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

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## ABSTRACT

Alfalfa is considered the most widely used fodder crop in the world, its main use is to feed cattle due to its high nutritional value, specifically in protein and digestible fiber. Currently the trend in agriculture is to decrease the application of chemicals and within them are fertilizers because they pollute soil and water, so the adoption of new technologies and others 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 timing and direct release of nutrients and synchronization or specific environmental response. Biofertilizers are important components of integrated nutrient management and play a key role in productivity, soil sustainability while protecting the environment, being a profitable, ecological and renewable source of plant nutrients to complement chemical fertilizers in the sustainable agricultural system. Nanotechnology and biofertilization practically allows the reduction in the application of chemicals contributing to the sustainability of agriculture, so this work aims to review the most relevant results on biofertilization, the use of nanotechnology and the evaluation of the nutritional composition of alfalfa when cultivated with the application of biofertilizers.

**Key words:** biofertilizers, nanomaterials, agriculture

# INTRODUCTION

The demand for food in the world is increasing exponentially, more so in developing countries where agricultural land and resources hardly contribute to the efficient production of crops necessary to satisfy such an urgent demand for food. There is a need to intensify agricultural production in a sustainable way through the efficient use of resources considering all the biochemical diversity of the agroecosystem and its potential to mitigate the adverse impacts of low soil fertility, abiotic stress, pathogens and pests [(1)]. Nutrients are essential for the growth and plant development and some of these 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 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 growth and development of the plants and improve the 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, and provide nutrients, antibiotics, hormones such as auxins, cytokinins, vitamins that enrich the root rhizosphere [(3)].

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

For all the information reported, this work aims to carry out a review of the relevant results on biofertilization and nanotechnology use in the alfalfa crop, illustrating how these technologies can lead to a reduction in chemical fertilizer application.

## Alfalfa, generalities, uses and applications

Alfalfa is a perennial legume representative of temperate regions and it is used mainly as feed for livestock, universally considered one of the highest quality forages.

It is a valuable crop because among the many agronomic and environmental advantages it has, are the preservation of soil fertility and biodiversity, protection against soil erosion, the climate change impact mitigation, pollution reduction by nitrates from groundwater, the reduction in the consumption of fossil fuels, the reduction in 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 to its high and stable yield as a nutritious herb (10). Its economic importance is based on the high potential for biomass production, higher than 80 t $ha^{-1}$ green and about 20 t $ha^{-1}$ of dry matter (11). Alfalfa forages are characterized by a high content of crude protein (12), well balanced with respect to amino acid. It is enriched with vitamins of vital importance and several essential microelements for the normal growth and development of animals. Alfalfa is the basic component in the feeding program for dairy cattle, as well as for cattle, horses, sheep and other kinds of cattle (13).

Alfalfa has also become interesting as a potential source of secondary metabolites. 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, until April 30 of the current year, 385 992 ha of green alfalfa have been sown, of which 384,693 ha have been harvested for a production of 15 360 646 and a yield of 39,930 t $ha^{-1}$ (16). 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 livestock (17,18). This plant, as forage, can be used in different ways, fresh, hay and silage mixed with one or more grasses (19,20).

## 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 the world population creates an increasing demand for food and to meet it, fertilizers are used that are defined as any substance that is used to increase soil productivity by promoting its fertility by adding nutrients, which helps in plant growth. Fertilizers that are composed of raw chemicals in solid or liquid form made in factories aimed at the nutritional requirements of plants are called, by definition, 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 various problems in nature, such as water acidification; ozone layer damage; the greenhouse effect; using them for a long time can change soil pH, the water eutrophication where the nutritional content in these environments increases, causing algae proliferation and, consequently, oxygen reduction in the water, which damages marine life [22]. A current solution to reduce the use of these fertilizers in agriculture is biofertilizer use [23].

Biofertilizers are microbial inoculants that contain live or latent cells of efficient strains of nitrogen-fixing microorganisms, phosphate solubilizers and cellulose decomposers. These are applied primarily to soils to improve their fertility and plant growth, by increasing the number and biological activity of beneficial microorganisms [3].

