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Review Article

New Insights on Biosynthesis of Nanoparticles Using Plants Emphasizing the Use of Alfalfa (*Medicago sativa* L.)

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Biological synthesis of nanoparticles (NPs) using alfalfa (*Medicago sativa* L.) and other plants has several advantages such as lower costs, reduction of pollution, and improvement of the environment and human health. Often, biosynthesis is used to synthesize Ag, Au, and ZnO NPs. Less often are also synthesized Cu and Fe NPs. Synthesis with plant extracts from their parts or callus cultures is a widely used method since extracts contain the most significant number of biomolecules. Synthesis with living plants (*in vivo*) provides NPs with improved properties for better interactions with plants but is used less often due to the long realization time, the need to control the plants' growing conditions, and difficulty in controlling the size and shape of the synthesized NPs. Here, we performed a systematic review of various methods for the biological synthesis of different metal NPs with different plants, to highlight advantages and disadvantages of mentioned methods. For discussion, results showed that biosynthesis of NPs allows obtaining NPs with reduced toxicity, and their size and shape depend on the type and number of biomolecules present in plants. Plant biomolecules determine the antibacterial and anticancer properties of NPs, as well as increasing the use of NPs in biomedicine, for better drug transport, therefore medicinal plants or sea plants are mostly used for biosynthesis. NPs which were synthesized in marine plants could be a very effective agent against water bacteria; therefore, if NP biosynthesis takes place in water, biological water purification is possible. Limitations of the study included a great methodological diversity of the synthesis, it is still difficult to systematize the synthesis methods, and it seems that each described study uses a different synthesis protocol; therefore, in future studies, it is necessary to clarify which method can provide the most efficient biosynthesis and develop a unified approach.

1. Introduction

Nanotechnology is a new science field, defined as the process of monitoring the shape and size of structures in nanoscales of 1 nm to 100 nm [1]. Development of nanotechnology started rapidly in the 20th century with several important discoveries of advanced microscopy (SEM and TEM) [2] and many widely used nanoparticles (NPs). The English scientist Faraday was the first who accidentally noticed tiny coloured particles of gold in solution while washing gold films. This physical magneto-optical phenomenon was later named the Faraday effect [3], and it is widely used in electromagnetic studies and considered the beginning of the synthesis and visualization of metal NPs [4].

NPs are most often classified by their morphology (spherical, crystalline, and flat), dimension of electron movement (0, 1D, 2D, and 3D), shape, chemical structure (inorganic, organic, and carbon-based NPs), or by the material they have been made of (metal NPs, ceramics NPs, and more) [5]. With the discovery of the nanoscale, it has been found that it strongly affects the physical and chemical properties of NPs, making their synthesis, use, and

interaction with living organisms significantly different from those of larger materials [6]. NPs have gained huge interest from scientists and specialists in almost all kinds of industries such as medicine, food, agriculture, cosmetics, information technologies, and more [3].

It has been established that NPs with physicochemical properties (novel silver NPs, mesoporous silica NPs, Fe_3O_4, etc.) support drugs by improving their solubility, degradation, clearance, and targeting due to small size and are an excellent tool for delivery of drugs and substances to multiple organs in plants, animals, or humans [7].

In addition, zinc and copper NPs have shown strong antibacterial properties, allowing the use of these NPs in agriculture, supplementing the composition of the soil fertilizers (material which is added to the soil to promote growth and productivity) for the prevention of bacterial and fungal infections in plants [8].

Nano-fertilizers with NPs such as potassium (K), calcium (Ca), magnesium (Mg) [8], copper (Cu), zinc (Zn), and iron (Fe) [9] at low to medium concentrations increase crops' harvest index, improve fruit quality, and stimulate plant growth [8]. The concentration of NPs plays a significant role in their effect on plants. It has been proven that NPs at high concentrations are toxic to plant cells [9]. An excellent example is a study with mung beans (*Vigna radiata* L.), which showed that Cu and Zn NPs at a concentration of 100–2000 mg/L reduce the content of minerals, flavonoids, and antioxidants [9]. And TiO_2 NPs added to Ag, Fe, and Cu ions at concentrations of 100 and 500 ppm negatively affect alfalfa (*Medicago sativa* L.) crops, reducing the crop's content of metabolites (phenols and flavonoids) and the size of the leaves and stem. It is worth mentioning that these high concentrations of TiO_2 cause a decrease in the content of nitrogen, boron, and potassium in the soil. Deficiency of these substances causes growth disorders in *Medicago sativa* L. crops [10].

World population growth and shortages in the amount of food are reasons why the use of NPs, despite their toxicity, should be considered, as it has been shown that small concentrations of different NPs improve food and water quality, provided that the use of NPs is strictly controlled and monitored [11]. Small concentrations of different NPs such as gold, zinc, and silver, 10 mg/L in the case of *Vigna radiata* L. [8] and 50 ppm used for *Medicago sativa* L. [10], promote seed germination and plant growth. Such doses encourage photochemical reactions, increase $NADPH^+$, H^+, and ATP for better CO_2 fixation, have a positive effect on DNA transcription, and increase the lipid metabolism rate and synthesis of peroxidases, catalases, and antioxidants, improving metabolism, even increasing the intensity of photosynthesis [12]. Surprisingly, silver NPs in different concentrations of 100–200 mg/L can limit bean yellow mosaic virus; the highest concentration is even able to completely reduce virus replication within 24 h. After 10 days of exposure to NPs, the amount of virus particles was reduced by 36%–82%, depending on the concentration of Ag NPs used. Ag NPs inhibit the production of viral envelope proteins, making it difficult for virus particles to come into contact with host cells [13].

The positive or negative effect of NPs depends on their method of synthesis [3]. Currently, dozens of methods of NP synthesis are actively used in research, which are based on physical (top-down), chemical (bottom-up), and biological methods [14].

Physical methods (top-down) involve the mechanical grinding of large materials down to the nanoscale (1–100 nm) [14]. Often used and widely described are methods such as mechanical ball milling, where an inert atmosphere is used to mill larger materials. Thermal decomposition, where the specific decomposition temperature breaks the chemical bonds of metallic materials, results in small-sized, semi-stable NPs [15]. The inert gas condensation method uses internal gas and a vacuum; the material is vaporized using thermal energy. Silver and platinum NPs are successfully obtained by this method. Laser ablation uses laser energy and vaporizes the material immersed in the solution; the heat causes condensation and the NPs later deposit from the vapor [16]. Hydrothermal synthesis uses high pressures (in autoclave) and temperatures. To synthesize specific NPs, it is necessary to follow a specific protocol by adjusting various parameters, but in general, this method has succeeded in synthesizing iron, gold, silver, and zinc oxide NPs [17].

Top-down methods are rarely used in recent studies because with these methods unstable NPs are produced in large quantities and control of the size and shape of NPs is almost impossible. It should be mentioned that the size and shape of NPs often affect their toxicity or magnetic and antibacterial properties which are the main factors that determine several NP interactions with living organisms. During the implementation of top-down methods, metal oxidation and free radicals occur, which are the main reasons for the toxicity of NPs [17]. It is important to emphasize that in every synthesis method, side products are formed alongside the NP, and also to carry out the synthesis, it is necessary to purchase expensive equipment [15].

Chemical methods (bottom-up) break down larger material using a solvent-induced chemical reaction. The sol-gel method is the most commonly used, where solid particles are dissolved and a gel is formed: a continuous network of resulting particles with a pore that is filled with liquid. The gel is then gradually dried and the NPs are deposited [14]. SiO_2 and ZnO NPs can be easily synthesized by this method [18]. Through spinning with a spinning disc reactor (SDR) the material is mixed with the solvent, the NPs are formed during the mixing, and then they settle and are dried. The reaction takes place without oxygen. The quality and size of NPs are affected by temperature and rotation speed [15]. The "water-in-oil" microemulsion method, in which the water and oil phases do not mix, is often used for the synthesis of iron NPs. The presence of a surfactant substance and collisions between water and oil initiate the breakdown of the material [14]. Since Faraday's experiment in 1857, the chemical reduction method is widely used for the synthesis of copper and gold NPs, which involves reducing the salt of the material with a reducing agent, such as polyol, sodium borohydride, hydrazine, ascorbic acid, or hypophosphite [14]. One of the biggest advantages of chemical methods is the precise control of

NP size and quantity, as well as shape, but these methods have many disadvantages: chemical methods make it possible to obtain small-sized NPs, which are produced in large quantities and end up in the environment, increasing pollution. People, animals, and plants can take them up, from air, water, or soil. Precisely the small size allows NPs to easily enter living organisms and move in them at the intercellular level. It should be noted that each chemical method uses various auxiliary substances, solvents, surfactants, etc. which are toxic and, together with NPs, enter the human, animal, or plant body; then allergic reactions or even severe intoxications can occur, which can lead to serious illnesses or dysfunctions [19].

To reduce the negative effects of NPs, it is necessary to improve their synthesis and use. It is important to mention that more than 4 tons of silver NPs and 5500 tons of zinc NPs are synthesized every year and the worst part is that a huge amount of it ends up in the environment due to their poor management and arbitrary use [11]. Due to their small size and other physical-chemical properties, NPs are easily able to interact with plants and other living organisms [10]. Of course, it is also important to remember that NPs are found in the environment, when they separate from natural objects, sand, soil, etc. Various NPs such as iron, zinc, and silicon are freely present in the air, water, and other living organisms [11]. The data mentioned above are reasons why it is necessary to look for ways to obtain NPs in a less toxic way.

Biological synthesis is a new, safer, and nontoxic approach [15]. The biological synthesis of NPs involves the production of NPs using natural material solvents, which are biomolecules, proteins, and enzymes present in the cells of living organisms, such as plants, algae, fungi, bacteria, and even yeasts, and various mammals or other animals. The most commonly used NP synthesis methods are those with fungi and bacteria [20].

Several dozens of edible and nonedible fungi as well as bacteria and algae are used in the synthesis of silver, cadmium, gold, and other NPs. This type of synthesis is effective because fungi, bacteria, and algae contain unique proteins, enzymes, and carbohydrates, with which synthesis is possible both outside and inside the cell or using extracts of fungi or algae [21]. Some of them have antibacterial and anti-inflammatory properties. The synthesized NPs can contain active substances of eukaryotes on their surface; therefore, the synthesized NPs also have enhanced anti-inflammatory properties. The biggest disadvantage of this type of synthesis is the need to maintain aseptic growing conditions to maintain continuous synthesis. Also, in synthesis with fungi and bacteria, controlling the size, shape, and number of NPs is almost impossible [22]. In contrast, algae are capable of synthesizing NPs of a stable size of 5–100 nm [20]. Yeasts are also able to produce stable NPs with a size of 30–40 nm because they contain compounds of photoheating and glutathione, which can reduce cadmium, zinc, silver, selenium, gold, nickel, and copper ions to NPs. However, the synthesis of all the abovementioned organisms is strongly influenced by external factors, temperature, humidity, water salinity, environmental pollution, and nutrient supply. It is important to study these methods of NP synthesis further because they usually occur in the environment, under uncontrolled conditions [20].

