Agric Res J **58** (5) : 821-827, *October* 2021
DOI No. 10.5958/2395-146X.2021.00117.4

EVALUATION OF THE MYCO-TOXICITIES OF SILVER SULFIDE NANOPARTICLES AGAINST PHYTOPATHOGENIC FUNGI

Anjali Sidhu[1]*, Garima Sethi[2], Anju Bala[3] and Radha Ahuja[1]

[1]Department of Soil Science, [2]Department of Chemistry, [3]Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana-141004, Punjab

ABSTRACT

Silver sulfide nanoparticles (Ag_2S NPs) are less explored under *in vitro* or *in vivo* conditions or as bio-compatible material in comparison to silver nanoparticles (AgNPs). The present study involved the synthesis of silver sulfide nanoforms by sonochemical approach using various sulfide ion sources and their characterization using UV-Vis, FTIR, TEM, DLS, Zeta potential, SEM and EDS techniques. All the synthesized samples were evaluated for their preliminary antifungal activity against phytopathogenic fungi *viz. Fusarium verticillioides, Bipolaris oryzae, Ustilago hordei* and *Uromyces viciafabia.* The Ag_2S-4 NPs obtained from sodium sulfide as sulfur source exhibited maximum bio potential with ED_{50} ≤13 µg/mL which was twofold less than ED_{50} value of Ag NPs (under similar size range) revealing better stability and bio potential of Ag_2S-4 NPs in comparison to AgNPs.

Keywords: Nanoform, Pathogenic fungi, Silver nanoparticles, Silver sulfide, Sonochemical irradiation

Metal sulfide nanoparticles (MS NPs) have not been adequately explored in agricultural field. Nanotoxicity is an important aspect which is needed to be addressed before going for *in vitro* activities. Sulfides of soft metals are their stable chemical form and therefore are hypothesized as most detoxified form (Sidhu *et al.,* 2017, Ahuja *et al.,* 2019, Ahuja *et al.,* 2020). MS NPs of various metals such as silver (Ag), iron (Fe), zinc (Zn), cadmium (Cd), manganese (Mn) and copper (Cu) etc., have been reported in terms of synthesis with varied nano morphologies (Sidhu *et al.,* 2017, Ahuja *et al.,* 2019, Ahuja *et al.,* 2020a, Ahuja *et al*., 2020b). Metal sulfides of transition metals are water insoluble and their nano dispersion in water makes them more appropriate in terms of application (Guo *et al.,* 2013), in finding new agrochemicals. Modifications as aqua formulations of insoluble materials (Sidhu *et al.,* 2017) are the essence of applied bio nanotechnological applications.

Silver sulfide (Ag_2S) is considered as one of the least toxic form of silver because of its slow release of Ag^+ ions (Levard *et al*., 2013) in environment. Long term stability of Ag_2S was studied by Lombi *et al.* (2016) who reported no transformation during 6 months under biosolid composting/storage conditions. Studies suggested long term stability of Ag_2S with significant concentrations (Donner *et al.,* 2015).

Silver nanoparticles (AgNPs) have wide applications and have been commercialized in various ways (Tran *et al.,* 2013). Antimicrobial properties of silver are well known since centuries with evidence of its use in ancient Egypt and Rome (Reidy *et al.,* 2013). Silver continues to be used in several forms to preserve fluids or treat various ailments. Exploitation of silver is strengthening its impact on phytopathological scenario in addition to its significant impact at commercial scale. So, it is always beneficial to resort to low/non toxic alternatives of existing forms.

Silver sulfide nanoforms have not been adequately reported as bio-compatible material in *in vitro* or *in vivo* applications. Kumari *et al.,* (2014) reported Ag_2S NPs obtained from sodium sulfide for antimicrobial evaluation against *E. coli* and *Staphylococcus aureus* showing a growth inhibition of more than 75% at 0.1 µg/mL. Ecological toxicity studies conducted on AgNPs proved the formation of Ag_2S as ultimate stage which was found to be less toxic and had low negative impact on soil micro flora owing to slow release of silver ions from Ag_2S. The low toxicity of Ag_2S NPs prompted us to explore them against various pathogens, in comparison to silver nanoform. The present paper presents the synthesis of Ag_2S NPs from different sources of sulfur and evaluation of their bio potential against various phytopathogenic fungi, relative to most heavily used and commercialized silver nanoparticles.

