


The sweet clover-*Sinorhizobium meliloti* system as a useful interaction for nitrogen fixation and as a soil improver. Review



Gabriel Gallegos Morales ^a

Omar Jiménez Pérez ^a

Juan Manuel Sánchez Yañes ^b

Perpetuo Álvarez Vázquez ^a

Francisco Castillo Castillo ^{c*}

^a Universidad Autónoma Agraria Antonio Narro. Departamento de Parasitología. Calzada Antonio Narro # 1923, Buenavista, Saltillo, 25315 Coahuila, México.

^b Universidad Michoacana de San Nicolás de Hidalgo. Instituto de Investigaciones en Química y Biología. Michoacán, México.

^c Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias. Campo Experimental Saltillo. Coahuila, México.

* Corresponding author: reyes.francisco@inifap.gob.mx

Abstract:

Faced with the challenges posed by the need for fertilizers to maintain agricultural production, a biological process of atmospheric nitrogen fixation occurs naturally, which is carried out by a group of symbiotic bacteria that form a very close association with plants of the legume group, among which is the sweet clover (*Melilotus* spp.). From an ecological point of view, this plant has an essential function due to its good ability to associate with native nitrogen-fixing bacteria of the genus *Sinorhizobium*. A fundamental aspect is that this plant species can grow normally in alkaline soils, which doubles its importance since, on the one hand, it fixes nitrogen, and on the other hand, it can be incorporated as green manure. With this, the physicochemical properties of the soil are improved, and the levels

of organic matter, which is in extremely poor condition in arid zone areas, are increased. Additionally, this species can withstand low temperatures and grow satisfactorily in winter. This paper presents a synthesis of the genus *Melilotus* and its symbiont *Sinorhizobium meliloti* and its importance as a potential natural soil improver.

Keywords: *Melilotus*, *Sinorhizobium*, Symbiont, Nitrogen, Soil.

Received: 05/07/2023

Accepted: 20/10/2023

Introduction

Within the Fabaceae family, weed plants have an enormous value in agricultural systems due to their ability to associate symbiotically with nitrogen-fixing bacteria^(1,2), which becomes an N supply and an improvement in soil quality, in addition to promoting the production of more protein-rich forages⁽³⁾; among these species is the sweet clover or *melilotus*⁽⁴⁾. Three species of *Melilotus* have been reported for Mexico⁽⁵⁾, where the species *Melilotus indica* (L.) is the most adapted or most common as a weed worldwide, in rustic environments such as temperate climates; it also develops in moderately saline areas, where traditional forage legumes cannot be successfully cultivated^(6,7). This weed is classified in the Fabaceae family⁽⁸⁾; its growth is widespread and can be present in crops such as wheat, tomato, soybeans, sorghum, beetroot, prickly pear, apple, corn, flax, chickpeas, fruit trees, beans, asparagus, citrus fruits, peas, rye, barley, safflower, squash, oats, cotton, alfalfa, grapes, and garlic⁽⁶⁾. The growth of *M. indica* associated with certain crops such as wheat is considered dangerous since the presence of coumarin in almost all parts of the plant is common, which causes the characteristic smell of the plant to be transmitted to the cereal, to the grains of the plant and later to the flour⁽⁸⁾. For this reason, it is considered a noxious weed in agriculture. Seeds of this species can also be found as foreign bodies in seeds of alfalfa, flax, and many other cereals, which limits their direct consumption. On the other hand, the fixation of N by soil microorganisms has an essential role in agriculture as it can replace or reduce the use of costly chemical fertilizers, reduce pollution in the environment, prevent soil fertility losses, and improve production costs. The legume-*Sinorhizobium* symbiosis offers an opportunity for bioremediation and the improvement and fertilization overexploited soils in agricultural and livestock areas⁽⁹⁾. The recovery of *Melilotus* seeds and the isolation of the bacteria associated with this legume could benefit the possibility of regenerating the quality of depleted or eroded soils by sowing the *M. indica* plant

inoculated with the nitrogen-fixing bacteria *Sinorhizobium meliloti*. Therefore, this paper presents a general review of the species *Melilotus* spp. and its symbiont *Sinorhizobium meliloti* as a potential improver of soil quality.

