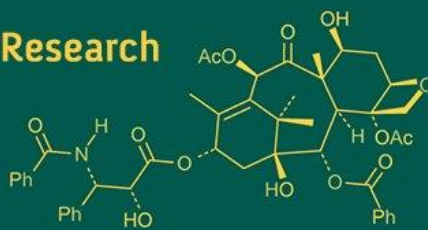
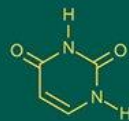
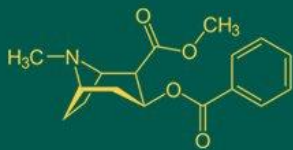


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Kadheeja Shahanoor Begum KK

Department of Nano Science and Technology, College of Agriculture, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, India

Naveen Kumar JR

Department of Nanotechnology, Institute of Engineering and Technology, Srinivas University, Mukka, Mangalore, Karnataka, India

Praveen BM

Department of Nanotechnology, Institute of Engineering and Technology, Srinivas University, Mukka, Mangalore, Karnataka, India

Reshmy Vijayaraghavan

Department of Nano Science and Technology, College of Agriculture, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, India

Corresponding Author:

Kadheeja Shahanoor Begum KK

Department of Nano Science and Technology, College of Agriculture, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, India

Green synthesis of silver nanoparticles from the leaf extract of *Ocimum sanctum* (Holy Basil/Tulsi), its characterization and incorporation in plant tissue culture medium for the performance of micropropagation in an open environment

Kadheeja Shahanoor Begum KK, Naveen Kumar JR, Praveen BM and Reshmy Vijayaraghavan

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Abstract

In this study, the possibility of performing plant tissue culture in an open environment was defined. In plant tissue culture technique the explants are grown *in vitro* in an artificial nutrient rich growth medium. As the chances of contamination are very high, plant tissue culture should be carried out carefully under controlled aseptic conditions inside a laboratory. Using the existing plant tissue culture media, plant tissue culture can be practiced only under aseptic conditions inside a well equipped laboratory by a trained and skilled technician with adequate knowledge and experience.

In this case the farmers cannot directly carry out the process of plant tissue culture by themselves and they also have to buy the tissue culture raised plantlets from the market paying lot of money. Hence, the study entitled "Green synthesis of Silver nanoparticles from the leaf extract of *Ocimum sanctum* (Holy Basil/Tulsi), its characterization and incorporation in plant tissue culture medium for the performance of micropropagation in an open environment" was carried out in the department of Nanotechnology, Institute of Engineering and Technology, Srinivas University, Mukka-574146, Mangalore, India during the period of 2021 to 2023. The idea of an artificial growth medium using which plant tissue culture can be carried out in an open environment was made possible with the incorporation of the green synthesized *Ocimum sanctum* mediated Silver nanoparticles in to the plant growth medium. The green synthesized Silver nanoparticles in the plant growth medium exhibited excellent antimicrobial properties which greatly reduced the chances of contamination.

Keywords: Green synthesis, silver nanoparticles, *Ocimum sanctum*, antimicrobial properties, plant tissue culture

1. Introduction

Plant tissue culture is a valuable technique used in modern agriculture and biotechnology for the propagation, conservation, and genetic improvement of plants. Plant tissue culture also called as micro propagation is a technique in which thousands of plants can be produced within a short period of time. Using this technique whole plants can be produced from any plant part by growing it in an artificial nutrient medium inside a test tube under sterile conditions. The plant part taken for tissue culture is called as explant and totipotency is the ability of a plant cell to grow into an entire plant. The plants produced by micro propagation are morphologically and genetically similar (identical) to the original plant from which they are produced hence they are called as somaclones. The existing media for plant tissue culture are MS media, White's media, Nitsch's media, Gamborg (B5) media.

Murashige and Skoog media is most commonly used for plant tissue culture. So it is called as standard tissue culture media. Murashige and Skoog media contains Inorganic nutrients (Macro nutrients such as Calcium, Magnesium and Nitrogen and Micro nutrients such as Copper, Iron and Zinc), Organic nutrients such as Vitamins and Amino acids, Growth hormones such as Auxins, Cytokinins and Gibberellins, Gelling agents like Agar and Gelatin and Carbohydrates like Sucrose.

