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Trichoderma asperellum Mediated Synthesis of Silver Nanoparticles: Characterization and its Physiological Effects on Tea [*Camellia sinensis* (L.) Kuntze var. *assamica* (J. Masters) Kitam.]

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A B S T R A C T

Silver nanoparticles (Ag NPs) were synthesized using culture filtrate of fungal antagonist *Trichoderma asperellum* for which silver nitrate was used as the precursor and were characterized by using UV-Vis spectroscopy, DLS, Zeta sizer, TEM and EDX. The physiological effects of Ag NPs on the host crop i.e. tea (clone-TV21) was evaluated at 100% concentration by introducing into the host with five different treatment methods *viz.* cutting treatment (one leaf bud cutting), injection method, foliar spray, soil application and seedling root dip treatment with 10 replicates for each. Observation on changes in chlorophyll content, moisture content, relative water content, total soluble sugar, total protein, lipid peroxidation (MDA content), secondary metabolites *viz.* phenol, alkaloid, and flavonoid were analyzed after 45 days of the treatment. Results showed that silver nanoparticles can induce the plants in increasing all the studied physiological parameters. Out of all the five treatments, foliar spray followed by seedling root dip treatment was found to be the best treatment for establishing silver nanoparticles in the plant system with maximum positive effects on all the parameters compared to other treatments and as control.

Introduction

In the field of agriculture and crop science, the particular interest of nanotechnology lies in agrifood applications including nanosizing of agrochemicals with an aim to improve efficacy and thus enable a reduction in the use of pesticides, biocides and veterinary medicines in food production, this may also enable a better control of applications as in slow-release pesticides (Kah *et al.,* 2012). Nano-sizing of active ingredients may also lead to the development of safer and more nutritious animal feeds (fortified with nano-sized supplements, antimicrobials, detoxifying substances). Nanotechnology also provides the tool and the technological platform for the study and transformation of biological systems *viz.* plants. But a few studies have focused on the effects and mechanisms of nanomaterials on plants. Since plants possess large size and high leaf area

and are of stationary in nature they have a greater chance of exposure to a wide range of NPs available in their surrounding environment (Dietz and Herth, 2011). The higher plants interact strongly with their atmospheric and terrestrial environments and so, when they come in contact with the nanoparticles, either artificially synthesized one or that from certain natural sources, are expected to be affected. Nano-fertilizers or nano-encapsulated nutrients are now considered as a better alternative to the commonly available fertilizers and it has been reported that nanoparticles can serve as "magic bullets", containing herbicides, nano-pesticides, fertilizers, or genes, which target specific cellular organelles in plants to release their content (Siddiqui *et al.,* 2015). These applied nano-fertilizers and nano-pesticides may undergo accumulation in plants and thus may have certain effects on the physiological and morphological parameters of plants. Many research works carried out by scientists all over the world underlines the positive as well as negative attributes of metal nanoparticles when applied in plants.

Silver nanoparticles (Ag NPs) are among the mostly used engineered nanoparticles in a wide range of consumer products and are expected to enter natural ecosystems including soil *via* diverse pathways (Anjum *et al.,* 2013). Also, silver ions and silver based composites are highly toxic for certain pathogenic microorganisms. Therefore, silver nanoparticles have been used in various types of pesticide formulations. So a number of studies on physiological parameters *viz.* chlorophyll content, carbohydrate, total protein and total phenolic content were made when host plants were exposed to silver nanoparticles. For most of the works, short duration non-hardy crops were selected like common bean, corn plants (Salama, 2012), *Vigna radiata (*Najafi and Jamei, 2014) and mustard plants (Pandey *et al.,* 2014). Whereas a very few works have been done by using other metal nanoparticles like gold, alumina, zinc, titanium, silicon etc. and no report of using hardy long duration crop is available.

Introduction and establishment of nanoparticles within plant system for studying its effect on the plant system is an important area of research but very little work has been done on this aspect. A few researchers have tried seed treatment and soil treatment for the introduction of nanoparticles within the plant system. Madvar *et al*., 2012 and Pandey *et al*., 2014 tried seed treatment and Mukherjee *et al.,* 2016 tried soil treatment for the introduction of nanoparticles within the plant system. Reports on other treatment methods for establishment of nanoparticles within the plant systems are not available.

