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Biosynthesis of silver nanoparticles using *Bacillus tequilensis* and its impact on tomato plant

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Abstract

Introduction: The field of nanotechnology is among the most common areas of modern material science. Recent advancements in nanotechnology and nanoscience have changed the methods of prevention, treatment and diagnosis of different diseases. Tomato (*Lycopersicum esculentum*) is an essential part of the Pakistani cuisine, and it has a demand all year round. The tomato crop grown by the orthodox agricultural methods cannot be grown all the year and has a very low yield.

Objectives: In this study, bacterial strain *Bacillus tequelensis* (NMCC34) was utilized to evaluate its potential to produce silver nanoparticles (AgNPs) and their effect on growth of tomato plant.

Methodology: The bacterial strain NMCC34 was refreshed and further identified through different morphological, molecular and biochemical testing. For the biosynthesis of AgNPs, the bacterial culture was mixed with 100 ml $AgNO_3$ solution at 1 mM aqueous solution. Tomato seeds were exposed to AgNPs solution in a single concentration of 50 ppm (1 ml). Data was recorded at different day's interval (Day 1, 2, 3, 6, 17 and 18).

Results: The plants in treated group (50 ppm AgNPs) have shown improvement in number of leaves, length, seed germination and leaves sizes as compared with control plants (untreated).

Conclusion: The current study revealed potential of silver nanoparticles to promote growth activity of tomato plant which can be exploited commercially for the economic benefit of mankind.

Keywords: *Bacillus tequelensis*; Silver nanoparticles; *Lycopersicum esculentum;* Agriculture

1. Introduction

Since the beginning of the last century, nanotechnology is known as a famous field of research. The term nanotechnology was coined by figurative Richard P. Feynman in 1960 [1]. In the past few decades, there have been special revolutionary developments made in the nanotechnology field.

The method by which nanoparticles are being synthesized is the basic step in adjusting its shape and size, chemical and physical properties to the desired levels of technology. There are different biological ways of synthesizing the silver nanoparticles including the use of plant extracts, microorganisms, waste materials and enzymes. The biologically synthesized AgNPs are eco-friendly which means that they are compatible with the environment. These biogenic AgNPs

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are more environment friendly than other ways of its synthesis because this approach relies on the natural resources like plants, bacteria, fungi, yeasts and algae [2].

Silver nanoparticles are among the important nanoparticles due to their unique chemical and physical properties. It is very important to have adequate knowledge of the properties of AgNPs in order to increase their potential applications. The AgNPs are both useful and hazardous which need to be qualified and quantified in order to understand its effects on the environment. Toxic properties are those which are hazardous. AgNPs have toxic properties that can be exploited in different applications such as antimicrobials against those microorganisms that cause diseases and infections. On the other hand, these toxic properties could be a health hazard. It is well known that silver nanoparticles are used in many commercial products like contraceptive equipment and female hygiene products which may have adverse effects on the reproductive system. It is used in several other products like textiles and cosmetics [3].

Silver nanoparticles are a group of nano materials with sizes ranging from 1–100 nm. The importance of the silver nanoparticles has recently increased due to their specific unique chemical, physical and biological properties. One of the amazing applications of silver nanoparticles was their use in optics by the manufacturers of glass at the time of Romans Empire around 4th century AD. Further research studies had been carried out by Henglein [4], who discovered that silver nanoparticles exhibit unique glass properties of catalytic effects, developed surfaces, high electric charge and surface plasma resonance (SPR). This is the reason that they are used in the manufacturing of optics, optical devices, electronics and sensor devices. Further advancements in the field of nanotechnology from past few decades have led to the synthesis and discovering of the unique properties of AgNPs. Although chemical synthesis is mostly used, but it has much adverse and hazardous effects on environment. Hence, the biological synthesis methods of silver nanoparticles have been discovered and developed. The biological synthesis has led to the discovery of new and improved properties regarding the size of the particles, its distribution and shape [5].