Using the existing plant tissue culture media, plant tissue culture can be practiced only under aseptic conditions inside a well equipped laboratory. An effective plant tissue culture protocol is essential for successful results, and it is influenced by many factors such as the plant's genotype and age, the physiological state of the explant, the composition of the growth media, the sterilization methods used, and the conditions in the culture and growth rooms (Kim *et al.*, 2017) [10].

In recent years, there has been growing interest in the application of nanotechnology in plant tissue culture, with silver nanoparticles emerging as a promising tool. Silver nanoparticles possess unique physicochemical properties that make them ideal for various applications in plant tissue culture, including enhancing micropropagation, improving plant regeneration, and combating microbial contamination. AgNPs showed a significant effect on the growth of *in vitro* growing olive shoots, which was highly, affected by plant genotype and AgNP concentrations (Mohamed *et al.*, 2024) [12]. Researchers have widely documented the successful use of metal and metal oxide nanoparticles (NPs) as antibacterial and antifungal agents (Wang *et al.*, 2017) [20]. These include nanoparticles such as gold (Au), silver (Ag), nickel (Ni), silicon (Si), copper (Cu), copper oxide (CuO), magnesium oxide (MgO), zinc oxide (ZnO), iron oxide (Fe₃O₄), aluminum oxide (Al₂O₃), and titanium dioxide (TiO₂) (Beyth *et al.*, 2015; Kim *et al.*, 2017) [3, 10].

Many studies have focused specifically on the use of silver (Ag) nanoparticles for surface sterilization in plant tissue culture. Their strong antimicrobial activity, combined with their generally low impact on plant growth, makes them a promising alternative to traditional sterilization techniques. Previous research has examined different concentrations of silver NPs and varying exposure times for a wide range of plant explants (Adebomojo and AbdulRahaman, 2020; Fakhreshani *et al.*, 2012; Gouran *et al.*, 2014; Nartop, 2019; Tung *et al.*, 2021a) [1, 7, 8, 13, 19]. A number of studies have demonstrated that nanoparticles can enhance callus induction. For instance, when *Solanum nigrum* leaf explants were cultured on MS medium supplemented with a relatively high concentration of silver nanoparticles (8 mg/L), researchers observed a marked increase in both callus formation (89%) and the average fresh weight of the callus (4.67 g per explant). The treatments also caused noticeable changes in callus appearance, resulting in friable, watery tissues that appeared white, greenish, or yellowish. These alterations may improve water solubility or allow for more targeted interactions. Nanoparticles can also be combined with various biologically active molecules, enabling highly specific delivery to biomolecules like proteins and nucleic acids, as well as to different cellular and tissue structures. Light microscopy showed that the nanoparticles were able to penetrate the callus and damage the cell walls, which ultimately increased water uptake and buildup within the callus (Ewais *et al.*, 2015) [6].

2. Materials and Methods

2.1 Chemicals and other materials

Analytical grade Silver nitrate, Potassium Nitrate, Calcium phosphate, Magnesium sulphate, Ferrous gluconate, Zinc oxide, Boric acid and Cupric chloride was purchased from Merck, Darmstadt, Germany. Sucrose, Rooting powder (FASTroots), Yeast extract and Agar was purchased from Hi-Media Pvt. Ltd., Nagpur, India.

Fresh and healthy Holy basil plants were collected from Chowki, a village located in the Kasaragod district of Kerala.

2.2 Preparation of instruments

The Apparatus used for this work were a 100 ml measuring cylinder, Florence flasks (250 ml), Beakers (50 ml), Florence flasks (1000 ml), a 1000 ml volumetric flask, Glass rods, Forceps, Spatula, Surgical blades and Erlenmayer flasks (250 ml).

All these Apparatus were properly cleaned and dried before starting the plant tissue culture experiment. A plastic bucket was filled with water and Surf excel (detergent) was added to it. All the Apparatus were immersed in the detergent solution for 15 minutes. After that the apparatus were cleaned properly using Nylon brushes. Then all the apparatus were immersed in another bucket filled with clean water and the detergent was washed away. At last the apparatus were washed using tap water and kept in clean tray. Then these apparatus were kept under the sunlight for 3 hours for drying. Then these properly cleaned and dried apparatus were used to carry out the plant tissue culture experiment.

