International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (3): 811-821
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Biotic Elicitation Based Enhancement of Some Secondary Metabolites and Antioxidants in *Sauropus androgynus* (L.) Merr.

Kirtana S. and N. Geetha
Dept. of Botany, Bharathiar University, Coimbatore-46, TN, India.

Received: 15 Mar 2019 / Accepted: 17 Apr 2019 / Published online: 1 Jul 2019
***Corresponding Author Email:** geethadrbot@gmail.com

Abstract

In the present investigation, an attempt was made to test the hypothesis that whether biotic elicitor may enhance some secondary metabolites and antioxidants in *S. androgynus*. For this, *B.subtilis* strain was purchased from MTCC, Chandigarh, India and maintained in LB medium. After reaching the log phase, the bacterial culture was centrifuged, and extracellular biomass was collected (supernatant) and used for synthesis of AgNPs. Among five concentrations of $AgNO_3$, a stable colour change was observed at 3mM concentration in 2:8 ratios ($AgNO_3$ and cell filtrate). The visual formation of AgNPs was confirmed by UV-Vis spectrophotometer, XRD and SEM analyses. Phytotoxicity analysis was carried out to understand the positive and negative effects of synthesized AgNPs on seed germination and also for dose fixation for treatment of leaf derived calli of *S. androgynus* with synthesized AgNPs. It was observed that there is no negative effect on germination rate for seeds by treatment with 50 mg/L, 100 mg/L, and 150 mg/L AgNPs concentrations, whereas germination percentage was reduced by treatment with 200 mg/L and 250 mg/L AgNPs concentrations. For callus induction, MS medium was prepared with 2.0 mg/L 2, 4-D, and 0.5 mg/L of BAP along with different concentrations of AgNPs such as 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L and 250 mg/L individually and young leaf of *S. androgynus* were collected from field grown plants. After sterilization, the explants were inoculated onto different media. After 30 days of culture maintenance, the leaf derived calli were removed and aqueous calli extract was prepared using 0.5 g tissue and used for determination of some secondary metabolites and antioxidants. It was observed that a significant enhanced level of total phenolics and total antioxidant capacity compared to control calli. Higher level of free radical scavenging activities such as DPPH and FRAP was found in calli treated with AgNPs compared to control calli. This is the first report to exhibit an enhancement in these plant components of *S. androgynus* using extracellular biomass of *B.subtilis* as elicitor.

Keywords
S. androgynus, *B.subtilis*, biotic elicitor, phytoxicity analysis, calli, secondary metabolites, antioxidants.

*****

INTRODUCTION

Many researchers have attempted to increase the yield of bioactive compounds through *in vitro* methods such as metabolic engineering, cell immobilization, optimization of nutrient medium, nutrient manipulations (1), addition of precursors and addition of elicitors such as methyl jasmonate and salicylic acid (2-3). Elicitors are biotic or abiotic origins that are defined as the biofactors or chemical molecules which can trigger a stress response as similar to the stress induced in the plants. Elicitors can modify both physiological and metabolic activities in plants by triggering the relevant signal transduction pathways which in turn increases the secondary metabolites production in plants (4). *Sauropus androgynus* (L.) Merr. is a multivitamin plant since it contains many nutritive values such as crude fiber, vitamin A, B, C, and K, protein and mineral salts.In addition to this, the plant has many secondary metabolites and antioxidants (5-6). In Tamil it is known as thavasi keerai and it is a green leafy perennial underutilized erect shrub belonging to the family *Phyllanthaceae* (7).

The use of elicitors as an approach for sustainable metabolite production which has been successfully reported in the formation of sanguinarine in *Papaver somniferum* cultures (8), saponins in *ginseng* cultures (9), tropane in *Datura stramonium* cultures (10), reserpine in *Rauwolfia serpentine* cultures (11), gymnemic acid in *Gymnema sylvestre* cultures (12) and taxol in *Taxus chinensis* cultures (13). Recently, Wee *et al*. (14) produced somatic embryos *S. androgynus* with increased level of papaverine and quercetin after two weeks of elicitation with methyl jasmonate and salicylic acid as elicitors. Nanobiotechnology is one of the important and emerging technological tools for the synthesis of nanoscale and materials for the development of eco-friendly and reliable methodology by using biological sources (15). In recent years there are several researchers demonstrated the synthesis of metallic nanoparticles such as silver, cadmium sulfide, gold, tin and Ni using the microbes (16-18).

Bacillus subtilis are recognized as safe and reliable probiotics strains that are non-pathogenic to humans and animals. Bacteriocin protein or polypeptide is produced during the process of growth and reproduction of this bacterium. This substance has a high antibacterial activity, broad antibacterial spectrum and tremendous thermal stability (19). Bacilli are generally used as plant-growth promoting rhizobacteria (PGPR) and they are efficient in improving seed quality such as seed germination, vigour index and nutritional quality such as protein content and carbohydrate content of sorghum (20).

In recent years various bacterial strains have been used as elicitors for the enhancement of plant secondary metabolites, for example the elicitor prepared from *B. subtilis* for production of plumbagin (21) and *B. subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Proteus aureus* have been used to enhance the forskolin content in *Coleus forskohlii* (22). Extracellular biosynthesis of AgNPs using bacterial *B.licheniformis* has been reported by Kalimuthu *et al*. (23). Despite evidence of success on usage of *B. subtilis* extracellular culture as elicitor in secondary metabolite production, the effect of biosynthesis of silver nanoparticles on the production of some antioxidants and secondary metabolites in *S. androgynus* has not been studied so far. Therefore, this study was undertaken to obtain extracellular synthesis of silver nanoparticles using *B. subtilis* and also to evaluate the effect of synthesized nanoparticles on some secondary metabolites contents and *in vitro* antioxidant activities of *S. androgynus* leaf derived calli.