MATERIALS AND METHODS

Instrumentation used

The size and morphology of prepared samples were studied by Hitachi Transmission Electron Microscope Hi-7650 at an accelerated voltage of 200 kV in EMN laboratory, Punjab Agricultural University, Ludhiana. TEM pictures obtained were employed in

*Corresponding author : anjalisidhu@pau.edu
Date of receipt: 07.02.2021, Date of acceptance: 10.09.2021

Image J software to study particle size distribution of nanoparticles and for each nanoparticle under analysis, diameter was determined, and average size was calculated. Scanning electron micrographs of prepared nanoparticles were recorded using SEM model Hitachi S-3400 in EMN laboratory, Punjab Agricultural University, Ludhiana. The analysis of particle size distribution of nanoparticles and Zeta potential determination was done using Litesizer 500 in Application Lab, Anton Paar, Gurgaon, India.

Synthesis of silver sulfide nanoparticles (Ag_2S-(1-4) NPs)

Silver nitrate solution (20 mL, 0.0003 M) with 2-3 drops of diluted triton-X as a surfactant was taken and to this 10 ml of 0.0003 M of thiourea/thioacetamide/ sodium thiosulfate/sodium sulfide was added dropwise under sonication. The obtained yellow colored solution was further irradiated with microwave radiations for 25 seconds and PVP (0.05 g) was added to cold solution during sonication, acting as a stabilizing agent. The sonication was further continued for 15 minutes to get PVP stabilized Ag_2S-(1-4) NPs (50 µg/ml).

Synthesis of silver nanoparticles (AgNPs)

Chemical reduction method was utilized for synthesis of silver nanoparticles, using sodium borohydride as a reducing agent (Aashritha, 2013). 1.0 mM silver nitrate (10 ml) was added dropwise (about 1 drop per second) to ice cold 5.0 mM sodium borohydride solution (10 ml) containing a drop of surfactant, Triton X, while stirring to get yellow colored solution (Nasrollahi *et al.,* 2011).

$$AgNO_3 + NaBH_4 \longrightarrow Ag + 1/2\ H_2 + 1/2\ B_2H_6 + NaHO_3$$

0.1g of polyvinyl pyrollidone K-30 dissolved in 5 ml of distilled water was added to synthesized silver nanoparticles and the solution was centrifuged at 10,000 rpm for 10 min and was resuspended in same amount of double distilled deionised water. This resulted in stable 0.5 mM solution of PVP-coated silver nanoparticles which were analyzed.

Antifungal evaluation of silver sulfide nanoparticles (Ag_2S-(1-4) NPs) and Silver NPs (Ag NPs)

All synthesized samples were tested for their preliminary *in vitro* antifungal activity against different fungi using poisoned food technique and spore germination method. Antifungal potential of Ag_2S-(1-4) NPs and Ag NPs at different concentrations (5,10, 15, 20, 25 µg/ml) against *Fusarium verticillioides* and *Bipolaris oryzae* was tested by Agar dilution technique (Nene and Thapliyal, 1997). Percent inhibition in growth was calculated by using the formula:

$$\text{Percent inhibition} = \frac{C - T}{T} \times 100$$

Where C = radial growth of the fungal colony in control

T = radial growth of the fungal colony in amended medium

The antifungal potential was expressed as ED_{50} values.

Ustilago hordei and *Uromyces viciafabia* were evaluated by spore germination inhibition technique. All the test samples Ag_2S-(1-4) NPs and Ag NPs were treated against these fungi. The numbers of spores germinated were counted and per cent spore germination inhibition was calculated by the following formula:

$$= \frac{\text{Spore germination in control} - \text{Spore germination in treatment}}{\text{Spore germination in control}} \times 100$$

RESULTS AND DISCUSSION

Characterization of silver sulfide nanoparticles (Ag_2S-(1-4) NPs)

The particle size of synthesized Ag_2S NPs was determined by TEM which indicated perfectly spherical shaped nanoparticles in all cases except Ag_2S-1(Fig.1 (a-d)) where distorted spherical shape with slight aggregation was observed. The particle sizes ranged from 8-20 nm. The smallest size was observed in case of Ag_2S-4 NPs (Fig. 1(a)) which was 8±1nm while in case of Ag_2S-2 (Fig. 1(b)) maximum size was observed to be 20±1 nm. The average sizes of all prepared samples are given in Table 1, which was in consonance with the reported literature (Kuznetsovaa *et al.,* 2017; Han *et al.,* 2012; Kumar *et al.,* 2015; Kim *et al.,* 2010) where the sizes varied from 9-20nm.