In the present study silver nanoparticles have been synthesized using *Trichoderma asperellum*, a potential fungal antagonist. The nanoparticles were then characterized for their size, shape, charge, composition etc. The effect of these biologically synthesized nanoparticles have been evaluated after introduction into the plant system and compared with the untreated or control plants.

Materials and Methods

Experimental Details

All the reagents and chemicals were of analytical grade and used without further purification. The pure culture of *Trichoderma asperellum* (ITCC no. 8886.13) was collected from preserved culture in *Surakshit* (A long-term preservation method of fungal biocontrol agents for which patent has been applied and published 977/KOL/2014) Department of Plant Pathology, AAU, Jorhat, Assam. Silver nitrate (HiMedia) was used as the precursor for synthesis of silver nanoparticles.

Synthesis of silver nanoparticles from culture filtrates of *T. asperellum*

Synthesis of silver nanoparticles was done by standardized method (Kaman, 2016). Freshly grown 7 days old fungal mat of *T. asperellum* was harvested and centrifuged at 5000 rpm for 10 minutes at 4˚C. 50 ml of *Trichoderma* supernatant was taken and treated with 50 ml of 1 mM $AgNO_3$ aqueous solution (as precursor) in a 250 ml Erlenmeyer flask. After adjusting the pH at 10, the whole mixture was kept in an orbital shaking incubator for 5 days under dark condition. Control experiments were conducted without the precursor. The formation of silver nanoparticles was monitored by using UV-Vis spectroscopy.

Characterization of silver nanoparticles

Characterization of silver nanoparticles was done by different type of equipments like UV-VIS Spectrophotometer (Eppendorf Biospectrometer), DLS (ZETA sizer, Nano series, Malvern instrument Nano Zs, 2000), Zeta sizer (ZETA sizer, Nano series, Malvern instrument Nano Zs, 2000), EDX and Transmission Electron Microscopy (JEM-2100) study at different institutes like Dept. of Plant Pathology, Assam Agricultural University, Jorhat, Assam, Department of Material Science, NEIST, Jorhat, Assam and SAIF, NEHU, Shillong, Meghalaya.

Treatment methods

To study the physiological effects of silver nanoparticles, Tea [*Camellia sinensis* (L.) Kuntze var. *assamica* (J. Masters) Kitam.] was selected as the host plant. The clone TV 21 developed at Tocklai Tea Research Institute, Jorhat, Assam was selected for this study. The study was done at the nursery (net house) of Experimental Garden for Plantation Crops (EGPC) at AAU, Jorhat. All the treatments were done at 100% concentration of silver nanoparticles and 10 sleeves were maintained for each treatment and control plants.

Cutting treatment

Healthy and freshly harvested cuttings (one-leaf bud cutting) from the primary shoots of mother tea bushes were taken for the treatment. Around 250 ml of aqueous solution of silver nanoparticles were taken in a beaker and the cuttings were dipped in that solution for half an hour. After treatment the cuttings were planted in the plastic bags (sleeves) containing soil. Cuttings were then gently sprayed with water.

Injection method

Healthy 5-6 months old seedlings were selected for the treatment and two ml of the silver nanoparticle aqueous solution was injected in the hard root of the seedlings by using a hypodermic syringe (BD Emerald needle syringe). The pores were then sealed by applying petroleum jelly and cuttings were then gently sprayed with water.

Foliar spray method

Leaves of the seedlings were sprayed with silver nanoparticles covering both the abaxial and adaxial surfaces by using a hand atomizer. Treated seedlings were then covered with perforated plastic bags to maintain the humidity on the foliage. At an interval of one day spraying was done with distilled water during the whole period of experimentation.

Soil treatment

Soil in the poly sleeves was treated with silver nanoparticles @ 25 ml per poly sleeve such that it gets wet up to a depth of 7 cm. Healthy

tea cuttings were planted in the poly sleeves where soil was treated with silver nanoparticles.