MATERIALS AND METHODS

Collection and maintenance of bacterial strain

B.subtilis strain (MTCC 441) was purchased from Microbial Type Culture Collection (MTCC), Institute of Microbial technology (IMTECH), Chandigarh, India. The subculture of strain was done using Luria Bertani (LB) medium. The OD of the broth was measured at 600 nm and then the culture was used for the further research.

Synthesis of silver nanoparticles using bacterial culture (Elicitor preparation)

To synthesize silver nanoparticles (AgNPs), the grown bacterial culture (OD=1) was centrifuged at 5000 rpm for 10 minutes at room temperature. After centrifugation, the supernatant (extracellular biomass) was collected and used for the synthesis of AgNPs. Different concentrations of silver nitrate solutions (1mM, 2mM, 3mM, 4mM, and 5mM) have been prepared and mixed with supernatant to find out the optimum concentration for the synthesis of AgNPs. Among five concentrations tried, 3mM concentration of silver nitrate ($AgNO_3$) was given stable colour change i.e pale yellowish to dark brown after 12 hours of dark incubation. Therefore, only this concentration was used for synthesis of AgNPs. To find out optimum ratio, both silver nitrate solution and supernatant in ratio were mixed such as 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1. Of these, 2:8 ratio only gave stable colour formation which

indicates the formation of AgNPs. So, in further studies, 20 mL of $AgNO_3$ (3mM) solution was mixed with 80 mL of supernatant. To avoid any photochemical reactions during this experiment, the prepared solutions were placed and maintained under dark condition at room temperature.

Characterization of synthesized silver nanoparticles

UV-VIS spectrophotometer analysis

The formation of AgNPs was primarily confirmed by visual colour change. The reduction of pure silver ions was observed by monitoring the UV-Vis spectrum of the reaction at different time intervals taking 1mL of the sample, compared with 1mL of distilled water used as blank.

X-Ray Diffraction analysis (XRD)

One of the most extensively used techniques for the characterization of NPs is X-ray diffraction (XRD). The obtained pellet was washed many times and redispersed in deionized water. The synthesized nanoparticles were coated on XRD grid for the XRD studies. The scanning is carried out with 2θ angle pattern. The image obtained was compared with the Joint Committee on Powder Diffraction Standards (JCPDS) for the structure of crystalline.

SEM analysis

Scanning electron microscopy (SEM) analysis of synthesized silver nanoparticles was done to find out structural characters of NPs.

Phytotoxicity analysis

Phytotoxicity analysis was carried out to understand the positive and negative effects of synthesized silver nanoparticles on seed germination and dose fixation for treatment of calli with synthesized AgNPs. For this, five different concentrations of silver nanoparticles (50mg/L, 100mg/L, 150mg/L, 200mg/L and 250 mg/L) were added in MS basal medium. Seeds of *Vigna radiata* L. were selected for *in vitro* germination. After, surface sterilization with 70% ethanol and the seeds were rinsed with distilled water 3-5 times and inoculated onto MS medium containing different concentrations of AgNPs. After one week of germination, percentage the seed germination was calculated. Shoot and root lengths were measured after two weeks of growth.

Treatment of *S. androgynus* derived calli with AgNPs

For callus induction, MS medium was prepared with 2.0 mg/L of 2, 4-Dichlorophenoxyacetic acid (2, 4-D), and 0.5 mg/L of kinetin and with different concentrations of AgNPs such as 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L and 250 mg/L individually. After media preparation, they were autoclaved at 121°C for 20 minutes. For callus induction, young leaf of *S. androgynus* were collected from field grown plants and washed with tap water to remove dust particles. Then the explants were treated with Tween 20 for 15 min followed by rinsing with distilled water until the Tween 20 is washed out. It was followed by sterilization with 0.1% (w/v) mercuric chloride for 10 min followed by rinsing with double distilled water thrice. Finally, the explants were sterilized inside the laminar air flow chamber using 70% ethanol for 8 to 10 min and then washed with sterile distilled water. The sterilized leaf explants were trimmed into 5-8 mm in length and inoculated onto different media and all the culture tubes were kept under 24±1°C, 50-60% relative humidity along with 16h photoperiod.

Determination of some secondary metabolites *and in vitro* antioxidants

After 30 days of treatment or callus induction with AgNPs, the calli were removed and washed with distilled water to remove the medium. Aqueous calli extract was prepared using 0.5 g tissue and used for determination of some secondary metabolites and antioxidants. The total phenol content of the sample was determined by following the method of Makkar (24). The flavonoid content was determined using the aluminum chloride methods with Rutin as a reference (25). The scavenging effect of calli extract on stable radical 2, 2-diphenypicrylhydrazyl (DPPH) was studied, employing the spectrophotometric method described by Braca *et al*. (26). The ferric reducing activity of the calli extract was estimated based on the ferric reducing ability of plasma (FRAP) assay developed by Pulido *et al*. (27). The antioxidant activity of calli extract was evaluated using the green phosphor molybdenum complex formation according to the method of Prieto *et al*. (28).

Statistical Analysis

Each assay was done three times using the same extract in order to determine their reproducibility. The data (means of three replicate determinations ± standard deviation) were subjected to a one-way analysis of variance (ANOVA) and Duncan's new multiple range test was used to determine significant differences. The P values <0.05 was considered as level of significance.

RESULTS AND DISCUSSION

Through biotic or abiotic elicitation, the enrichment of plant secondary metabolites in cell suspension cultures or calli culture has become a potential biotechnological approach for large-scale and commercialized production of bioactive compounds (4). The study of effect of bacterial elicitors on the cell suspension culture of *Coleus forskohlii*, enhances the focus towards yield increase of forskolin in shorter phase of time (22). Keeping this background

in mind, in the present investigation, an attempt was made to test the hypothesis whether the given biotic elicitor enhance some secondary metabolites and *in vitro* antioxidants of *S.androgynus* or not.