Green synthesis of NPs using plants is divided into two groups: first, synthesis with extracts prepared from different parts of the plant (leaves, stems, bark, roots, seeds, flowers fruits, and callus cultures); second, the synthesis of NPs *in vivo* in cells of independently growing plants [23]. Medicinal plants, especially alfalfa (*Medicago sativa* L.), are often used for the synthesis of NPs because they contain unique synthesis-promoting biomolecules such as phenols, flavonoids, proteins, vitamins minerals, and glycolates. These biomolecules are not only able to successfully break down various larger materials but also give alfalfa anti-inflammatory and antioxidant effects. Alfalfa extract can destroy free radicals or prevent their formation. Alfalfa is also able to reduce oxidative stress. If alfalfa is used for the synthesis of NPs, the obtained NPs will also acquire some of the mentioned properties [24]. Not only alfalfa, but also other medicinal plants, for example, *Salvadora oleoides Decne* [25], *Buxus papillosa* C. K. *Schneid* [26], *Anagallis arvensis* (L.) [27], and *Alhagi maurorum* [28], mostly in the roots and leaves, contain dozen different secondary metabolites, such as terpene, glycoside, and sesquiterpenoid derivatives, as well as glucocleomin and emotin, which provide antioxidant properties, allowing the development of new bioactive pharmaceuticals [25]. Triterpenoids, alkaloids, steroids, phenols, flavonoids, and saponins also have been discovered in plants which demonstrate the pharmacological effects, including enzyme inhibition, antioxidant, antispasmodic, antimicrobial, and anthelmintic effects [27–29]. Methanol, dichloromethane, and n-hexane have been discovered in *Buxus papillosa* stem parts which give anticancer activities against five different human carcinoma cell lines [26]. *Anagallis arvensis* (L.) [27], *Alhagi maurorum* [28], and other plants also have a high *in vivo* and *in vitro* pharmacological activity observed directly in fresh plant material and its crude extracts and/or its parts. Because of these bio-properties, these medicinal plants are used to treat various ailments including wound healing, lung problems, kidney stones, urinary tract infections, gout, and rheumatic diseases [27–29].

Plant-initiated synthesis of NPs has several important and promising advantages such as lower costs, reduction of pollution, and an improvement of the environment and human health [30]. However, it also has many disadvantages, for example, its realization requires a longer time and an adapted place, and the growth conditions of plants or other living organisms must be controlled as well. Synthesis using plants also involves complex extract preparation procedures, and of course, the size of the synthesized NPs is difficult to predict as it is affected by many factors [31].

Despite the challenges, green synthesis has huge potential and broad prospects [30]. It is important to continue analysing the already available methods, emphasize their shortcomings, and highlight their advantages, in order to find the best, most suitable for the environment and people, and cheapest methods of biological synthesis of NPs [31].

In this systematic review, we present the currently available knowledge of suitable methods for the biological synthesis of NPs using different species of plants including

crops, above all alfalfa (*Medicago sativa* L.) seedlings, and their callus cultures. Currently, there is a lack of information in the scientific literature, about NP biological synthesis with plants, especially about *in vivo* NP synthesis, so one of the main tasks of this review is to gather as much information as possible about the biosynthesis of various NPs with medical plants. We review the least complex, cheapest, and newest biosynthesis approaches and analyse the specific features of each method and its advantages and disadvantages. Our analysis offers tailored and novel insights into how to improve methods of biological synthesis of NPs with living plants to produce NPs with the best physical and chemical properties for applications in agriculture, medicine, and environmental improvement.

2. Materials and Methods

The present systematic review was framed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [32].

2.1. Literature Search. Published articles were selected from Web of Science, Scopus, ISI, PubMed, Embase, and MEDLINE by searching for all available articles on the biological synthesis of NPs (up to February 2023). Searches were made using the following keywords: "biosynthesis of different nanoparticles," "biosynthesis of nanoparticles with plant's extracts," "*Medicago sativa* L. and biosynthesis of various nanoparticles," "biosynthesis of nanoparticles using extracts from different plant's callus cultures," "biosynthesis of nanoparticles using extracts from *Medicago sativa* L. callus cultures," "plant-mediated *in vivo* synthesis of nanoparticles," and "*in vivo* biosynthesis of nanoparticles with *Medicago sativa* L." One investigator screened the titles, abstracts, and methods of the published articles to find the most suitable articles for this study.

Inclusion and exclusion criteria were applied to each article, and the article's availability and relevance to the topicality of this review were checked.

2.2. Inclusion and Exclusion Criteria. The inclusion of scientific articles in this study was evaluated according to certain criteria: (1) biosynthesis of NPs using plants; (2) biosynthesis of NPs using *Medicago sativa* L.; (3) studies in the English language with complete protocols of laboratory-based biological synthesis methods; and (4) original articles published with full-text literature and properly cited references. The following studies were excluded: (1) studies with incomplete data on biosynthesis methods or achieved results with noncited references; (2) published conference proceedings; and (3) published review articles, letters, or repetitions of previously published articles.

2.3. Data Extraction. The authors' names, year of publication, types of methods used to synthesize NPs, plant species used in the synthesis, plant part, NP type, shape, and size, precursor solution, characterization techniques, additional catalyst(s) or control variables of biosynthesis, and applications of NPs were noted in the form of a best-suited method for the biological synthesis of NPs using plants.

3. Results

A total of 100 articles were found; among them, 90 non-duplicate publications were identified. After the screening of titles and abstracts, 13 publications were excluded; then, another 21 published papers were excluded after the full-text screening of 77 published articles. The remaining 56 studies were included in this systematic review (Figure 1). These results are very significant, particularly for antimicrobial activity, because 32 of 56 studies studied the biosynthesis of different NPs to test the nanoparticle antimicrobial activity. All statistical analyses that were conducted confirmed statistically significant antimicrobial activity of different NPs in 32 of 56 studies.

3.1. Study Characteristics. The study characteristics of the analysed studies showed that 31 experimental studies used plant extracts of different plant parts for the biosynthesis of various NPs. In each of the studies, the sample size for obtaining the extract was chosen very differently. It varied from 1 gram [33, 34] to 1 kilogram [35] of different parts of plants. Most often, 20–25 grams of biomass was used for obtaining an extract [36–38]. The specific techniques for extraction and analysis also were chosen very differently. Grinding [39, 40], sieving [41], centrifugation [33], mixing [42], sonication [34], stirring [36, 43–45], and dehydration [46] were most often used. Information about these methods and the most important facts are summarized briefly in Table 1. Mainly just the leaves of the different plant species, including *Medicago sativa* L., were used to obtain the extract (15 studies out of 60). The whole plant was used to obtain the extract in five studies; of these, three used alfalfa. One study with *Medicago sativa* L. and two with other plant species used seeds to obtain the extract. Passion fruit from *Passiflora edulis* Sims, black tea from *Camellia sinensis* (L.) Kuntze, stem from *Euphorbia neriifolia* L., and bark from *Mimosa tenuiflora* (Willd.) Poir. were applied in one study each. To obtain the extract, one study used both leaves and flowers. Nuts, leaves, and gel from multiple plants were used also in one study; fruit and peel were used in two studies. One study obtained extract from callus cultures (culture of dedifferentiated plant cells induced on media, containing relatively high auxin concentrations under in vitro conditions [64]) of *Medicago sativa* L., which were obtained from seeds. Sixteen other studies also used callus culture extract for NP biosynthesis, which was obtained from various plants species and their parts, for example, from leaves (5 studies), from whole plants/seedlings or plantlets (6 studies), from seeds (2 studies), from leaves and petioles (2 studies), and from flowers (1 study). The most important facts about biosynthesis with extract from callus cultures are summarized briefly in Table 2. Table 2 provides information also about sample size and techniques used for extract extraction and analysis. The mass of callus cultures varied widely between

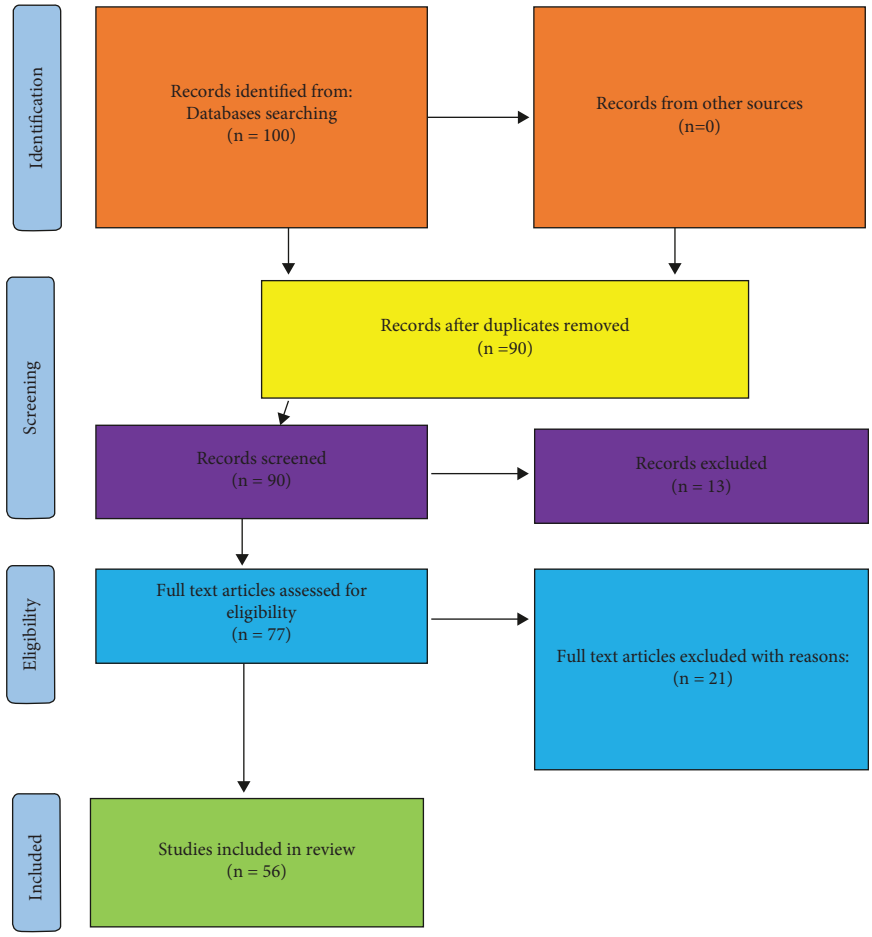

Figure 1: PRISMA flowchart of scientific studies analysed in this systematic review.

studies, most often from 20 g [68] to 50 g [71]. Often the callus culture biomass was not disclosed [65, 76, 77]. In most of the studies, plant biomass was crushed in sterile distilled, boiled water [38, 71, 72, 75]; biomass heating was also often used [61, 77, 78]. After obtaining the extract, it was visually evaluated, often its colour changed to dark green, and in almost every study, the obtained extract was filtered [71, 75, 77]. *In vivo* biosynthesis of different NPs using *Medicago sativa* L. was used in five studies. *In vivo* biosynthesis of different NPs using *Brassica juncea* (L.) Czern. was used in four studies. *In vivo* biosynthesis of different NPs with *Festuca rubra* L. and *Sesbania drummondii* (Rydb.) Cory was used just in one study each. The most important facts about *in vivo* synthesis of NPs are summarized briefly in Table 3. Figure 2 shows the numerical distribution of the studies included in this systematic review.

