA). The explants then were incubated under standard culture environments (25 ± 2^0C temperature under a 16–8 h light/dark regime with 40–50 mol $m^{-2}s^{-1}$ illumination supplied by the cool fluorescent lamp) (Sandhya et al. 2021).

Growth kinetics:

C. longa in vitro-grown plants were used for biomass growth on MS liquid media loaded with different concentrations of sucrose. (3, 6, and 9 percent w/v). The growth and biomass increase were investigated using a homogeneous shoot mass of 1.3g as the starting inoculum, for 40 days in MS liquid medium. At regular intervals of 5 days, the increase in biomass was recorded and reported as grammes of fresh weight (gFW)

Quantification of curcuminoids:

The cultures are examined for biomass and curcuminoid production at an interval of 5 days for 40 days. HPLC analysis was carried out to quantify the curcuminoids in the elicited and control plants according to the protocol of Wu et al. (Wu et al. 2015). Before HPLC analysis, the extracts were filtered using a 0.22 m filter membrane. Shimadzu HPLC system with a UV-VIS detector attached to a C18 column was used for analysis of curcuminoids. The solute had an absorption maximum of 425 nm and was composed of acetonitrile, water, and 5% acetic acid. The flow rate was 1 ml per minute and the injection volume was 20µl with a flow rate of 1ml. min-1.

Elicitor treatment:

ZnO, CuO, and Ag nanoparticles have been utilized in the current investigation as elicitors to increase the synthesis of curcuminoids from *C. longa in vitro* shoot cultures. The stock solutions of elicitors was made at a standardized dosage of 50 mg each of Ag, CuO, and ZnO, which was then dissolved in 50 ml of distilled water. The mixture was then mixed in a sonicator for ten min, with a 5-minute break, and then sonicated once again for 10 minutes. The nanoparticles were augmented in the media prior to setting the pH and sterilized by autoclaving. In the current study Zinc oxide (30 mg, 60 mg, 120 mg/L), Silver (0.5 mg, 3 mg/L), and Copper oxide (1, 2 and 5 mg/L) were applied as an elicitor to increase the production of curcuminoids in C. longa shoot culture.

Results and Discussion:

Rhizome bud explants were used to establish aseptic cultures of *C. longa* on MS media enriched with 2.5mg BAP L^{-1} and 1.5 mg NAA L^{-1}. In plant growth initiation, rhizome buds responded similarly to other Zingiberaceae species (Kewu et al. 2015, Kim et al. 2004). The importance of BAP and NAA in establishment of *C. longa in vitro* cultures in the present study was in accordance with the earlier reports (Sathiyabama et al. 2016; Kumar et al. 2010). To investigate the biomass increase, the development of *in vitro* plants was assessed on MS medium augmented with various concentrations of sucrose (3, 6 and 9 percent).

Fig: 1 In *vitro* grown *plants of C. longa* on MS medium supplemented with 6% (w/v) sucrose

The 30th day of incubation, or mid-stationary phase, had the maximum biomass accumulation (3.31g) in MS liquid medium supplemented with 6 percent (w/v) sucrose (Fig.2).The initial inoculum was 1.3g. In *Curcuma* sp., the beneficial effects of sucrose at a greater concentration of 6% on micro propagation have previously been documented. It was shown that higher concentrations of the carbon source sucrose (6% w/v) promoted the development of aerial multiple shoots and roots (Shi et al. 2015). This is crucial for the development of *in vitro* cultures from rhizome bud explants. The sucrose concentration of 9 % (w/v) has shown to impede growth. For growth studies the cultures were regularly harvested every 5 days till 40th day, at which point they entered the decline phase.