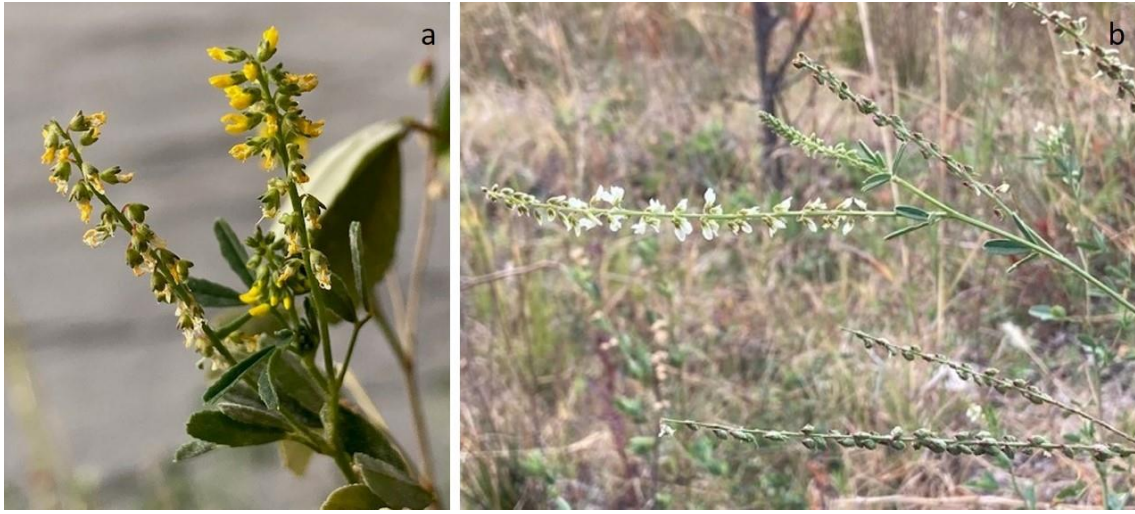
Origin and distribution

In the legume or Fabaceae family, the genus *Melilotus* includes different species, generally known as sweet clovers⁽⁷⁾. Its origin is found in Europe and Asia⁽¹⁰⁾. During the conquest, it dispersed and adapted abundantly in America and Australia, although today, it has a cosmopolitan distribution. It is noted that in Mexico⁽⁵⁾, in most states, it is recorded as a weed and is considered an exotic plant, with the species *Melilotus indica* (L.) being the most widely distributed⁽⁸⁾ compared to the other two species present (*M. albus* and *M. officinalis*). It has been recorded in Aguascalientes, Baja California Norte, Baja California Sur, Distrito Federal, Oaxaca, Querétaro, Sinaloa, Sonora, Tlaxcala, Veracruz, Durango, Guanajuato, Hidalgo, Jalisco, Estado de México, Michoacán, Morelos, Nuevo León, Chiapas, Chihuahua, Coahuila, and Colima⁽¹¹⁾.

Botanical characteristics

The species *Melilotus indica* is characterized as an herbaceous, annual or biannual, erect, highly-branched, 30-50 cm tall plant with a taproot, with compound, trifoliolate leaves, remarkably similar to those of traditional alfalfa, slightly dentate margin; with variable size from 1 to 2 cm long by 3 to 5 cm wide, obtuse or rounded apex and attenuated base. It has a stem with lanceolate stipules. Inflorescence arranged in clusters of 30 to 70 and small flowers 3 to 5 mm long with very short pedicels, with yellow or white corolla 1-3 mm long, in thin clusters (Figure 1), starting from the axil of the upper leaves and being longer than these, with the banner longer than the other petals, and a group of 10 stamens distributed in 9 that form a bundle and one that is free^(12,13).

Figure 1: Inflorescence of two species of melilot, yellow (*Melilotus indica*) and white (*M. albus*)



The fruit is a subglobose legume, about 3 mm, apiculate, hairless, yellowish-green, with transverse wrinkles, containing one or two smooth, yellowish seeds of 1.5 mm in diameter and globose surface (Figure 2). Flowering usually occurs in May and can last all summer. The plant has a slightly bitter taste; when it dries, it emits an intense coumarin aroma⁽¹³⁾.

Figure 2: Fruits of green (a) and mature (b) melilots



In the seedling stage, it is characterized by having a hypocotyl measuring 11 to 42 mm, greenish, smooth, and cotyledons of oblong to elliptical blade 4 to 8 mm long and 2 to 4 mm wide, without pubescence and without epicotyl. Alternate leaves, the first simple and the second compound (Figure 3).