3.2. Systematic Review

3.2.1. Advantages, Disadvantages, and Future Prospects of Nanoparticle Biosynthesis. Biological synthesis of NPs is now increasingly used because it has many significant advantages over physical and chemical methods. The biosynthesis of NPs with plants does not use any chemical solvents which can cause extra toxicity to synthesized NPs. Metal NPs already have some toxicity from the metal elements themselves which often prevents their use in the health sector or the food industry [11].

With biosynthesis, it is possible to even improve the properties of NPs by changing their natural toxicity from high to low by changing the ratio of the solution (amount of extract: amount of precursor) or amount of precursor in the plant's medium [33, 41, 60, 62, 65, 71].

Biosynthesis with plant extracts or with grown plans (*in vivo*) is much easier to implement than physical or chemical synthesis or even biosynthesis with microorganisms (algae, bacteria, and fungi) because it has a low cost, rapidity, high yield, and biocompatibility [83, 85].

One of the principal advantages of NP biosynthesis with extracts is the ability to obtain NPs of different sizes and shapes. Synthesis with plant extracts and also with living plants (*in vivo*) [86, 87] provides NPs with improved antibacterial and anticancer properties due to plant biomolecules which give NPs better interactions with plants or other organisms. The biggest disadvantage of plant extract methods is a long and complicated realization process with the need to control multiple parameters. Especially, biosynthesis with callus culture cells requires realization, on average, for 1-2 days because often the extract with the precursor is left for 24 h to reduce the metal ions [56–59].

Table 1: Synthesis of different nanoparticles using *Medicago sativa* L. and other plant extracts.

Nr.	Plants	Plant's part/ simple size	Nanoparticle	Shape	Nanoparticle size (nm)	Precursor solution	Characterization/ extraction techniques	Additional catalyst(s) or control variables	Applications of NPs	Reference
1	*Medicago sativa* L.	The whole plant/ undetermined quantity of dry biomass	Au	Multiple shapes	30–60	$KAuCl_4$	SEM, TEM, AFM, HAADF/grinding and sieving for extraction	Temperature, amount of extract, ambient light, pH	—	Montes et al. [41]
2	*Medicago sativa* L.	Seeds/1 g dry weight	Ag	Spherical, hexagonal	5–51, 86–108	$AgNO_3$	UV-Vis, TEM, SEAD, XRD, AFM, SEM, XPS, GC–MS/ centrifugation for extraction	Temperature, amount of extract, ambient light, pH	Antibacterial properties	Lukman et al. [33]
3	*Medicago sativa* L.	Leaves/ undetermined quantity of dry biomass	ZnO	Hexagonal	13.94 ± 1.08	$Zn(NO_3)_2$	XRD, TEM, EDX/ mixing for extraction	Time, centrifugation parameters, amount of extract, precursor	Antimicrobial activity	Król et al. [42]
4	*Medicago sativa* L	The whole plant/ 1 g of grass powder	Ag	Spherical	50–55	$AgNO_3$	UV-Vis, TEM, FTIR, XRD/sonication for extraction	Ethanol, time, ratio of extract, centrifugation parameters, temperature	The effect of Ag NPs on germination, seedling growth	Song et al. [34]
5	*Medicago sativa* L.	The whole plant/ 25 g of the powder	Ag	Face-centred cubic (fcc) crystalline	15–35	$AgNO_3$	FTIR, FESEM, EDS, TEM, XRD, UV-Vis, DLS/stirring for extraction	Time, centrifugation parameters, amount of extract, precursor, pH	Cytotoxicity, antifungal, antioxidant, antibacterial, photodegradation potential	Bidaki et al. [36]
6	*Impatiens balsamina* L., *Lantana camara* L.	Leaves/10 g dry weight	Ag	—	<24	$AgNO_3$	UV-Vis, TEM/ stirring for extraction	Time, amount of extract	Antimicrobial activity	Aritonang et al. [45]
7	*Mimosa tenuiflora* (Willd.) Poir.	Bark/15 g of biomass	Au	Multiple shapes	20–200	$HAuCl_4$	TEM, XRD, UV-Vis/ grinding for extraction	Time, ratio of extract, Ag precursor concentration, pH	Cytotoxicity, cellular uptake, catalysis	Rodríguez-León et al. [47]
8	*Stigmaphyllon ovatum* (Cav.) Nied.	Leaves	Ag, Au	Multiple shapes	15–80	$AgNO_3$, $HAuCl_4$	UV-Vis/for extraction	Time, ratio of extract, Ag, Au precursor concentration	Anticancer potential	Elemike et al. [48]
9	*Fagonia indica* Burm.f.	The whole plant/ undetermined quantity of dry biomass	Ag	Spherical	10–60	$AgNO_3$	UV-Vis, SEM, XRD, DLS/stirring for extraction	Time, amount of extract, Ag precursor concentration, pH	Anticancer activity	Ullah et al. [44]
10	*Blumea balsamifera* L.	Leaves/1.3 kg dry biomass	Cu	Spherical	30–55	$CuSO_4$	UV-Vis, SEM, EDX, SPR, GC-MS/ grinding for extraction	Time, amount of extract, Cu concentration	Antioxidant activity	Ginting et al. [35]

Table 1: Continued.

Nr.	Plants	Plant's part/ simple size	Nanoparticle	Shape	Nanoparticle size (nm)	Precursor solution	Characterization/ extraction techniques	Additional catalyst(s) or control variables	Applications of NPs	Reference
11	*Azadirachta indica* A. Juss. (neem)	Leaves/ undetermined quantity of dry biomass	ZnO	Hexagonal	10–70	Zn $(CH_3COO)_2.2H_2O$	XRD/stirring for extraction	Time, amount of extract	Antibacterial analysis, photocatalytic analysis	Haque et al. [43]
12	*Origanum dictamnus* L., *Salvia officinalis*, *Elaeagnus rhamnoides* L., *Calendula officinalis* L.	Leaves/ undetermined quantity of dry biomass	Ag	Spherical	76.90	$AgNO_3$	DLS, UV-Vis, XRD, XRF, TEM, FTIR/ grinding for extraction	Time, ratio of extract, Ag precursor concentration	Antioxidant activity	Siakavella et al. [40]
13	*Magnolia champaca* L.	Leaves/60 g of biomass	Ag	Spherical	164.6	$AgNO_3$	SEM/grinding for extraction	Time, amount of extract	Active caspase-3 expression	Antony et al. [39]
14	*Vaccinium arctostaphylos* L.	Fruit/10 g of dried sample	Ag	Spherical	7–16	$AgNO_3$	UV-Vis, SEM, TEM, FTIR, XRD/ dehydration for extraction	Time, amount of extract, Ag precursor concentration	Antibacterial analysis	Khodadadi et al. [46]
15	*Camellia sinensis* (L.) Kuntze	Black tea/1.5 g of black tea	Ag	Orthorhombic-shaped	1–100	$AgNO_3$	UV-Vis, SEM, XRD/ stirring and heating for extraction	Time, amount of extract, Ag precursor concentration, pH	Antimicrobial activity	Mohamed et al. [49]
16	*Aloe Vera* (L.) Burm.f., *Terminalia arjuna* (Roxb.) Wight & Arn.	Leaves, gel, nuts/ 5 g of dried plant	ZnO	Octahedral	280–500 29–75	Zn $(CH_3CO_2)_2$	SEM, FTIR, UV-Vis/ stirring for extraction	Time, centrifugation parameters, amount of extract	Antimicrobial ability	Dar et al. [50]
17	*Azadirachta indica* A. Juss.	Leaves/ undetermined quantity of dry biomass	Ag	Spherical	3–5	$AgNO_3$	SPR, UV-Vis, XRD, FTIR, TEM/ suspension for extraction	Time, amount of extract	Antibacterial and photo catalyst ability	Zanjage et al. [51]
18	*Ananas comosus* (L.) Merr. (pineapple), *Passiflora edulis* Sims. (passion fruit)	12 g of fruit peel	Au	Spherical	300–700 <100	$HAuCl_4$	UV-Vis, XRD, FTIR/ stirring for extraction	Time, centrifugation parameters, amount of extract	Anticancer activity	Pechyen et al. [52]
19	*Spinacia oleracea* L. (spinach), *Musa acuminata* Colla. (banana)	Leaves, peel/20 g of fresh leaves	Fe	Multiple shapes	240–430, 20–50, 10–70	$FeCl_3$	UV-Vis, TEM. FTIR, AFM XRD/stirring and heating for extraction	Time, amount of extract, Ag precursor concentration	Antibacterial potential	Tyagi et al. [37]
20	*Galphimia glauca* Bartl.	Leaves/20 g of leaves	Ag	Spherical	17–40	$AgNO_3$	UV-Vis, FTIR, AFM, SEM, EDS, HR-TEM, XRD, TGA/heating for extraction	Time, ratio of extract, pH	Antioxidant, antimicrobial and antiproliferative activity	Chakraborty et al. [53]
21	*Pistacia chinensis* Bunge.	Seeds/1 kg	Au	Spherical	10–100	$HAuCl_4$	UV, FT-IR, AFM/ soaking in methanol for 10 days for extraction	Time, amount of extract	Enzyme inhibitory screening, analgesic activity, sedative activity on mice	Alhumaydhi [54]

Table 1: Continued.

Nr.	Plants	Plant's part/ simple size	Nanoparticle	Shape	Nanoparticle size (nm)	Precursor solution	Characterization/ extraction techniques	Additional catalyst(s) or control variables	Applications of NPs	Reference
22	*Malva parviflora* L.	Leaves/ undetermined quantity of biomass	Ag	Spherical	12–63	$AgNO_3$	AFM, UV-Vis, EDX/ macerating for extraction	Time, centrifugation parameters, amount of extract	—	Oraibi et al. [55]
23	*Euphorbia serpens* Kunth.	The whole plant/ 25 grams of the powder	Ag	Spherical	30–80	$AgNO_3$	UV-Vis, SEM, TEM, FTIR, XRD, FCC/ centrifugation for extraction	Time, amount of extract, Ag precursor concentration	Antibacterial analysis	Ahmad et al. [56]
24	*Hibiscus rosasinensis* L.	Leaves, flower, bark/2.5 g of biomass	Ag	Spherical	51	$AgNO_3$	SEM, UV-Vis, FTIR/ stirring and heating for extraction	Time, amount of extract, Ag precursor concentration	Antimicrobicidal activity	Periasamy et al. [57]
25	*Coriandrum sativum* L.	Leaves/10 g	ZnO	Rod-shaped, polycrystalline	100	Zn $(CH_3CO_2)_2$,	XRD, SEM, TEM/ dilution with distilled water for extraction	Time, amount of extract, Zn precursor concentration, pH	Antibacterial use as fertilizer	Ukidave et al. [58]
26	*Aloe vera* (L.) Burm.f., *Mentha arvensis* L. (mint), *Coriandrum sativum* L. (coriander), *Cymbopogon citratus* (DC.) Stapf, (lemongrass)	Leaves/25 g	Ag	Multiple shapes	10–22	$AgNO_3$	UV-Vis, (XRD), (TGA), (SEM), (EDX)/heating for extraction	Time, amount of extract, pH	Antibacterial activity	Ahmad et al. [59]
27	*Capsicum chinense* Jacq.	Leaves/18.04 g, 3.33 g and 24.3 g of fresh chopped stems, roots, and leaves	Au. Ag	Multiple shapes	20–100	$HAuCl_4$, $AgNO_3$	UV-Vis, FTIR-ATR, XPS, UHR-SEM-EDX, TEM/striation for extraction	Time, amount of extract	Antibacterial activity	Lomelí-Rosales et al. [60]
28	*Euphorbia neriifolia* L.	Stem/ undetermined quantity of biomass	Au	Face-centred cubic structure, spherical	22–25	$AuCl_4$	UV-Vis, FTIR, SEM, TEM, XRD, SPR/ striation for extraction	Time, amount of extract	Antibacterial and antifungal activity	Ul Haq et al. [43]
29	*Bunium persicum* (Boiss.) Pimenov & Kljuykov	Seeds/ undetermined quantity of biomass	Ag	Multiple shapes	35–77	$AgNO_3$	UV-Vis, SEM. FTIR, AFM EDX/striation for extraction	Time, amount of extract, Ag precursor concentration	Antibacterial activity	Khan et al. [61]
30	*Apium graveolens* L.	Leaves/50 g	Au	Multiple shapes	4–15	$HAuCl_4$	TEM, UV-Vis, SEM-EDX, XRD, FT-IR/Dilution with distilled water for extraction	Time, ratio of extract, centrifugation parameters	Antidiabetic potential	Panchamoorthy et al. [62]
31	*Plumeria alba* L.	Leaves/20 g	Ag	Spherical	26.43	$AgNO_3$	FTIR, AFM, SEM, TEM, XRD, UV-Vis/ dilution with distilled water for extraction	Time, ratio of extract, centrifugation parameters	Physiochemical, pharmacological properties, anticancer and antimicrobial activity	Rudrappa et al. [63]