Some of the advantages that biofertilizers have is that they are profitable and ecological, gradually improving the quality of soils. The microorganisms contained in the biofertilizer promote the supply of nutrients to the plants and, therefore, their development, growth and physiological regulation are ensured. Added to this, the crop yield can increase by 10 to 25 % and with its use; the plants are less prone to soil diseases. Among the main limitations that biofertilizers have, it can be pointed out 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 are [22]:

1- Nitrogen fixing biofertilizer: *Rhizobium*, *Azotobacter*, *Azospirillium*, *Bradyrhizobium*.
2- Phosphorus solubilizer biofertilizer: *Bacillus*, *Pseudomonas*, *Aspergillus*.
3- Phosphorus mobilizing biofertilizer - *Mycorrhiza*.
4- Plant growth promoter biofertilizer: *Psuedomonas*, *Trichoderma*.

The effects of the aforementioned biofertilizers in nitrogen fixation terms in the soil is carried out through the root nodules of the legume crop, making the $N_2$ available to the plant. Other microorganisms that can be used as biofertizates are: Azolla which is a heterogeneous fern with seven species that are endosymbiont with *Anabaena azollae*, a nitrogen fixing cyanobacterium [25] and blue green algae can fix nitrogen in the anaerobic environment due to one cell specialized 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 the soil. Dehygrogenates are involved in the soil oxidation process and they are used as an indicator of its microbial activity [28].

Some studies have been carried out on alfalfa 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 the absorption of nutrients from plants and increase the productivity of crops. 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 (broth of yeast mannitol with the addition of agar, sodium alginate, calcium chloride, glycerol or ferric chloride and combinations thereof). For the survival of L3 Si, during a storage time of 150 days the formulation of the medium containing glycerol was applied in combination with agar or sodium alginate, which was used as liquid inoculant. The alfalfa seeds were pre-inoculated with four formulations (yeast mannitol broth (YMB), YMB with agar (1 g $L^{-1}$), YMB with 1 or 5 g $L^{-1}$ of sodium alginate) for three months. Seeds pre-inoculated and stored for a month produced successful alfalfa plants. The nitrogen content in alfalfa obtained from seeds pre-inoculated one month before sowing increased varied between 3.72-4.19 % [29].

The ability of 17 rhizobacteria strains to improve the physiology, nutrient absorption, growth and performance of alfalfa plants grown under desert agricultural conditions in Saudi Arabia was studied [30]. The 17 rhizobacteria 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 the relative water content; chlorophyll a; chlorophyll b; carotenoid content; content of N, P and K; plant height; leaf-stem relationship; and fresh, dry dough. *Acinetobacter* pittiiJD-14 was more effective in increasing the fresh and dry mass of alfalfa by 41 and 34 %, respectively, compared to non-inoculated control plants. However, all strains improved crop characteristics compared to control plants, indicating that these strains of desert rhizobacteria could be used to develop an ecological biofertilizer for alfalfa and possibly other crop plants to improve production sustainable in arid regions.

## Evaluation of the nutritional composition of alfalfa (*M Sativa*) when grown with the application of biofertilizers

Six doses of fermented cattle manure biofertilizers were used in a biodigester (0, 25, 50, 100, 200 and 400 $m^3$ $ha^{-1}$) and five repetitions. The biofertilizer chemical characteristics the 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 results, it was obtained that the best absorption of N, K, Ca and Mg occurred with the dose of 400-m3 $ha^{-1}$. In the case of N, it was 22 % more than in the control and it was linear with the increase in biomass. The Cu, Mn and Zn micronutrient levels did not have significant differences between the doses applied, as did the concentrations of crude protein [(31)].

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

## 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 Sciences, in 1974 [(33)]. Although the term "nanotechnology" has long introduced in multiple disciplines, the idea that nanoparticles (NPs) could be of interest in agricultural development is a recent technological innovation and it is still in progressive development [(34)].

NPs are organic, inorganic or hybrid materials with at least one of their dimensions ranging from 1 to 100 nm (at the 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 ecological nature and production process simplicity 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 point out both positive and negative effects on the growth and development of plants when using NPs and the impact of these on plants depends on the composition, concentration, size and chemical and physical properties, as well as the plant species [38].

For the exploitation of green nanotechnology, plant species number and microorganisms, including bacteria, algae and fungi, they 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 (Z), and copper (Cu), can be synthesized within living plants, such as *Brassicajuncea*, *M. sativa*, and *Heleanthusannus* [36].