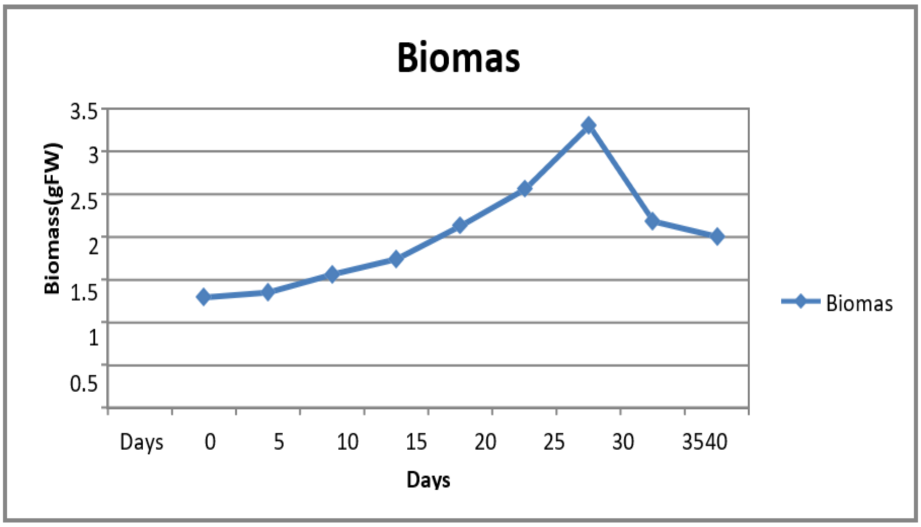

Fig: 2 Biomass increase in shoot cultures of *Curcuma longa*

The upsurge in biomass and the curcuminoid production in the shoot cultures of *C. longa* were analyzed simultaneously. According to the HPLC study, after 30th day of culture all the three curcuminoids displayed maximum production, it followed the same pattern as the growth of shoot cultures. The quantification of the curcuminoids showed maximum production of Curcumin (41.314gFW-1), Demethoxy curcumin (76.7514g FW-1) and Bisdemethoxy curcumin (36.14gFW-1) after 30 days of culture (Fig.3). The findings of this investigation were in corroboration with earlier findings (Ferrari et al 2016, Chen et al 2017), which indicated that curcumin was the primary component when tested *in vitro*.

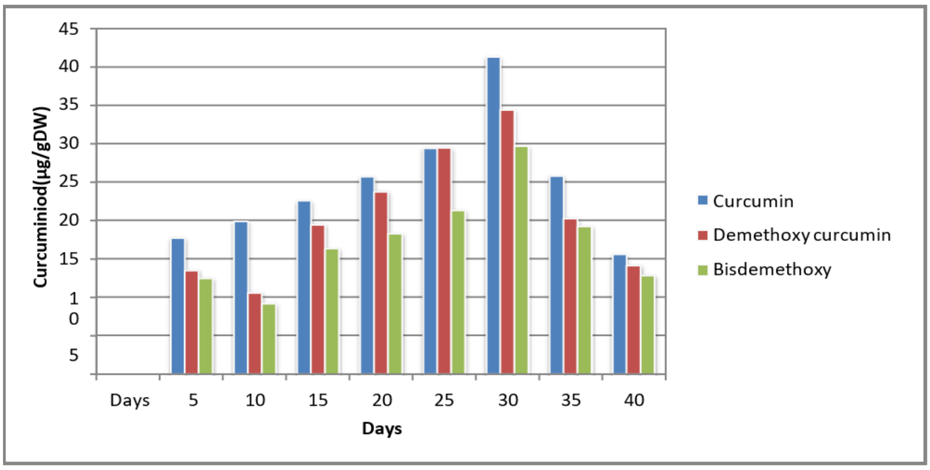

Fig: 3 Curcuminoid production in control shoot cultures of *Curcuma longa*

Elicitation with Zinc oxide nanoparticles (ZnONPs):

Owing to its distinctive optical, catalytic, band gap, and high surface area to volume ratio features, zinc oxide nanoparticles (ZnONPs) are used in a wide variety of applications. From all kinds of nutrients, zinc nanoparticles had a great interest in investigations due to its importance for plant as it is involved in many growth processes (Werayut et al. 2005). ZnONPs were used to treat *C. longa* shoot cultures at varied doses (30, 60, and 120 mg L^{-1}), and the results showed maximum biomass increase on 30th day (5.5 gFW) and it is more than control plant cultures in MS liquid medium without nanoparticles. All the concentrations of ZnONPs exhibited increase in the biomass till 30th day. However, ZnONPs 60mg L^{-1} showed maximum biomass increase compared to other concentrations (30mg, 120mg) (Fig.4). ZnONPS elicitation had an optimistic impact on curcuminoid production. The ZnO concentration of 60mgL^{-1}displayed maximum enhancement in the production of all the three curcuminoids. The curcumin content was 412.32µg/gDW which was 10 fold higher than the control plants. Demethoxy curcumin content was 160.25 µg/gDW, which was 4.8-fold higher than control plant. Bisdemethoxy curcumin content was 72.31 µg/gDW, 2-fold higher than control plant.

