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Confocal microscopic and nuclear magnetic resonance (NMR) spectroscopic studies of tea leaves treated with green synthesized silver nanoparticles

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Abstract
Biological synthesis of silver nanoparticles was done using culture filtrate of *Trichoderma asperellum*, a potential fungal antagonist taking 1Millimolar silver nitrate solution as the precursor. The synthesized nanoparticles were characterized using UV-VIS Spectrophotometer, Dynamic Light Scattering, Zeta sizer, Transmission Electron Microscope and Energy Dispersive X-ray analysis. The results showed a characteristic Surface Absorption Band at 420 nm which confirmed the formation of silver nanoparticles. The charge of silver nanoparticles was found to have a negative potential value of -1.34 mV and indicated stable on dispersion. The nanoparticles were uniform and well-dispersed in nature with a spherical shape. The average particle size recorded was 8.26 nm with a size range from 4-14 nm and the biosynthesized material contained 32.18% silver, 10.16% oxygen, and 57.66% carbon. The Silver nanoparticles were introduced into 4-5 months old tea seedlings by foliar spray technique and control was maintained by spraying distilled water. After 45 days of treatment leaf samples were collected from both the treated and control plants and subjected to Nuclear Magnetic Resonance Spectroscopic and Confocal Microscopic studies. Presence of silver nanoparticles was detected inside the treated leaf sample and some extra metabolites were reported in treated sample than that of the control sample.

Keywords: Silver nanoparticles, green synthesis, nuclear magnetic resonance, confocal microscopy

Introduction
Green or biological synthesis of metal nanoparticles, recently, has gained vast interest in the field of nanotechnology. Similarly study on its effects on the environment, plant system, animals and humans are also attracting scientists around the globe. 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 [1]. 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 [2]. Hence an effort was made through this study to know about the metabolic changes that occur in the host plant after application of fungus mediated green synthesized silver nanoparticles. Nuclear Magnetic Resonance (NMR) spectroscopy is a realistic and suitable approach to have a general view of all the metabolites present in an organism under certain conditions. In this study, NMR was done for complete metabolomic analysis of the treated and untreated samples, to ascertain any changes in plant metabolites that occurred in the host plants after application of silver nanoparticles (Ag NPs). Confocal microscopy is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of adding a spatial pinhole placed at the confocal plane of the lens to eliminate out-of-focus light. It enables the reconstruction of three-dimensional structures from sets of images obtained at different depths (a process is known as optical sectioning) within a thick object. It uses airy scan detector (area detector) which captures all of the light coming from the sample thus, image quality is significantly improved and also possible to carry out an extremely sensitive or extremely fast scan. Similar works done suggested that nanoparticles can be visualized by reflection on a confocal microscope;

inferred as dark areas in an auto fluorescent background (either natural or induced), although with some limitations depending on cell types. The results of present work showed the presence of nanoparticles both in the extracellular space and within some cells [3].

2. Experimental Details

2.1 Synthesis and Characterization of Silver Nanoparticles

Synthesis of silver nanoparticles was done by standardized method [4]. 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.

2.2 Application of silver nanoparticles on host

Leaves of the 4-5 months old seedlings were sprayed with silver nanoparticles covering both the abaxial and adaxial surfaces by using a sprayer (Plate 1). 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.

Plate 1: Foliar spray of seedlings with silver nanoparticles

Seedlings in control were maintained by using sterile distilled water in place of silver nanoparticles.

After 45 days of treatment leaf samples were collected from the treated and control seedlings. The leaves were oven dried and kept for NMR studies and for confocal microscopic studies fresh samples were collected.

2.3 Nuclear Magnetic Resonance (NMR) Spectroscopic studies

NMR studies were carried out at SAIF, NEHU, Shillong. ^{1}H NMR or Proton NMR method was followed for the analysis. The dried leaf samples were extracted by using deuterated methanol ($MeODd_4$) and the extraction was subjected to analysis in the NMR spectroscopy (Bruker 400 Ultra Shield).

