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ENHANCED PRODUCTION OF SECONDARY METABOLITES BY SILVER NANOPARTICLE APPLICATION IN CALLUS CULTURES OF *LAVATERA CASHMERIANA*: AN ENDEMIC MEDICINAL PLANT OF KASHMIR HIMALAYA

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Abstract

Lavatera cashmeriana Cambess, endemic and endangered plant of Kashmir Himalaya with a widespread range of medicinal importance. Traditionally it is used as mild laxative, anti-inflammatory, analgesic and also used to cure skin irritation in pregnant ladies. There is a deficiency of research efforts on its proliferation and production of medicinally important secondary metabolites under field and *in vitro* conditions. In this study 24hr treatment of different concentrations (2.5mg^{-l}, 5mg^{-l}, 7.5mg^{-l}, 10.0mg^{-l}, 12.5mg^{-l}) of silver nanoparticles (AgNP) in combination with plant growth hormones BAP(2.0mg^{-l}) 2,4-D(1.0mg^{-l}) and NAA(1.0mg^{-l}) to callus cultures were tested for enhancement of secondary metabolite production. Among the different concentration AgNP (7.5mg^{-l}) along with above plant growth regulators showed maximum increase in production of bioactive compounds: phenols (17.24mg^{-g}), alkaloids (9.23mg^{-g}) flavonoids (27.65mg^{-g}) as compared to the control. However the further increase in concentrations of AgNP (10.0mg^{-l}) proved lethal to the callus.

Key words: *Lavatera cashmeriana*, nanoparticles, *in vitro* cultures, secondary metabolites, growth hormones.

Introduction

Lavatera cashmeriana belongs to malvaceae family and oftenly called as Kashmiri tree mallow and, in English as, wild hollyhock (Rakshanda *et al.,* 2012). Only few species of malvaceae have been studied *in vitro* with the most representative example being cotton (*Gossypium* spp.) (Davidonis and Hamilton, 1983; Trolinder and Goodin, 1987). Members of Malvaceae are commonly documented for their various pharmacological effects including anti-inflammatory (Hossain *et al.,* 2007), antioxidative (Guder and Korkmaz, 2012) antibacterial effects (Razavi *et al.,* 2011). *L. cashmeriana* is generally used in traditional folk medicine (Kaul, 1997) and is also being integrated in numerous Unani medicinal arrangements. It is used in throat problems, given as a mild laxative and its roots are being significantly harvested and supplied in market as crude drug (Rakshanda *et al.,* 2012). It has endangered status with fabulous traditional uses and people use it for various therapeutic purposes that pose danger to its existence; however, there is very scanty information available in the literature about its in-depth scientific studies including *in vitro* techniques for conservation and isolation of important secondary metabolites. The development of callus and cell suspension cultures will reduce the time requisite for plantlet growth and production of bioactive compounds that are either formed in limited quantities in parental plants or difficult to produce under laboratory conditions (Ali *et al*., 2013).

Production of a various groups of secondary metabolites like alkaloids, glycosides, coumarins, flavanoids, steroids, etc. has made plants specifically important for the formulation of diverse pharmaceutical compounds (Premila *et al.,* 2016). In stress conditions, plant cells use either endogenous enzymes or non

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enzymatic components including phenolics and flavonoids that deactivate the injurious effects of Reactive Oxygen Species (ROS) (Valco *et al.,* 2006). The non-enzymatic organization participates in stimulus of the immune system, quenching toxic-free radicals, interaction with cell cycle arrest, regulation of gene expression, enzyme activation and apoptosis initiation (Joo *et al.,* 2010). These compounds play a defensive function during stress conditions, pathogenic attacks, UV radiation and physiological damage. The antioxidant or defensive capability of plant tissue is because of bioactive compounds, most notably phenolics and flavonoids. Usually, a strong relationship exists between phenolics and flavonoids with antioxidant activity (Amid *et al.,* 2011).

The distinctive properties of nanoparticles have extended their application to different fields. However, in the field of tissue culture and medicinal plant biotechnology, the use of nanoparticles is relatively new and needs additional research. The knowledge of how these nanoparticles manage plant growth and developmental processes is a poorly explored area and needs more research. To the best of our knowledge, there is no prior report on enhanced production of secondary metabolites by nanoparticle elicitation in callus cultures of *L. cashmeriana.*

Therefore, the present study aimed to examine the possible effects of AgNPs on production of desired secondary metabolites, including total phenolic content (TPC), total flavonoid content (TFC) and total alkaloid content (TAC) in *L. cashmeriana.*