Microbial synthesis of silver nanoparticles and their characterization

UV-Vis spectroscopy

In the present study, among various concentrations tried for synthesis of AgNPs, 3mM concentration gave colour change after 12h incubation at room temperature. The colour change from pale yellowish to dark brown indicated the formation of AgNPs (Fig.1). The colour transform arises because of the coherent oscillation of the electrons on the surface of nanoparticles resulting in surface Plasmon resonance (29). After colour change, the development of Plasmon Resonanace Band was confirmed by UV-Vis spectroscopy (Fig. 2).

XRD analysis

The XRD analysis of silver nanoparticles showed six strong intense peaks corresponding to the 32.10, 46.11, 27.70, 65.02, 38.13 and 44.638 Bragg's reflection based on the fcc (face centered cubic) structure of AgNPs (Table 1 and Fig. 3). The broadening of Bragg's peak indicates the development of silver nanoparticles. The mean size of AgNPs was calculated using Debye-Scherrer's equation (30). The size of the nanoparticles was found to be about ~17 nm. Our result is similar to the result of Alhussain *et al*. (31) who synthesized AgNPs using *B. subtilis*.

SEM analysis

The SEM image of AgNPs was due to connections of hydrogen bond and electrostatic interactins between the bioinorganic capping molecules bound to the AgNPs. In the present study, the AgNps were found to be mondispersed (Fig. 4) and the dispersed character of nanoparticles without the formation of aggregates indicating stabilisation of nanoparticles by a capping agent (32).

Phytotoxicity analysis

There are numerous phytoxicity studies who reported both positive and negative effects on AgNPs on higher plants especially seed germination, root elongation, cell division, growth and metabolic processes (33-34). In the present study, the reason for the incorporation of phytotoxicity analysis is to test the hypothesis that the synthesized AgNPs will affect the seed germination of *Vigna radiata* in positive or negative manner and also to determine the dosage of AgNPs to be used as elicitor on *S. androgynus* leaf callus induction medium. In the present investigation, it was found that a significant increase in germination rate for seeds as well as for root elongation, when treated with 50 mg/L, 100 mg/L, and 150 mg/L AgNPs concentrations, whereas no significant effect on germination percentage and root growth was observed with 200 mg/L and 250 mg/L AgNPs concentration (Fig. 5 and Table 2). This observation indicates the positive effect of lower concentrations of AgNPs on seed germination and root growth. Seed germination and root elongation is a rapid and widely used acute phytotoxicity test owing to sensitivity, simplicity, low cost and suitability for unstable chemicals (35).

Effect of biotic elicitor on secondary metabolites

Free radicals are fundamental of any biochemical process and represent as an indispensable part of aerobic life and metabolism (36). The most common reactive oxygen species (ROS) include peroxyl radicals, superoxide (O_2^-) anion, reactive hydroxyl ($OH^·$) and radical's hydrogen peroxide. The nitrogen derived free radicals are nitric oxide ($NO^·$) and peroxynitrite anion. Majority of the diseases/disorders are mainly associated to oxidative stress produced due to free radicals (37). Herbal drugs containing free radical scavengers are known for their therapeutic action (38). Among the numerous naturally occurring antioxidants; ascorbic acid, carotenoids, flavonoids, and phenolic compounds are more efficient (39).

Total phenolics and total flavonoids

Phenolics and flavonoids are potent antioxidants found in plants that contribute to antioxidative mechanisms, including free radical scavenging, lipid peroxidation and chelating metal of ions (40). In the present investigation, the very important two plant secondary metabolites such as total phenolics and total flavonoids were quantitatively determined in calli grown on medium containing different concentrations of extracellularly synthesized silver nanoparticles of *B. subtilis* along with control calli (Fig. 6 and 7). Of various concentrations of silver nanoparticles containing media, the calli grown on media containing 150 mg/L, 200 mg/L and 250 mg/L AgNPs (291.90 ± 12.8 mg GAE/g of callus extract, 200.48 ± 18.86 mg GAE/g of callus extract and 1175.71 ± 8.92 mg GAE/g of callus extract, respectively) showed significant level of total phenolics compared to control calli (151.43 ± 7.95 mg GAE/g of callus extract). The calli grown on media containing different concentrations of AgNPs showed an enhanced level of flavonoids. However, there is no significant variation between the contents of control and treated calli. Our observation is in agreement with the results of Wee *et al*. (14) who observed increased level of these two metabolites in *S. androgynus* plants under elicitation condition.

***In vitro* antioxidants**

Antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electron or hydrogen from substances to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can create chain reactions, when the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants cease these chain reactions by removing free radical intermediates and inhibit other oxidative reactions, (41-42).

Free radical scavenging activity on DPPH

The DPPH is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by antioxidant (43). It has been extensively used to test the ability of compounds as free-radical scavengers or hydrogen donors and to evaluate the antioxidative activity of plant extracts and foods (44-45). DPPH assay was measured based on the antioxidant's ability of crude extracts to quench the stable free radical DPPH by donating either electron or hydrogen atoms (46), It was noted that the calli grown on media containing 150 mg/L, 200 mg/L and 250 mg/L AgNPs (19.29 ± 7.19 µg/mL, 18.84 ± 7.84 µg/mL and 16.76 ± 2.0 µg/mL) showed significant amount of free radical scavenging activity (Fig. 8). This result is accordance with the result of Wee *et al*. (14).