UV-Vis studies were performed by measuring the absorbance of the aqua-dispersed Ag_2S NPs in the 200–800 nm region. The maximum absorption between 270-295 nm (Fig. 2) indicated the presence of Ag_2S nanoparticles (Xaba *et al.,* 2017). The λ_{max} of Ag_2S NPs was in good agreement with the previously reported optical properties of Ag_2S NPs (Kuznetsovaa *et al.,* 2017). Optical properties of the Ag_2S NPs were measured in freshly prepared dispersions as well as in the same dispersions after regular intervals of 15 days till 4 months. There were no significant diversions from the starting values revealing long shelf life of prepared nanoparticles.

SEM-EDS was performed to test the surface morphology and elemental composition of Ag_2S NPs. The SEM-EDS spectrum confirmed the presence of silver and sulphur in the elemental ratio of 2:1. The

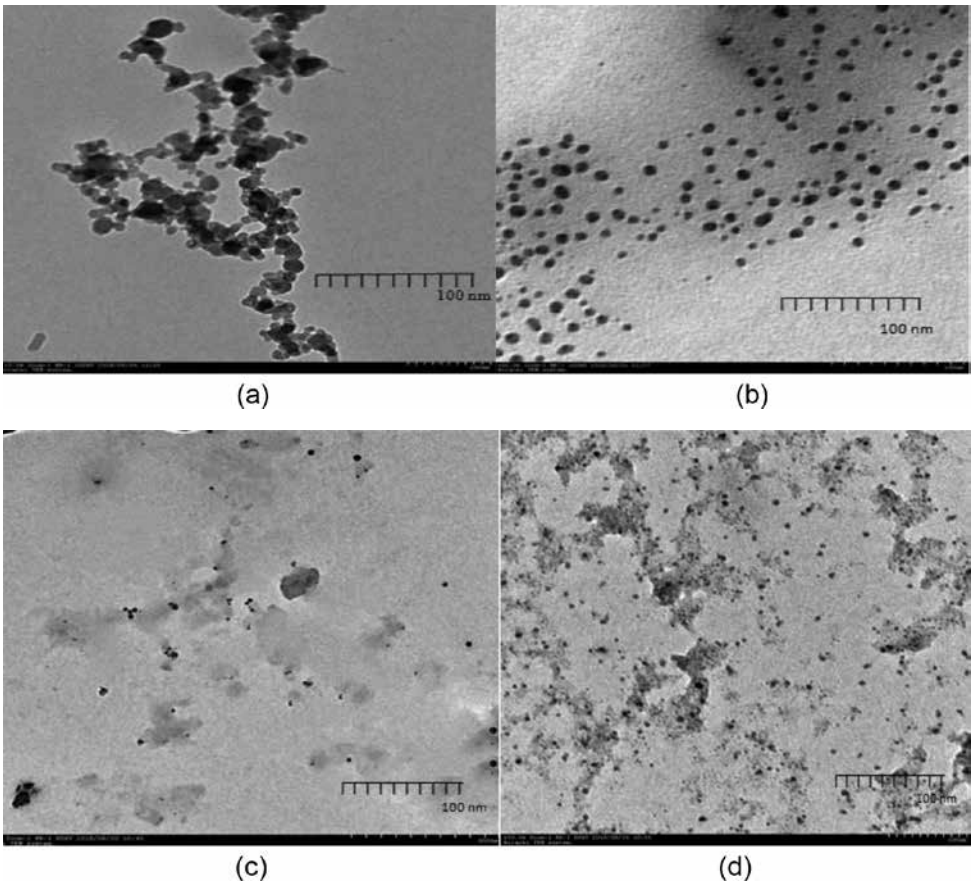

Fig. 1. (a-d) TEM image of sample Ag_2S-(1-4) NPs

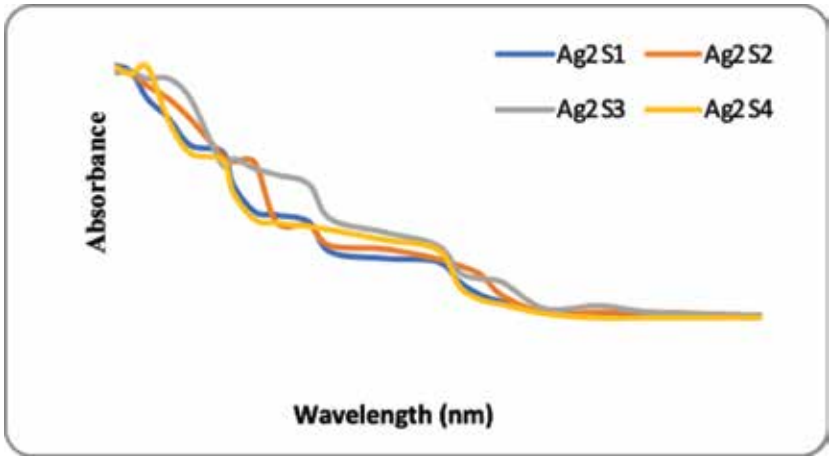

Fig. 2. UV-Vis spectra Ag_2S-(1-4) NPs

SEM micrograph of sample Ag_2S-1 revealed spherical morphology(Fig. 3(a)) and their elemental composition showed by EDS spectra was 34.46 % S and 65.54 % Ag (Fig. 4 (a)). The micrograph of sample Ag_2S-4 showed that the particles were spherical and smooth (Fig. 3(d)) in nature and the EDS spectrum confirmed the presence of sulfur and silver in elemental ratio of (32.57% S, 67.43%Ag), (33.17% S and 66.83% Ag) and (33.89 % S, 66.11%Ag), respectively (Fig. 4 (b-d)).

The DLS size distribution of synthesized silver sulfide nanoparticles Ag_2S-(1-4) exhibited hydrodynamic diameter of 224.7 ± 12.5 nm, 228.4 ± 45.25 nm, 143.74 ± 29 nm and 113.25 ± 10.58 nm, respectively. DLS showed results of the mean diameter