Silver nanoparticles are widely employed in agriculture to enhance crop production. In various plant species, low doses of silver nanoparticles are reported to increase seed germination. At moderate doses of silver nanoparticles, improved chlorophyll content as well as photosynthetic efficiency was observed in various species. Increase in carbohydrate content was reported due to the application of silver nanoparticles in plants. Growth of several species accelerated at lower concentrations of silver nanoparticles. However, at higher concentrations, a marked inhibition has been reported in different growth biomarkers. Photosynthesis was reported to be reduced at high silver nanoparticle concentrations. Application of different concentrations of silver nanoparticles markedly reduced reactive oxygen species. The anti-oxidative defence system and proline content of several crops has been found to enhance after the exposure to silver nanoparticles. Application of silver nanoparticles also enhanced abiotic stress tolerance. Recently a positive role of silver nanoparticles has been revealed under salt stress. The beneficial role of silver nanoparticles has been reported in saffron and soybean under flooding stress [6].

2. Research Methodology

2.1. Experiment Design

The sample size for the experiment was total of 12 small glasses or containers filled with peat moss and soil in a ratio of 2:1. These containers were divided into two groups: one was the control group 1 (C1) and the other was the control group 2 (C2). All the containers were labelled. The seeds were sown at a depth of 1 cm in the center of the pots. The C1 group was treated with AgNP solution while the C2 was without treatment under same conditions provided. The experiment was conducted for 3 weeks in the green house of National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre (NARC). Two of the containers from C1 group germinated the seeds on 6th day. And one from the C2 germinated its seeds on 7th day. On 15th day, all the pots germinated the seeds except one from C2 group. The seeds were watered each day. The physical parameters were measured on day 17.

2.2. Material and Method

The materials used in the experiment were 10 autoclaved glass slides, 10 ml phosphate–buffered saline PBS, vortexes, petri plates and MRS agar provided by NIGAB labs.

2.3. Refreshing of Bacillus strain

The previously isolated and identified bacterial strain NMCC34 was refreshed. In this procedure, a drop of TSA media was added to a petri plate and a colony of identified bacteria was mixed with it. The plate was then incubated at 37°C

for 48 hours. When the colony had grown it was further identified through different morphological, molecular and biochemical testing.

2.4. Gram staining

Gram-staining test was done for morphological identification of the bacillus specie. A single colony was taken from incubated plate with the help of a loop and put on the labelled glass slide. Now a small drop of water was added and mixed to the sample. Then the colony was fixed with heat flame. Heat should be hand bearable. Now crystal violet stain (primary stain) was added to the fixed slide and washed off with water after 10 seconds with water. Further iodine solution was added to the smear for 10-60 seconds and again washed with water to remove the excess. Now add decolorizer for 1-2 seconds and then wash with water. Now flood the smear with safranin (counter stain) for 5-10 seconds and wash again. The slide was air dried and examined under the microscope (Fig 1) [7].

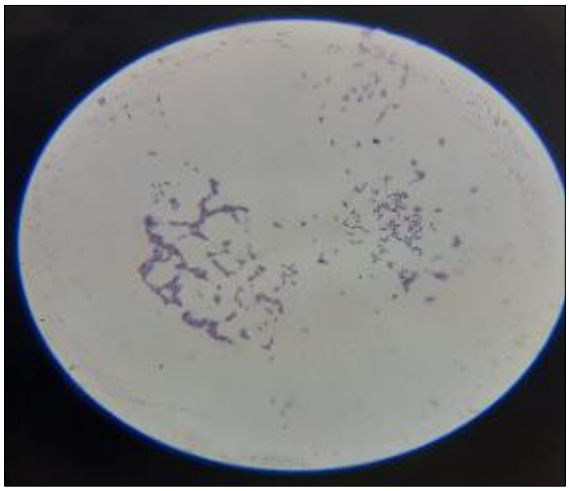

Figure 1 Gram staining

2.5. Catalase test

A small drop of H_2O_2 is placed on dry, clean, grease free slide. Now pick a single colony from incubated plate and mix it with H_2O_2 drop. The formation of bubbles indicated that the colony is catalase positive (Fig 2). No bubble formation will indicate negative results [8].