Figure 3: Morphotypical characteristics of *Melilotus* plants. Lanceolate leaves and Branched stem



Taxonomy of *Melilotus indicus*

The species *Melilotus indicus* (L.) All. is included in the following taxa: Class: Equisetopsida; Subclass: Magnoliidae; Superorder: Rosanae; Order: Fabales; Family: Fabaceae; Genus: *Melilotus* (L.) Mill; Species: *indicus* [*Melilotus indicus* (L.) All.]. Other synonyms given to the species are: *Sertula indica* (L.) Kuntze and *Sertula melilotus* var. *indica* (L.) Lunell⁽⁸⁾.

Generalities, impact, and importance of *Melilotus indicus* on crops

Melilotus species are undesirable, considered weeds when they grow together with cereal crops, with annual growth, mainly in wild environments of temperate climates⁽¹⁴⁾; due to the production of coumarin that gives it a characteristic aroma, there is a great diversity of names given to it, such as sweet clover or small-flowered melilot, small melilot, scented melilot, scented clover, royal crown, yellow sweet clover, king's narrow crown, scented cart⁽¹⁵⁾. In addition, it is noted that this species can be considered a good forage plant⁽¹⁶⁾ and

a good plant source for production and incorporation as green manure. It is also noted that it can be a good option for soil improvement and nitrogenation as it has the ability to make symbiotic associations with nitrogen-fixing microorganisms naturally.

Due to the presence of coumarin, transmitted as a characteristic smell to cereals or grains and later transmitted to flour during milling, *Melilotus* spp. plants are listed as a weed (Figure 4), which is undesirable to find among seeds intended for human consumption, and for this reason, it has been declared as a weed in many countries, including Mexico, where it is considered exotic⁽¹¹⁾.

Figure 4: Sweet clover crop plants and their association with maize crops



“El Bajío” Experimental Field. Antonio Narro Autonomous Agrarian University in Buenavista, Saltillo, Coahuila, Mexico

It has been reported that, among different species of *Melilotus*, the coumarin content varies between varieties, ecotypes, and individuals of the same species⁽¹⁷⁾. This presence of coumarin also varies throughout the plant’s growth cycle, where it is mentioned that its presence is maximum in new leaves or buds, or as a response to stress from pests and diseases (biotic factors), as well as salinity, alkalinity, nutritional deficiencies in the soil, etc. (abiotic factors)⁽¹⁸⁾. Its relevance is also due to its ability to fix atmospheric nitrogen symbiotically, which allows it to be a protein store, which is another important factor that allows it to be chosen as forage; in addition, it reduces production costs since it reduces the work of applying and purchasing fertilizers, which leads to an improvement in the chemical properties of the soil.

Biological and ecological importance of *Melilotus*

Species belonging to the genus *Melilotus* have recently received particular attention due to their use in response to the need for a broader range of legume species suitable for saline soils^(19,20). Cultivars that have shown considerable potential have been released, such as *Melilotus albus* cultivar Jota Medik⁽²¹⁾. The potential of *M. siculus* (Turra) Vitman ex B. D. Jacks. (Syn. *M. messanensis*) as a grass species has also been described as a cultivar^(22,23).

As already mentioned, *Melilotus* spp. contributes indirectly to the nutrition process of cultivated plants by allowing symbiosis or association with rhizobacteria, allowing this utilization to a large extent due to the beneficial fixation of atmospheric nitrogen, in addition to promoting greater solubility and conductivity of nutrients⁽²⁴⁾. This biological nitrogen fixation results from the enzymatic conversion of gaseous nitrogen to ammonium; this is a characteristic of all prokaryotes, and very specifically of the genera of free-living nitrogen-fixing rhizobacteria associated with legumes⁽²⁵⁾, such as the group of species of the genus *Sinorhizobium*, which mainly associate with legumes⁽²⁶⁾.

The symbiotic relationship formed by the plant species *Medicago sativa* and the beneficial bacteria *Sinorhizobium meliloti* is a reference model to know and explore the mechanisms that interact in molecular expression, through which the legumes-rhizobia symbiosis develops, and how these are regulated, making it possible to lay the foundations to address the manipulation and improvement of symbioses for practical purposes in an agro-economic sense⁽²⁷⁾.