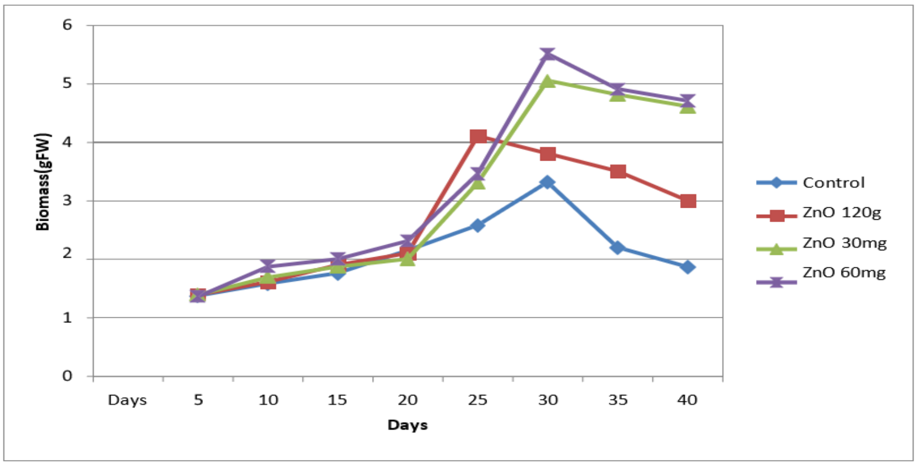

Fig: 4 Effect of various concentrations of ZnONPs on growth (biomass) of *Curcuma longa* shootcultures

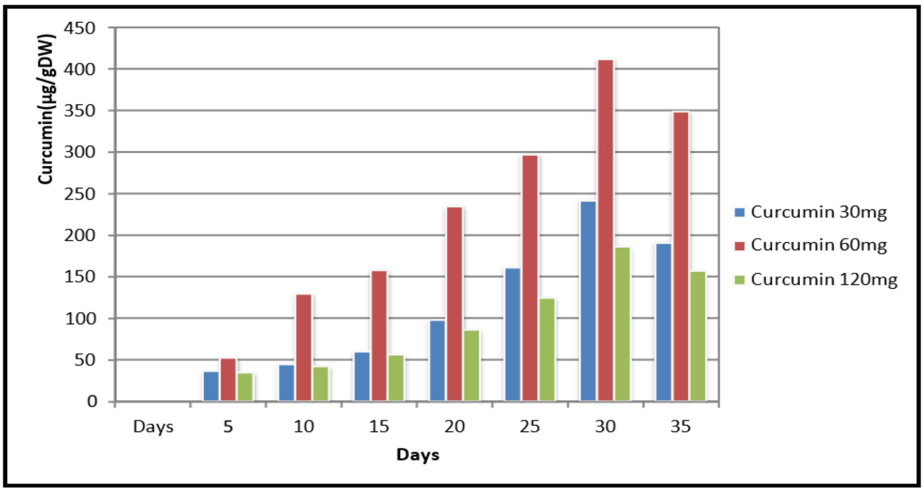

Fig: 5 Curcumin production in ZnONPs (30mg, 60mg, and 120mg) treated shoot cultures of *Curcuma longa*

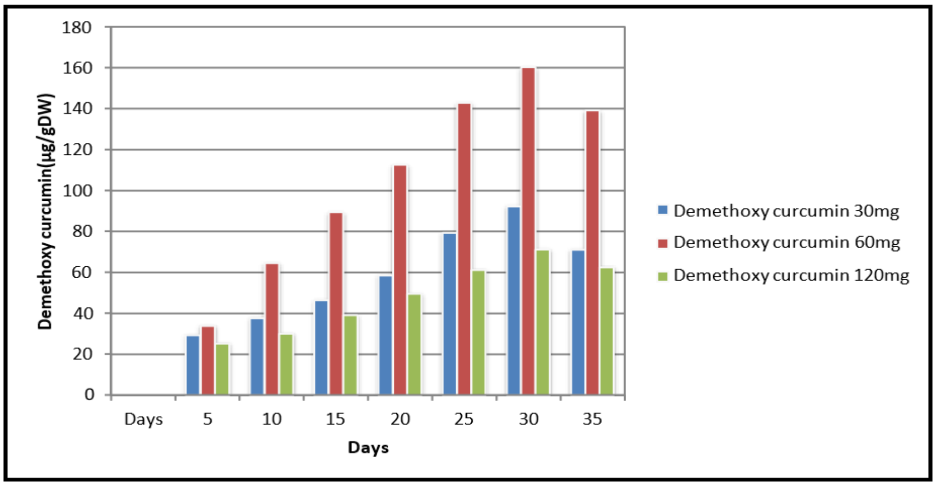

Fig: 6 Demethoxy curcumin production in ZnONPs (30mg, 60mg, and 120mg) treated shoot culturesof *Curcuma longa*