Figure 2 Catalase test

2.6. Oxidase test

A filter paper was placed on a clean petri plate. Add a drop of oxidase reagent on this filter paper. Pick a colony from plate and rub on the place where the reagent is placed. The change in color indicates the positive result (Fig 3). No color change will indicate negative results [9].

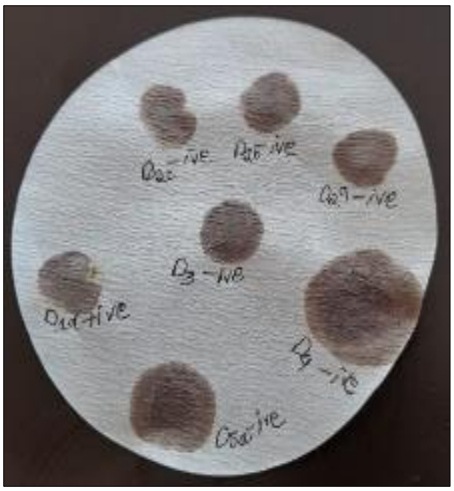

Figure 3 Oxidase test

2.7. Molecular Identification

Table 1 Biochemical testing

Sample No.	Catalase	Oxidase	Gram staining
1	-	-	-
2	-	-	+
3	+	-	-
4	+	+	+
5	-	+	+
6	+	+	-
7	_	-	-

+, present; -, absent

Table 2 Biochemical testing results of *Bacillus tequelesis*

Strain ID	Catalase	Oxidase	Gram staining
NMCC 34	-	+	+

2.7.1. DNA Extraction

The eppendorf tubes were labeled according to the sample number. Previously isolated colony was picked in sterilized environment and mixed with 20 µl of T.E buffer in eppendorf tubes. It was then placed in thermo cycler for 10 minutes at 95°C. The tubes were then centrifuged at 13000 rpm for 3 minutes. The palette was discarded and the supernatant was transferred in new autoclaved tubes for further use.

Bacillus strain was selected using 16S rRNA gene sequencing.

Table 2 Primers sequencing

S NO.	Primers	Sequence
1	9F	5-GAGTTTGATCCTGGTCAG-3
2	1510R	5-GGCTACCTTGTTACGA-3

2.8. PCR

Label the tubes and make the master mix recipe given in the table 3. After labeling the tubes, 19 µl of PCR water was added to each of them. Then forward and reverse primers were added using pipette 2 µl from each (table 2). Then 25 µl of master mix was added to it. DNA template was added to the same tube and mixed to make sure that the solution sets at the bottom of the PCR tube.

2.8.1. Master Mix recipe for PCR

The master mix recipe for PCR is given in table 3.

Table 3 Reagents for master mix

Reagent	Quantity for 1 reaction mixture vol. 50µl
DNA	2
Primer forward	2
Primer reverse	2
Master mix (ready to use)	25
PCR water	19

2.8.2. Preparing the samples

These PCR tubes were then placed in thermocycler and the temperature was adjusted in the following criteria as given in table 4:

Table 4 Steps of PCR

Steps	Temperature (°C)	Time (Minutes)
Denaturation	94	3
Annealing	50	1
synthesis	72	6

The time taken was almost three hours. It may vary depending on the number of cycles.

2.9. Gel electrophoresis

The gel electrophoresis was carried out by following standard operating procedure.

100 ml of 1x T.E buffer was taken in a conical flask and the 2 gm of agarose was added in it. The mixture was then heated until the solution turns transparent. Then 4 µl of ethidium bromide was mixed into it.