Table 2: Synthesis of different nanoparticles using *Medicago sativa* L. and other plant extracts from callus cultures.

Nr.	Plants	Plant's part/simple size	Nanoparticle	Shape	Nanoparticle size (nm)	Precursor solution	Characterization/ extraction techniques	Additional catalyst(s) or control variables	Applications of NPs	Reference
1	*Medicago sativa* L.	Callus cultures from seeds/ a known mass	Ag	Spherical, disk, irregular	2–50	$AgNO_3$	UV-Vis, TEM, FTIR/ fresh callus soaked in nonaerated silver nitrate for extraction	Time, room temperature, no light exposure, Ag precursor concentration, ratio of pH	—	Hegazy et al. [65]
2	*Carica papaya* L.	Callus cultures (CC) of shoot-tips and leaf-bits/20 g	Ag	Spherical	60–80	$AgNO_3$	FTIR, SEM, XRD/ crushing in sterile distilled water for extraction	Time, room temperature, amount of extract, Ag precursor concentration	—	Mude et al. [66]
3	*Sesuvium portulacastrum* L.	CC of whole plant/20 g	Ag	Spherical	5–20	$AgNO_3$	UV-Vis, TEM, FT-IR/ centrifugation for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature	Antimicrobial activity	Nabikhan et al. [67]
4	*Arachis hypogaea* L.	CC of leaves/ 20 g	Au	Spherical	31–50	$HAuCl_4$	UV-Vis, XRD, EDS, SAED/centrifugation for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature	—	Raju et al. [68]
5	*Justicia jendaracia* (Burm.f)	CC of seedlings/25 g	Ag	Spherical	50–65	$AgNO_3$	UV-Vis, SEM/ crushing in sterile distilled water for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature	Antibacterial activity	Namasivayam et al. [69]
6	*Michelia champaca* L.	CC of fresh flowers/25 g	Ag, Au	Multiple shapes	1–200	$HAuCl_4$, $AgNO_3$	UV-Vis/for extraction TEM, XRD, DLS, ICP-OES, FTIR/ crushing in sterile distilled water for extraction	Time, ratio of extract and precursor, centrifugation parameters, temperature	Antimicrobial activity	Indira-Iyer and Panda [70]

Table 2: Continued.

Nr.	Plants	Plant's part/simple size	Nanoparticle	Shape	Nanoparticle size (nm)	Precursor solution	Characterization/ extraction techniques	Additional catalyst(s) or control variables	Applications of NPs	Reference
7	*Linum usitatissimum* L.	CC of whole plant/10 g of CC	Ag	Spherical	19–24 49–54	$AgNO_3$	UV-Vis, FT-IR, XRD TEM, FT-IR/crushing in sterile distilled water for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature	Bactericidal activity	Anjum and Abbasi [38]
8	*Taxus yunnanensis* Zucc.	CC of whole plant/50 g	Ag	Spherical	6.4–27.2	$AgNO_3$	UV-visible, TEM, XRD, FTIR/crushing in sterile, boiled distilled water for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature, pH	Antibacterial activity, anticancer activity	Xia et al. [71]
9	*Couroupita guianensis* Aubl.	CC of leaves/ 10 g	Ag	Spherical	30.38–88.32	$AgNO_3$	UV-visible, SEM, AFM, FTIR/crushing in sterile, boiled distilled water for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature, pH	Antimicrobial potential	Kumar et al. [72]
10	*Viola canescens* Wall.	CC of leaves, petiole/12 g	ZnO	Hexagonal	0.9	$Zn(NO_3)_2$	UV-visible, XRD, FTIR, SEM/heating for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature	Antibacterial activity	Khajuria et al. [73]
11	*Hyptis suaveolens* (L.) Kuntze	CC of whole plant/20 g	Ag	Spherical	12–25	$AgNO_3$	UV-visible, EDS, SEM, TEM, XRD, FTIR/heating for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature	Anticancer activity	Botcha and Prattipati [74]
12	*Cinnamomum camphora* Nees.	CC of plantlets/ 1 g	Ag	Spherical	5.47–9.48	$AgNO_3$	UV-Vis, TEM, SEM-EDX, DLS, FT-IR, XRD/ incubation under different types of light for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature, pH	Antibacterial activities	Aref and Salem [64]

Table 2: Continued.

Nr.	Plants	Plant's part/simple size	Nanoparticle	Shape	Nanoparticle size (nm)	Precursor solution	Characterization/ extraction techniques	Additional catalyst(s) or control variables	Applications of NPs	Reference
13	*Lithospermum erythrorhizon* Siebold, Zucc.	CC of whole plant/0.1 g of dry simple	Ag, Au	Spherical	20–50	$AgNO_3$, $HAuCl_4$	UV-Vis, XRD, TEM, EDX/boiled water for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature	Anticancer activity	Shkryl et al. (2021) [75]
14	*Alhagi maurorum* Medik.	CC of seeds/ a known mass	Ag	Spherical	16–30	$AgNO_3$	UV–VIS, FTIR, XRD, TEM/stirring for extraction	Time, amount of extract and precursor, temperature	Antimicrobial activity	Sehrawat et al. [76]
15	*Solanum incanum* L.	CC of leaves and petioles/ a known mass	Ag	Spherical, crystallographic	15–60	$AgNO_3$	UV-Vis, FT-IR, XRD TEM, FT-IR/heating for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature	Antimicrobial, cytotoxic efficacy	Lashin et al. [77]
16	*Bunium persicum* (Boiss.) Pimenov & Kljuykov	CC of seeds/ 20 g	Ag	Spherical	35–77	$AgNO_3$	UV-visible, SEM, XRD, FTIR, EDX, TGA/heating for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature, pH	Antibacterial activity	Khan et al. [61]
17	*Randia aculeata* L	CC of leaves/ a known mass	Ag	Spherical	10–40	$AgNO_3$	UV-VIS, DLS, TEM, FTIR/heating for extraction	Time, amount of extract and precursor, centrifugation parameters, temperature, pH	Antibacterial, antiproliferative activity	Bernabé-Antonio [78]

Table 3: *In vivo* synthesis of different nanoparticles using *Medicago sativa* L. and other plants.

Nr.	Plants	Nanoparticle	Shape	Nanoparticle size (nm)	Precursor solution	Characterization techniques	Additional catalyst(s) or control variables	Applications of NPs	Conclusion	Reference
1	*Medicago sativa* L.	Au	Icosahedral	2–20	$HAuCl_4$	XAS, TEM, EDS	Nutrient solution with agar-agar, 1 g/200 Au, 5.8 pH, 12 h photoperiod, 25/18°C day/night	—	Synthesis of AuNPs in living plants was confirmed	Gardea-Torresdey et al. [79]
2	*Medicago sativa* L.	Ag	Nanocrystalline	2–35	$AgNO_3$	SSRL, XANES, EXAFS, SEM, EDS, STEM, HAADF	Medium with chloride salts, $AgNO_3$ concentrations (0, 40, 80, 160, 320 mg/l), pH 5.8, 12 h light/12 h dark photoperiod at 25 and 18°C	—	Synthesis of Ag NPs in living plants was confirmed	Gardea-Torresdey et al. [80]
3	*Medicago sativa* L. *Brassica juncea,* (L.) Czern.	Ag	Multiple shapes	~50	1,000 ppm $AgNO_3$	TEM, EDX, PIXE, AAS	Concentration of Ag nitrate (500, 1,000, 2,500, and 10,000 ppm) The exposure times (24, 48, 72 h)	—	Synthesis of Ag NPs in living plants was confirmed	Harris and Bali [81]
4	*Medicago sativa* L. *Brassica juncea.* (L.) Czern *Festuca rubra* L.	Ag	Multiple shapes	Multiple sizes	$AgNO_3$	TEM, TEM X-ray, ICP-OES	Hoagland's solution, replaced every 7 days, a cycle of 30 days, fluorescence lamps with a 16:8-h (light/dark) photoperiod, 22°C ± 2°C.	Plant metabolism parameters	Synthesis of Ag NPs in living plants was confirmed	Marchiol et al. [82]
5	*Medicago sativa* L. Three types of Iranian alfalfa plants (Nikshahri, Hamedani, and Yazdi)	Au	Crystalline	Multiple sizes	$HAuCl_4$	UV-Vis, SEM, XRD	MS medium (25 ± 2°C 16 h, fluorescent light)	—	Synthesis of AuNPs in living plants was confirmed	Keshavarzi et al. [83]
6	*Brassica juncea* (L.) Czern.	Au, Ag, Cu	Multiple shapes	5–50	$HAuCl_4$, $AgNO_3$, CuCl	AAS, TEM, STEM, EDX, HAADF	Metal rich soil was produced, after 9 weeks of growth, biomass was harvested	—	Synthesis of Au, Ag, and Cu NPs in living plants was confirmed	Haverkamp et al. [84]

Table 3: Continued.