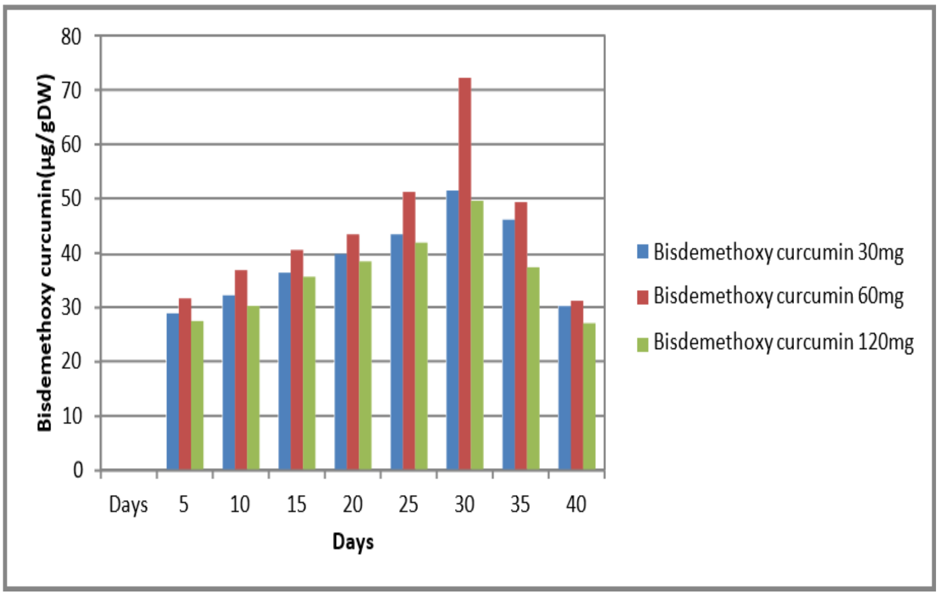

Fig: 7 Bisdemethoxy curcumin production in ZnONPs (30mg, 60mg, and 120mg) treated shootcultures of *Curcuma longa*

Elicitation with silver nanoparticles (AgNPs):
Nanoparticles of Silver (AgNPs) are the most explored MNPs as elicitors of plant Secondary Metabolite production in *in vitro* cultures of

several plant species (Chung et al. 2018; Yasur et al. 2013). *C. longa* shoot cultures were treated with AgNPs at varied concentrations (0.5 mg and 3 mg/L) and the results showed maximum biomass increase (4.65gFW) on 30th day and it is more than control plant cultures in MS liquid medium. With both the concentrations of AgNPs there was an increase in the biomass on 30th day. The AgNPs at 3mg/L concentration displayed higher biomass increase compared to other concentration (0.5mg) (Fig.8). Higher concentration (3mg) of AgNPs gave maximum production of all the three curcuminoids. Maximum enhancement was observed on 30th day. The curcumin content was 311.95 µg/gDW which was 8 fold higher than the control plants (Fig: 9). Demethoxy curcumin content was 135.19 µg/gDW 3.97-fold higher than control plant (Fig.10). Bisdemethoxy curcumin content was 78.45 µg/gDW1.5 fold higher than control plant (Fig.11).

