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REVIEW ARTICLE

Biofertilization and nanotechnology in alfalfa (*Medicago sativa* L.) as alternatives for a sustainable crop

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ABSTRACT

Alfalfa is considered the most used forage crop in the world, its main use is for cattle feeding, due to its high nutritional value, specifically in protein and digestible fiber. Currently, the trend in agriculture is to reduce the application of chemicals and among them are fertilizers that pollute soil and water, so the adoption of new technologies and other not so new is becoming a good habit among farmers. Nanotechnology in the plant system allows the development of new fertilizers to improve agricultural productivity and the release of mineral nutrients in nanoforms, which has a wide variety of benefits, including the timing and direct release of nutrients, as well as synchronizing or specifying the environmental response. Biofertilizers are important components of integrated nutrient management and play a key role in soil productivity and sustainability. While protecting the environment, they are a cost-effective, environmentally friendly and renewable source of plant nutrients to supplement chemical fertilizers in the sustainable agricultural system. Nanotechnology and biofertilization allow in a practical way the reduction in the application of chemicals, contributing to the sustainability of agriculture, so this work aims to review the relevant results on biofertilization, the use of nanotechnology and the evaluation of the nutritional composition of alfalfa when grown with the application of biofertilizers.

Keywords: Agriculture; Biofertilizers; Nanomaterials; Nanomaterials

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1. Introduction

The demand for food in the world is increasing exponentially, more so in developing countries where land and agricultural resources hardly contribute to efficient crop production needed to meet such an urgent demand for food. There is a need to intensify agricultural production in a sustainable manner through efficient use of resources considering the full biochemical diversity of the agroecosystem and its potential to mitigate the adverse impacts of low soil fertility, abiotic stresses, pathogens and pests[1].

Nutrients are essential for plant growth and development and some of them are not available in the soil, due to many factors such as leaching, degradation by protolysis, hydrolysis and decomposition, so it is necessary to reduce the loss of these nutrients during fertilization and increase crop production through new technologies[2]. One of these technologies is nanotechnology and nanomaterials (NMs), because nanofertilizers could have effective qualities for crops, such as being able to release nutrients according to demand, controlled release of chemical fertilizers that regulate plant growth and development and improve target activity[2].

Another technology is the application of biofertilization. Biofertilizers are used to supplement chemical fertilizers mainly to maintain soil fertility. These fertilizers are organic, biodegradable, contain microorganisms, provide nutrients, antibiotics, hormones such as auxins, cytokinin, vitamins that enrich the root rhizosphere[3].

Legumes contribute to the sustainability of agriculture: they reduce mineral fertilizers, thus decreasing N_2O production and increasing N_2 fixation, renew and enrich soil fertility due to their deep rooting systems, rapidly decompose their root biomass and accumulate in the soil[4]. Alfalfa (*Medicago sativa* L.) has the ability to accumulate significantly greater amounts of nitrogen than other legumes through its deep rooting system and, in addition, fixes atmospheric N_2 by 40 to 80% through biological fixation of this element[5].

Therefore, the objective of this work is to review the relevant results on biofertilization and the use of nanotechnology in alfalfa cultivation, illustrating how these technologies can lead to a reduction in the application of chemical fertilizers.

2. Alfalfa, generalities, uses and applications

Alfalfa is a perennial legume, representative of temperate regions and is mainly used as livestock feed, and is universally considered one of the highest quality forages. It is a valuable crop because among its many agronomic and environmental advantages are the preservation of soil fertility and biodiversity, protection against soil erosion, mitigation of climate change impacts, reduction of nitrate contamination of groundwater, reduction of fossil fuel consumption, reduction of greenhouse gas emissions, among others[6–9].

This legume (alfalfa) has a set of variable morphological and physiological characteristics of importance in world agriculture and contributes with its high and stable performance as a nutritious grass[10]. Its economic importance is based on its high biomass production potential, exceeding 80 $t \cdot ha^{-1}$ green and about 20 $t \cdot ha^{-1}$ dry matter[11]. Alfalfa forages are characterized by a high crude protein content[12], well balanced with respect to amino acid. It is enriched with vitally important vitamins and several microelements essential for normal animal growth and development. Alfalfa is the basic component in the feeding program for dairy cattle, as well as for cattle, horses, sheep and other livestock[13].