2.4 Confocal Microscopic Studies

The confocal microscopic study was done at Nanotechnology department of IIT, Guwahati. A thin slice of the specimen was mounted on a glass slide and a fluorescent dye was put and covered by a cover slip. After that, it was observed under the microscope (Zeiss Confocal and Super-resolution microscopy, Model LSM 880 with Airy scan).

3. Results and Discussion

3.1 Synthesis and Characterization of Silver nanoparticles

For the green synthesis of silver nanoparticles, supernatant of *T. asperellum* was exposed to 1mM aqueous solution of Ag NO_3, the color of supernatant changes from green to yellowish brown to brown after 192 hours of reaction which confirms the formation of silver nanoparticles. A characteristic, SPR absorption band was observed in the supernatant of *T. asperellum* treated with 1mM Ag NO_3 at 420 nm. DLS analysis showed that biosynthesized nanoparticles have an average size of 68 nm with a polydispersity index (PDI) of 0.857. Transmission Electron Microscopy (TEM) micrographs at 20,000 X magnifications revealed that the average size of nanoparticles were 8.26 nm with roughly spherical shape. TEM micrographs also indicated that nanoparticles were relatively uniform in nature and well separated from each other having no agglomeration. 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%] [5]. The fungal media *i.e.* PDB used for culturing *T. asperellum* might be the source of carbon and oxygen in the biosynthesized material.

3.2 NMR Spectroscopic Studies

Silver nanoparticles treated leaf samples were subjected to complete metabolomic analysis in NMR spectroscopy. The interpretation of the NMR spectra obtained, showed production of extra metabolites *i.e.* alkenes in the silver nanoparticles treated samples (Fig.1), which were not recorded in the spectra of untreated leaf samples (Fig. 2). Alkenes are a group of plant hormones that control plant growth and other changes in tissues.