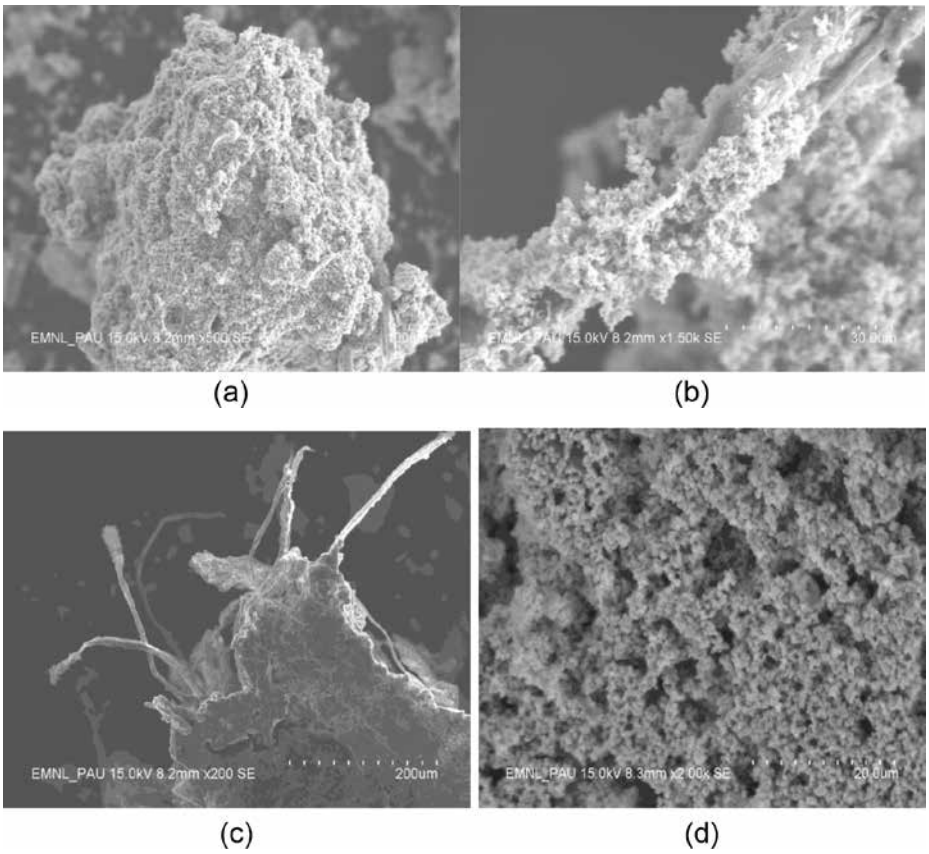

Fig. 3(a-d) SEM images of Ag_2S-(1-4) NPs

Table 1. Size and optical properties of synthesized silver sulfide NPs

Sample No.	Sulfide ion source	λ_{max}(nm)	Average size (nm)
Ag_2S-1	Thiourea	279	18±2
Ag_2S-2	Thioacetamide	295	20±1
Ag_2S-3	Sodium thiosulfate pentahydrate	273	11±3
Ag_2S-4	Sodium sulfide	270	8±1