Alfalfa has also become interesting as a potential source of secondary metabolites, because it is considered an alternative of phytoestrogens useful in health (human food ingredient and supplements), so its growth has become widespread in different continents, due to its high adaptability to different types of soils, pH values and environmental conditions, as well as the possibility of sustainable and ecological production[14,15].

In Mexico[16], 385,992 ha of green Alfalfa have been planted, of which 384,693 ha have been harvested for a production of 15,360,646 and a yield of 39,930 $t \cdot ha^{-1}$. In this country, the main use of alfalfa is to feed dairy cattle in arid, semi-arid and temperate regions. The crop is cut at medium intervals to harvest the highest forage yield per year per unit area, as well as for its good crude protein content, digestibility and degree of acceptance by cattle[17,18]. This plant can be used as fodder in different ways, fresh, hayed and ensiled in mixture with one or more grasses[19,20].

3. Biofertilization in alfalfa

The development of a country is directly proportional to the amount of food or nutrients available to the population. The growing increase in world population creates an ever-increasing demand for food and to supply it, fertilizers are used which are defined as any substance used to increase the productivity of the soil, promoting its fertility by adding nutrients, which aids in plant growth. Fertilizers that are composed of crude chemicals in solid or liquid form made in factories targeted to the nutritional requirements of plants are, by definition, called a chemical fertilizer. Nitrogen (N), phosphorus (P) and potassium (K), called NPK, are normally present in these chemical fertilizers along with other nutrients[21].

The excessive use of chemical fertilizers has generated several problems in nature such as, for example, water acidification; damage to the ozone layer; the greenhouse effect; using them for a long time can change the pH of the soil, eutrophication of the water where the nutritional content in these environments increases, causing algae proliferation and, consequently, the reduction of oxygen in the water, which damages marine life[22]. A current solution to decrease the use of these fertilizers in agriculture is the use of biofertilizers[23].

Biofertilizers are microbial inoculants containing live or dormant cells of efficient strains of nitrogen fixing, phosphate solubilizing and cellulose decomposing microorganisms[3]. These are intended to be applied primarily to soils to improve soil fertility and plant growth by increasing the number and biological activity of beneficial microorganisms[3].

Some of the advantages of biofertilizers are that they are cost-effective and environmentally friendly, gradually improving soil quality. The microorganisms contained in the biofertilizer promote the supply of nutrients to the plants, thus ensuring their development, growth and physiological regulation. In addition, crop yields can increase by 10 to 25% and plants are less prone to soil diseases. Among the main limitations of biofertilizers are that they act more slowly than chemical fertilizers; they are difficult to store due to their high sensitivity to changes in temperature and humidity; they cannot replace other fertilizers completely; and the scarcity of particular or local strains of microorganisms reduces their availability[24]. The types of biofertilizers available[22] are:

1. Nitrogen fixing biofertilizer: *Rhizobium*, *Azotobacter*, *Azospirillium*, *Bradyrhizobium*.
2. Phosphorus solubilizing biofertilizer: *Bacillus*, *Pseudomonas*, *Aspergillus*.
3. Phosphorus mobilizing biofertilizer - Mycorrhiza.
4. Biofertilizer plant growth promoters: *Psuedomonas*, *Trichoderma*.

The effects of the above mentioned biofertilizers in terms of nitrogen fixation in the soil is carried out through the root nodules of the leguminous crop, making N_2 available to the plant. Other microorganisms that can be used as biofertilizers are: *Azolla* which is a heterogeneous fern with seven species that are endosymbionts with *Anabaena azollae,* a nitrogen fixing cyanobacterium[25] and blue green algae can fix nitrogen in the anaerobic environment due to a specialized cell called heterocyst[26].