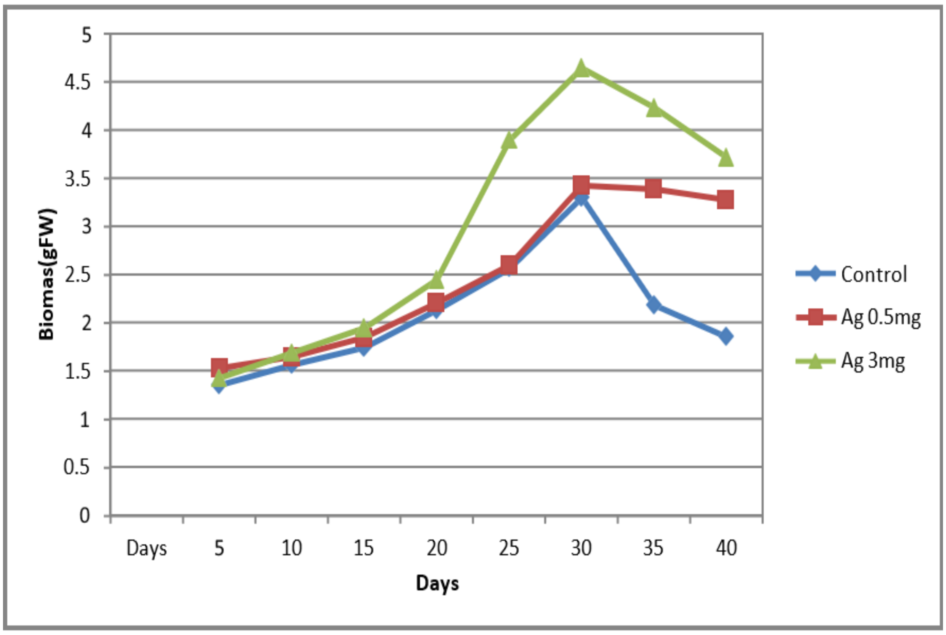

Fig: 8 Effect of various concentrations of AgNPs on growth (biomass) of *Curcuma longa* shootcultures

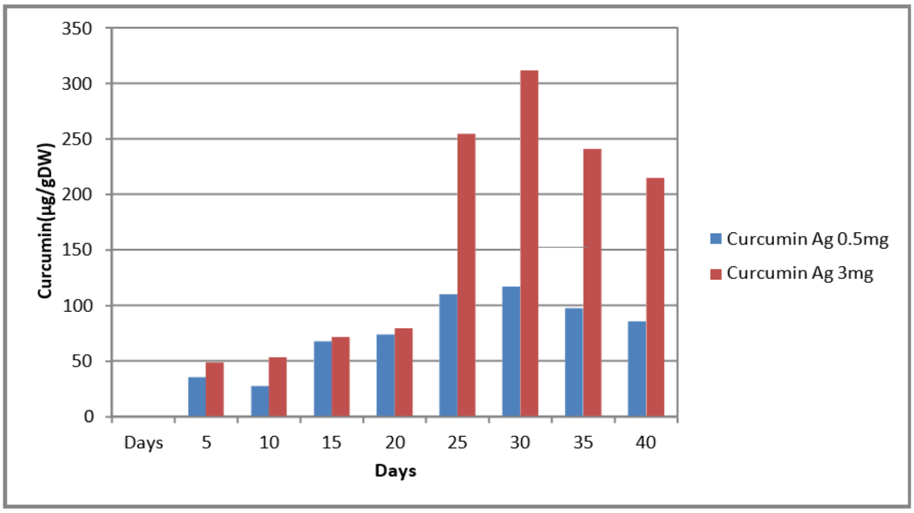

Fig: 9 Curcumin production in Ag (0.5mg, 3mg) treated shoot cultures of *Curcuma longa*

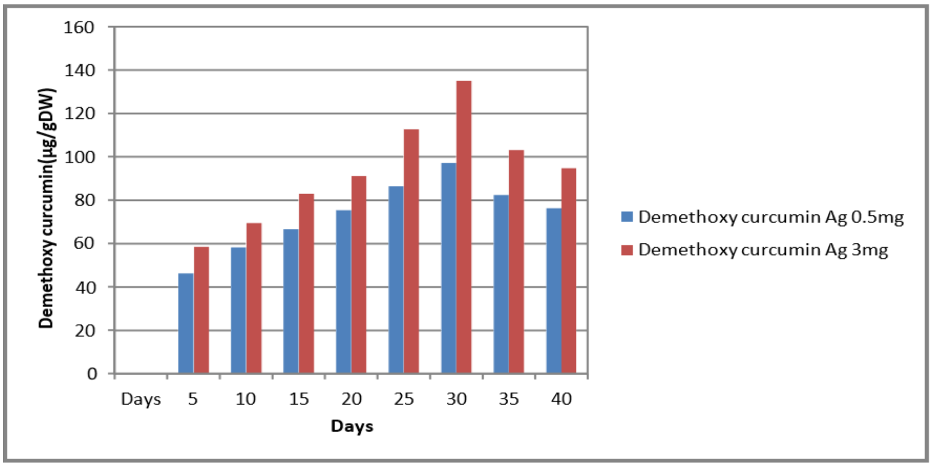

Fig: 10 Demethoxy curcumin production in Ag (0.5mg, 3mg) treated shoot cultures of *Curcumalonga*