The solution was added in the gel electrophoretic tank avoiding the bubble formation while pouring the solution. When the solution gets solidify, the combs and stoppers were removed and the plate was placed in the gel tank. 8 µl of the PCR product in the well was added.

The time, voltage and current were adjusted and the tank was then covered. The electrodes were connected, and the formation of bubbles indicated the start of the procedure (Fig 4).

Connect the electrodes and wait for the bubble formation. The bubble formation on both sides of tank indicates the start of procedure. After almost 45 minutes, the sample has migrated towards their respective electrodes which can be seen through the lid.

The power supply was disconnected and after approximately 10 minutes, the gel was removed carefully. The gel was then placed on the UV transilluminator for visualizing DNA and proteins.

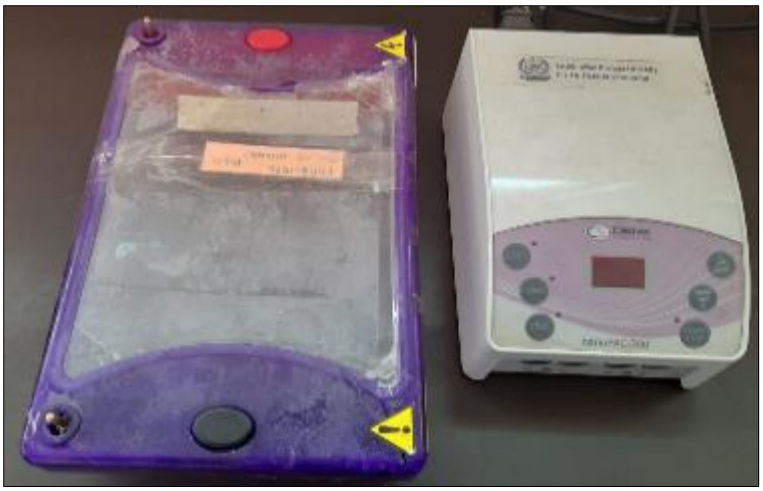

Figure 4 Apparatus for gel electrophoresis

2.10. Biosynthesis of AgNPs

Biosynthesis of AgNPs using *Bacillus tequilensis* were recognized by using the experimental methods given by Wang et al., [10] and Das et al., [11]

The silver nanoparticles were synthesized by inoculating previously isolated bacterial colony (Fig 5) into 250 ml Erlenmeyer flask containing 100 ml nutrient broth. The flasks were then incubated in a rotating incubator at 120 rpm for 24 hours at 37 °C. After incubation, the culture was centrifuged at 1000 rpm for 10 minutes. The supernatant obtained was further used for synthesis of AgNPs (Fig 5). The supernatant was mixed with 100 ml $AgNO_3$ solution at 1 mM aqueous solution. It was then incubated at 200 rpm for 1 hour at 60°C. After incubation, the samples were then visually observed for the formation of AgNPs. The samples showed color change from yellow to brown (Fig 6) which indicated the reduction of Ag ions by the bacteria [11].