Relationship of *Sinorhizobium* and *Melilotus*

Under natural conditions, there is a very specific symbiotic relationship between the species of the genus *Sinorhizobium*, which is characterized by the formation of nodules in some legumes, settling within their roots, where they proliferate, differentiate and fix nitrogen⁽²⁸⁾. In this sense, a very specific symbiotic relationship is found between *Melilotus* plants and the genus *Sinorhizobium meliloti*^(26,29,30), as shown in Figure 5. Currently, there are few descriptions of legume-type plants associated with a greater number of nitrogen-symbiotic species⁽²⁴⁾.

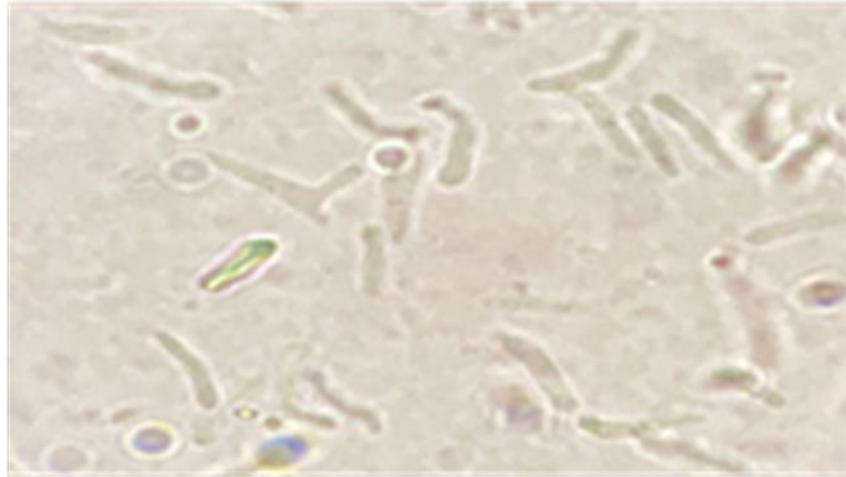
Figure 5: Melilot plants with nodules characteristic of the bacteria *Sinorhizobium* under natural conditions growing as wild weeds



N assimilation is carried out by an enzymatic process where a change from atmospheric nitrogen to ammonium occurs. This process is a primary characteristic of prokaryotes and is distributed in different genera of bacteria with the same ability to fix N naturally⁽²⁵⁾. Generally, the artificial incorporation of nitrogen as a chemical fertilizer inhibits nodule formation and atmospheric nitrogen fixation in plants with the presence of nodules. It is mentioned that the nitrogen fixation process is very energetically expensive⁽³¹⁾. Atmospheric nitrogen fixation contributes about 90 million tonnes annually for legume crops such as soybeans, red clover, and peas⁽³⁰⁾.

The interaction between the plant and the bacteria begins with the signaling or synthesis in the roots and the exudation of flavonoids, which are chemical recognition signals between the two organisms⁽³²⁾. Phenolic compounds start the expression in bacteria of the genes involved in the nodulation process, which allows the synthesis and secretion of lipochitins called nodulation factors^(32,33,34), which, when interacting in the root, cause morphological changes in the plant according to the type of legume⁽³⁵⁾. Once the bacteria invade the root cells of the plant, they proliferate and differentiate as bacteroids (Figure 6), which are responsible for nitrogen fixation inside the cell; these bacteroids are surrounded by a plant-derived peribacteroid membrane, which constitutes a new organelle called a symbiosome. The plant contributes carbohydrates to the bacteroid for its metabolism through the phloem, and the bacteroid contributes ammonium to the plant in the form of different amino acids^(36,37). The verification of the cell morphology allows to observe the bacteroids that are characteristic of these bacteria existing within the root cells. This form of bacteroid is due to the lack of a defined shape (Figure 6) as they lack a cell wall; therefore, they are considered amorphous⁽³⁸⁾.

Figure 6: *In vivo Sinorhizobium meliloti* bacteroids obtained from nodules and observed under the 100X compound microscope.