Ferric reducing antioxidant power (FRAP)

FRAP result indicates the reducing ability of complex ferric ion to the ferrous ion which in turn shows the antioxidant capacity of the calli grown on different concentrations of AgNPs i.e for 100 mg/L AgNPs (469.19 ± 6.97 µMol Fe (II) E/g of callus extract), 150 mg/L AgNPs (431.92 ± 5.85 µMol Fe (II) E/g of callus extract) and 200 mg/L AgNPs (416.06 ± 7.86 µMol Fe (II) E/g of callus extract) (Fig. 9). These results were in line with wee *et al*. (14) for tissue of *S. androgynous* and Ghasemzadeh *et al*. (47) for tissue culture of *Zingiber officinale*.

Phosphomolybdate or total antioxidant capacity (TAC)

The assay is based on the fact that molybdenum (VI) is reduced to molybdenum (V) in the presence of reducing agent (antioxidant), forming a green phosphomolybdate (V) complex, which can be evaluated spectrophotometrically at 765 nm. This assay involves an electron transfer mechanism. Many natural products, including phenols and flavonoids, can cause this reduction. The result clearly indicates that the calli grown on nanoparticles containing media 50 mg/L AgNPs, 100 mg/L AgNPs, 150 mg/L AgNPs, 200 mg/L AgNPs and 250 mg/L AgNPs (61.10 ± 8.98 mg AAE /g of callus extract, 70.62 ± 4.03 mg AAE /g of callus extract, 78.80 ± 4.45 mg AAE /g of callus extract, 72.93 ± 4.36 mg AAE /g of callus extract and 69.30 ± 2.98 mg AAE /g of callus extract, respectively) had higher total antioxidant capacity when compared to control calli (57.91 ± 11.66 mg AAE /g of callus extract) (Fig. 10). Plant antioxidants are natural products that inhibit the adverse effects of Reactive Oxygen Species (ROS) in plant cells (48). Antioxidant capacity of calli grown on media containing different concentrations of AgNps may well be related to the proportion of total phenolics (49-50) and total flavonoids (51) that constitute it.

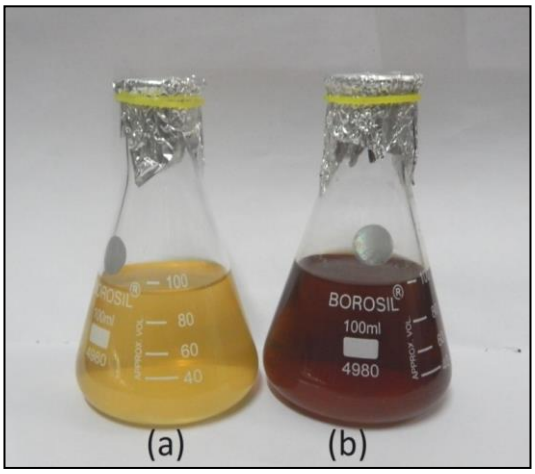

Fig.1 (a) Reaction mixture before colour change (b) Reaction mixture after colour change

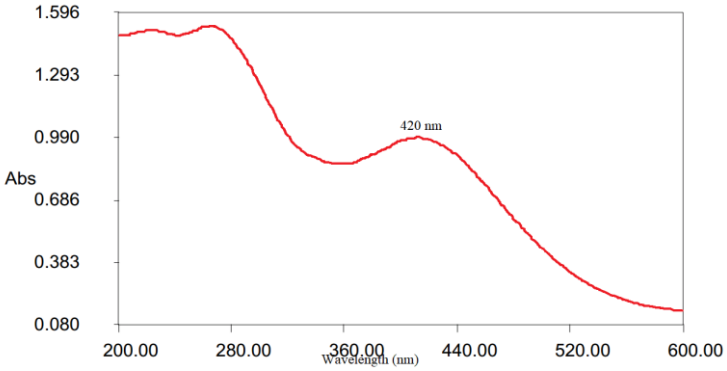

Fig. 2 UV-VIS spectrophotometer analysis of biosynthesized AgNPs

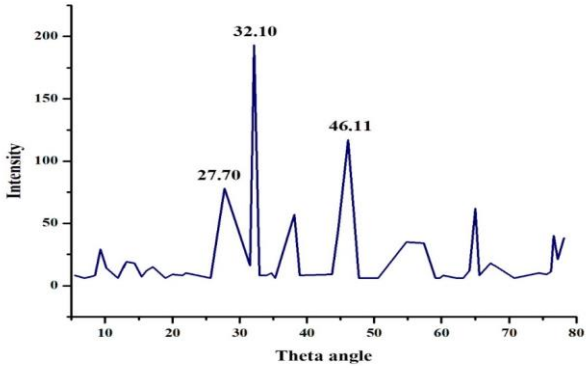

Fig.3 XRD of AgNPs

Table 1. Values of XRD

PEAK NO.	2 THETA (deg)	d (A)	FWHM (deg)	INTENSITY (counts)
1	32.1066	2.78558	0.48670	193
2	46.1150	1.96678	0.51590	117
3	27.7018	3.21768	0.50940	78
4	65.0258	1.43314	0.34830	62
5	38.1333	2.35805	0.93330	57
6	44.638	2.02837	0.40960	45