2.3 Preparation of the leaf extract of *Ocimum sanctum* (Holy Basil/Tulsi)

Fresh and healthy Holy basil plants were washed properly for three times using distilled water to remove dirt and soil. Then the Holy basil plants were kept for drying in dark for 15 days. After 15 days, the leaves of the dried Holy basil plants were powdered using a domestic mixer grinder. Then the plant broth was Prepared by adding 50 gram of this dried powder into 200 ml deionized distilled water and kept this solution to boil for 15 minutes at 60 °C. Then the resulting solution was filtered thoroughly for 3 times using clean filter papers and made sure that no insoluble material appeared in the plant broth. Then the filtrate was immediately collected in a 250 ml Erlenmayer flask.

2.4 Green synthesis of Silver nanoparticles from the leaf extract of *Ocimum sanctum* (Holy Basil/Tulsi)

The Silver nanoparticles were green synthesized from the leaf extract of *Ocimum sanctum* using the protocol of (Mallikarjuna *et al.*, 2011; Tailor *et al.*, 2020; Parmar and Singh, 2024; Shinde *et al.*, 2024) [11, 17, 14, 16].

Soon after the filtrate was formed, 100 ml Silver nitrate (AgNO₃) solution was immediately added to 150 ml of the filtrate. After that the resulting mixture was gently shaken for 15 minutes in a shaking incubator and kept undisturbed on the table inside the laboratory for 2 hours. Then the resulting sample was subjected to UV-Visible Spectrophotometry and monitored at regular intervals of time in order to identify the characteristic absorption properties of Silver nanoparticles.

2.5 Preliminary characterization of the Green synthesized Silver nanoparticles (AgNPs)

UV-Visible spectrophotometry was carried out for the preliminary characterization of the Silver nanoparticles. In order to carry out UV-Visible spectrophotometry, first the sample (mixture of 150 ml filtrate and 100 ml AgNO₃) was diluted with Millipore water for 20 times. 150 micro litre of the sample was added to 2850 micro litre of the Millipore water for the preparation of 3000 micro litre or 3 ml of 20 times diluted sample with Millipore water. Millipore water was used as the blank solution. The absorbance value of the sample at different wavelength ranges, 300-320 nm, 320-340

nm, 340-360 nm, 360-380 nm, 380-400 nm, 400-420 nm, 420-440 nm, 440-460 nm, 460-480 nm, 480-500 nm, 500-520 nm, 520-540 nm and 540-560 nm was observed using Shimadzu UV-1780 UV-Vis Spectrophotometer.

2.6 Separation of the Green synthesized Silver nanoparticles (AgNPs) from the sample

After the preliminary confirmation of the formation of Silver nanoparticles, these nanoparticles were separated from the sample (mixture of 100 ml AgNO₃ and 150 ml filtrate) using an Eppendorf SE Centrifuge 5430 R (Eppendorf AG, Barkhausenweg 1, 22339 Hamburg, Germany). The sample containing Silver nanoparticles were centrifuged at 7500 rpm for 20 minutes at 24 °C to separate the *Ocimum sanctum* mediated Silver nanoparticles. The Silver nanoparticles were washed twice in deionized water at 7500 rpm for 10 minutes at 24 °C. Then the supernatant was decanted and the Silver nanoparticles settled at the bottom of the falcon tubes were carefully transferred to a clean petri dish. The Silver nanoparticles were further dried in a hot air oven at 60 °C for 24 hours. The Silver nanoparticles thus obtained was subjected to Scanning Electron Microscopy (SEM) analysis.

2.7 Physicochemical characterization

UV-Visible spectrophotometry was used for the preliminary characterization of the Silver nanoparticles. Scanning electron microscopy (SEM) was used to examine the morphology of the green synthesized Silver nanoparticles, including their size and shape, and to help link these structural features with their material properties. Dried and purified Silver nanoparticles were imaged using a field emission scanning electron microscope (FESEM) (TESCAN MAIA3 XMH, Brno, Czech Republic) at the Sophisticated Analytical Instrument Facility (SAIF), Mahatma Gandhi University, Kottayam. The elemental makeup of the nanoparticles was analyzed using energy dispersive X-ray spectroscopy (EDX) with a microprobe attached to the SEM.