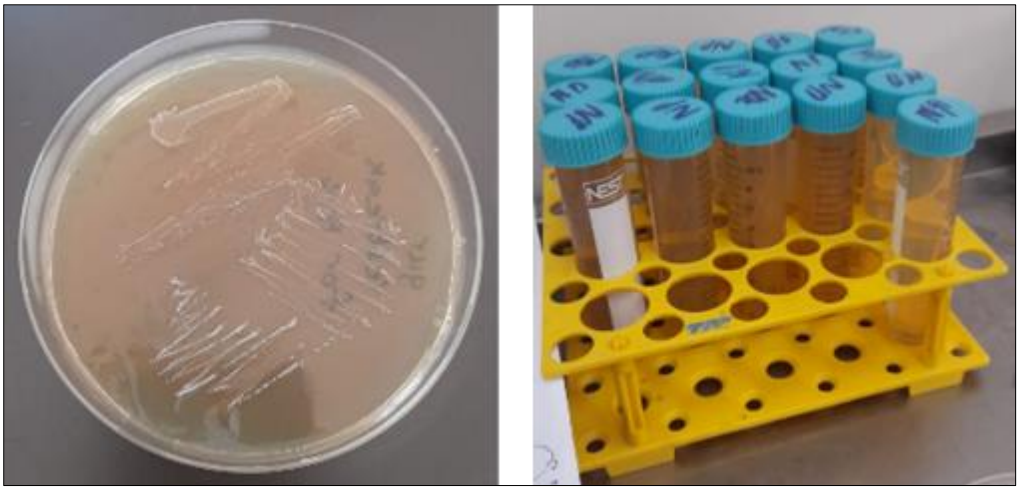

Figure 5 Bacterial Culture and AgNPs Solution

Figure 6 AgNP solution showing color change

2.11. Method of treatment

Tomato is widely used as a model crop due to its diverse physiological, cellular, biochemical, molecular, and genetic studies. It can be easily grown in greenhouses or growth chambers [12].

2.11.1. Seeds preparation

Tomato seeds grown for the experiment were provided by NIGAB labs. Peat moss was purchased from wholesale nursery and seed shop. Sterilized tomato seeds (Fig 7) were provided by NARC. Around 60-70 seeds were sown in the experiment 4 in each pot/container. The pots were filled with peat moss and soil in a ratio of 2:1. The seeds were sown in each pot and covered with a cm of soil. The pots were watered immediately after the seeds were sown. The experiment was carried out in a greenhouse at an average temperature of 24-29 °C [12].

Figure 7 Tomato Seeds

2.11.2. Dose preparation

Previously prepared AgNPs were used in a single concentration of 50 ppm. The 50 ppm concentration was prepared by taking 50µg of powdered AgNPs and was added to 50 ml of distilled water. This working solution was then given to the seeds.

2.11.3. Treatment to tomato seeds

Lycopersicum esculentum or tomato seeds were exposed to AgNPs solution. Control group 1 was treated by giving 1ml AgNP solution close to the seedlings. Both the groups were watered on daily basis with water quantity of 20 ml per pot.

The pots of both groups were placed in the same location to keep the environment conditions same for both groups (Fig 9).

Figure 8 Day 1 Seeds are sown

Figure 9 Treatment with AgNP Solution

3. Results

The viability of previously identified bacterial strain was checked through biochemical testing. Seven samples were used for biochemical testing (Table 1). The biochemical characterization of bacterial strain *Bacillus tequelensis* NMCC34 is listed in table 2 and the results were catalase negative, oxidase positive and gram positive.

The bacterial strain *Bacillus tequelensis* has retained crystal violet dye that can be seen violet/purple under the microscope which shows that it is a gram-positive bacterium as shown in fig 1. The catalase test for *Bacillus tequelensis* was negative as it shows no bubble formation on the glass slide (Fig 2). The oxidase test result obtained was positive as it has shown blue color change within 10-15 seconds (Fig 3).

Fig 8 and 9 shows that tomato seeds were sown and treated with biosynthesized AgNPs solution. On the next day, all the containers were watered as shown in fig 10. On day 3, no germination was noticed, and containers were monitored (Fig 11). On 6th day two of the seeds germinated from C1 treated group and two from C2 untreated group as shown in fig 12. Both the groups were watered and on 17th day all the plants from C1 treated group have grown (Fig 13). Growth parameters of both the groups were measured on day 17 as shown in fig 14.

Based on our findings, C1 treated seeds have shown better growth parameters (Table 5) comparing to C2 negative control group. Although the germination time for both the groups was almost the same but one of the sample seeds from

C1 germinated a day before the C2 group. All the seeds germinated in about 2 weeks except the one from C2 group which did not germinate at all. The biosynthesized AgNPs using *Bacillus tequilensis* have shown an overall positive impact on the growth of tomato plant and the seed germination percentage was high in the C1 group.