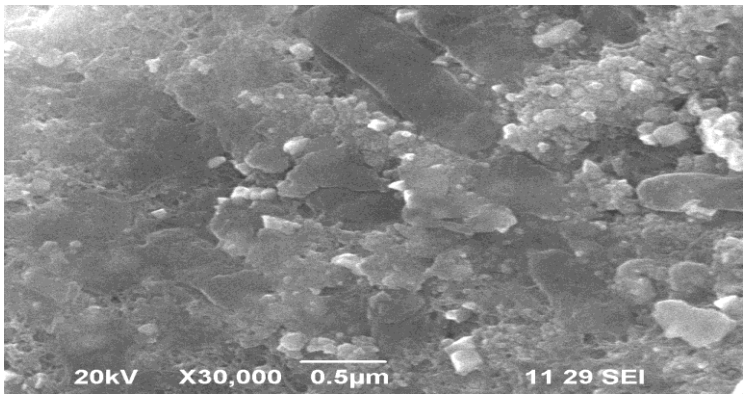

Fig. 4 SEM of silver nanoparticles

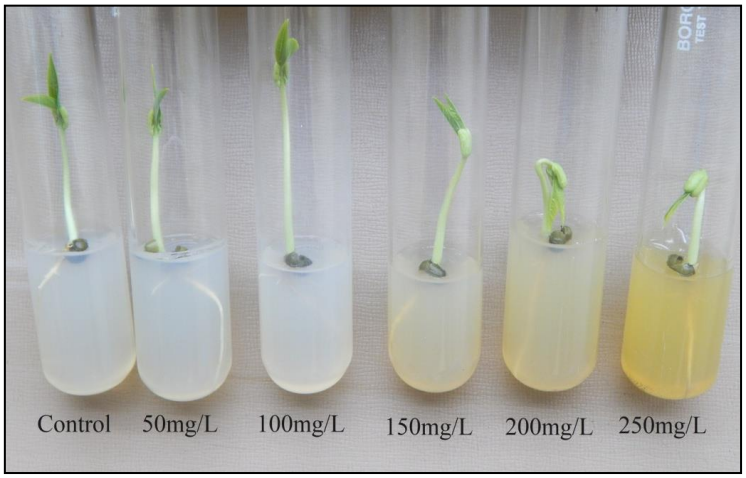

Fig. 5 AgNPs treated seedlings of *Vigna radiate*

Table 2. Influence of various concentrations of AgNPs on germination percentage

Treatment	No of seeds taken (N)	No of seeds germinated (Gf)	Germination (%)
Control callus(MS basal)	10	10	100
MS basal+50 mg/L AgNPs	10	9	90
MS basal+100 mg/L AgNPs	10	10	100
MS basal+150 mg/L AgNPs	10	9	90
MS basal+200 mg/L AgNPs	10	4	40
MS basal+250 mg/L AgNPs	10	4	40