2.8 Preparation of the Culture medium

For the preparation of the culture medium, 3.5 grams of Potassium Nitrate salt, 3.5 grams of Calcium phosphate salt, 3.5 grams of Magnesium sulphate salt, 1 gram of Ferrous gluconate salt, 1 gram of Zinc oxide salt, 1 gram of Boric acid powder and 2.25 grams of Cupric chloride salt were properly weighed using a weighing balance and dissolved in 500 ml distilled water by continuous stirring using a clean glass rod. To this solution, 15 grams of Sucrose, 5 grams of Rooting powder (FASTroots) and 1.5 grams of Yeast extract were added and stirred using a magnetic stirrer (LABQUEST by BOROSIL) at 22 °C for 30 minutes. To the resulting solution (mixture), 4 ppm green synthesized Silver nanoparticles were added (Tung *et al.*, 2021b) ^[18] and subjected to probe sonication for 60 minutes at 24 °C. The volume of the solution thus obtained was made upto 1000 ml by adding distilled water and 50 ml of the resulting solution was kept undisturbed in a beaker to grow Onion. To the remaining 950 ml of the solution, molten agar was added and boiled at 100 °C for 10 minutes. Then the culture medium is allowed to cool.

The pH of the prepared culture medium was observed using a pH meter.

2.9 Pouring of the culture medium

100 ml of the prepared culture medium was carefully poured into Six 250 ml florence flasks each and the florence flasks were cotton plugged. 35 ml of the prepared culture medium which does not contain agar was carefully poured into Four 50 ml beakers each to grow onions.

2.10 Preparation of explants

The part of the plant taken for tissue culture is called as the explant. Jack fruit seeds, Onions and stem cuttings of Rose plant were chosen to perform plant tissue culture. Healthy and disease free Jack fruit seeds and stem cuttings of Rose plant were collected from Chowki, a village in the Kasaragod district of kerala. Fresh Onions were bought from a local vegetable market located in the Kasaragod town of Kerala. Soon after the collection, the Jack fruit seeds and the stem cuttings of Rose plant were kept in a beaker containing clean water to avoid the entrance of air bubbles, contaminants and microbes through the exposed or cut parts. This will also avoid browning due to phenolic oxidation. The dust particles and other contaminants present in the Onions were properly removed using a clean tissue paper. Mild detergent, fungicide or an antibiotic was not used for the surface sterilization of the explant. The stem cuttings of the Rose plant was properly cut into smaller pieces using a pair of scissors and immediately placed in a beaker containing deionized water.

2.11 Inoculation of the explant

The prepared culture medium has Silver nanoparticles of excellent antimicrobial property in it. So the explants were easily transferred into the culture medium in open environment itself. The Jack fruit seeds were transferred into 250 ml Florence flasks containing 100 ml of the culture medium. Stem cuttings of the Rose plant were also transferred into 250 ml Florence flasks containing 100 ml of the culture medium. Onions were placed in 50 ml beakers containing 35 ml of the culture medium and 50 ml beakers containing 35 ml of clean water to compare the growth of Onion roots and time taken for sprouting in normal water and the prepared culture medium.

2.12 Incubation for growth

After the Inoculation of the explant, the Florence flasks containing Jack fruit seeds were kept undisturbed in the table for 22 days. The beakers containing Onion were kept undisturbed on the table for 20 days. The Florence flasks containing the stem cuttings of Rose plant were incubated in the plant incubator (growth chamber) for 30 days. The temperature maintained inside the plant incubator was 26 °C. The explants (stem cuttings of Rose plant) were subjected to 16 hours light alternating with 8 hours darkness. After 7 days, all cultures with either bacterial or fungal contaminants were removed and the remaining cultures were allowed to grow further. As the culture medium contained rooting powder (FASTroots) in it, after 36 days of incubation rooting was induced in the shoots which formed after 3 to 4 weeks of incubation of the explants (stem cuttings of Rose plant). After complete 6 weeks of incubation, the shoots were rooted. Jack fruit seeds started to germinate in the culture medium after 17 days. After 22 days, the Jack fruit seeds were completely germinated. The formation of roots in Onions kept undisturbed in the culture medium started after 5 to 6 days and the Onions started to sprout in the culture medium after

12 days. After 20 days, the Onions were properly sprouted to produce new plants.

2.13 Acclimatization of plantlets and transfer to pots

Once the plants were properly grown *in vitro* in the culture medium (nearly 3 to 4 cms in height), then these plants were directly transferred into the soil for further growth and development. After 4 weeks of growth in the soil, many new leaves appeared in the tissue culture grown rose plant and Jack fruit plant. In Onions the plant shoot had grown into appreciable height.