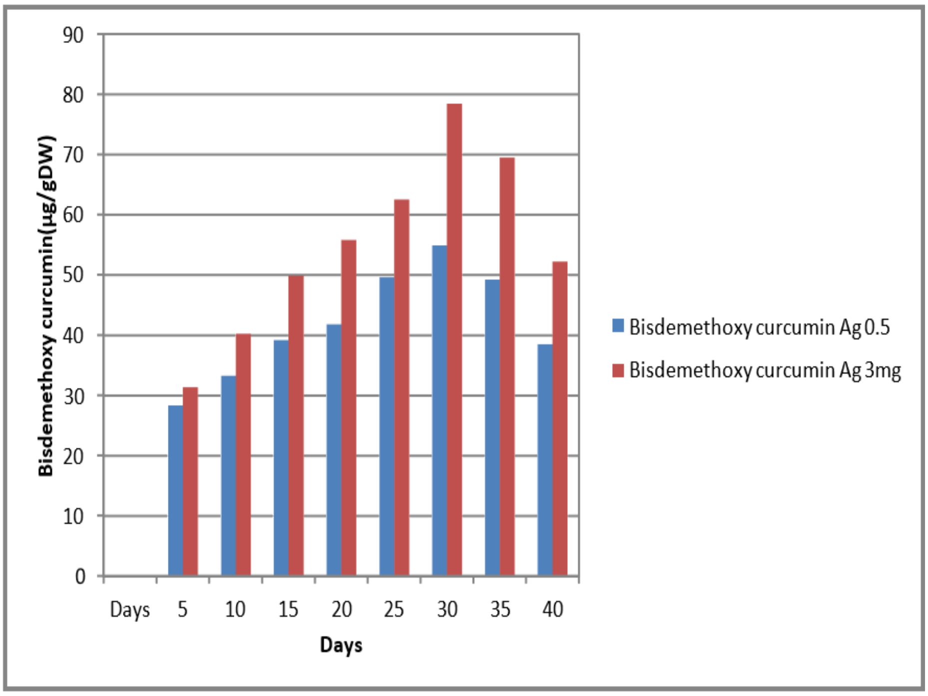

Fig: 11 Bisdemethoxy curcumin production in Ag (0.5mg, 3mg) treated shoot cultures of *Curcuma longa*

Elicitation with copper oxide nanoparticles (CuONPs):

Plants (Sumaira et al. 2022) CuONPs were applied to shoot cultures at various doses to treat (1,2and 5 mg\L). With all concentrations of CuONPs there was an increase in the biomass on 30th day (4.31gFW) and it is more than control shoot cultures (Fig.12). The CuONPs at 2mg concentration showed maximum biomass increase compared to other concentrations (1mg, and 5mg). CuONPS elicitation shows lower curcuminoid production Copper (CuO) is a crucial component of plant nutrients and is crucial to both the general and complex metabolism of compared to other nanoparticles (ZnO and Ag). CuONPs (2mg) concentration gave maximum production of curcuminoids on 30th day. The curcumin content was 236.15 µg/gDW which was 6 folds higher than the control plants (Fig.13). Demethoxy curcumin content was (102.57 µg/gDW) 3.06-fold higher than control plant (Fig.14). Bisdemethoxy curcumin content was (69.45 µg/gDW) 2.34-fold higher than control plant (Fig.15).

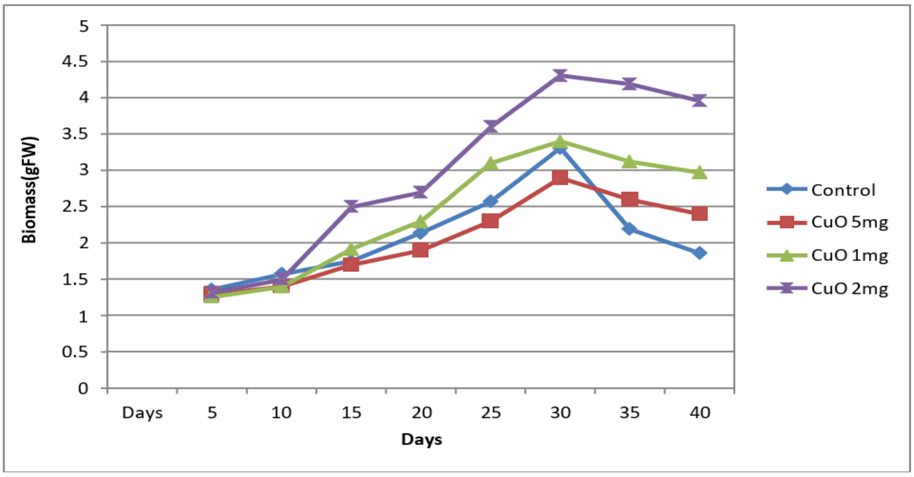

Fig: 12 Effect of various concentrations of CuONPs on growth (biomass) of *curcuma longa* Shoot cultures