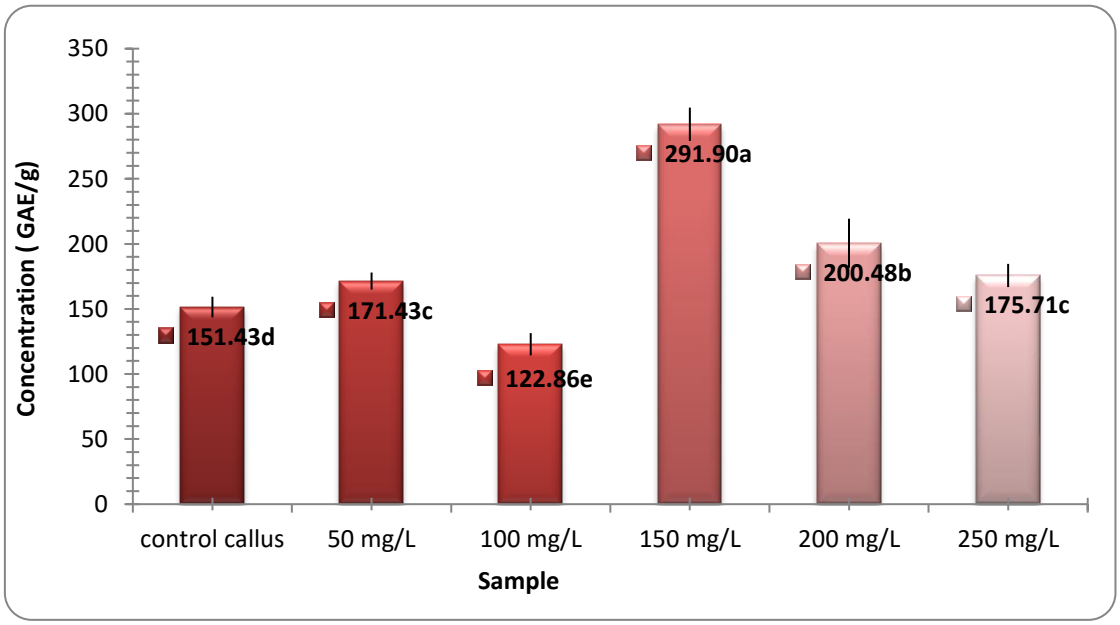

Fig. 6 Content of total phenolics

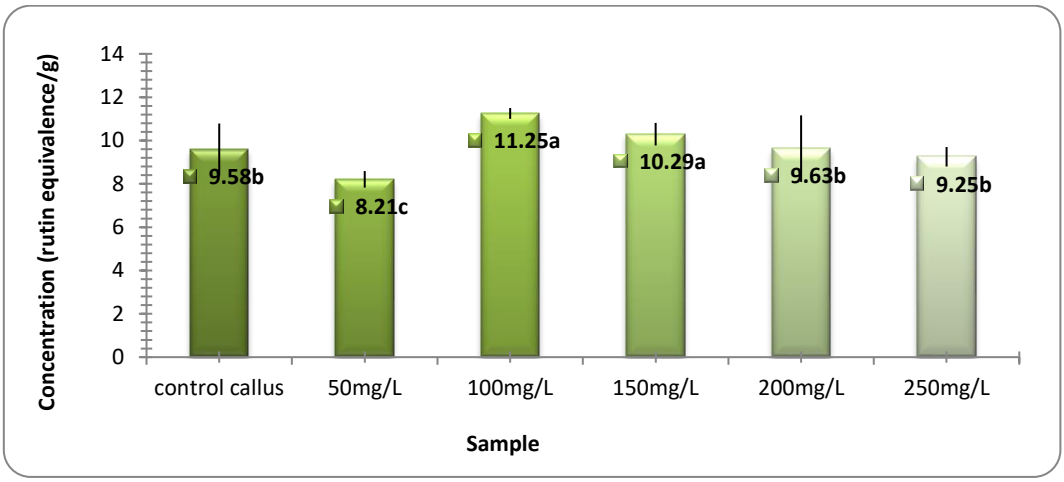

Fig. 7 Content of total flavonoids

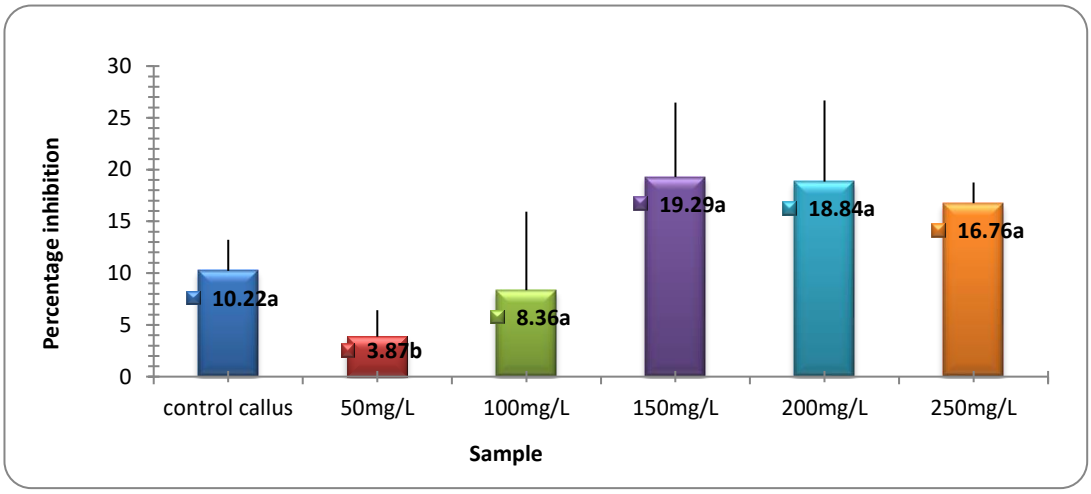

Fig. 8 DPPH radical scavenging activity

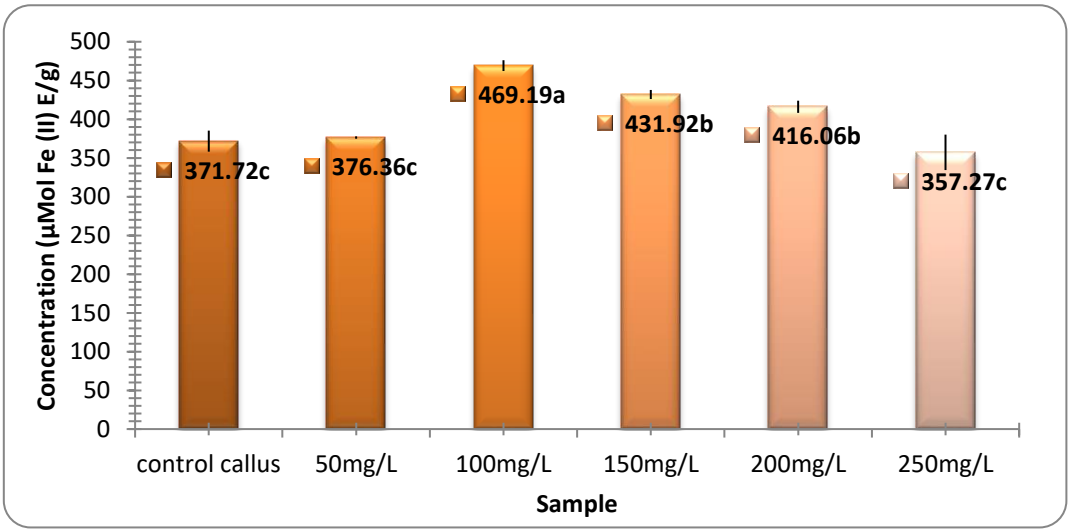

Fig. 9 Ferric reducing antioxidant power (FRAP)

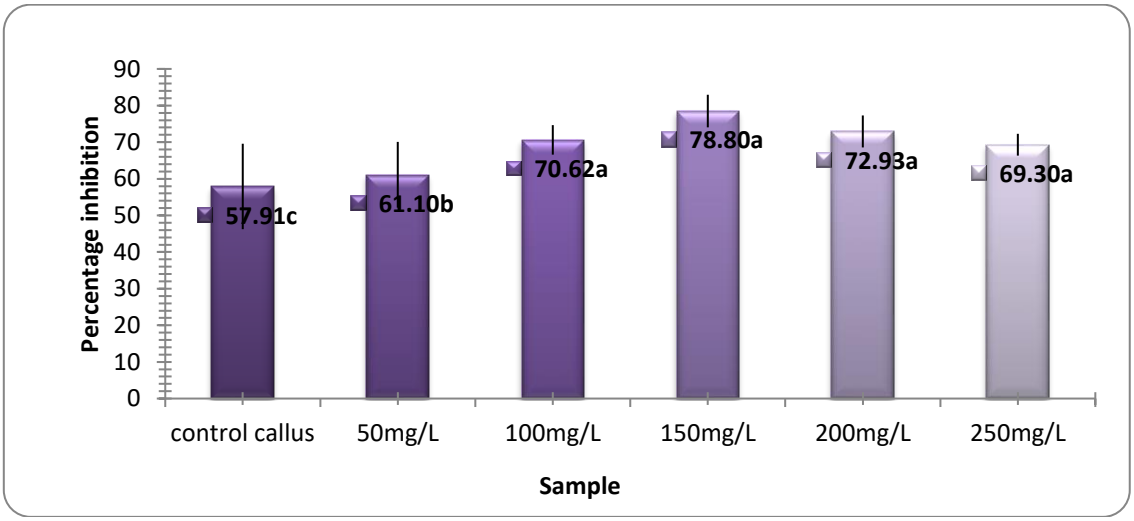

Fig.10 Phospho molybdenum activity

CONCLUSION

In conclusion, this is the first report to exhibit an enhancement in some secondary metabolites such as total phenolics and total flavonoids and an elevated total antioxidant capacity and scavenging activities for DPPH and FRAP free radicals in *S. androgynus* when their calli treated with different concentration of AgNPs as elicitor. The described protocol will need further study to understand its metabolic pathway behind this enhancement.