Phosphate-solubilizing bacteria produce organic and inorganic acids such as gulconic acid and ketogulconic acid that solubilize phosphorus[27]. Gluconic acid produces a carboxyl and hydroxyl group, this group will function as a chelator of Fe^{2+}, Al^{3+} and Ca^{2+}, which will reduce soil pH. It is also important to mention that there is a positive interaction between *Gluconacetobacter spp* and *Burklderia spp* to increase dehydrogenase activity in soil. Dehydrogenases are involved in the soil oxidation process and are used as an indicator of soil microbial activity[28].

In Alfalfa, some studies have been conducted using organic cultivation, which includes the use of biofertilizers. The application of liquid microbial inoculants to legume seeds is a sustainable agricultural practice that can improve plant nutrient uptake and increase crop productivity. After application to legume seeds the inoculants should provide long-term survival of rhizobia in the final product and to study the survival of *Sinorhizobium (Ensifer) meliloti* L3 Si, ten different media formulations of microbial inoculants (yeast mannitol broth with the addition of agar, sodium alginate, calcium chloride, glycerol or ferric chloride and combinations thereof) were examined. For survival of L3 Si, for a storage time of 150 days, the medium formulation containing glycerol in combination with agar or sodium alginate was applied, which was used as a liquid inoculant. Alfalfa seeds were pre-inoculated with four formulations (mannitol yeast broth (YMB), YMB with agar (1 $g \cdot L^{-1}$), YMB with 1 or 5 $g \cdot L^{-1}$ sodium alginate) for three months. Seeds pre-inoculated and stored for one month produced successful alfalfa plants. Nitrogen content in alfalfa obtained from seeds pre-inoculated one month before sowing increased between 3.72%–4.19%[29].

The ability of 17 rhizobacterial strains to improve physiology, nutrient uptake, growth and yield of alfalfa plants grown under desert agricultural conditions in Saudi Arabia was studied by some authors[30]. The 17 rhizobacterial isolates were confirmed as plant growth promoting rhizobacteria by classical biochemical tests and using 16S rDNA gene sequence analysis, the strains were identified as Bacillus, Acinetobacter and Enterobacter. Inoculation of alfalfa with any of these 17 strains improved relative water content; chlorophyll a; chlorophyll b; carotenoid content; N, P and K content; plant height; leaf-to-stem ratio; fresh and dry mass. Acinetobacter pittiiJD-14 was more effective in increasing alfalfa fresh and dry mass by 41 and 34%, respectively, compared to uninoculated control plants. However, all strains improved crop characteristics compared to control plants, indicating that these desert rhizobacterial strains could be used to develop an environmentally friendly biofertilizer for alfalfa and possibly other crop plants to improve sustainable production in arid regions.

4. Evaluation of the nutritional composition of alfalfa (*M. sativa*) when grown with the application of biofertilizers

Six biofertilizer doses of cattle manure fermented in a biodigester (0, 25, 50, 100, 200 and 400 $m^3 \cdot ha^{-1}$) and five replicates were used. The chemical characteristics of the biofertilizer were: 0.300 g N (Nitrogen) L^{-1}; 0.057 g P (Phosphorus) L^{-1}; 0.188 g K (Potassium) L^{-1}; 0.105 g Ca (calcium) L^{-1}; 0.057 g Mg (magnesium) L^{-1}, 1 mg Mn (manganese) L^{-1}; 1 mg Fe (iron) L^{-1}, and 1 mg Zn (zinc) L^{-1}. As a result, the best absorption of N, K, Ca and Mg was obtained with the dose of 400 $m^3 \cdot ha^{-1}$. In the case of N, it was 22% more than in the control and was linear with the increase in biomass. The levels of the micronutrients Cu, Mn and Zn did not differ significantly among the doses applied, as did the crude protein concentrations[31].