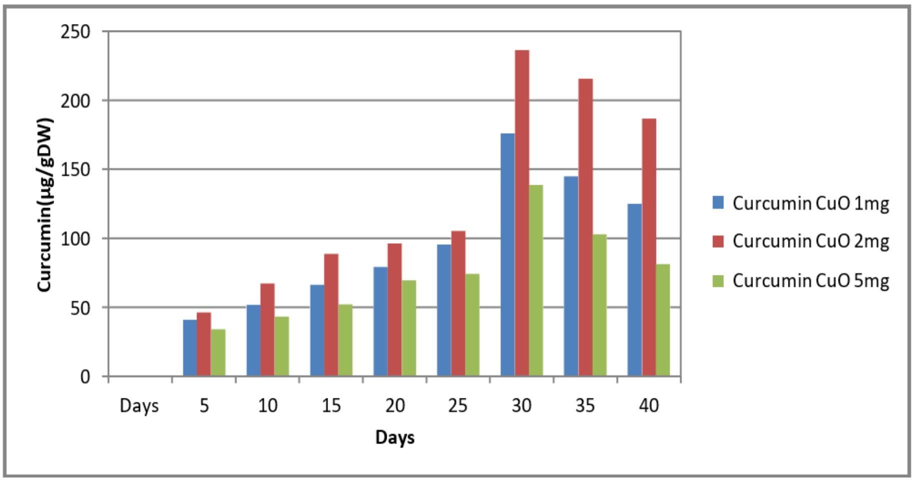

Fig: 13 Curcumin production in CuO (1mg, 2mg, and 5mg) treated shoot cultures of *Curcuma longa*

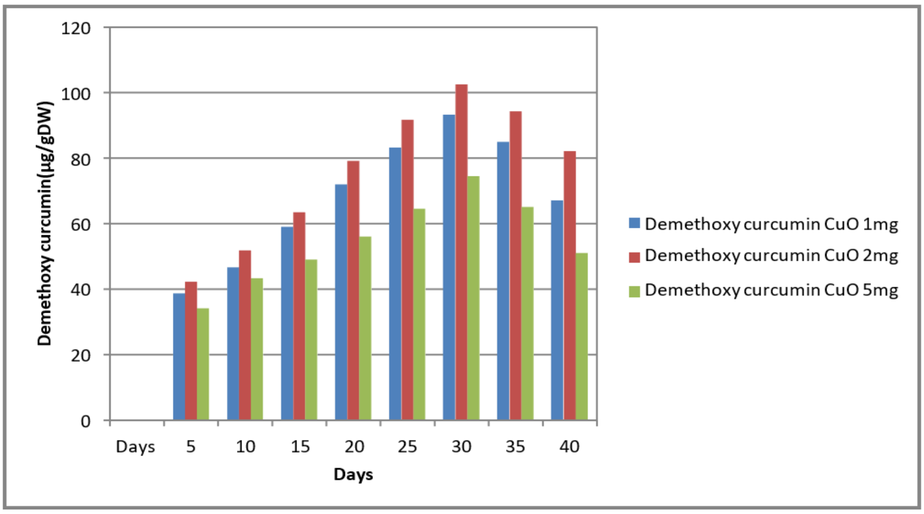

Fig: 14 Demethoxy curcumin production in CuO (1mg, 2mg, and 5mg) treated shoot cultures of *Curcuma longa*