ACKNOWLEDGEMENTS

The authors are thankful to UGC-SAP and DST-FIST for financial support for department instrumentation to carry our research work.

REFERENCES

1. Prasad A., Mathur A., Singh M., Gupta M. M., Uniyal G. C., Lal R. K. and Mathur A. K., Growth and asiaticoside production in multiple shoot cultures of a medicinal herb, Centella asiatica (L.) Urban, under the influence of nutrient manipulations. J Nat Med, 66 (2): 383-387, (2012)
2. Chodisetti B., Rao K., Gandi S. and Giri A., Gymnemic acid enhancement in the suspension cultures of Gymnema sylvestre by using the signaling molecules—methyl jasmonate and salicylic acid. In Vitro Cell Dev Biol Plant, 51 (1): 88-92, (2015)
3. Pawar K. D., Yadav A. V., Shouche Y. S. and Thengane S. R., Influence of endophytic fungal elicitation on production of inophyllum in suspension cultures of Calophyllum inophyllum L. Plant Cell Tissue Organ Cult (PCTOC), 106 (2): 345-352, (2011)
4. Netala V. R., Kotakadi V. S., Gaddam S. A., Ghosh S. B. and Tartte V., Elicitation of gymnemic acid production in cell suspension cultures of Gymnema sylvestre R. Br. through endophytic fungi. 3 Biotech, 6 (2): 232, (2016)
5. Padmavathi P. and Rao M. P., Nutritive value of Sauropus androgynus leaves. Plant Foods Hum Nutr, 40 (2): 107-113, (1990)
6. Kanchanapoom T., Chumsri P., Kasai R., Otsuka H. and Yamasaki K., Lignan and megastigmane glycosides from Sauropus androgynus. Phytochemistry, 63 (8): 985-988, (2003)
7. Benjapak N., Swatsitang P. and Tanpanich S., Determination of antioxidant capacity and nutritive values of Pak-Wanban (Sauropus androgynus L. Merr.). Warasan Witthayasat Mokho, (2008)
8. Eilert U., Kurz W. G. W. and Constabel F., Stimulation of sanguinarine accumulation in Papaver somniferum cell cultures by fungal elicitors. J Plant Physiol., 119 (1): 65-76, (1985)
9. Lu M., Wong H. and Teng W., Effects of elicitation on the production of saponin in cell culture of Panax ginseng. Plant Cell Rep, 20 (7): 674-677, (2001)
10. Zabetakis I., Edwards R. and Hagan D., Elicitation of tropane alkaloid biosynthesis in transformed root cultures of Datura stramonium. Phytochemistry, 50 (1): 53-56, (1999)
11. Harisaranraj R., Suresh K. and Babu S. S., Production of reserpine in somatic embryos of Rauwolfia serpentina cultured in bioreactors by the induction of elicitor (Methyl Jasmonate). Glob J Biotechnol Biochem, 4 (2): 143-147, (2009)
12. Veerashree V., Anuradha C. M. and Kumar V., Elicitor-enhanced production of gymnemic acid in cell suspension cultures of Gymnema sylvestre R. Br. Plant Cell Tissue Organ Cult (PCTOC), 108 (1): 27-35, (2012)
13. Wang C., Wu J. and Mei X., Enhancement of taxol production and excretion in Taxus chinensis cell culture by fungal elicitation and medium renewal. Appl Microbiol Biotechnol, 55 (4): 404-410, (2001)
14. Wee S. L., Alderson P. G. and Yap W. S. P., Establishment of Plantlet Regeneration System from Nodal, Internodal and Leaf Explants of Sauropus