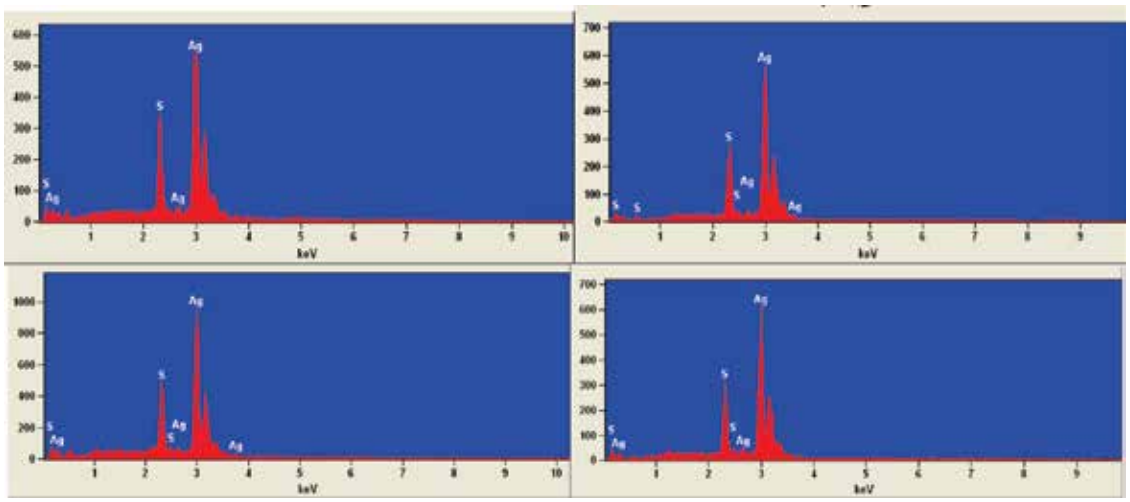

Fig. 4 (a-d) SEM-EDS spectrum of Ag_2S-(1-4) NPs

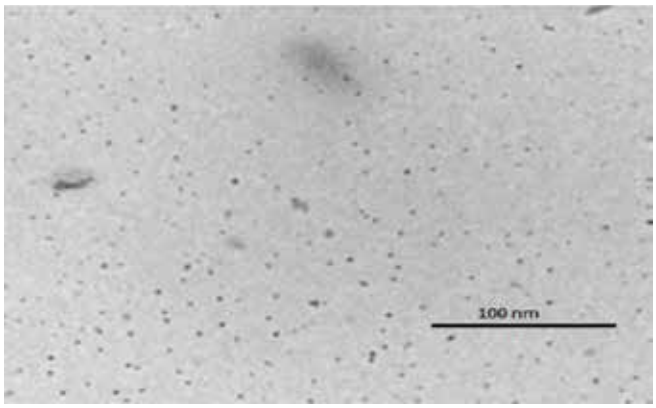

Fig. 5. TEM analysis of unloaded Ag NPs

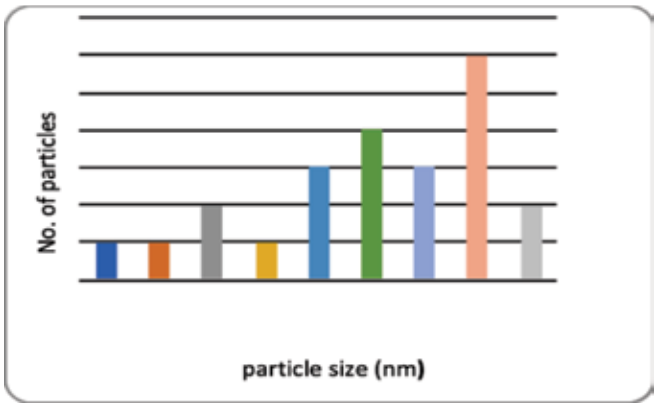

Fig. 6. Particle size distribution of Ag NPs

in the higher end of the size distribution obtained by TEM. The particle size, which is derived from the DLS measurement, is not the true size, but rather this method considers solvation layer around the particle to be a part of it. Because of this, the particles, when measured using DLS, look bigger, than their actual size. Also larger particles tend to cover smaller ones, so, the DLS measurement is quite broad. The zeta potentials of **Ag_2S-(1-4)** were -14.6 mV, -12.0 mV, -13.7 mV and -16.2 mV respectively. This negative value of zeta potential confirmed high repulsions among particles and indicated the formation of stable Ag_2S NPs.

Characterization of AgNPs nanoparticles

Typical chemical reduction methodology was used for the synthesis of silver nanoparticles (AgNPs) using silver nitrate as source of silver and sodium borohydride as reducing agent with Triton X as surfactant, in ice cold conditions. The prepared AgNPs studied by TEM (Fig. 5) depicted that spherical shape of nanoparticles which were uniformly distributed without any agglomeration with size between 8.6-8.8 nm (Fig. 6). Maximum absorption peak was observed at 412 nm (λ max) as observed by UV–Vis spectra (Fig. 7).

Antifungal activity of Ag_2S-(1-4) NPs and Ag NPs

The synthesized Ag_2S-(1-4) NPs and Ag NPs were screened *in vitro*, against *Ustilago hordei* and *Uromyces viciafabia* by spore germination inhibition technique and antifungal potential was expressed as ED_{50} values (Table 2). The mean of three replicate tests performed for each antifungal test, calculated using Analysis of Variance (ANOVA). Antifungal potential of Ag_2S-(1-4) NPs and Ag NPs against *Fusarium verticillioides* and *Bipolaris oryzae* was also studied using PDA method, whose ED_{50} values and percent inhibition values were also calculated (Table 2). Both Ag_2S-(1-4) NPs and Ag NPs were compared for their antifungal evaluation.

The antifungal potential of both Ag_2S NPs and Ag NPs were multifold higher as compared to the standard fungicides Carboxin, Bavistin and Captan. The antifungal potential of Ag_2S NPs was better than