Table 5 Plant response to treatment and growth measurements

S. #	Height (cm)		No. of tillers		Flowering		No. of leaves		Leaves size			
									Width (cm)		Length (cm)	
	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2
1	2	0.9	2	1	--	--	4	2	0.5	0.4	1.6	1.2
2	1.1	1	2	1	--	--	2	2	0.4	0.3	1.1	0.9
3	0.8	0.7	1	1	--	--	2	2	0.3	0.3	0.8	0.4
4	0.7	0.4	1	1	--	--	2	2	0.3	0.2	0.7	0.4
5	0.6	0.4	1	1	--	--	2	2	0.3	0.2	0.6	0.3
6	0.5	--	1	--	--	--	2	--	0.3	--	0.4	--

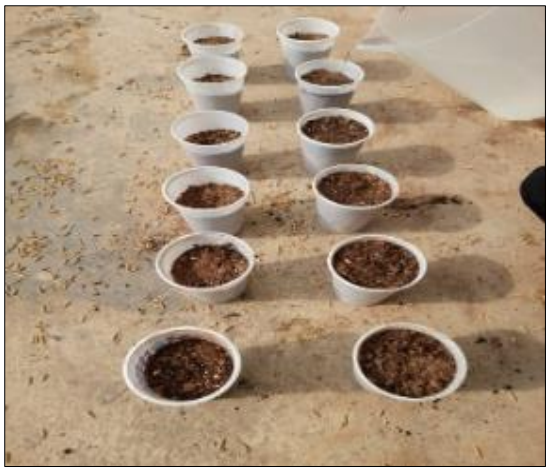

Figure 10 Day 2: All the containers were monitored and watered

Figure 11 Day 3: Watered; No germination

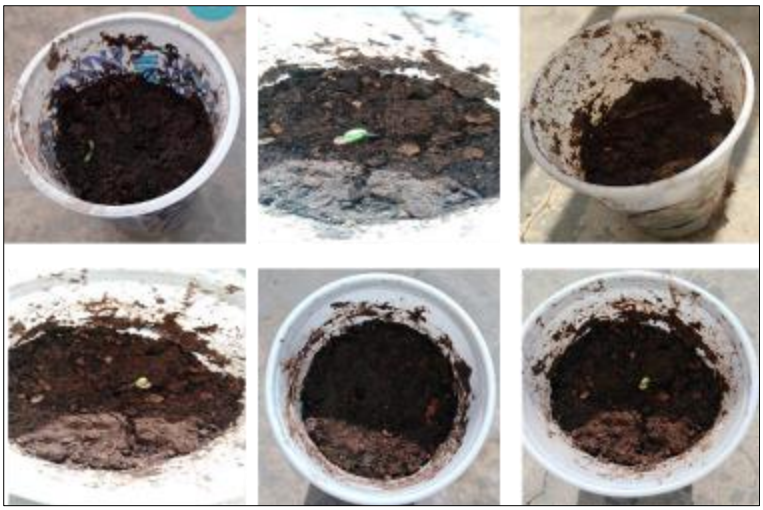

Figure 12 Day 6: Seeds germinated

Figure 13 Day 17 C1 Group

Figure 14 Day 17 C2 Group

4. Discussion

In this study, bacterial strain *Bacillus tequelensis* (NMCC34) was utilized to evaluate its potential to produce nanoparticles and effect of these bacterial synthesized silver nanoparticles on tomato plant's growth. The AgNPs have shown growth promoting potential. The strain has ability to reduce Ag metal ions into silver nanoparticles which can be used for improving plant growth. This method is sustainable and eco-friendly and is advantageous over standard chemical method. Hence this mechanism of bacteria is used in the synthesis of AgNPs. Bacterial cell wall electrochemically interacts with positively charged metal ions (Ag^+) as it is negatively charged and consequently, caused bioreduction of metal ions to metal nanoparticles. Thus, it plays a crucial role in the synthesis of AgNPs [13]. The physical and chemical methods of silver nanoparticles production generate lots of toxic chemicals and heat whereas bacterial synthesis of nanoparticles is economic and environmentally safe technique. The plant group treated with silver nanoparticles (50 ppm) showed improvement in plant length. Significant improvement was recorded in length of C1 group as compared with control plants (C2 untreated). There was also an improvement observed in the number of leaves in comparison to control plants.