androgynus (Sweet Shoot). Asian Journal of Biotechnology, 7 (2): 46-59, (2015)
15. Gilaki M., Biosynthesis of silver nanoparticles using plant extracts. J Biol Sci, 10 (5): 465-467, (2010)
16. Bruins M. R., Kapil S. and Oehme F. W., Microbial resistance to metals in the environment. Ecotoxicol Environ Saf, 45 (3): 198-207, (2000)
17. Beveridge T. J., Hughes M. N., Lee H., Leung K. T., Poole R. K., Savvaidis I., ... and Trevors J. T., Metal-microbe interactions: contemporary approaches. Adv Microb Physiol, (Vol. 38: pp. 177-243), (1996)
18. Sastry M., Ahmad A., Khan M. I. and Kumar R., Biosynthesis of metal nanoparticles using fungi and actinomycete. Curr Sci, 85 (2): 162-170, (2003)
19. Lu Y. and Foo L. Y., Antioxidant activities of polyphenols from sage (Salvia officinalis). Food Chem, 75 (2): 197-202, (2001)
20. Prathibha K.S. and Siddalingeshwara K.G., Effect of plant growth promoting Bacillus subtilis and Pseudomonas fluorescence as Rhizobacteria on seed quality of sorghum. Int J Curr Microbiol App Sci, 2 (3): 11-18, (2013)
21. Komaraiah P., Amrutha R. N., Kishor P. K. and Ramakrishna S. V., Elicitor enhanced production of plumbagin in suspension cultures of Plumbagorosea L. Enzyme Microb Technol, 31 (5): 634-639, (2002)
22. Swaroopa G., Anuradha M. and Pullaiah T., Elicitation of forskolin in suspension cultures of Coleus forskohlii (Willd.) Briq. using bacterial elicitors. J. Indian Bot. Soc, 92 (1and2): 97-100, (2013)
23. Kalimuthu K., Babu R. S., Venkataraman D., Bilal M. and Gurunathan S., Biosynthesis of silver nanocrystals by Bacillus licheniformis. Colloids Surf B Biointerfaces, 65 (1): 150-153, (2008)
24. Makkar H. P. S., Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rumin Res, 49 (3): 241-256, (2003)
25. Zhishen J., Mengcheng T. and Jianming W., The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem, 64 (4): 555-559, (1999)
26. Braca A., De Tommasi N., Di Bari L., Pizza C., Politi M. and Morelli I., Antioxidant principles from bauhinia t arapotensis. J Nat Prod, 64 (7): 892-895, (2001)
27. Pulido R., Bravo L. and Saura-Calixto F., Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J Agric Food Chem, 48 (8): 3396-3402 (2000)
28. Prieto P., Pineda M. and Aguilar M., Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem, 269 (2): 337-341, (1999)
29. Kaliappan N. D. and Viswanathan P. K., Pharmacognostical studies on the leaves of Plectranthus amboinicus (Lour) Spreng. Int. J. Green Pharm (IJGP), 2 (3), (2008)
30. Borchert H., Shevchenko EV., Robert A., Mekis I., Kornowski A., Grubel G., Robert A. and Weller H., Determination of nanocrystal sizes: a comparison of TEM, SAXS, and XRD studies of highly monodisperse CoPt3 particles. Langmuir, 21 (5): 1931-6, (2005)
31. Alhussain A. J. A., Abd F. G. and Alkaim A. F., Biological synthesis and characterization of silver nanoparticles using Bacillus subtilis. JGPT, 9 (9):239-244, (2009)
32. Priya A. M., Selvan R. K., Senthilkumar B., Satheeshkumar M. K. and Sanjeeviraja C., Synthesis and characterization of CdWO4 nanocrystals. Ceram Int, 37 (7): 2485-2488, (2011)
33. Lin D. and Xing B., Phytotoxicity of nanoparticles: inhibition of seed germination and root growth. Environ Pollut, 150 (2): 243-250 (2007)
34. Lin D. and Xing B., Root uptake and phytotoxicity of ZnO nanoparticles. Environ Sci Technol, 42 (15): 5580-5585, (2008)
35. Abdel-Azeem E. A. and Elsayed B. A., Phytotoxicity of silver nanoparticles on Vicia faba seedlings. N Y Sci J, 6 (12): 148-156, (2013)
36. Tiwari A. K., Imbalance in antioxidant defence and human diseases: Multiple approach of natural antioxidants therapy. Curr sci, 1179-1187, (2001)
37. Gutteridge J. M. and Halliwell B. Invited review free radicals in disease processes: a compilation of cause and consequence. Free Radic Res, 19 (3): 141-158, (1993)
38. Hakiman M. and Maziah M., Non enzymatic and enzymatic antioxidant activities in aqueous extract of different Ficus deltoidea accessions. J Med Plant Res, 3 (3): 120-131, (2009)
39. Duh P. D., Tu Y. Y. and Yen G. C., Antioxidant activity of water extract of Harng Jyur (Chrysanthemum morifolium Ramat). LWT- Food Sci Technol, 32 (5): 269-277, (1999)
40. Barros L., Ferreira M. J., Queiros B., Ferreira I. C. and Baptista P., Total phenols, ascorbic acid, β-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. Food chem, 103 (2): 413-419, (2007)
41. Ames B. N., Shigenaga M. K. and Hagen T. M., Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci, 90 (17): 7915-7922, (1993)
42. Shenoy R. and Shirwaikar A., Anti-inflammatory and free radical scavenging studies of Hyptis suaveolens (Labiatae). Indian drugs, 39 (11): 574-577, (2002)
43. Lu Y. and Foo L. Y., Antioxidant activities of polyphenols from sage (Salvia officinalis). Food chem, 75 (2): 197-202, (2001)
44. Porto C., Calligaris S., Celotti E. and Nicoli M. C., Antiradical properties of commercial cognacs assessed by the DPPH test. J Agric Food Chem, 48 (9): 4241-4245, (2000)
45. Soares J.R., Dins T.C.P., Cunha A.P. and Almeida L.M., Antioxidant activity of some extracts of *Thymus zygis*. Free Radical Research, 26: 469, (1997)
46. Sahin F., Gulluce M., Daferera D., Sokmen A., Sokmen M., Polissiou, M., ... and Ozer, H., Biological activities

of the essential oils and methanol extract of Origanum vulgare ssp. vulgare in the Eastern Anatolia region of Turkey. Food control, 15 (7): 549-557, (2004)

47. Ghasemzadeh A., Jaafar H. and Karimi E., Involvement of salicylic acid on antioxidant and anticancer properties, anthocyanin production and chalcone synthase activity in ginger (Zingiber officinale Roscoe) varieties. Int J Mol Sci, 13 (11): 14828-14844, (2012)
48. Matkowski A., Plant in vitro culture for the production of antioxidants—a review. Biotechnol Adv, 26 (6): 548-560, (2008)
49. Sultana B., Anwar F. and Przybylski R., Antioxidant potential of corncob extracts for stabilization of corn oil subjected to microwave heating. Food Chem, 104 (3): 997-1005, (2007)
50. Oliveira I., Valentao P., Lopes R., Andrade P. B., Bento A. and Pereira J. A., Phytochemical characterization and radical scavenging activity of Portulaca oleraceae L. leaves and stems. Microchem J, 92 (2): 129-134, (2009)
51. Khan M. A. and Pradhan D., Antiurolithc activity of Ceropegia bulbosa extract in rats. Der Pharmacia Sinica, 3 (1): 148-152, (2012)