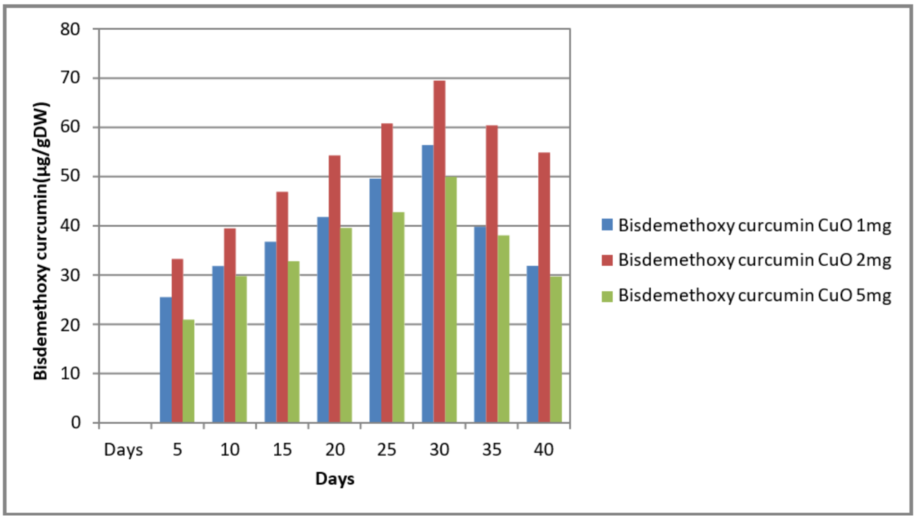

Fig: 15 Bisdemethoxy curcumin production in CuO (1mg, 2mg, and 5mg) treated shoot cultures of *Curcuma longa*

Our results confirmed that ZnONPs proved to be the best elicitor for curcuminoid production in *C. longa* displaying maximum biomass increase at $60mgL^{-1}$ and also enhancing the accumulation of all the three curcuminoids. The AgNP also had a positive effect on growth and curcuminoid production at $3mgL^{-1}$, however, lower concentration 0.5 mgL^{-1} did not affect the biomass production, but curcuminoid production was increased to a certain extent, which is lesser than $3mgL^{-1}$. Among the three classes of nanoparticles investigated in the present study CuONPsproved to be least potent for biomass production and for invoking enhanced production of curcuminoids. CuONPs at $2mgL^{-1}$concentration proved to be the best for biomass increase and curcuminoid production, higher concentration $5mgL^{-1}$ had a negative effect on growth, however,the curcuminoid production was increased slightly in comparison to unelicited control. Curcuminoids represent a class of therapeutically important bioactive compounds and are important component of the pharmaceutical, nutraceutical, cosmetic industries. Due to theirimmense health benefits, there is an upsurge in demand for curcuminoids in world market (Cao et al. 2022). However, production of curcuminoids from plants has its limitations owing to its triploid nature, and transmittance of pathogens in the field maintenance via rhizome replantation (Taghavi et al. 2021). *In vitro* production of curcuminoids using the plant tissue culture technique has great advantages. Elicitation is a useful tool for production of secondary metabolites at industrial levels. The elicitation of *in vitro* cultures activates the plant defense system and enhances the accumulation of secondary metabolites in higher quantities. Nanoparticles significantly contribute towards higher plant yield, and enhanced concentrations of therapeutically valuable secondary metabolites (Khan et al. 2021). Nanoparticles on the basis of their size, concentration, and mode of application has increased plant performance in a range of medicinal plants. There is a rejuvenated interest for use of plant secondary metabolites asprincipal components of modern-day drugs.

In order to increase the generation of industrially significant secondary metabolites in plant cell and organ cultures, nanoparticles (NPs) induce oxidative stress (Pandita 2022; Zaeem et al. 2022). There are numerous reports of various classes of NPs acting as Nano-elicitors of secondary metabolites, including metallic NPs (silver and copper), metallic oxide NPs (copper oxide, iron oxide, zinc oxide, and silicon dioxide) (Khan et al. 2021).

Our findings support those of Perveen et al. (2022), who reported on the biosynthesis of Copper, Iron, and Zinc nanoparticles by Eriobotrya japonica seed extract and their impact on the production of alkaloids, tannins, and flavonoids from shoot cultures of *C. roseus*. Under copper and zinc nanostress, propagating shoots produced vincristine more quickly.

In order to determine the effectiveness of CuO nanoparticles as elicitors in stimulating the formation of Phenols, anthocyanin's, and flavonoids in Zataria multiflora Boiss under both in vitro and greenhouse settings, similar results were published by (Asadollahei et al. 2022). Their results indicated that there was increased production of these bioactive compounds in nanoparticle treated cultures in comparison to untreated controls. Our findings can pave way for production of pharmaceutically important curcuminoids at commercial level optimizing all the conditions after nanoelicitation.

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