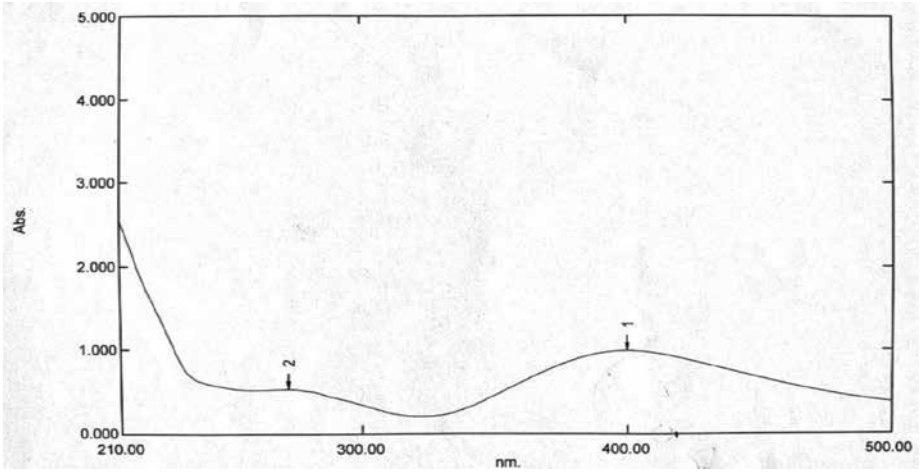

Fig. 7. UV analysis of Ag NPs

Table 2. Antifungal potential of aqua-dispersed Ag_2S-(1-4) NPs and Ag NPs

Sample	ED_{50} value (µg/ml)			
	Fusarium verticillioides	Bipolarisoryzae	Ustilagohordei	Uromycesviciafabia
Ag_2S-1	13	14	11	14
Ag_2S-2	24	20	10	12
Ag_2S-3	17	15	12	15
Ag_2S-4	11	13	8	11
Ag-NPs	24	27	18	24
Carboxin	-	-	78	70
Bavistin	26	-	-	-
Captan	-	37	-	-

Fig. 8. Antifungal activity against *Fusarium verticillioides* of a) Ag_2S-1 b)Ag_2S-2c) Ag_2S-3 d) Ag_2S-4 e)Ag NPs

Fig. 9. Antifungal activity against *Bipolaris oryzae* of a)Ag_2S-1 b) Ag_2S-2 c) Ag_2S-3 d) Ag_2S-4 e) Ag NPs

Ag NPs (Fig. 8-9). The results of augmented mycocidal potential of Ag_2S NPs endorse our earlier claims on increase in antifungal potential of bioactive metal ions on combining with sulfide ions. Sulfur is a macronutrient with known antifungal potential. Combination of sulfur with silver further synergized its impact on fungal hyphae and thus reduced growth of the fungus.

Silver species are found to be able to penetrate inside biological membranes, can change physiological, molecular and biochemical conditions of organisms and have long term toxic effects on environment. Soil microbial biomass and diversity can be greatly affected by silver exposure. It may decrease the growth of plants and invertebrate reproduction of soil. Contrary to reduced toxicities in soil and slow release of Ag ions are inflicted by Ag_2S. Low concentrations of silver exhibited no effects on microbial biomass (Judy *et al.,* 2015) and nitrifier abundance (Doolette *et al.,* 2016). Vardar *et al*., (2019) in their recent studies on cyto-toxicity and geno-toxicity of silver sulfide quantum dots revealed their hypo-toxicity on lung fibroblast V79 cell line advocating their biocompatibility.

The toxic potential of silver favors the replacement of silver with other alternatives. The formulations of bioactive metals as their sulfides approve to be a fruitful strategy as revealed by Sidhu *et al.* (2017), Ahuja *et al.* (2019), Ahuja *et al.* (2020). So Ag_2S NPs can be recommended as potential antifungal agents after intensive investigatory studies.

Authors' contribution

Conceptualization of research work and designing of experiments (AS); Execution of field/lab experiments and data collection (GS); Analysis of data and interpretation (AB, RA); Preparation of manuscript (AS, RA).

LITERATURE CITED

Aashritha S 2013. Synthesis of silver nanoparticles by chemical reduction method and their antifungal activity. *Int Res J Pharmacy* **4**(10): 111-13.

Ahuja R, Sidhu A and Bala A 2019. Synthesis and evaluation of iron(II) sulfide aqua nanoparticles (FeS-NPs) against *Fusarium verticillioides* causing sheath rot and seed discoloration of rice. *Eur J Plant Pathol* **155**(1): 163-71.

Ahuja R, Sidhu A and Bala A 2020a. Effect of iron sulfide nanopriming in reducing *Drechslera* seed rot and seedling mortality in rice. *Agric Res J* **57**(1): 60-65.

Ahuja R, Sidhu A and Bala A 2020a. Manganese sulfide

nanospheres as mycocidal material and priming agent for fungi-infested rice seeds. *Phytopathology* ***168****(11-12):* 678-87.

Donner E, Scheckel K, Sekine R, Popelka-Filcoff R S, Bennett J W, Brunetti G R, Naidu S P, McGrath and Lombi E 2015. Non-labile silver species in biosolids remain stable throughout 50 years of weathering and ageing. *Environ Pollut* **205**: 78-86.

Doolette C L, Gupta V V S R, Lu Y, Payne J L, Batstone D J, Kirby J K *et al.* 2016. Quantifying the sensitivity of soil microbial communities to silver sulfide nanoparticles using metagenome sequencing. *PLoS ONE* **11** 114-24.