Seedling root dip treatment

Roots of the selected seedlings of age 5-6 months were washed properly in running tap water to remove all the adhered soil particles. Rooted seedlings were then dipped in the silver nanoparticle aqueous solution for 30 min. and were planted carefully in the poly sleeves. After planting, seedlings were gently sprayed with water.

Control

Soils and seedlings in control were maintained by using sterile distilled water in place of silver nanoparticles.

Estimation of physiological parameters

Total chlorophyll determination

Total chlorophyll content of the seedlings under experimentation was determined by the method described and standardized by McKinney (1941).

Total soluble sugar determination

Total soluble sugar of the plants under experiment was determined by following the method described and standardized by Yemm and Willis (1954).

Total protein estimation

The total soluble protein content was estimated by using standard Lowry method (Lowry *et al.*, 1951).

Total phenol determination

The total phenol content was estimated by using the method of Singleton (1999).

Total alkaloid determination

The total alkaloid content of the plants under experiment was estimated by using the method of Harborne (1973).

Flavonoid determination

The plants under experimentation were subjected for the estimation of total flavonoid content.

The method of Woisky and Salatino (1998) was followed for the purpose.

Leaf moisture content determination

The moisture content of the samples was estimated by the standard procedure given by Association of Analytical Communities (2000).

The moisture content of the test samples was calculated by using the following equation:

$$\text{Moisture (\%)} = [(W_1 - W_2) / (W_1)] \times 100$$

Where, W_1= Weight (gm) of the sample before drying

W_2= Weight (gm) of the sample after drying

Relative Leaf Water Content (RLWC) determination

The RLWC of the samples were estimated by following the standard method of Yamasaki and Dillenburg (1999). The weights of the dried samples were then recorded.

$$\text{RLWC (\%)} = [(FW - DW)/ (TW - DW)] \times 100$$

Where, FW= Fresh weight (gm) of the sample
DW= Dry weight (gm) of the sample
TW= Turgid weight (gm) of the sample

Lipid peroxidation Determination (MDA Content)

The lipid peroxidation level was measured in terms of Malondialdehyde content (MDA), a product of lipid peroxidation by following the method of Heath and Packer (1968).

Results and Discussion

Fungus mediated green synthesis of silver nanoparticles

Trichoderma asperellum was purposefully selected for synthesis of silver nanoparticles based on its higher efficacy, rapid multiplication rate and huge biomass production. For the green synthesis of silver nanoparticles, when supernatant of *T. asperellum* was exposed to 1mM aqueous solution of $AgNO_3$ the color of supernatant changes from green to yellowish brown to brown after 192 hours of reaction (Plate 9 b). The fungal supernatant of *T. asperellum* without $AgNO_3$ retains its original color (Plate 9 a). The color change in the supernatant from green to brown confirms the formation of silver nanoparticles. The color change observed during this study was due to the Surface Plasmon Resonance (SPR) phenomenon. Generally, the metal nanoparticles have free electrons, which help in the formation of the absorption band. It happens due to the united vibration of the electrons of metal nanoparticles in resonance with light waves.

A possible mechanism for the conversion of silver ions into nano form by using fungal biomass could be the extracellular reduction of silver ions in the solution followed by precipitation on to the cells. This may be the reason for the gradual change in color of the silver nitrate treated *Trichoderma* supernatant from green to brown (Tripathi *et al.,* 2013 and Vahabi *et al.,* 2011).

Characterization of biosynthesized silver nanoparticles

UV-VIS Spectrophotometer analysis

UV-VIS Spectroscopy of the $AgNO_3$ treated with *T. asperellum* was carried out at different wavelengths and showed maximum absorption at the critical wavelength (300-500 nm). Metal nanoparticles have free electrons, which give the Surface Plasmon Resonance (SPR) absorption band, due to the combined vibration of electrons of metal nanoparticles in resonance with a light wave. In the present study a characteristic, SPR absorption band was observed in the supernatant of *T. asperellum* treated with 1mM $AgNO_3$ at 420 nm (Fig. 1). No absorption band was observed in control i.e. supernatant of *T. asperellum* without 1mM $AgNO_3$ (Fig. 2). Earlier works reported that for silver nanoparticles SPR band occur at 300-500 nm (Basavaraja *et al.,* 2007). SPR band for silver nanoparticles synthesized by using *Fusarium semitectum* (Basavaraja *et al.,* 2007), *T. harzianum* (Singh and Raja, 2011) and *Rhizopus stolonifer* (Rahim *et al.,* 2017) were reported at 420 nm.

Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) is a technique used in material physics for determining the size distribution profile of nanoparticles in suspension or polymers in solution. This technique was used in present study to determine the size distribution profile of nanoparticles present in the final solution after ultrasonication. DLS also determines polydispersity, hydrodynamic sizes and aggregation of particles in the suspension. DLS analysis showed that biosynthesized nanoparticles have an average size of 68 nm (Fig. 3) with a polydispersity index (PDI) of 0.857 indicating the nanoparticles were poly dispersed in nature. PDI is dimensionless with a value between 0 and 1, which is scaled such

that values with 0.10 or less are considered highly mono dispersed and above 0.10 are polydispersed (Hughes *et al.* 2015).

Zeta potential

Nanoparticles are very small in size because of which they are energetically very unstable. Therefore the particles undergo agglomeration to stabilize themselves. So there are some potential charges on the surface of nanoparticles which makes them stable. To study the stability of the biosynthesized silver nanoparticles Zeta potential was determined and recorded the charge as -1.34 mV (Fig. 4).

It indicated that synthesized silver nanoparticles were highly stable and do not have an affinity to agglomerate. Nanoparticles with Zeta Potential values between +30 mV and -30 mV typically have high degrees of stability and the large negative zeta potential value (above -0.50 mV) indicates higher electrostatic repulsion among silver nanoparticles and stable on their dispersion (Zhang *et al.* 2009).

Transmission Electron Microscopy (TEM)

Transmission Electron Micrograph (TEM) micrographs (Plate 10 a-c) obtained at an accelerating voltage of 200 kV with 20,000 X magnifications revealed that the nanoparticles were formed in the size range of 4-14 nm with an average size of 8.26 nm with shape roughly spherical. TEM micrographs also indicated that nanoparticles were relatively uniform in nature and well separated from each other having no agglomeration. TEM micrographs recorded in this study for biosynthesized silver nanoparticles coated with copper indicated that the nanoparticles were pure in form without any impurities. The electron diffraction pattern (ED) indicated the crystalline nature of synthesized material (Plate 11).

Energy Dispersive X-ray analysis (EDX)

Energy Dispersive X-ray analysis (EDX) was done at an accelerating voltage of 200 kV using the TEM. EDX spectrum revealed that the synthesized nanoparticles contain elements *viz.* silver (32.18%), oxygen (10.16%) and carbon (57.66%) which is shown in the figure 5. The presence of carbon and oxygen in the sample between 0 and 4 keV confirms the presence of stabilizers composed of alkyl chains. The results are in accordance with the findings of Kaushik and Joshi (2015). The fungal media i.e. PDB used for culturing *T. asperellum* might be the source of carbon and oxygen in the biosynthesized material.

Estimation of physiological parameters

After 45 days of treatment, samples were collected from the treated seedlings as well as from the control. Samples were then subjected for studying the effects on host physiology by maintaining five replications per treatment.

Chlorophyll content

Data presented in Table 1 shows the leaf chlorophyll content in all the five different treatments followed to apply silver nanoparticles in tea *viz*. cutting treatment, injection method, foliar spray, soil treatment, seedling root dip treatment, and control. Significantly higher chlorophyll content (1.71 mg/g fresh wt.) was recorded in plants where nanoparticles were sprayed. Chlorophyll content recorded for foliar spray (1.71 mg/g fresh wt.) and seedling root dip method (1.68 mg/g fresh wt.) were statistically at par with each other but significantly differs from injection method (1.66 mg/g fresh wt.), cutting treatment (1.22 mg/g fresh wt.), soil treatment (1.08 mg/g fresh wt.) and control (1.04 mg/g fresh wt.). Chlorophyll content recorded in injection method, soil treatment,

cutting treatment and control were not differing significantly. Higher chlorophyll content observed in the treated seedlings might be due to the triggering of more chlorophyll production, as application of silver nanoparticles enhances the nutrients absorbing ability of the host plant. More absorption ability directly influences the plant to uptake more nitrogen and magnesium from the soil which leads to more production of chlorophyll (Lee *et al.,* 2011). Pandey *et al.* (2014) studied the effects of silver nanoparticles on chlorophyll content in mustard plants and found increased chlorophyll content with increasing concentration of silver nanoparticles.