Materials and Methods

Calli Cultures Development

Seeds of *Lavtaera cashmeriana* were used as explants to develop callus cultures. Practically, 3-4 explants (seeds) were inoculated on the solid surface of Murashige and Skoog (Murashige and Skoog, 1962) media, initially supplemented with 1.0mg^{-1} NAA, 1.0 mg^{-1} 2,4-D and 2.0 mg^{-1} BAP. The MS media was augmented with 30 g l^{-1} sucrose and solidified with 8 g^{-1} the pH was adjusted to 5.7 and all the media were autoclaved at 121°C for 20 min. All cultures were maintained in a growth room at temperature of 25 ± 1°C under a 16/8-h photoperiod with light intensity that ranged from ~40 to 50 µmol m^{-2} s^{-1}, provided by fluorescent tube lights (20 W, Toshiba FL20T9D/19; 380-780 nm). To study the effects of different concentrations of nanoparticle on production of secondary metabolites fresh calli were collected after 30 days of explant incubation (Fig. 1). The calli were transferred to fresh MS media supplemented with (0, 2.5, 5.0, 7.5 mg^{-1}) of silver nanoparticles in combination with pgrs for 24hrs. The MS media without AgNPs was used as control. The MS media augmented with AgNP (7.5mg^{-1}) in combination with growth hormones significantly enhanced the secondary metabolites (Table 1).

Table 1: Different concentrations of silver nanoparticle applied for the production of secondary metabolites in callus cultures of *L. cashmeriana.*

Treatments	Nanoparticle and PGRS (mg^{-1})
To (control)	MS+1.02,4-D+2.0BAP+1.0NAA
T_1	MS+2.5AgNP+1.02,4-D+2.0BAP+1.0NAA
T_2	MS+5.0AgNP+1.02,4-D+2.0BAP+1.0NAA
T_3	MS+7.5AgNP+1.02,4-D+2.0BAP+1.0NAA
T_4	MS+10.0AgNP+1.02,4-D+2.0BAP+1.0NAA
T_5	MS+12.5AgNP+1.02,4-D+2.0BAP+1.0NAA

Quantification of Non-Enzymatic Compounds

The Total phenol content (TPC) in each sample was determined by using the recent methods of (Ahmad *et al.*, 2014). For extract preparation dried calli samples were grounded by using a mortar and pestle. Precisely, 10 mg of the powdered samples was mixed with 10 ml of ethanol and kept for 1 week with periodic shaking. These solutions were centrifuged (14,000 rpm) for 15 min and the supernatant was used for the determination of TPC and total flavonoid content (TFC). Briefly, 0.1 ml (2 N) Folin- Ciocalteu reagent was mixed with 0.03 ml of extract and 2.55 ml of sterile distilled water. Before incubation for 6 min, the mixture was centrifuged (10,000 rpm; 14 min) and then filtered through a 45-µm membrane in a UV-visible spectrophotometer (Shimadzu-1650, Japan) cuvette. The absorbance of the resulted mixture was measured at 760 nm. Gallic acid (Sigma; 1.0-10 mg/ml; R2 = 0.9878) was used for plotting the standard calibration curve. Results as Gallic acid equivalent (GAE)

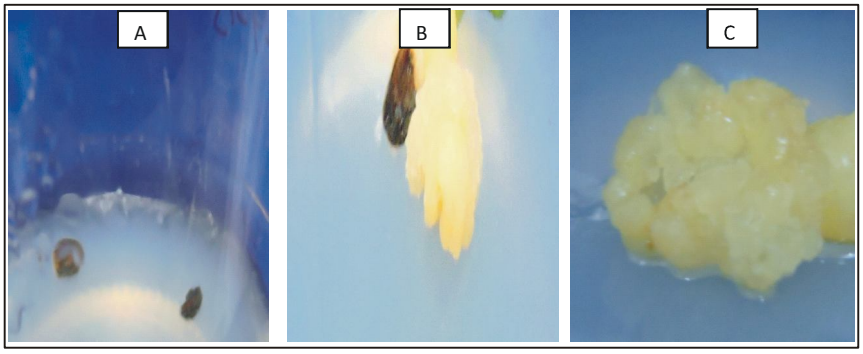

Fig. 1: Induction of friable callus after 30 days of inoculation from seed explants of *L. cashmeriana* on MS medium supplemented with 2mg/l BAP+1mg/l 2,4-D+1mg/l NAA (A-C).

mg/g of DW were obtained from % TPC by using the following equation:

%Total phenolic content ¼ 100 = 100×(AS-AB)/(CF×DF)

Where AS is the absorbance of the sample and AB is the absorbance of the blank. CF is the conversion factor from standard curve and DF is the dilution factor. The TFC in the dried samples was determined by using the method of (Ahmad *et al.*, 2014). The methanolic extract (0.25 ml) of the treated samples was mixed with sterile distilled water (1.25 ml) and 0.075 ml $AlCl_3$ (5%; w/v). Before incubation (5 min) and centrifugation (10,000 rpm; 14 min), the solution was mixed with 0.5 ml of NaOH (1M). The absorbance was checked at 510 nm with a UV-visible spectrophotometer (Shimadzu-1650PC, Japan). Rutin (Sigma; 1.0-10 mg/ml; R2 = 0.9866) was used for plotting the standard calibration curve. The total flavonoid content was expressed as rutin equivalent (RE) mg/g-DW of extracts.