Synthesized nanofertilizers have a specific use to regulate nutrient release based on 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 the efficiency of fertilizers and raises production cost [39]. On the other hand, nitrogen fertilizer nanoformulations synchronize the N-fertilizer release with its demand for absorption by crops. Consequently, nanoformulations avoid undesirable nutrient losses through direct internalization by crops and thus avoid the interaction of nutrients with soil, water, air, and microorganisms [36].

Micronutrient deficiency not only lowers crop productivity, but also affects human health through 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 sense, the use of nanoformulated micronutrients for the slow or controlled release of nutrients would stimulate the absorption process by plants, promote the growth and productivity of crops and contribute to maintaining soil health [41]. For example, in zinc-deficient soils, the application of nano zinc oxide at low doses positively influences growth and physiological responses, such as sprouting and root elongation, fresh dry weight, and photosynthesis in many species of plants compared to the control [42, 43].

## Nanotechnology applications in alfalfa cultivation

Boron (B) is among the nutrients that are necessary for plant growth and yield production, and it can improve the nutritional properties of forage crops. However, at higher levels it can be toxic and adversely affect plant growth and forage quality. B concentration in plants is affected by different parameters, such as fertilization with this same micronutrient, the soil, the climate, the plant species, etc. For all this, the effects of different treatments of B in alfalfa on B concentration of and on the content of pigments, including chlorophyll b, total and carotenoids, were studied. The experimental treatments were: 1) six types of soil (S1-S6), 2) B sources, including fertilization with boricacide (B1) and nano boron (B2), and 3) number of sprays (zero, one, two and three times). The results indicated that the type of soil, B source and the number of fumigations significantly affected ($P \leq 0.01$) the B concentration in alfalfa and the content of pigments. Spraying three times significantly increased B concentration of as it resulted in an increase of 207.81 % compared to the control treatment and also increased the content of pigments ($P \leq 0.05$) 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 NPs ($K_2SO_4$) 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 based on 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 $m^{-1}$ using $CaCl_2$ $2H_2O$: NaCl (2:1) mixed with Hoagland's solution. Three treatments of $K_2SO_4$ NPs were applied consisting of 1/4, 1/8 and 1/10 of the K level in full strength Hoagland solution (235 mg $L^{-1}$). The highest shoot dry weight, relative yield, and root length and root dry using $K_2SO_4$ NPs obtained weight in both genotypes at the 1/8 level. The different doses of $K_2SO_4$ NPs significantly affected the Na/K ratio and the concentrations of Ca, P, Cu, Mn and Zn in the plant tissue. The application of $K_2SO_4$ NPs at a rate of 1/8 improved the physiological response of the plant to salt stress by reducing electrolyte leakage, increasing the content of catalase and proline, and increasing the activity of antioxidant enzymes. These results suggest that KNPs application may have a better efficiency than conventional K fertilizers to provide adequate plant nutrition and overcome the negative effects of salt stress on alfalfa [(45)].

The toxicity of zinc oxide nanoparticles (ZnONPs) in seed germination/root elongation and the absorption of ZnONPs and $Zn^{2+}$ in alfalfa (*M. sativa*), cucumber (*Cucumis sativus* L.) and tomato seedlings (*Solanum lycopersicum* L.) was investigated [(46)]. The seeds were treated with ZnONPs at 0-1 600 mg $L^{-1}$ as well as 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 in alfalfa and tomato decreased by 40 and 20 %, respectively. With 250 mg of $Zn^{2+}$ $L^{-1}$, only tomato germination was reduced compared to 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. NPs could be harvested industrially. For example, alfalfa plants grown in an environment rich in AuCI4 showed metallic gold absorption. AuNPs can be separated mechanically by dissolving organic material (plant tissue) after harvest [(47)]. Alfalfa plants can also absorb Ag from a solid medium rich in this element with the subsequent formation of Ag NPs [(48)].

## CONCLUSIONS

- 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, which is why 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.
- In sustainable agriculture and the protection of the environment against pollution is essential, so the application of nanotechnology ensures better management and conservation of inputs for the production of agricultural food. This advanced technique represents a significant benefit for agricultural productivity, since nanoparticles are an efficient platform for the transfer of genes and biomolecules to plants from engineering.

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