On the other hand, the effect of *S. meliloti* strain ENRRI A12 and chicken manure (0, 2, 4, 6, 6, 8 and 10 $t \cdot ha^{-1}$) on alfalfa cultivar (*M. sativa*) "Hegazi" was studied under pot and field conditions. In the pot experiment, *S. melioti* inoculation and chicken manure levels significantly increased plant height, root fresh and dry mass, and nodule number and dry weight. In the field experiment, both *S. melioti* and chicken manure significantly increased plant density, fresh forage yield and protein content, and significantly decreased crude fiber percentage. Fresh forage yield and chicken manure level were highly correlated ($r > 0.99$)[32].

5. Nanotechnology in alfalfa

Nanotechnology is one of the latest technological innovations. The term "nanotechnology" was first coined by Norio Taniguichi, a professor at Tokyo University of Science, in 1974[33]. Although the term "nanotechnology" has long been introduced in multiple disciplines, the idea that nanoparticles (NPs) could be of interest in agricultural development is a recent technological innovation and is still under progressive development[34].

NPs are organic, inorganic or hybrid materials with at least one of their dimensions ranging from 1 to 100 nm (nanoscale). NPs that exist in the natural world can be produced from photochemical reaction processes, volcanic eruptions, forest fires, erosion, plants and animals or even by microorganisms[35]. The production of NPs derived from plants and microorganisms has become an efficient biological source of green NPs attracting additional attention from scientists in recent times due to their environmentally friendly nature and the simplicity of the production process compared to the other routes[36].

NPs, depending on their properties, interact with plants causing various morphological and physiological changes. The efficiency of NPs is determined by their chemical composition, size, surface coverage, reactivity, and most importantly, the dose at which they are effective[37]. Researchers report both positive and negative effects on plant growth and development when using NPs and the impact of NPs depends on the composition, concentration, size, chemical and physical properties, as well as the plant species[38].

For the exploitation of green nanotechnology, a number of plant species and microorganisms, including bacteria, algae and fungi, are currently being used for the synthesis of NPs. For example, the plant species *M. sativa* and *Sesbania* are used to formulate gold nanoparticles. Similarly, inorganic nanomaterials, made of silver (Ag), nickel (Ni), cobalt (Co), zinc (Zn) and copper (Cu), can be synthesized within living plants, such as Brassicqjuncea, *M. sativa* and Heleanthusannus[36].

Synthesized nanofertilizers have a specific use to regulate nutrient release according to crop requirements, while minimizing differential losses. For example, conventional nitrogen fertilizers are characterized by large losses to the soil through leaching, evaporation or even degradation of up to 50%–70%, which ultimately reduces fertilizer efficiency and raises the cost of production[39]. On the other hand, nitrogen fertilizer nanoformulations synchronize the release of N-fertilizer with its uptake demand by crops. Consequently, nanoformulations prevent undesirable losses of nutrients through direct internalization by crops and thus avoid nutrient interaction with soil, water, air and microorganisms[36].

Micronutrient deficiency decreases not only crop productivity, but also affects human health through the consumption of micronutrient-deficient foods. For example, iron deficiency causes anemia, impaired growth, reproductive health problems, and even decreased cognitive and physical performance in humans[40]. In this regard, the use of nano-formulated micronutrients for slow or controlled release of nutrients would stimulate the process of plant uptake, promote crop growth and productivity, and also contribute to maintaining soil health[41]. For example, in zinc deficient soils, application of nano zinc oxide at low doses positively influences growth and physiological responses such as shoot and root elongation, fresh dry weight and photosynthesis in many plant species compared to control[42,43].

6. Nanotechnology applications in alfalfa cultivation

Boron (B) is among the nutrients that are necessary for plant growth and yield production and can improve the nutritional properties of forage crops. However, at higher levels, it can be toxic and negatively affect plant growth and forage quality. The concentration of B in plants is affected by different parameters, such as fertilization with this same micronutrient, soil, climate, plant species, etc. For all these reasons, the effects of different B treatments in alfalfa on B concentration and pigment content, including chlorophyll, b, total and carotenoids, were studied. Experimental treatments were: (1) six soil types (S1–S6); (2) B sources, including boricicide (B1) and nano boron (B2) fertilization; and (3) number of sprays (zero, one, two and three times). Results indicated that soil type, B source and number of sprays significantly ($P \leq 0.01$) affected alfalfa B concentration and pigment content. Spraying three times significantly increased B concentration as it resulted in 207.81% increase compared to the control treatment and equally increased pigment content ($P \leq 0.05$) including chlorophyll, b, total and carotenoids compared to the other treatments[44].