3. Results and Discussion

3.1 Green synthesis of Silver nanoparticles from the leaf extract of *Ocimum sanctum* (Holy Basil/Tulsi)

When 100 ml of Silver nitrate was added to 150 ml of filtrate, very soon the colour of the mixture started to change from green to light Yellow to light brown to dark brown. This is due to the reduction of Ag^+ ions to elemental silver (Ag^0) and formation of colloidal silver nanoparticles (AgNPs), accompanied by nucleation and growth. This pattern of colour change is regarded as a typical early indication of nanoparticle synthesis in many green synthesis studies (Brahmachari *et al.*, 2014) [4].

3.2 Physicochemical characterization

The preliminary confirmation of the formation of Silver nanoparticles was obtained through UV-Visible spectrophotometry. The absorption peak (surface plasmon resonance peak) was found to be around 430 nm as seen in Table 1 and Fig 1. This is consistent with the characteristic SPR bands reported for plant mediated silver nanoparticles. Indeed, other studies using *Ocimum sanctum* extracts have reported SPR absorption peaks in the range of 420-430 nm, confirming the presence of nanoscale silver (Deepan *et al.*, 2023) [5].

Scanning Electron Microscopy analysis revealed the formation of spherical shaped silver nanoparticles as seen in Fig 2. Average particle size of the Silver nanoparticles was found to be 42 nm. This aligns with the earlier literature claiming that green synthesized AgNPs commonly fall in the size range of 10-50 nm depending on synthesis conditions (Shahzadi *et al.*, 2025) [15].

The energy dispersive X-ray analysis (EDAX) revealed the predominance of silver atoms with 68 percent by weight. This strongly supports the effective reduction of Ag^+ to Ag^0 and dominance of silver in the nanoparticles. The presence of additional elements (O, Cl, Ca) is likely due to residual phytochemicals such as organic molecules and minerals from Tulsi leaf extract that act as capping and stabilizing agents. Such capping is an indication of green synthesis, which relies on plant phytochemicals to reduce metal ions and to stabilize the formed nanoparticles (Shahzadi *et al.*, 2025) [15].

Table 1: The absorbance value of the green synthesized Silver nanoparticles at different wavelength ranges

Wavelength range (nm)	Absorbance value of Silver nanoparticles (a.u)
300-320 nm	0.31
320-340 nm	0.26
340-360 nm	0.14
360-380 nm	0.28
380-400 nm	0.37
400-420 nm	0.48
420-440 nm	0.59
440-460 nm	0.46
460-480 nm	0.35
480-500 nm	0.23
500-520 nm	0.21
520-540 nm	0.16
540-560 nm	0.11

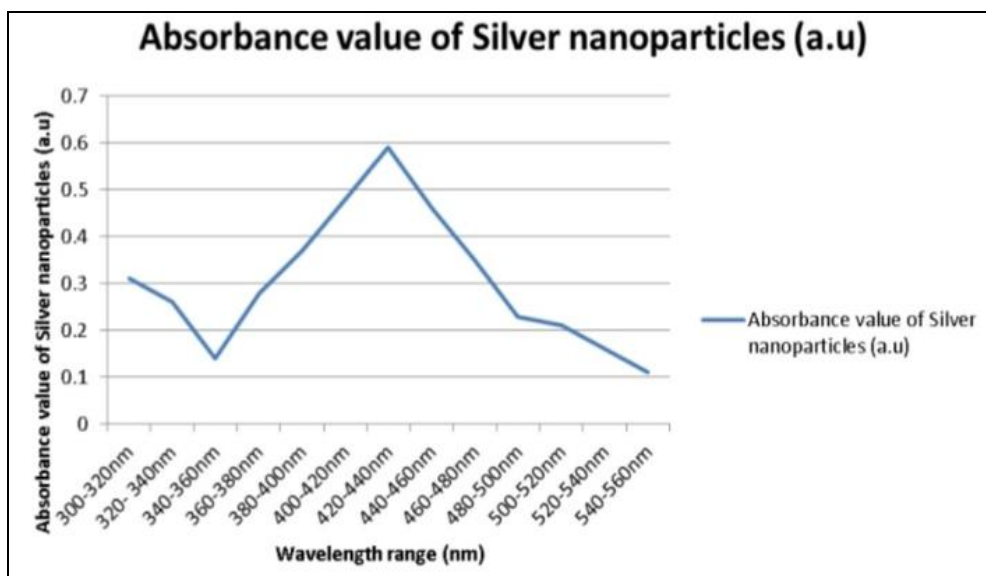


Fig 1: Graphical representation of the absorbance value of Silver nanoparticles at different wavelength ranges