Guo L, Panderi I, Yan D D, Szulak K, Li Y, Chen Y, Ma H, Niesen D B, Seeram N, Ahmed A, Yan B, Pantazatos D and Lu W 2013. A comparative study of hollow copper sulfide nanoparticles and hollow gold nanosphere on degradability and toxicity. *ACA Nano* **7**:8780–93.

Han L, Lv Y, Asiri A M, Al-Youbi A O, Tu B and Zhao D 2012. Novel preparation and near-infrared photoluminescence of uniform core-shell silver sulfide *J Mater Chem* **22**: 7274-79.

Judy J D, Kirby J K, Creamer C, McLaughlin M J, Fiebiger C, Wright C,Timothy R and Cavagnaro Bertsch P M, 2015. Effects of silver sulfide nanomaterials on mycorrhizal colonization of tomato plants and soil microbial communities in biosolid-amended soil. Environmental Pollution *Environ Pollut* **206:** 256-63.

Kim Y and Walsh D 2010. Metal sulfide nanoparticles synthesized via enzyme treatment of biopolymer stabilized nanosuspensions. *Nanoscale* **2**: 240-47.

Kumar B, Smita K, Cumbal L, Debut A, Camacho J, Hernández-Gallegos E, Chavez-Lopez M G, Grijalva M, Angulo Y and Rosero G 2015. Pomosynthesis and biological activity of silver nanoparticles using *Passiflora tripartita* fruit extracts. *Adv Mater Lett* **6**: 127-32.

Kumari P, Chandran P and Khan S K 2014. Synthesis and characterization of silver sulfide nanoparticles for photocatalytic and antimicrobial applications. *J Photochem Photobio B: Biology* **141**: 235–40.

Kuznetsovaa Y V, Rempela S V, Popova I D, Gerasimovc E Y and Rempela A A 2017. Stabilization of Ag_2S nanoparticles in aqueous solution by MPS. *Colloids and Surfaces A: Physicochem Eng Aspects* **520**: 369-77.

Levard C, Hotze E M, Colman B P, Dale A L, Truong L, Yang X Y andLowry G V 2013. Sulfidation of silver nanoparticles: natural antidote to their toxicity. *Environ Sci Tech* **47**:13440-48.

Lombi E, Donner E, Taheri S, Tavakkoli E, Jamting A K, McClure S, Naidu R,Miller B W, Scheckel K G, Vasilev K 2013. Transformation of four silver/silver chloride nanoparticles during anaerobic treatment of wastewater and post-processing of sewage sludge. *Environ Pollut* **176:**193-207.

Nasrollahi A, Pourshamsian K and Mansourkiaee P 2011. Antifungal activity of silver nanoparticles on some of fungi. *Int J Nano Dim* **1**(3): 233-39.

Nene and Thapliyal 1997. *Fungicides in Plant Disease Control*, Medtech, New Delhi, India.

Reidy B, Haase A, Luch A, Dawson K and Lynch I 2013. Mechanisms of silver nanoparticle release, transformation and toxicity: a critical review of current knowledge and recommendations for future studies and applications *Materials* ***6:*** *2295-2350*.

Sidhu A, Barmota H and Bala A 2017. Antifungal evaluation studies of copper sulfide nano-aquaformulations and its impact on seed quality of rice (*Oryzae sativa*). *Appl Nanosci* **7**: 681-89.

Tran Q H, Nguyen V Q and Le A T 2013. Silver nanoparticles: Synthesis, properties, toxicology, applications and perspectives. *Adv Nat Sci: Nanosci Nanotechnol* **4**: 1-20.

Vardar D O, Sevtap A, İbrahim H H, Havva Y, Nursen B 2019. An *in vitro* study on the cytotoxicity and genotoxicity of silver sulfide quantum dots coated with meso-2, 3-dimercaptosuccinic acid. Turkish Journal of Pharmaceutical Sciences *Turk J Pharm Sci* **16:** 282-89.

Xaba T, Moloto M J, Nchoe O B, Nate Z and Moloto N 2017. Synthesis of silver sulfide nanoparticles through homogeneous precipitation route and the preparation of the Ag_2S-chitosan nanocomposites for the removal of iron(II) ion from wastewater. *Chalcogenide Lett* **14**: 337-46.