A greenhouse study was conducted to explore the effect of various doses of potassium sulfate (K_2SO_4) NPs on alfalfa growth and physiological response under salt stress. A salt-tolerant genotype (Me-sa-Sirsa) and a salt-sensitive genotype (Bulldog 505) were selected on the basis of germination under salt and planted in pots containing 2 kg of sand. The two genotypes were subjected to salt levels of 0 and 6 $dS \cdot m^{-1}$ using $CaCl_2 \cdot 2H_2O$:NaCl (2:1) mixed with Hoagland's solution. Three treatments of K_2SO_4NPs consisting of 1/4, 1/8, and 1/10 of the K level in full-strength Hoagland solution (235 $mg \cdot L^{-1}$) were applied. The highest shoot dry weight, relative yield, root length and root dry weight in both genotypes were obtained when using K_2SO_4NPs at the 1/8 level. The different doses of K_2SO_4NPs significantly affected the Na/K ratio and Ca, P, Cu, Mn and Zn concentrations in plant tissue. Application of K_2SO_4 NPs at a rate of 1/8 improved plant physiological response to salt stress by reducing electrolyte leakage, increasing catalase and proline content, and increasing antioxidant enzyme activity. These results suggest that the application of KNPs may have better efficiency than conventional K fertilizers in providing adequate plant nutrition and overcoming the negative effects of salt stress in alfalfa[45].

The toxicity of zinc oxide nanoparticles (ZnONPs) on seed germination/root elongation and uptake of ZnONPs and Zn^{2+} in alfalfa (*M. sativa*), cucumber (*Cucumis sativus* L.) and tomato (*Solanum lycopersicum* L.) seedlings was investigated by the literature[46]. Seeds were treated with ZnONPs at 0–1,600 mg·L^{-1} as well as at 0–250 mg·L^{-1} of Zn^{2+} for comparison purposes. The results showed that at 1,600 mg·L^{-1} of ZnONPs, germination in cucumber increased by 10% and germination of alfalfa and tomato was reduced by 40 and 20%, respectively. With 250 mg Zn^{2+} L^{-1}, only tomato germination was reduced with respect to the controls. The highest Zn content was 4,700 and 3,500 mg·kg^{-1} dry weight (DW), for alfalfa seedlings germinated in 1,600 mg·L^{-1} of ZnONPs and 250 mg·L^{-1} of Zn^{2+}, respectively.

Alfalfa in nanotechnology has also been used to obtain NPs. Scientists have found a way to grow and harvest gold (Au) from crop plants. The NPs could be harvested industrially. For example, alfalfa plants grown in an environment rich in $AuCl_4$ showed uptake of metallic gold. AuNPs can be mechanically separated by dissolving the organic material (plant tissue) after harvest[47]. Alfalfa plants can also adsorb Ag from a solid medium rich in this element with subsequent formation of Ag NPs[48].

7. Conclusions

(1) The indiscriminate and unbalanced use of chemical fertilizers, especially urea, together with chemical pesticides and the lack of organic fertilizers leads to a considerable reduction in soil health, so the use of biofertilizers is on the rise in various countries and crops. The cultivation of microbial communities induces high productivity with negligible energy investments and, therefore, significantly reduces the effects on the environment.

(2) In sustainable agriculture and environmental protection against pollution is critical, so the application of nanotechnology ensures better management and conservation of inputs for agricultural food production. This advanced technique represents a significant benefit for agricultural productivity, as nanoparticles are an efficient platform for the transfer of genes and biomolecules to plants from engineering.

Conflict of interest

The authors declared no conflict of interest.

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