Total Soluble Sugar (TSS) content

Data presented in Table 1 shows that the tea seedlings treated by foliar spray (28.5%) of silver nanoparticles has highest TSS content than all the other treatments and control. TSS content recorded for foliar spray (28.5%), seedling root dip treatment (28.33%) and soil treatment (28.17%) were statistically at par with each other but significantly differs from control (21.17%). TSS content recorded in injection method (23.67%) also significantly differs from control. Cutting treatment (23.17%) and control were statistically at par with each other. This increase in TSS content may be due to improvement in absorption and utilizing abilities of the plant after application of silver nanoparticles which ultimately improves the complete plant health. Krishnaraj *et al.* (2012) reported that TSS being the primary metabolite is directly involved in the plant growth promotion and biologically synthesized silver nanoparticles interact with plant growth and metabolism.

Total soluble protein

Total soluble protein content was found to be significantly influenced by different treatment methods, except the cutting treatment (Table 1). Similar to the above-mentioned tests foliar spray (25.4%) was found to be the best treatment with significantly highest protein content. The total soluble protein content for foliar spray (25.4%), injection method (24.40%), soil treatment (24.20%) and seedling root dip method (24.25%) were statistically at par with each other but significantly differs from cutting treatment (19.70%) and control (19.00%).

The increase in protein content is due to that after application of silver nanoparticles, plant undergoes a mild stress condition, and any biotic or abiotic stresses result in the production of various stress related proteins. These stress proteins are responsible for safeguarding the plants up to a certain range.

Total phenol

Phenol content of plant samples from different treatment methods showed that the phenol content was increased in the seedlings treated with silver nanoparticles (Table 2). Significantly highest phenol content (21.80%) was recorded in the foliar spray treated seedlings. Phenol content recorded for foliar spray (21.80%), seedling root dip treatment (21.30%) and injection method (20.40%) were statistically at par with each other but significantly different from control (18.20%). No significant difference in phenol content was recorded in soil treatment (19.40%), cutting treatment (18.70%) and control.

Total alkaloid

Data presented in Table 2 shows the alkaloid content of plant samples treated by different methods. Alkaloid content was found increased in all the treated seedlings, but there was no significant difference in alkaloid content among the treated and the control seedlings. The alkaloid content recorded for

different treatments were 4.01%, 3.96%, 3.83%, 3.82%, 3.81% and 3.80 % for foliar spray, seedling root dip treatment, injection method, cutting treatment, soil treatment, and control respectively.

Total flavonoid

Results of total flavonoid content presented in Table 2 shows a significant influence of different treatment methods *viz.* cutting treatment, injection method, foliar spray, soil treatment and seedling root dip treatment on it. Flavonoid content in foliar spray (6.60%) and root dip treated seedlings (6.40%) was statistically at par with each other and significantly differ from other treatment and control.

Flavonoid content of soil treated seedlings (5.80%) and injection method (5.70%) is statistically at par with each other and significantly differs from control. There was no significant difference among cutting treatment (5.60%) and control (5.20%). Phenol, alkaloid, and flavonoid are the secondary metabolites which have role in the plant defense system. Silver nanoparticles when introduced in the plant stimulate its anti-oxidant system which ultimately improves plant's resistance to adversities by the production of defense-related compounds (Najafi and Jamei, 2014).

Total leaf moisture content

Leaf Moisture Content of the treated and control plants is presented in Table 3. Leaf Moisture Content was found to be significantly highest in the seedlings treated with foliar spray (68.27%) of silver nanoparticles. For other treatments leaf moisture content recorded were 64.64%, 63.47%, 63.09%, 62.90%, and 62.03% for soil treatment, seedling root dip treatment, injection method, cutting treatment and control respectively. But these treatments had no significant difference among them and control.