Total alkaloid content (TAC)

Total alkaloid was determined according to the method given by (Fazel *et al*., 2008). Plant sample was centrifuged in methanol (*i.e.*, 1g/10ml) at 10,000 rpm for 10 minutes. The supernatant collected and the residue was re-extracted and centrifuged. The supernatant thus collected was evaporated till dryness and the dried residue was then dissolved in 2N HCl. One ml of this solution was transferred to a separating funnel and then 5 ml of BCG solution along with 5 ml of phosphate buffer were added. The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The extracts were collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm.

The standard curve was prepared by using 0.2, 0.4, 0.6, 0.8 and 1.0 ml of caffeine. Using standard graph the total alkaloid content was calculated and the unit of total alkaloid is expressed in the terms of mg/100g sample.

Results

Effect of Nanoparticles on Phenolics, Flavonoids and Alkaloids in Calli Cultures of *L. cashmeriana*

In the present study, 5 different treatments of AgNP were applied to investigate their effects on secondary metabolite production. Phenolic, flavonoid and alkaloid components of the non-enzymatic defense system were investigated in the callus cultures in response to various concentrations of AgNPs. Maximum production of TPC (17.24GAE-mg/g-DW) was observed when AgNP ($7.5mg^{-l}$) was added to the medium as compared to the control (4.2 GAE-mg/g-DW). Among various concentrations of AgNP used in the experiment AgNP ($7.5mg^{-l}$) showed maximum increase in TFC (25.66 RE-mg/g-DW) as compared to control (17.08 RE-mg/g-DW). Silver nano particle 7.5mg/l was the most efficient nano particle concentration that caused an enhancement in TAC (9.23mg/100g of sample) than control (5.7mg/100g of sample). However, the higher concentrations of AgNP proved lethal to the calli cultures. It shows that NP at optimum concentration caused a significant increase in all the secondary metabolites examined in the given experiment (Table 2).

Table 2: Effect of different concentrations of silver nanoparticle and PGRs on total phenol, flavonoid and alkaloid contents in *L. cashmeriana* callus grown in *in vitro* culture conditions.

Silver nano-particle and PGRs con-centration (mg^{-l})	*L. cash-meriana* TPC (GAE-mg /g-DW)	*L. cash-meriana* TFC (RE-mg /g-DW)	*L. cash-meriana* TAC (mg/100g of sample)
Control	4.20	17.08	5.7
2.5	10.45^{c}	21.41^{c}	6.45^{c}
5.0	12.84^{b}	24.85^{b}	7.80^{b}
7.5	17.24^{a}	27.65^{a}	9.23^{a}
10.0	0.00	0.00	0.00
12.5	0.00	0.00	0.00
G. Mean	13.51***	24.64***	7.83***
S.E.M	0.276	0.14	0.36
C.D.1%	0.113	0.1	0.08

TPC= Total phenolic content, TFC= Total flavonoid content, TAC= Total alkaloid content

Discussion

In this study, application of AgNP considerably influenced secondary metabolite production in calli cultures of *L. cashmeriana*. However, there are no prior reports available concerning the impact of NPs on the development of plant cell, tissue organ culture and their subsequent positive or negative effects on secondary

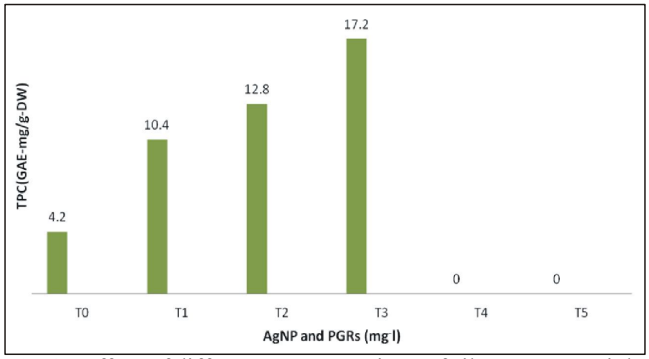

Fig. 2: Effect of different concentrations of silver nanoparticle along with PGRs on total phenol content (TPC) of callus cultures of *L. cashmeriana.*