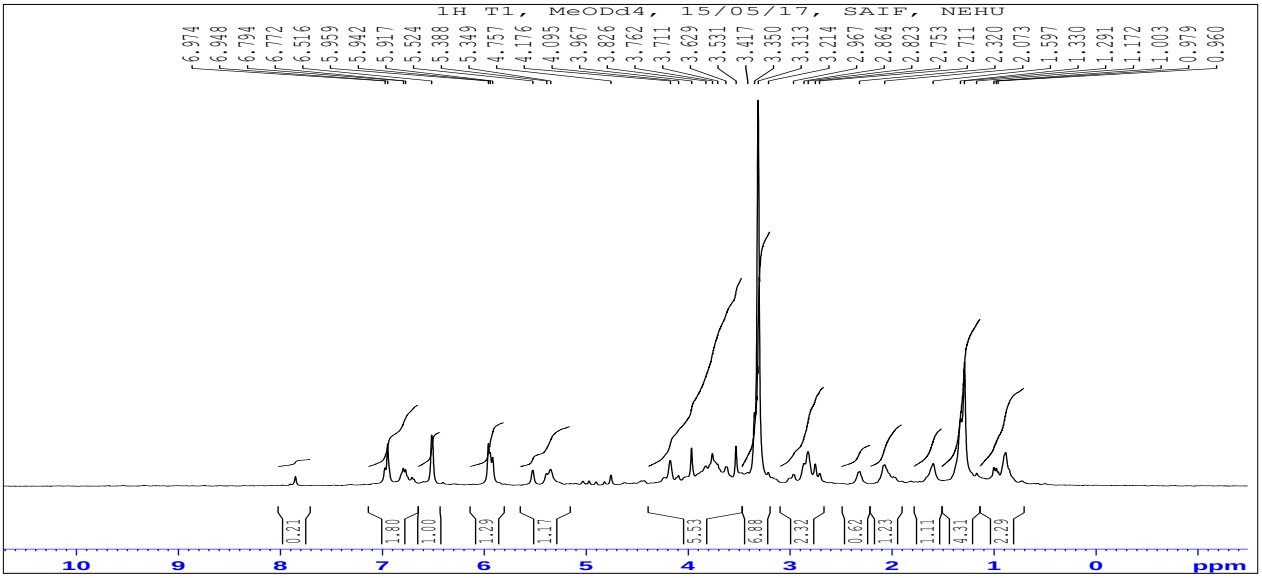


Fig 1: NMR spectra of green synthesized silver nanoparticles treated leaf sample

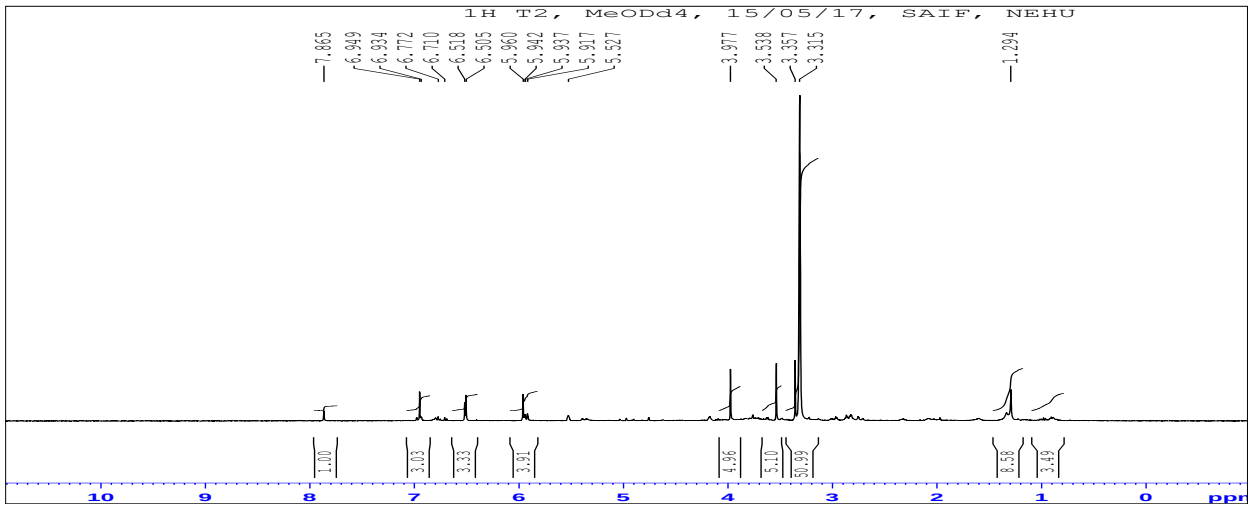
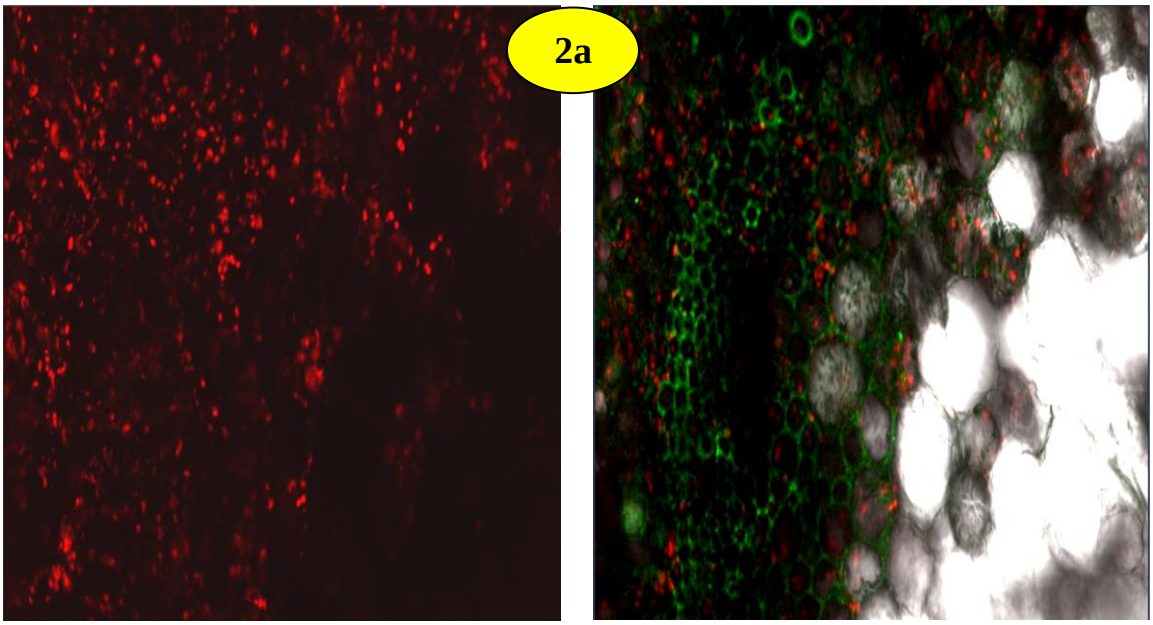