Nr.	Plants	Nanoparticle	Shape	Nanoparticle size (nm)	Precursor solution	Characterization techniques	Additional catalyst(s) or control variables	Applications of NPs	Conclusion	Reference
7	*Sesbania drummondii* (Rydb.) Cory	Au	6–20	Spherical	$HAuCl_4$	TEM, XANES, EXAFS	Sterile agar-water medium seedlings were grown at 25 ± 2°C; 16 h light and 8 h dark regime of cool fluorescent light for 6 d	—	Synthesis of AuNPs in living plants was confirmed	Sharma et al. [85]
8	*Brassica juncea* (L.) Czern.	Ag	2–35	Multiple shapes	$AgNO_3$, $Na_3Ag(S_2O_3)_2$ Ag $(NH_3)_2$ NO_3	XANES, AAS, TEM	100 mL pots, standard Hoagland solution (pH = 5.5), solution was replaced every 2-3 days. A high-pressure sodium lamp (600 W) on a 16 h light/8 h dark cycle for 5 weeks, growth in Ag solution for 2 days	—	Synthesis of Ag NPs in living plants was confirmed	Haverkam, Marshall [86]
9	*Brassica juncea* (L.) Czern.	Ag, Au	Spherical	2–100	$AgNO_3$, Ag $(NH_3)_2$ NO_3 or $HAuCl_4$	TEM, XAS	Hoagland (pH 5.5 solution), replaced every 1-2 days, 16 h light/8 h dark cycle, growth in metal ion solution for 24 h	—	Au and Ag NPs were found in leaves, stems, and roots	Beattie, Haverkamp [87]

Also, an important factor is the average growth time of seedlings, which often is 15 days [68], and the average growth time of callus culture, which lasts from 30 days [38] to 45 days [74]. Therefore, the implementation of the experiment may take several months.

Perhaps the time involved is the main reason why extracts from callus cultures are not used as often for the biological synthesis of NPs as extracts from only parts of the plant, such as leaves and fruits. Obtaining an extract from plant parts does not require time to grow plants in the laboratory and time to grow callus cultures; for example, only a couple of hours were required to synthesize Fe NPs from *Musa acuminata* (Colla) banana extract, where centrifugation at 8000 rpm for 30 min was used to speed up the reaction [37]. The biggest disadvantage of the *in vivo* method is the need to spend a lot of time implementing all the steps, starting with seed preparation and ending with plant tissue analysis.

In general, each study about all three of the NP biosynthesis methods reviewed in this review had a unique synthesis methodology. For example, in NP synthesis with extracts from plant parts and callus cultures, each study had a different method protocol where the amount of precursor was changed constantly, for instance, 90 mL of silver nitrate (1 mM) [33], 45 mL of 10^{-3} M aqueous $AgNO_3$ [34], 100 mL of Zn $(NO_3)_2$ [42], 100 mL of 10^{-4} M $HAuCl_4$ [41], or 9 mL of 1 mM chloroauric acid [62]. The amount of extract, for example, 20 mL of cell suspension [68] or 100 μL of extract [38], and the temperature are also changed frequently, for instance, from 28°C [72] to at least 60°C [73]. Also often changed are the centrifugation speeds, for example, 10,000 rpm for 15 min [74] or 20,000 × g for 20 min [75], and the pH of the extract medium [61, 64, 71, 72, 78].

There is a reason to think that these differences in the number of solutions, photoperiod, or reaction exposure time are the factors that determine the properties of the synthesized NPs and made different results for each study [85].

Biologically synthesized NPs have great potential for use in the future, as their cytotoxicity, antifungal, antioxidant, antibacterial, and photodegradation potential, and activity are already being actively studied in order to find out the possibilities of using NPs for the development of agriculture, medicine, or other industries [33, 65, 71]. The effect of different NPs on germination and seedling growth on multiple plants has also been discussed in multiple studies [34].

3.2.2. Plants Mediating the Biosynthesis of Nanoparticles, Their Properties, and Applications. Medicago sativa L. has been chosen for NP biosynthesis because it is widely used in agriculture as a feed product for farm animals [34, 42]. Approximately 32.2 million hectares of *Medicago sativa* L. are planted annually worldwide [36]. Alfalfa has minerals, secondary metabolites such as saponins, flavonoids, and phenolic compounds [42], carbohydrates, vitamins, amino acids, and other organic compounds which can positively affect the synthesis and properties of NPs [33]. Lucerne (another name for alfalfa) is also broadly used in medicine as a cure for kidney problems and oral central nervous system disorders [42].

Other plants used for biosynthesis also contain highly active phytochemicals and enzymes that participate in oxidation or reduction reactions [88] such as multiple vitamins [55, 59], triterpenoids [45], polyphenols, carbohydrates [44], flavonoids [35, 54, 56], such as catechins and catechin gallates [49], phytosterols, ellagic acid [47], essential oils [45], phenols, such as gallic acid, gallic acid, and quercetin [54], carbohydrates, proteins, alkaloids [44, 46], glycosides, iridoid glycosides, phenylethanoids, oligosaccharides, quinine, saponins [56], steroids, sesquiterpenoids, tannins [86], ketones, aldehydes, and amides [72, 74]. These molecules give the plants anticancer, antimicrobial, antioxidant, antidiabetic, anti-inflammatory, anthelmintic, antiulcer, antiuretic [82], and cytotoxic activity [35], antibacterial properties [56, 58], and the ability to break down various antigens or invasive substances such as NPs [71]. Plant extract biomolecules are capping and stabilizing agents; they prevent aggregation, determine the speed of synthesis, and affect the properties of NPs [44].

Most of the plants used in the biosynthesis of NPs are broadly available because they are widely used in medicine to strengthen the immune system or to treat various diseases such as multiple cancers, diabetes, rheumatic arthritis, ulcers, skin burns, indigestion [35, 40, 47, 58, 82], asthma, malaria, diarrhoea, allergies [53], fever, cough, and respiratory disease [65] or to regulate blood sugar and blood pressure [46], and they have antioxidant, antibacterial, and anticancer properties [34]. Animal and human foods are produced from *Ananas comosus* (L.) Merr. (pineapple), *Passiflora edulis* Sims (passion fruit) [52], *Spinacia oleracea* L. (spinach), *Musa acuminata* Colla (banana) [37], *Mentha arvensis* L. (mint), *Coriandrum sativum* L. (coriander), and *Cymbopogon citratus* (DC.) Stapf (lemongrass) [59]. In the production of household items or ornamental decorations are used *Plumeria alba* L. [63], *Magnolia champaca* L. [39], and *Terminalia arjuna* (Roxb.) Wight & Arn. for natural silk [50]. Most of them are grown in nurseries or large plantations to meet world demand for them. It has been observed in studies that NPs obtained in biosynthesis can receive some of the properties from plants such as antimicrobial activity [35, 37, 39, 44, 46, 50, 53, 55–58, 61–63, 88], antioxidant activity [35, 37, 44], and cytotoxicity for antigens such as different cancers. Bio-NPs have easy cellular uptake, and they can initiate catalysis [40, 47, 48, 52] or initiate apoptosis by activating caspase-3 expression [39, 49, 68, 70, 73].

Also, plant extracts from plant organs and callus cells, plant cells, and tissues are rich in biomolecules: glucose, fructose, ascorbic acid, citric acid, and total polyphenols, which are also major metal reducers in *in vivo* synthesis of NPs [82]. *Brassica juncea* (L.) Czern., *Festuca rubra* L., and *Sesbania drummondii* (Rydb.) Cory, like alfalfa, are commonly used in agriculture or the food industry [78, 84, 85].

3.2.3. Biosynthesis of Nanoparticles Using Extracts from Different Parts of Medicago sativa L. and Other Widely Used Plants. Five studies used an extract of *Medicago sativa* L. as a reducing agent for the biosynthesis of Ag [33, 34, 36], ZnO

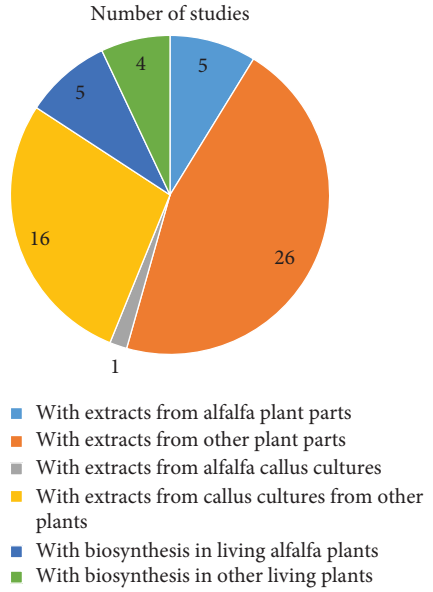

Figure 2: Numerical distribution of the studies included in this systematic review.

[42], and Au NPs [41]. $AgNO_3$ as a precursor was used in three studies to produce Ag NPs. Zn $(NO_3)_2$ was used to produce ZnO NPs [42], but $KAuC_{l4}$ was used to make Au NPs [41]. The most important facts about NP biosynthesis with *Medicago sativa* L. extract are shown in Table 1. A short scheme of the synthesis process is shown in Figure 3.

In order to be able to synthesize NPs, an extract must be obtained first. Most of the time, plant parts are dried at high temperatures (378.15–338.15 K) [34]. Depending on the method, drying can take up to several days [34]. Then, biomass is ground into a powder [36]. Grinding and sieving speed can be modified for each study individually [41]. Then 1 g [34], 15 g [41], 25 g [36], or a freely chosen quantity of alfalfa powder or an aqueous extract [42] can be mixed either with 60% ethanol [34] or 500 mL of methanol [36]. The mixture can be kept at room temperature for 72 h [36] or sonicated at a power of 4×104 Hz for 30 min at 323.15 K [34]. Alfalfa parts can be heated at 60°C for 2 h in ultrapure water in a 10 : 100 (w/v) ratio, to make an extract [42]. To obtain an extract from alfalfa seeds, seeds must first be sterilized in 1% H_2O_2 and deionized water. Exudate from 1 g of seed mass should be obtained by soaking seeds in a medium and centrifugation [33].

To synthesize silver NPs, $AgNO_3$ solution can be used in different concentrations of 2×10^{-3} mol/L [34], 16 mg dissolved in 10 mL of deionized distilled water [34], or 0.1 M and 0.01 M [36]. The amount of alfalfa extract can also be changed from 2 mL [33] to 10 mL [36] or $AgNO_3$ and alfalfa extracts can be used in different ratios: 1 : 4, 1 : 2, 1 : 1, 2 : 1, and 4 : 1. Mixing or centrifugation rotation speed of both solutions can be changed as well (150, 250, 350, and 450 rpm) [34]. All mentioned parameters affect the synthesis temperature. Therefore, the temperature affects the incorporation of alfalfa extract biomolecules into the precursor. It is also important to control the lighting during the synthesis or storage of the extract because light can affect the properties of NPs (amount, shape, or other) [34]. To change the properties of NPs, synthesis is often regulated by changing the pH of the environment. With 0.1 M nitric acid (HNO_3), the environment is regulated to pH 2, and with sodium hydroxide (NaOH) it is adjusted to pH 11. In an acidic environment (pH 2), the synthesis of NPs is limited, or does not occur at all, but in an alkaline environment (pH 11), the synthesis is accelerated and is always observed [42].

To synthesize Zn NPs, 1 g of zinc nitrate and *Medicago sativa* L. extract can be stirred magnetically at 50°C for 3 h. Centrifugation ($7000 \times g$ for 15 min) and heating at 110°C for 1 h are necessary [42].