metabolism in *L. cashmeiana*. Herewith, the enhancement of secondary metabolites by the use of AgNP from the seed-derived calli of *L. cashmeriana* was developed for the first time. Different growth parameters are affected by NPs depending on the plant species, age, physiological state and types of tissues (Vannini *et al.,* 2013). A lot of contradictory reports are cited in literature on the absorption, translocation, accumulation, biotransformation and toxicity of NPs in various plant species (Ma, 2010). The function of free radicals in elicitor-induced increase of secondary metabolites has been broadly experimented in the biosynthesis of indole alkaloids in *Catharanthus roseus* cell cultures (Zhao, 2001). Numerous reports on plant cell cultures have publicized that elicitation can boost the production of secondary metabolites. Methyl jasmonate (MeJA) was documented as an efficient elicitor that could enhance the production of paclitaxel in *Taxus candensis* and *T. cuspidate* (Kethum *et al.,* 1999), gymnemic acid in *Gymnema sylvestre* and anthocyanin in *Tulipa gesneriana* (Saniewski *et al.,* 1998), (Veerashree *et al.,* 2012). Cell suspension culture of *H. perforatum* showed the double fold increase in the production of both hypericin and pseudohypericin as compared to control cell suspension cultures (Gadzovska *et al.,* 2012). MeJA and AgNPs stirred the production of essential oils *in A. millefolium L.* plants, viewing the protective reaction of plants against elicitation effects of MeJA and AgNPs. Earlier, the uses of AgNPs showed inhibitory effect on seed germination and mean shoot length in *Hordeum vulgare* and *Linum usitatissimum* (El-Temsah and Joner, 2010; Sharma *et al.,* 2012; Salama, 2001). In contrast, AgNPs influenced the growth of *Brassica juncea*, *Phaseolus vulgaris* and *Zea mays* (Salama, 2012; Sharma *et al.,* 2012). In this study, the addition of different concentrations of AgNPs to MS media was found encouraging for improved production of phenolics, flavonoids and alkaloids in the calli cultures of *L. cashmeriana* (Fig. 2, 3, 4). Reports on the production of

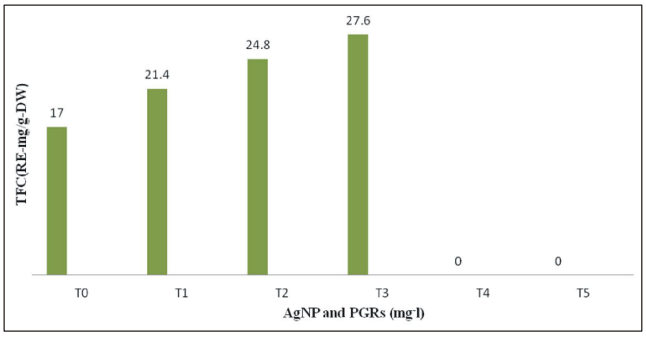

Fig. 3: Effect of different concentrations of silver nanoparticle along with PGRs on total flavonoid content (TFC) of callus cultures of *L. cashmeriana*.

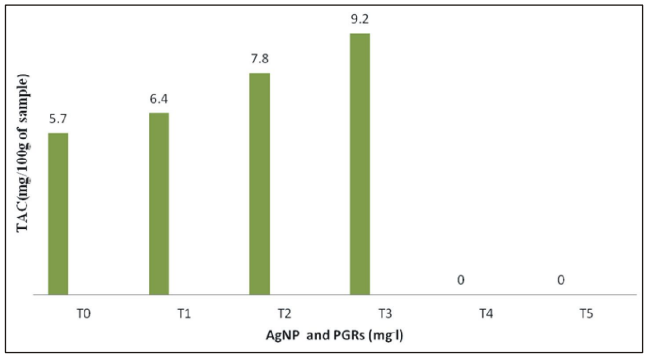

Fig. 4: Effect of different concentrations of silver nanoparticle along with PGRs on total alkaloid content (TAC) of callus cultures of *L. cashmeriana.*

TPC, TFC and TAC in *L. cashmeriana* cultures *in vitro* are not available in the literature. However, TPC, TFC and TAC have been reported in the *in vitro* cultures of other medicinal plants (51, Parsaeimehr, 2010; Costa, 2012). Reports are available that both elicitors and PGRs alone or in combination not only affect the organogenic capacity but also the synthesis and production of primary and secondary metabolites. Callus cultures of *Cicer arietinum* showed higher quantities of TPC than other tissues (Naz and Iqbal, 2008). (Ghasemzadeh *et al.,* 2010) documented enhanced production of phenolics and flavonoids in two varieties of *Zingiber officinale*. The results of the present study propose that the calli cultures of *L. cashmeriana* are a rich source of bioactive compounds and have a potential for marketable applications. Moreover, the NP taken up in this experiment has potential for enhanced morphological variations in the callus cultures of *L. cashmeriana*. Lastly, it can be concluded that NPs makes the practical application of nanotechnology in the branch of plant sciences with respect to the intend of restricted environment for *in vitro* safe secondary metabolite production.

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Conflict of interest

Authors have no conflict of interest.

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