Fig 2: NMR spectra of untreated leaf sample

3.3 Confocal microscopic study

Silver nanoparticles treated and untreated leaf samples were subjected to confocal microscopic study. Silver nanoparticles were seen inside the treated leaf tissues clearly (Plate 2 a), whereas no such observations were made in the untreated leaf sample (Plate 2 b).



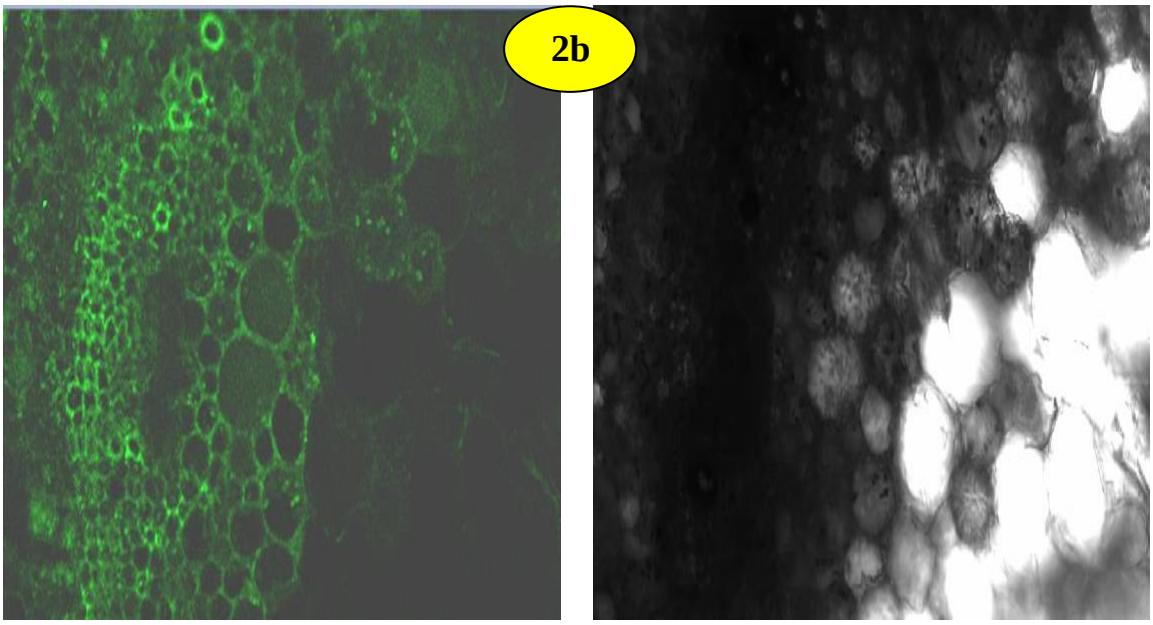


Plate 2: Confocal microscopy images of (a) green synthesized silver nanoparticles treated leaves (b) untreated leaves

4. Conclusion

An attempt was made to synthesize silver nanoparticles from organic source *i.e.* culture filtrate of fungus *Trichoderma asperellum*, which was characterized using various equipments. The synthesized nanoparticles were sprayed on tea seedlings and after 45 days of treatment leaf samples were collected from both the treated and control plants which were subjected to Nuclear Magnetic Resonance Spectroscopic and Confocal Microscopic studies. Presence of silver nanoparticles was detected inside the treated leaf sample and some extra metabolites were reported. The study carried out here is a preliminary one and it opens up many future lines of research on further extensive experimentation on proper delivery mechanism of nanoparticles in plants; and simple or complex microscopical techniques for scanning the whole movement of nanoparticles within the plant system.

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