To synthesize Au NPs, potassium tetrachloroaurate salt (3 mM $KAuCl_4$) and alfalfa extracts at a 4 : 1 ratio were used to produce gold NPs. The solution was stirred for 8 h, and the pH was adjusted with a ROSS semi-micro pH electrode (8115BN ORION) filled with Ag/AgCl (ORION 900011). After synthesis, the solution was centrifuged and dried to precipitate the NPs [41]. Centrifugation or high temperature is used for drying, in almost every protocol of NP biosynthesis with plant extracts. Shape and size are easier to determine for dry NPs.

Ag NPs with spherical [33, 34], face-centred cubic crystalline [36], and hexagonal [33] shapes and different sizes from 5 to 108 nm [33], ZnO NPs with a hexagonal shape and average size of 13.94 ± 1.08 nm [42], and Au NPs with decahedral and icosahedral shapes and size from 30 to 60 nm have been synthesized with alfalfa extract [41].

Table 1 also demonstrates key facts about 26 studies in which extracts from plants other than *Medicago sativa* L. were used for biological synthesis of different NPs. In study identification through databases, it was evident that there are many of these studies. When evaluating them, only the latest studies from the years 2019 (3 studies) [45, 47, 48], 2020 (4 studies) [35, 40, 43, 44], 2021 (8 studies) [37, 39, 46, 49–53], and 2022 (11 studies) [41, 43, 54, 56, 58–63] were included in

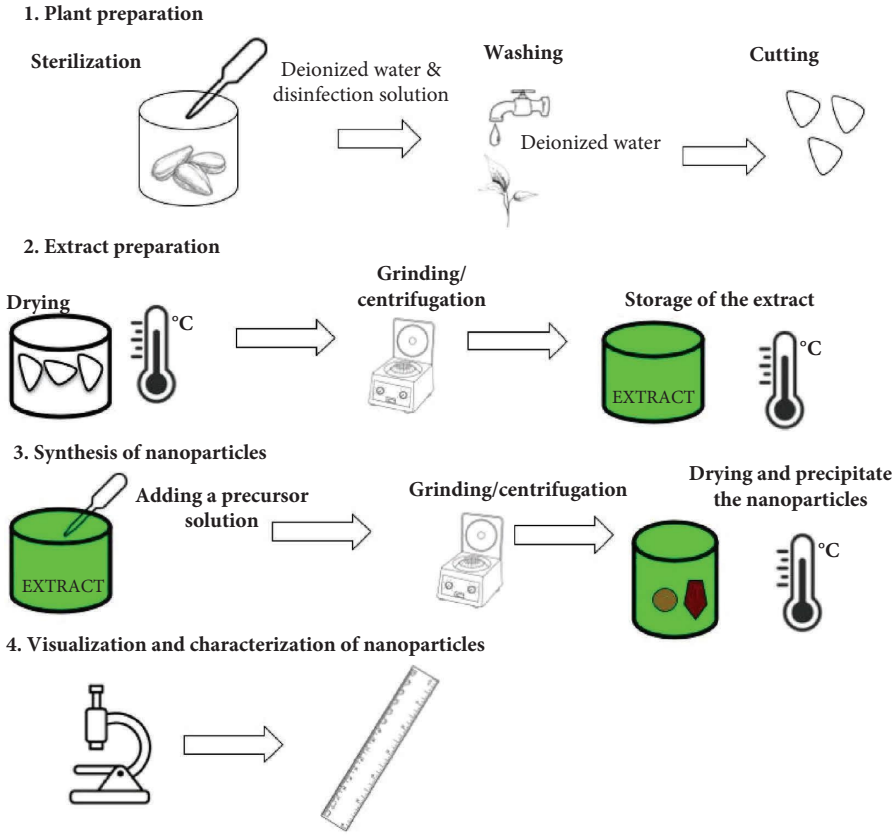

Figure 3: Biological synthesis of nanoparticles with plant extracts.

this review. These statistics show that the biosynthesis of NPs with extracts obtained from different parts of the various plants is the most commonly applied method of biological synthesis of NPs. Analysis of these studies revealed reasons for the most frequent use of this method.

In the reviewed studies, the smallest size of NPs was 1 nm, for Ag NPs obtained from *Camellia sinensis* (L.) Kuntze black tea extract [49]. Ag NPs had multiple shapes: spherical [34–36, 43, 44, 58, 66, 67, 72, 74, 75, 79, 87, 88], orthorhombic [49], or a face-centred cubic structure [43]. Small Au NPs with a size of 4 nm were also obtained from *Apium graveolens* L. leaf extract [34]. Au NPs had a size range from 15 nm [33] to 700 nm [52]. ZnO NPs synthesized from *Azadirachta indica* A. Juss. (neem), *Aloe vera* (L.) Burm. f., and *Terminalia arjuna* (Roxb.) Wight & Arn. showed sizes from 10 to 75 nm [50, 58, 88] and rod-shaped, polycrystalline [58], octahedral [50], and hexagonal shapes [88]. With *Blumea balsamifera* L. extract from leaves, Cu NPs with sizes from 30 to 35 nm and a spherical shape were obtained [35]. With a size of 20–430 nm and multiple shapes, Fe NPs were obtained from *Spinacia oleracea* L. (spinach) and *Musa acuminata* Colla (banana) extracts [37].

As in the alfalfa studies, in these studies, the synthesis procedure also began with extract extraction. In almost every study, extract preparation was unique but generally similar. A certain amount of plant biomass was taken and at first washed in distilled water [45]. Then it was ground, or mixed with ethanol [47] or methanol [54], or boiled with distilled water [52, 88] for a certain amount of time, from a few minutes to hours at controlled temperature to speed up the reaction [40]. The extract can mostly be stored at room temperature or in the refrigerator or at a controlled temperature. Filtration was also used frequently almost in all studies. It should be noted that plant extract to successfully synthesize Ag or Au NPs can also be obtained from Hibiscus rosa-sinensisL. [57] and *Mimosa tenuiflora* (Willd.) Poir tree bark [47]. Extracts from the bark are obtained less often because it too needs to be boiled at 80–90°C [42] or kept in ethanol for 15 days at room temperature to obtain an extract [85].

In these studies, the synthesis of Ag, Au, and ZnO NPs proceeded similarly to studies with alfalfa extract. $AgNO_3$ [39, 40, 44–49, 51, 53, 55–57, 59–61, 63], $HAuCl_4$ [47, 48, 52, 54, 60, 62], and Zn $(CH_3CO_2)_2$ [50, 58, 88] were used as precursors. These studies also confirmed that silver and gold NPs are most often synthesized with plant extracts. The amount and consistency of the precursor can be very different, for example, 10 mg of stock solution [45], 100 mL of aqueous leaf extract [47], 5 mg/mL of aqueous extract, and $AgNO_3$ (1 M) [40] or 0.1698 g of $AgNO_3$ in 1000 mL of distilled water [52]. Each study used unique ratios of precursor to extract, so we have not listed them all here. The solution's pH can be controlled with solid potassium carbonate (K_2CO_3) [49] or hydrogen peroxide (H_2O_2) [54].

Three studies obtained Zn NPs. Extract of 21.94 μg [88], or 2.9 g [50], or 50 mL of 0.02 M [58] Zn $(CH_3COO)_2.2H_2O$ can be mixed into 50 mL of water and stirred for 20 min at

35°C, and then it can be mixed by vigorous stirring [76] or a magnetic stirrer for 1 h at room temperature [50] and dried at 80°C for 2–4 h. After that, the dried powder can be calcined at 250°C for 4 h to obtain ZnO NPs [50, 88]. NaOH can be used for pH control [58]. ZnO NPs with sizes from 10 [88] to 500 nm were obtained [72]. ZnO NPs had different shapes: hexagonal [60], octahedral [50], rod-shaped, and polycrystalline [58].

One study synthesized multiple-shaped Fe NPs from spinach leaf and banana peel extract with a size range from 10 to 430 nm. To make an extract, 20 g of fresh spinach leaves was boiled, stirred, and then filtered. Fresh banana peel (100 g) was washed, cut into pieces, and dried and then banana peel powder (8.3 g) was extracted with 125 mL of deionized and distilled and stirred for 30 min at 60°C. Lastly, centrifugation took place for 30 min at 8000 rpm. $FeCl_3$ was dissolved in 40 mL of deionized water to create a 0.1 M $FeCl_3$ solution. The solution ratio was 2 : 1 (0.1 M $FeCl_3$ solution: biomass extract). The solution was put at 60°C for 30 min and strung magnetically. ZnO NPs showed antimicrobial activity against the two common bacteria *Bacillus subtilis* and *Escherichia coli* [37].

Cu NPs were also obtained in one study. *Blumea balsamifera* fresh leaves were cut, dried, and ground with a blender. Then 2.5% of $CuSO_4$ and 0.1% of *Blumea balsamifera* extracts were mixed at a ratio of 1 : 2. After that, strirring took place for 30 min and the solution was stored for 24 h.

Centrifugation at 4000 rpm for 10 min with distilled water and ethanol was used to collect and clean Cu NPs. Spherical shapes and sizes from 30 to 55 nm were shown in NPs in visualization analysis [35].

3.2.4. Biosynthesis of Nanoparticles Using Extract from Callus Culture Cells of Medicago sativa L. and Other Widely Used Plants. One study with alfalfa and 16 with other plants are shown in Table 2; they used very similar methods for synthesizing Ag, Au, and ZnO NPs. There was only one difference: the extract for the synthesis of NPs was obtained from previously grown callus cultures. A short scheme of the synthesis process is shown in Figure 4.

At the end of 2022, only one study in which *Medicago sativa* L. callus culture extract was used for the synthesis of Ag NPs was found in the databases. This study was published long ago in 2014; we did not find more recent studies with alfalfa. NPs with sizes from 2 to 50 nm and spherical, disk, and irregular shapes were successfully synthesized. NP synthesis with callus cultures takes more time because at first the callus culture must be grown, and only then can the extract or callus cell suspension be obtained from them.

First, sterilized alfalfa seeds were transferred to Petri dishes with Whatman no. 3 filter paper. Seeds were germinated in the dark at a temperature of 25°C for 7 days. Then, 3–5 mm hypocotyls were planted on callus induction MS (Murashige and Skoog) medium. Explants were incubated in a growth chamber at a constant temperature of 25 ± 2°C. In addition, three subcultures were maintained (each subculture after 3 weeks). MS medium contained 3% sucrose, 2 mg/L 2.4 D, 1 mg/L BA, and 0.8% agar. The pH of the medium was adjusted to 5.8 ± 0.2, and the semi-solid medium was autoclaved for 20 min at 121°C [65].

The callus mass used in Ag NP synthesis was not reported in this study [65]. Also, in some studies with other plants, callus mass was not specified [61, 64, 73, 74, 76, 78, 83]. However, 9 out of 16 studies reported different callus weights from 1 g [75] to 50 g [71]. Several studies used 20 g [66, 67]. In the study with *Arachis hypogaea* L., 20 mL of callus cell suspension was used [68].

To synthesize Ag NPs with alfalfa callus cultures, silver nitrate (0.1 M) was mixed into callus cells. $AgNO_3$ puts the callus in a metal stress condition; as a result, bio-secretions and bio-sorption are observed. This means that the biomolecules and enzymes in the callus cells were activated and could act on the added metal. Also, in culture, the pH was adjusted (pH at 2, 5, 7, 9, 10, and 11) in non-pH-adjusted bulk material [65].