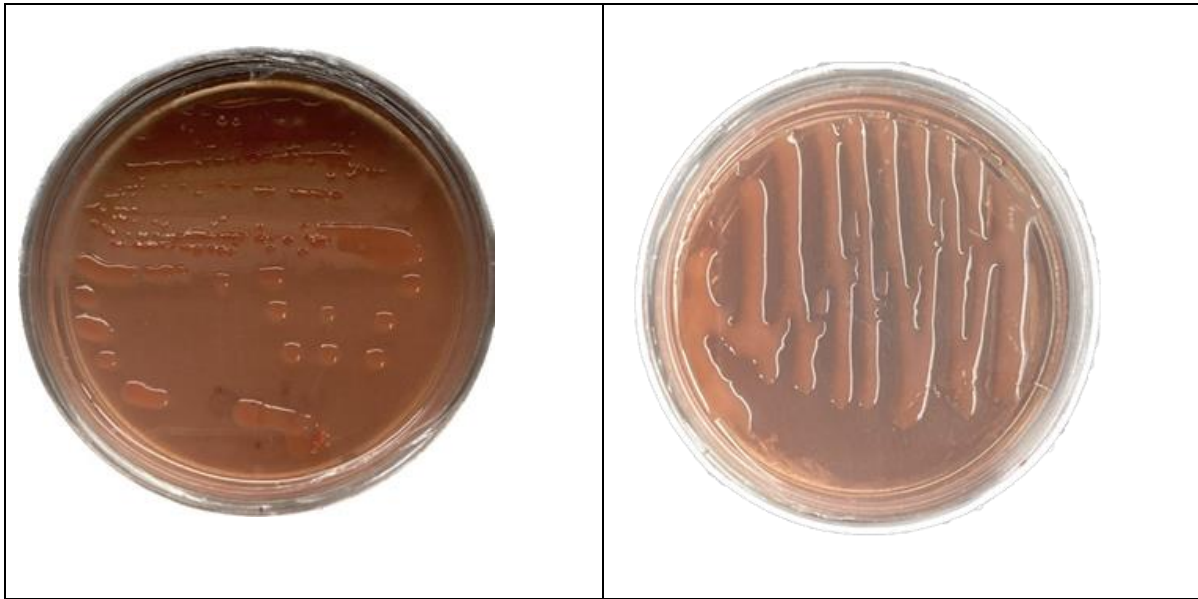
Characteristics of *Sinorhizobium meliloti*

The characteristics of these bacteria are that they are bacillary in shape, belong to the Gram-negative group, do not form spores, and are heterotrophic and aerobic. The bacteria *S. meliloti* are very capable of thriving both in a complex and competitive environment, such as the rhizosphere, and intracellularly once the association is established. Due to their complex and large genome size, these microorganisms are highly versatile, which gives them a great metabolic capacity with advantages of colonizing different niches in nature⁽³⁹⁾. Generally, the bacterial cell of *Sinorhizobium* has dimensions between 0.5-1.0 x 1.2-3.0 μm , with the presence of large plasmids, megaplasmids, quite common in these species, where symbiotic genes are located in some cases⁽⁴⁰⁾. Their use in agricultural systems would bring benefits such as: the reduction of production costs as the use of chemical fertilizers decreases, the increase in agricultural production, and the contribution to the remediation of overexploited, alkaline, or low organic matter soils⁽⁴¹⁾.

When wanting to isolate *S. Meliloti*, nodules that are generally reddish, which indicates that they contain leghemoglobin, and present in the secondary roots of sweet clover plants should be collected, washed with soap and water, and disinfected with chlorine and washed several times with sterile distilled water, to subsequently macerate the nodule in a sterile tube and seed the resulting liquid into the culture medium through a bacteriological loop. For this purpose, it is common to use the growth medium based on the yeast mannitol agar-Congo red and incubate at 28 °C for two days until the red growth of the typical colonies of the genus is observed. Subsequently, it is purified by streaking in the same culture medium until isolated colonies are obtained in the culture⁽²⁴⁾. The main characteristic of the colonies

of these bacteria on mannitol agar is that they are of the mucoid type with an elevation and smooth edges (Figure 7). Certain tests help to identify the bacteria better, such as the Gram stain test, which must be negative (-), presence of flagella, polysaccharide (KOH) production positive, sodium chloride growth positive, indole production positive, and acidic pH growth positive ⁽⁴²⁾.

Figure 7: Isolation by reseeded of rhizobacteria from sweet clover nodules



a) Streak seeding of the maceration of melilot nodules and growth of colonies typical of the genus *Sinorhizobium* spp. in mannitol agar culture. b) Rhizobacteria purified by simple streaking.

Diversity of symbiotic nitrogen rhizobacteria

As a group, rhizobacteria are very diverse in terms of genera, species, and molecular phylogenetic relationships. It is noted that they include six genera (*Allorhizobium*¹, *Azorhizobium*², *Bradyrhizobium*³, *Mesorhizobium*⁴, *Rhizobium*⁵, and *Sinorhizobium*⁶), each with different species, and target plant species, as described below in Table 1^(43,44,45).