Table.1 Effect of different methods of application of green synthesized silver nanoparticles on chlorophyll content, TSS and Total protein content of tea

Treatment	Chlorophyll content (mg/g fresh weight)	TSS (%)	Total protein (%)
T_1: Cutting treatment	1.22^{abcd}	23.17 $(28.73)^{de}$	19.70 $(26.35)^{e}$
T_2: Injection method	1.66^{abc}	23.67 $(29.00)^{d}$	24.40 $(29.60)^{ab}$
T_3: Foliar spray	1.71^{a}	28.50 $(32.27)^{a}$	25.40 $(30.26)^{a}$
T_4: Soil treatment	1.08^{abcde}	28.17 $(32.01)^{abc}$	24.20 $(29.47)^{abcd}$
T_5: Seedling root dip treatment	1.68^{ab}	28.33 $(32.14)^{ab}$	24.25 $(29.53)^{abc}$
T_6: Control	1.04^{cdef}	21.17 $(27.35)^{ef}$	19.00 $(25.84)^{ef}$
SEd (±)	0.30	0.71	0.72
CD (0.05)	0.63	1.46	1.49

Table.2 Effect of different methods of application of green synthesized silver nanoparticles on total phenol, alkaloid and flavonoid content of tea

Treatment	Total phenol (%)	Alkaloid (%)	Flavonoid (%)
T_1: Cutting treatment	18.70 (25.62)^{cde}	3.82 (11.24)	5.60 (13.69)^{cde}
T_2: Injection method	20.40 (26.85)^{abc}	3.83 (11.24)	5.70 (13.81)^{cd}
T_3: Foliar spray	21.8 (27.83)^{a}	4.01 (11.54)	6.60 (14.89)^{a}
T_4: Soil treatment	19.4 (26.13)^{bcd}	3.81 (11.24)	5.80 (13.94)^{c}
T_5: Seedling root dip treatment	21.30 (27.49)^{ab}	3.96 (11.39)	6.40 (14.65)^{ab}
T_6: Control	18.20 (25.25)^{def}	3.80 (11.24)	5.20 (13.18)^{ef}
SEd (±)	0.73	0.29	0.26
CD (0.05)	1.52	NS	0.54

Table.3 Effect of different methods of application of green synthesized silver nanoparticles on leaf moisture content, RLWC content and MDA content of tea

Treatment	Leaf moisture content (%)	RLWC (%)	MDA content (µmol/g fresh weight)
T_1: Cutting treatment	62.09 (52.48)^{bcde}	71.40 (57.70)^{de}	70.81
T_2: Injection method	63.09 (52.54)^{bcd}	73.85 (59.20)^{cd}	70.83
T_3: Foliar spray	68.27 (55.67)^{a}	85.30 (67.46)^{a}	71.40
T_4: Soil treatment	64.64 (53.49)^{b}	77.10 (61.41)^{b}	71.30
T_5: Seedling root dip treatment	63.47 (52.70)^{bc}	76.17 (60.73)^{bc}	71.20
T_6: Control	62.03 (51.94)^{bcdef}	66.80 (51.82)^{f}	70.82
SEd (±)	0.95	0.90	0.50
CD (0.05)	1.96	1.87	NS

* Data are mean of five replications
* Data in parentheses are angular transformed values

Plate.1, 2 & 3 Pure culture of *Trichoderma asperellum* (a. In PDA slant, b. In PDA plate), Mass culture of *Trichoderma asperellum* in Potato Dextrose Broth (PDB) & Supernatant of *T. asperellum*

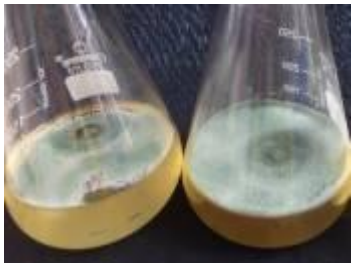




Plate.4, 5 & 6 Treatment of cuttings with silver nanoparticles, Injecting of seedlings with silver nanoparticles & Foliar spray of seedlings with silver nanoparticles



Plate.7, 8 & 9 Seedling root dip treatment with silver nanoparticles, Soil treatment with silver nanoparticles & Vials containing the supernatant of *T. asperellum* in aqueous solution of 1 mM $AgNO_3$ at the beginning of the reaction (a) and after 5 days of reaction (b)