A total of 16 studies were found in which Ag [38, 61, 64, 66, 67, 71, 72, 74–76, 78, 82], Au [59, 68, 75], and ZnO [52] NPs were synthesized using callus culture. Unfortunately, even here, half of the studies were conducted more than 5 years ago (2009–2017) [38, 39, 50, 69, 70, 72]. However, it has also been possible to find new studies from 2019 [61, 64, 73–77] to 2023 [78].

The studies obtained Ag NPs with a size of 1 to [67] 200 nm [77]. The size diversity of the Ag NPs was wide [66, 67]. A spherical [41] and crystallographic [77] shape was observed in the Ag NPs. Au NPs were synthesized with sizes from 31 to 50 nm [68] and spherical shapes [75]. ZnO NPs showed an average size of less than 0.9 nm and a hexagonal shape [73].

To obtain callus cultures, shoot-tips and leaf-bits [66], whole plants [71, 75], leaves [68, 78], seedlings [83], fresh flowers [52], petioles [40, 82], plantlets [38, 64, 67, 74], and seeds [62, 72, 73, 77] were used.

Callus cultures in these studies were obtained according to a similar principle, but there are differences in each study, for example, most studies used MS medium for growing cultures. However, the composition of the medium was often changed by choosing different nutrients [73, 74]. There are also exceptions where W'' medium [75] or Gamborg's medium [66] was used, which have a completely different chemical composition and implementation.

The composition of the callus culture medium is very important; if the cells are supplied with enough hormones and other supplements, then the callus cells will be able to grow as quickly as possible and their composition will contain the maximum number of plant-specific biomolecules such as polysaccharides, polyphenolic compounds, and proteins which can influence the synthesis of NPs [74, 75].

3.2.5. In Vivo Biosynthesis of Nanoparticles Using Independently Growing Medicago sativa L. and Other Widely Used Plants. In vivo biosynthesis of NPs is the last biosynthesis method reviewed in this systematic review. The most important facts about the five studies that demonstrated *in vivo* synthesis of Ag [80, 81, 84] and Au [86, 87] NPs in *Medicago sativa* L. plants and *in vivo* synthesis of Ag

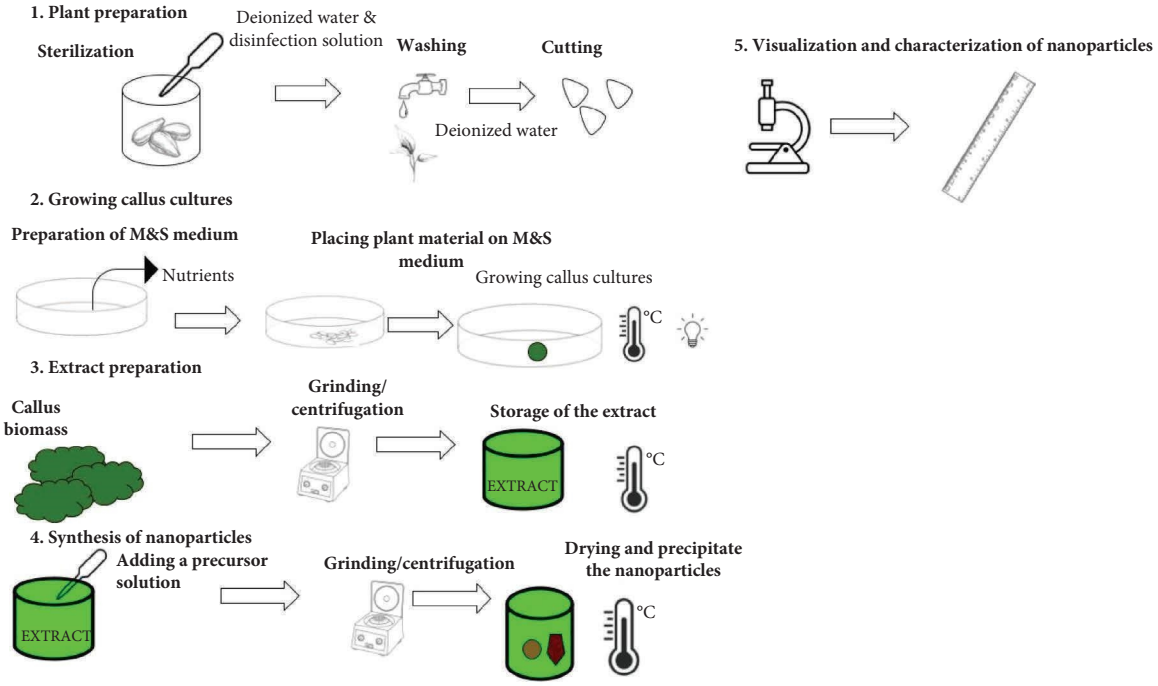

Figure 4: Biological synthesis of nanoparticles with plant callus culture extracts.

[78, 81, 82, 86, 87], Au [85, 87], and Cu NPs [78] with *Brassica juncea* (L.) Czern. are shown in Table 3. One study demonstrated *in vivo* synthesis of Au NPs with *Sesbania drummondii* (Rydb.) Cory [85] and another study demonstrated the synthesis of Ag with *Festuca rubra* L. [82]. Six studies confirmed the synthesis of Ag NPs from silver nitrate, five studies showed the synthesis of Au NPs from $HAuCl_4$, and one study demonstrated Cu NP synthesis from CuCl [84]. In two studies, different precursors ($AgNO_3$) [85, 86], $Na_3Ag\,(S_2O_3)_2$ [85, 86], and $(Ag\,(NH_3)_2NO_3)$ [86] were used for the synthesis of Ag NPs.

Medicago sativa L. has been used successfully to synthesize icosahedral [79] and crystalline Au NPs of multiple sizes [83]. Part of the synthesized Ag NPs showed nanocrystallinity and a size of 2–35 nm [80]. The other half of the NPs had multiple sizes and shapes [81, 82]. With *Brassica juncea* (L.) Czern. plants, most of the studies showed successful *in vivo* biosynthesis of both silver and gold NPs of various shapes and sizes [78, 86]. One study obtained Ag and Au NPs with a spherical shape and sizes of 2–100 nm.

With *Festuca rubra* L. plants, multiple-sized and shaped Ag NPs have been successfully synthesized [82]. A study with *Sesbania drummondii* (Rydb.) Cory obtained Au NPs with a spherical shape and size of 6–20 nm [85].

To synthesize Ag and Au NPs with *Medicago sativa* L., the seeds need to first be sterilized with 3% formaldehyde for 15 min [86] or with captan solution (2 g/L) [78] and washed three times with deionized water [86]. Then a certain number of seeds (100) [78] need to be moved to a medium containing various chemical elements of the most important plant nutrients, such as Fe, Zn, Cu, Mg [86], or chloride salts ($MnCl_2.4H_2O$ and $FeCl_3$) [78], and others in different concentrations. For Au NP synthesis, a pH of 5.8 can be maintained for all media. Then agar-agar, 1 g per 200 mL, and gold (III) from potassium tetrachloroaurate need to be added to the medium at concentrations of 0, 5, 10, 20, 40, 80, 160, and 320 ppm. For Ag NP synthesis, 5 g of Difco Bacto agar was added to each litre of nutrient solution and silver nitrate ($AgNO_3$); concentrations of 0, 40, 80, 160, and 320 mg were used [80]. All media were set in a growth chamber for a 12 h light/12 h dark photoperiod at 25 and 18°C, respectively, for 2 weeks when Au NPs were synthesized [86] and for 9 days when Ag NPs were synthesized [78].

A simple scheme of *in vivo* biosynthesis of NPs in living plants is shown in Figure 5.

Some studies show a simpler approach for the *in vivo* synthesis of Ag NPs with *Medicago sativa* L. plants. Alfalfa seeds can be grown hydroponically in demineralized water, under artificial light for 4 weeks. Plants without any damage can be placed in Petri dishes with silver nitrate at concentrations of 500, 1000, 2500, and 10,000 ppm. The time of exposure can vary between 24, 48, and 72 h [81].

Another option is to transfer alfalfa, *Brassica juncea* (L.) Czern., or *Festuca rubra* L. seedlings after 15 days of germination to a hydroponic system (1 L pots) [82] which contains a half-strength modified aerated Hoagland's solution. The nutrient solution needs to be replaced every 7 days. All plants need to be grown on a laboratory bench lit for 30 days. It is necessary to provide an average photosynthetically active radiation (PAR) at the top of the plants of 500 μmol/m^2/s with a 16 : 8 h (light/dark) photoperiod with fluorescence lamps. The ambient temperature needs to be maintained at 22 ± 2°C. After the removal of nutrient solution plants, it is necessary to wash roots three times with deionized water. After washing, plants need to be put in 1000 ppm $AgNO_3$ (99.9999% salt; Sigma-Aldrich, St. Louis,

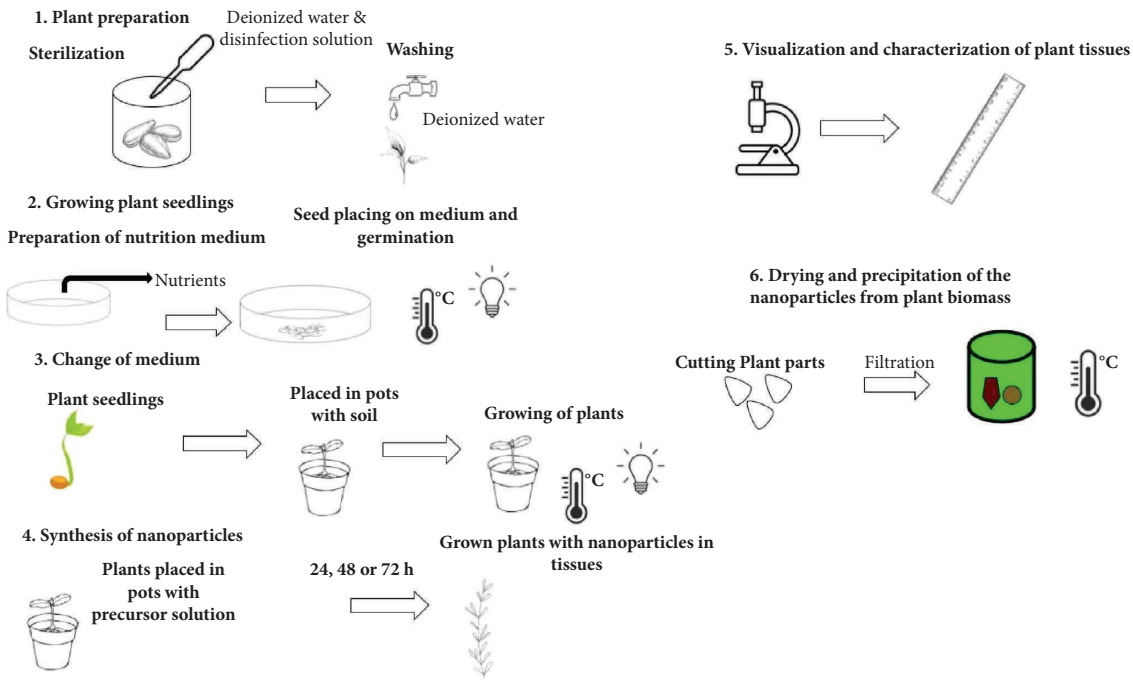

Figure 5: *In vivo* biological synthesis of nanoparticles with independently growing plants.