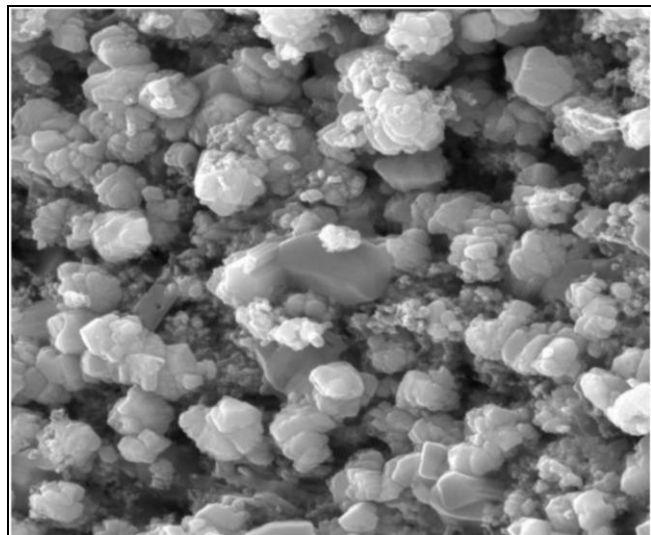


Fig 2: Scanning Electron Microscopy image of the green synthesized Silver nanoparticles.

3.3 Plant tissue culture in an open environment using the prepared culture medium

Plant tissue culture was successfully performed using the prepared culture medium in an open environment. Healthy and disease free Rose plants were grown from the stem cuttings, Jack fruit plants were grown from its seeds and Onion plants from Onions. This suggests that the Silver nanoparticles acted effectively as antimicrobial agents, preventing microbial contamination. This aligns with the recent reports demonstrating that biogenic AgNPs can suppress bacterial and fungal contamination *in vitro* culture media, improving explant survival and plantlet establishment (Alfarraj *et al.*, 2023) [2]. Biogenically synthesized AgNPs significantly reduced microbial contamination and promoted callus growth compared to controls in a study on *Rumex nervosus* explants (Alfarraj *et al.*, 2023) [2]. Similarly, another study demonstrated that low concentrations (2-5 ppm) of biogenic AgNPs added to the culture medium of *Rosa indica* (rose) and *Cestrum nocturnum* (night queen) effectively prevented bacterial and fungal contamination without negatively affecting plant morphology, physiology or genetic stability (Jahangir *et al.*, 2025) [9]. The pH of the culture medium maintained was 5.7. Plants generally take up nutrients at a pH ranging from 5.6 to 5.8.

The Onions started to form roots after 5 to 6 days in the culture medium. After 12 days, the Onions started to sprout in the culture medium. After 20 days, the Onions were properly sprouted and a 3 to 4 cm long stem was formed in the Onions as seen in Fig 3. These sprouted Onions with many number of roots were directly transferred into the soil for its further growth as seen in Fig 4. After 4 weeks in the soil, these sprouted Onions had grown and matured into a well developed shoot system. The shoots were grown into appreciable height (20 to 25 cms) as seen in Fig 5. We had successfully sprouted Onions and grown fibrous roots of Onions in the culture medium (Agar was not used in the culture medium).

Jack fruit seeds started to germinate in the culture medium after 17 days. After 22 days, the Jack fruit seeds completely germinated in the culture medium and the seedlings were 3 to 4 cms in length as seen in Fig 6. These seedlings were further grown in the soil. After 4 weeks, these seedlings were grown and developed into small healthy plants with more number of leaves as seen in Fig 7.

In the culture medium, the stem cuttings of Rose plant developed shoots after 3 to 4 weeks of incubation. After 36 days of incubation, rooting was induced in the shoots. The shoots were properly rooted after 6 weeks of incubation as seen in Fig 8. The shoots were 3 to 4 cms in length when it was transferred into the soil for further growth. After 4 weeks since these tissue culture raised plantlets (Rose plant) were directly transferred into the soil, they had grown into healthy and disease free plants with more number of leaves. These plants were nearly 25cms in length as seen in Fig 9. Flowering occurred in none of these plants.