Plate.10a, b & c TEM micrograph of green synthesized silver nanoparticles from *T. asperellum*, TEM micrograph of green synthesized silver nanoparticles from *T. asperellum* (b-c)

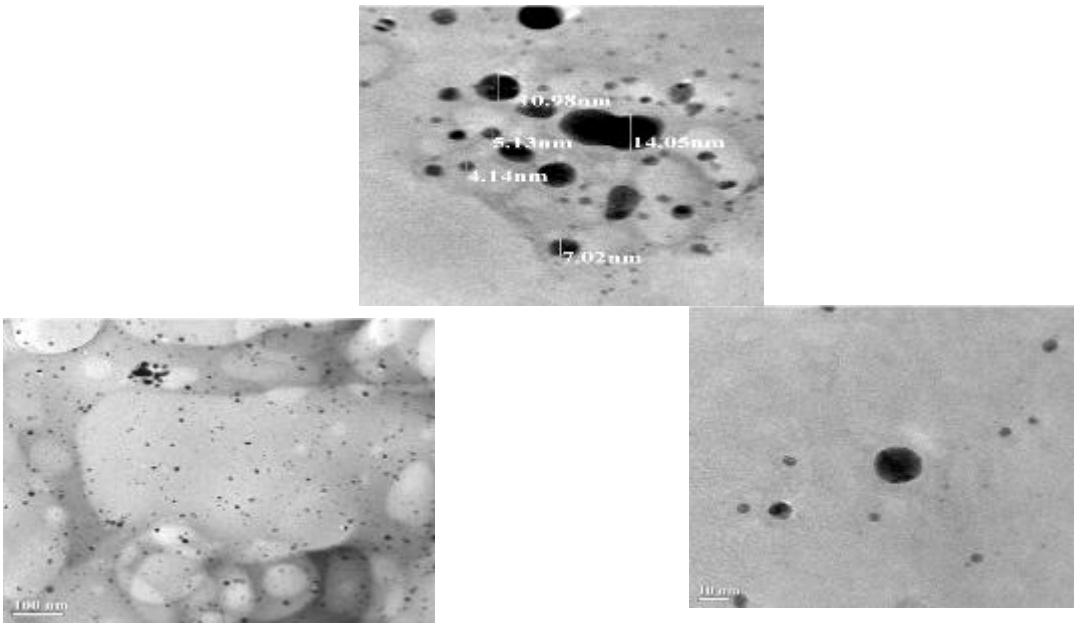


Plate.11 ED pattern of green synthesized Ag NPs from *T. asperellum*

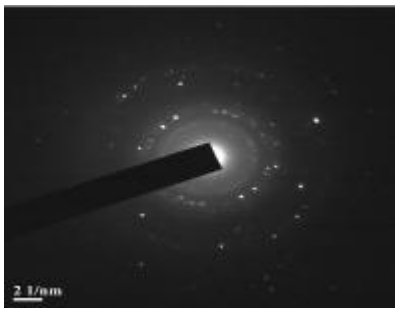
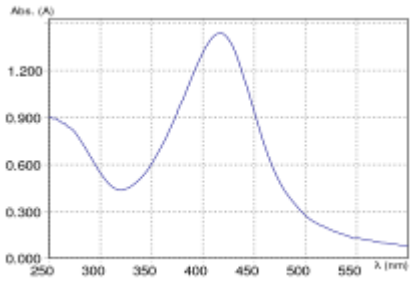


Fig.1&2 UV –VIS absorption spectra of green synthesized Ag NPs from *T. asperellum* & UV – VIS spectra of supernatant of *T. asperellum*



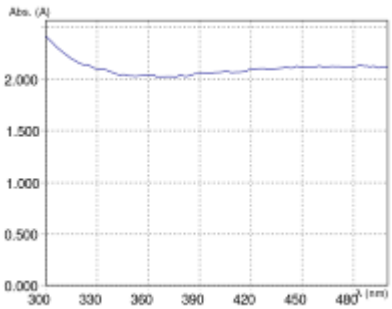
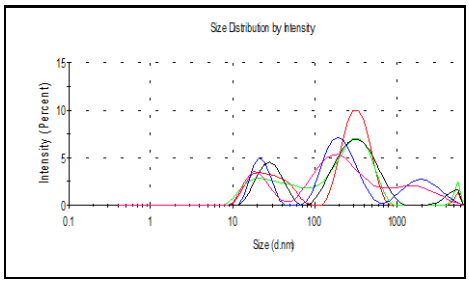