MO, USA) solution with deionized water (control). After 24 h, the analysis of plant tissue can be started [81].

In one study, three types of *Medicago sativa* L. (Nikshahri, Hamedani, and Yazdi) were used for *in vivo* biosynthesis of Au NPs; 70% alcohol for 30 s was used for seed disinfection. Then seeds were washed with distilled water and treated with 30% sodium hypochlorite and shaken for 12 min. After washing, seeds were placed on MS medium and were kept in the growth chamber at 25 ± 2°C and with 16 h fluorescent light. Three-week-old seedlings were transferred to the second medium that contained 25 mL of water with 1, 2, and 3 mM chloroauric acid [83].

Au, Ag, and Cu NPs were synthesized in *Brassica juncea* (L.) Czern. plants by a different approach. Dried agricultural soil was enriched with gold chloride solution, silver nitrate, and copper chloride solutions. The seeds were sown on soil that had stood for 2 weeks with metals. Healthy plants were left in each pot. After 9 weeks of growth, 100 ml of 4 g l^{-1} potassium cyanide solution was irrigated onto each pot. After 14 days, biomass was harvested and dried and sieved [78].

To synthesize Au NPs from *Sesbania drummondii* (Rydb.) Cory, seeds were sterilized with 85% H_2SO_4 for 40 min, rinsed for 30 min, and again sterilized in 0.1% $HgCl_2$. After that, seeds were plated in a sterile agar-water medium growth chamber (25–28°C; dark for 4 days followed by 16/8 h of light and dark cycle, respectively). Three-week-old seedlings (8–10 cm long shoots and 4–6 cm long roots) were transferred individually to flasks (200 mL), with 75 mL of sterile deionized water and 0–200 mg/L potassium tetrachloroaurate (pH adjusted to 4.8). Seedlings were grown in a growth chamber at 25 ± 2°C in a 16 h light and 8 h dark regimen of cool fluorescent light for 6 days. Controls were set up with 0 mg/L potassium tetrachloroaurate or with negligible exposure to 100 mg/L potassium tetrachloroaurate [85].

One other way to synthesize Ag NPs is to germinate seeds of *Brassica juncea* (L.) Czern. in deionized water and then put seeds in 100 mL pots filled with glass wool with a hydroponic system in standard Hoagland solution (pH = 5.5). The solution was changed every 2–3 days and held on a 16 h light/8 h dark cycle for 5 weeks of growth. After that, the Hoagland solution was replaced with deionized water, and the system was flushed for 2 days. Then a silver solution ($AgNO_3$, $[Ag(NH_3)_2]NO_3$, or $Na_3[Ag(S_2O_3)_2]$) was circulated through the system. After that, randomly selected plants were harvested at selected time intervals (8 h, 12 h) for each experimental group [86]. In this study, it was found that if the plant is kept in the precursor solution for too long, metal particles accumulate on the roots and the penetration of metal ions into the plant is blocked. The concentration of the precursor also affects the penetration of the metal into the plant tissue and thus also the number of synthesized NPs [86].

Also, Au NPs have been synthesized by *Brassica juncea* (L.) Czern. plants, with a similar method as for Ag NP synthesis. In this study, $HAuCl_4$ was used as a precursor, and the plants were kept in the precursor solution for a certain time (24 h) [87]. All studies on *in vivo* synthesis have found that NPs are found in plant tissues of leaves, stems, and roots [78–83, 85–87].

To confirm the successful synthesis of NPs and to determine their properties, various visualization techniques were used in all studies included in this systematic review. Most often, UV-visible spectrum analysis, transmission electron microscopy (TEM), scanning electron microscopy (SEM), X-ray diffraction (XRD) analysis, Fourier-transform

infrared (FTIR) spectroscopy, and others were used [33, 34, 36, 41, 42].

It is important to emphasize that the NP visualization methods differ in each study, offering a unique, complicated analysis of plant tissue and NP properties [87].

4. Discussion

In this systematic review, an extensive analysis of studies on methods of biological synthesis of Ag, Au, Fe, and Cu NPs is presented. Using plants biologically, NPs can be synthesized by three methods [59, 61, 83]. First, the most commonly used is the biosynthesis of NPs using extracts from various plant parts, for which there were dozens of studies in the databases; we had to select the newest and most suitable ones from 2019 [45, 47, 48] to 2022 [63]. Method 2 is synthesis with plant extracts from callus cultures, which is used less often, but often enough to find new publications [61, 75–78]. Fewer studies were found on the *in vivo* biosynthesis of NPs. Also, these studies were much older than 5 years [78, 80, 82, 84–87].

All three synthesis methods are time-consuming. All studies have to start with the preparation, sterilization, or washing of the plants, and often the plant stock is grown for several months [36, 60, 65, 78, 82, 87]. A growth-stimulating medium must be prepared both for *in vivo* synthesis [86] and for growing callus cultures [77]. It is necessary to use many different microelements and supplements, which significantly increases the cost of the synthesis process [75, 77, 79]. This problem is perfectly solved if the extract is obtained from previously purchased plants, their fruits, or other organs. Of course, the biochemical composition of such plants is uncontrolled and it may affect the properties of the synthesized NPs [46, 52].

However, the synthesis of NPs with extracts is not so fast and simple either. The preparation of the extract can take a lot of time, up to several hours or days [50, 75]. Centrifugation is often used to prepare the extract, and proper storage of the extract, in the dark and at a certain temperature, must be ensured [50, 55, 62, 63].

In all three methods, the precursor is added in liquid form by adding and mixing it into the extract or medium on which the plants will grow [42, 82]. Ag and Au NPs of various sizes and shapes can be successfully synthesized by all three methods discussed [34, 83]. Fe NPs can be synthesized with *Spinacia oleracea* L. (spinach) leaf extract or *Musa acuminata* Colla (banana) peel extract [37]. Cu NPs can be synthesized from *Blumea balsamifera* L. leaf extract [35] and synthesized *in vivo* by *Brassica juncea* (L.) Czern. [78]. ZnO NPs can be synthesized both with plant extracts [50] and with extracts of callus cultures [73].

Ag, Au, ZnO, Fe, and Cu are some of the most widely occurring metals in the environment, and their NPs are widely used in medicine, the food industry, agriculture, and elsewhere. It is important to synthesize these NPs as ecologically as possible to reduce their toxicity and negative effects and to further improve their positive properties [3, 7–9].

The sizes of synthesized NPs are in the entire nanoscale (1–100 nm) [49] and even larger nanomaterials have been discovered [52]. NPs are also very different in shape. A spherical shape is very common; more than 30 studies reported on spherical shape. The variety of shapes and sizes is most directly influenced by the parameters used in the synthesis, such as the amount of precursor, temperature, medium composition, and others. In our review, we observed that the synthesis protocol is unique in each study. Each synthesis method has basic steps that overlap, such as making an extract or adding a precursor. The differences can be observed precisely in the nuances, for example, the parameters of temperature, photoperiod, or concentration of added substances often change.

An important influence on the properties of the synthesized NPs is the environmental pH, which is often different in studies [36, 58, 59, 61, 72, 79]. Changes in pH are claimed to affect the properties of NPs [71, 78].

Several studies claim that the biosynthesis of NPs is influenced by many external factors: environmental temperature, air humidity, and light intensity. These factors act on the synthesis of biomolecules in plants and various plant biomolecules that are precursor solvents. The more different biomolecules there are in the plant, the faster and better the biosynthesis will be [35, 39, 45, 46, 49, 52, 53, 55, 59]. Unfortunately, there is still a lack of information about which biomolecules are the most important and which of the genes or their molecular mechanisms account for the efficiency of biosynthesis of NPs or the properties of the synthesized NPs.

Medicago sativa L. is used in all three plant-related NP biosynthesis methods due to its composition and wide applicability in agriculture [33, 34, 36, 41, 65, 70, 79–81, 83].

Studies have also established that biologically synthesized NPs can also be widely used for other purposes, such as antibacterial and anticancer use, but to achieve this, the obtained extract with NPs or plant tissue with NPs must be dried and the NPs must be precipitated from them in the form of a powder or at least a clean solution [43, 48, 61, 64, 74–78].

Due to its wide availability, *Medicago Sativa* L. and other plants can biosynthesize various NPs in the environment. Plants freely break down metals in the surrounding environment, which have arrived there with waste. This is how the uncontrolled synthesis and spread of NPs occurs. Therefore, environmental pollution is also increased even more [19, 20].

Biosynthesis of various NPs can take place not only on land but also in water, as metal pollution is also present in water. Various marine algae such as *Gracilaria textorii* (Suringar) Hariot [89], due to its chemical composition and applications in raw materials for agars and medical applications, fertilizer, food for humans and shellfish, and the bioremediation of eutrophication seawater, can also be used for biosynthesis of NPs. The synthesized NPs would have significantly reduced toxicity and could be used in biomedicine [89].

Also, in all three methods, very diverse visualization and characterization methods of NPs are used, which are generally very diverse and complicated. It would be necessary to write a separate review about the characterization of NPs to go deeper into the challenges, advantages, and disadvantages of NP visualization and characterization methods [36, 63–65, 78, 80].

In general, the biosynthesis of NPs should continue to be used in research to discover new factors that can both favourably and unfavourably affect the properties of NPs.

After this review, it can be seen that there is currently a lack of research on which NPs, using plants, would be obtained by all three biosynthesis methods at the same time, in one study. Then it would be easier to compare these methods if the same conditions were used for all three at the same time for growing plants, extract extraction, selection of precursor concentration, or selection of other factors.

5. Conclusion

To reduce the toxicity of NPs and improve their properties, chemical synthesis methods should be replaced by biological synthesis methods as they do not use chemical and toxic substances as NP solvents. Biosynthesis by plants is the most common method used to synthesize Ag, Au, ZnO, Cu, and Fe NPs. Synthesis with plant extracts from plant parts or callus cultures is a widely used method since extracts contain the most significant number of biomolecules. Synthesis with living plants (*in vivo*) provides NPs with improved properties for better interactions with plants but is used less often due to the long realization time, need for control of the plants' growing conditions, and difficulty in controlling the size and shape of the synthesized NPs. Each of the proposed synthesis methods offers a unique realization of the synthesis process, which allows easy changes to several parameters that also affect the properties of the synthesized NPs. This systematic review provides a detailed and unique insight into protocols of NP biosynthesis allowing us to understand the unique contributions of each method to science. Future studies are required to clarify which molecules can provide the most efficient biosynthesis and how plant molecular mechanisms account for the properties of the synthesized NPs. It is necessary to conduct a study that would compare all three methods, offering them similar conditions; then it would be easier to analyse which of these methods is the most effective. At the moment, this has not been fully clarified, as each method has been performed separately and has its advantages and disadvantages.

Data Availability

The data presented in this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

LJ was responsible for investigation, conceptualization, methodology, visualization, and original draft preparation. IK, IP, MJ, and RG were responsible for review and editing. All authors have read and approved the final manuscript.

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