Improvement in seed germination with application of nanoparticles in the C1 group has been recorded as compared to C2. C1 group has shown better leaves sizes as compared to C2. Mehta et al., [14] reported improvement in biomass and germination due to the of nanoparticles' application. The production of root exudates is promoted by the application of silver nanoparticles which may facilitate plant-microbes interactions and hence play its role to improve growth of plant [15]. When silver nanoparticles were applied on Fenugreek, growth parameters showed increment. In the current study bacterial silver nanoparticles showed the growth promotional activity which indicates that these bacterial nanoparticles can be commercially used for the benefit of humankind in an economic and eco-friendly manner.

Enormous applications of nanoparticles in several fields of modern society make it inevitable that they are studied and brought into use in the ecosystem. The world population has increased manifold in the past century and with this increase in population there is a huge rise in demand for food. There is a huge pressure on the agriculture sector of the world to meet this demand. With this increase, it is very important as well that innovative methods of agriculture are brought into use to increase the yield of the crops. Compared to the developed countries of the world the yield of crops in Pakistan is less, however, recently Pakistan has started to focus on the innovative technology to increase the yields of the crops to meet the rising demand of food. Tomato is an essential part of the Pakistani cuisine and it has a demand all year round. The tomato crop grown by the orthodox agricultural methods cannot be grown all the year and has a very low yield. With the Nano particles technology the yield can increase incredibly and the time of growth of the tomato plant also increases. This method of growing crops is cost effective and it is very eco-friendly. The crop grown with the use of nano-particles is good for human health as compared to the chemical methods of agriculture [16].

Little was known about nanoparticles till very recently. However, over the last few decades a lot of research has taken place due to increased public funding and more attention, as a result both the negative and positive effects of nanoparticles have been explored. Although in some cases the nanoparticles that are released in the air as pollutants and consumed by humans and living things this can be a health hazard. On the other hand, various types of nanoparticles have positive affects if it is properly applied. In the field of agriculture it has been found through various studies that if properly synthesized and applied, the nanoparticles adds nutritional value to the fruits and vegetables grown with its help. It has been found that nanoparticles have a large surface to volume ratio due to which they are more effective to be used to address the challenges which are not properly met by the physical, chemical and biological pesticides and their control methods. The nanoparticles offer novel agrochemical agents that help in increasing the crop productivity.

Nanoparticles are currently being researched and tested for its increased use and better application according to its characteristics and usability. Most of the experiments carried out on the impacts of AgNPs are carried out in the laboratories and done under controlled environment which is different than the real fields; therefore, it is important that more research is carried out directly in the field. The conditions in the field will give a better and clearer picture about the application of AgNPs in the field of agriculture.

5. Conclusion

The biosynthesized silver nanoparticles have potential to improve tomato plant growth. The agriculture industry can play a major role in the economy of a country. Tomato prices have hit a record several times in Pakistan due to less supply and high demand. So, there is need for the solution of fulfilling the demand. It can be done through innovation and development of the nano technology in the agriculture sector. There is need to identify some other microbes as well their potential use in synthesizing the AgNPs.

It can be concluded at the end of our research that the silver nano particles have overall positive impact on the tomato crop. This research was conducted to find the potential of the use of biosynthesized silver nano particles using bacillus specie in the field of agriculture. The results highlighted that nano particles have potential to be beneficial for the agriculture industry. This is an innovative method and in order to utilize it properly, more detailed research is required on its application in the cultivation of crops.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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