Fig.3&4 DLS pattern of green synthesized Ag NPs from *T. asperellum* & zeta potential analysis of Ag NPs from *T. asperellum*



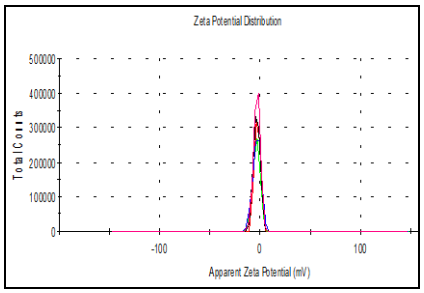
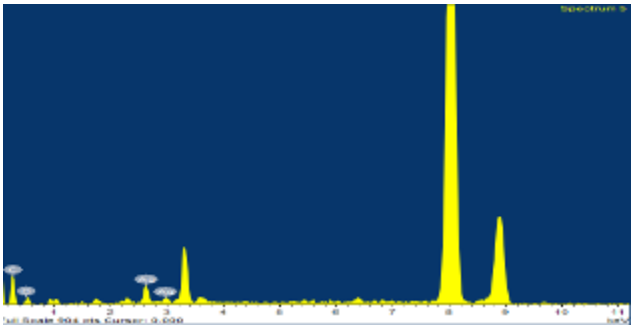


Fig.5 EDX pattern of green synthesized silver nanoparticles from *T. asperellum*

Processing option : All elements analyzed Number of iterations : 1

Element	Line	k factor	Absorbtion corrn.	Weight%	Weight% sigma	Atomic%	Standard
C	K_SERIES	1.706	1.0000	57.66	6.69	83.72	Standardless
O	K_SERIES	1.353	1.0000	10.16	2.71	11.08	Standardless
Ag	L_SERIES	2.072	1.0000	32.18	7.37	5.20	Standardless
Totals				100.00			



Relative Leaf Water Content (RLWC)

Table 3 depicts the information on relative leaf water content (RLWC) of the plant samples treated with silver nanoparticles by different methods and showed that all the treated plants had significantly higher RLWC than the control. The maximum RLWC was observed in the foliar spray (85.3%) treated seedlings which significantly differ from control (66.80%) and all the other treatments. RWC recorded for other treatments were 77.10%, 76.17%, 73.85%, 71.40% for soil treatment, seedling root dip treatment, injection method, cutting treatment and control. RLWC of cutting treatment and injection method was statistically at par with each other. Similarly, soil treatment and seedling root dip treatment methods have statistically at par values of RLWC. The increase in Leaf Moisture Content and Relative Leaf Water Content in treated plants might be due to the increase in root length of the silver treated seedlings as reported by many workers (Vaninni *et al.,* 2014). The increased root length helps the plant to absorb more water from the soil resulting in increased turgid weight of treated seedlings and ultimately increasing the plant moisture content.

Lipid peroxidation (MDA content)

Malondialdehyde (MDA) content of tea seedlings treated by different methods is presented in Table 3. The application of silver nanoparticles had no significant effect on the seedlings. The MDA content recorded for different methods was 71.40%, 71.30%, 71.20%, 70.83%, 70.82% and 70.81% for foliar spray, soil treatment, seedling root dip treatment, injection method, cutting treatment and control respectively, which had no significant difference among them. Metal

nanoparticle treatment can increase the MDA content in host plants (Song *et al.*, 2012). The increase in MDA content signifies that the treated seedlings undergo a mild stress condition. However, in the present study, the enhancement in MDA content through lipid peroxidation was statistically non-significant on the tea seedlings.

The highest increase in all the plant metabolites reported in seedlings treated by foliar spray is due to more absorption of silver nanoparticles from stomata and leaf epidermis. The transport of minerals and nutrients is faster in foliar treated plants (Rachel and Sirisha, 2016).

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