The prepared culture medium successfully supported the growth of plants *in vitro* in an open environment. The plants were also properly rooted in the culture medium because of the presence of rooting powder (FASTroots) in it. The plants grown *in vitro* in the prepared culture medium were resistant to bacterial or fungal infection because of the presence of the green synthesized Silver nanoparticles in the culture medium. During the growth of the plant, the plant roots absorbed the Silver nanoparticles along with other micro nutrients and macro nutrients present in the medium. The presence of Silver nanoparticles in the culture medium had helped to raise the tissue culture grown plantlets into disease free and healthy plants which were highly resistant to microbial contamination.



Fig 3: Onion kept in the prepared culture medium got rooted and sprouted within one week whereas the Onion kept in water only got rooted within one week



Fig 4: The rooted and sprouted Onion from the culture medium was transferred to the soil for further growth



Fig 5: Onions developed shoot system during its growth in the soil



Fig 6: The Jack fruit seeds kept in the culture medium got germinated and further grown into small plants after 38 days



Fig 7: The Jack fruit plant grown in the culture medium was transferred to soil for its further growth

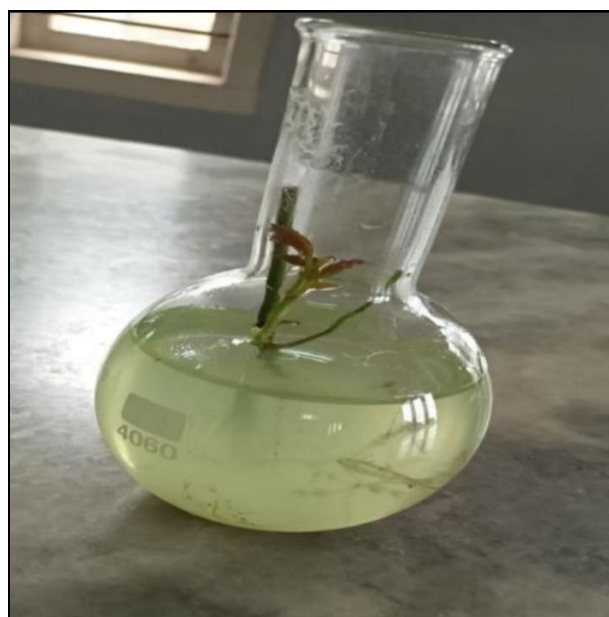


Fig 8: A new healthy plant was formed from the stem cutting of Rose plant kept in the culture medium

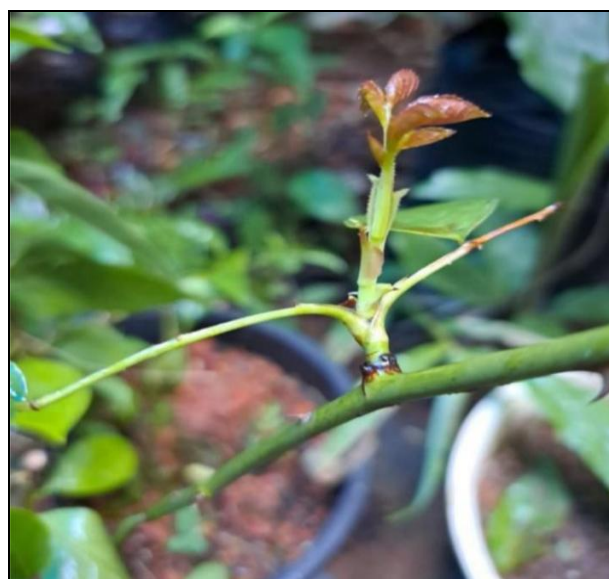


Fig 9: The Rose plant grown in the culture medium was transferred into the soil for its further growth

4. Conclusion

The Preparation of culture medium will help the farmers to carryout the process of plant tissue culture in open environment by themselves. This will greatly reduce the dependency of farmers towards the market for buying quality plantlets, there by improving the agricultural sector.

As the silver nanoparticles present in the culture medium exhibits excellent antimicrobial property, the chances of contamination will be nearly zero. This property of the culture medium eliminates the use of certain chemicals such as 70% Ethanol, Chromic acid, antibiotics etc in the plant tissue culture technique. It will greatly reduce the complexity of plant tissue culture technique and makes this method very easy to perform.

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