Table 1: Nitrogen-fixing bacteria and their associated species

Rhizobacteria			Rhizobacteria				
Genus	Species	Crop	Genus	Species	Crop		
<i>Allorhizobium</i>	<i>undicola</i>	<i>Neptunia natans</i>	<i>Azorhizobium</i>	<i>caulinodans</i>	<i>Sesbania rostrata</i>		
<i>Bradyrhizobium</i>	<i>Japonicum</i>	<i>Glycine max</i>	<i>Rhizobium</i>	<i>hainanense</i>	<i>Sesbania herbacea</i>		
	<i>elkanii</i>	<i>Glycine max</i>		<i>hautiense</i>	<i>Vicia</i>		
	<i>liaoningense</i>	<i>Glycine max</i>		<i>leguminosarum</i>	(peas)/ <i>Trifolium</i>		
	<i>yuanmingense</i>	<i>Lespedeza</i>		<i>mongolense</i>	(clover) <i>Medicago ruthenica</i> / <i>Phaseolus vulgaris</i> <i>Phaseolus vulgaris</i> / <i>Leucaena</i> <i>Neptunia natans</i>		
<i>Mesorhizobium</i>	<i>Loti</i>	<i>Lotus</i>	<i>Sinorhizobium</i>	<i>arboris</i>	<i>Acacia</i>		
	<i>amorphae</i>	<i>Amorpha</i>		<i>fredii</i>	<i>senegal</i> / <i>Prosopis chilensis</i>		
	<i>cicero</i>	<i>fruticosa</i>		<i>kostiens</i>	<i>medicae</i>	<i>Glycine max</i>	
	<i>huakuii</i>	<i>Cicer</i>		<i>meliloti</i>	<i>saheli</i>	<i>Acacia senegal</i> / <i>Prosopis chilensis</i>	
	<i>mediterraneum</i>	<i>arietinum</i>		<i>terangae</i>		<i>Medicago spp</i> <i>Medicago sativa</i> <i>sesbania</i> <i>Sesbania</i> / <i>Acacia</i> <i>Glycine max</i>	
	<i>plurifarium</i>	<i>Astragalus</i>		<i>C. mediterranium</i>		<i>xinjiangense</i>	
				<i>Leucaena</i>			

Melilot response to nodulation

Bioassays carried out under greenhouse conditions to determine the efficiency of nodule formation showed that, when sowing melilot seed inoculated with *Sinorhizobium meliloti*, the latter induced the formation of nodules mostly cylindrical and branched (Figure 8), characteristics representative of symbiotic nodules of *S. meliloti*⁽⁴⁰⁾. It is reported that *Sinorhizobium meliloti* induces the formation of pinkish nodules in seed-generated *Melilotus* spp. plants, both in pots and naturally⁽⁴⁶⁾.

Figure 8: Roots of melilot plants with the presence of lobulated pinkish nodules of *Sinorhizobium meliloti*



Some agroecological characteristics of melilot plants

Sweet clovers or melilots can develop in saline soils⁽¹²⁾ poor in organic matter, with alkaline pH, at temperatures ranging from temperate to cold, where it has been observed that in some areas of occasional or irregular cold, they withstand temperatures of at least 0 °C and manage to grow normally during winter at temperatures below 15 °C, so this make this plant agronomically interesting for soil remediation during the winter seasons. It is a very competent weed, manages to develop favorably among plants, and fruits before or after the formation of crop fruits; it particularly excels in garlic, onion, corn, oats, sorghum, and wheat crops (Figure 9).

Figure 9: Melilot plants surviving winter frosts that damage other weeds

Nitrogen fixation by *Melilotus* spp

Generally speaking, most rhizobia symbiotically fix atmospheric N_2 in amounts of up to $200 \text{ kg ha}^{-1} \text{ year}^{-1}$ of N in the nodules of plants of the Fabaceae family under specific conditions of temperature, pH, humidity, content of inorganic N, Fe, Co, Mo, and P in the soil. Among the best-known genera with this function are: *Azospirillum*, *Bacillus*, *Beijerinckia*, *Azotobacter*, and *Pseudomonas*. N_2 fixation by free-living bacteria in associated meadows of ryegrass (*Lolium perenne*) and *Melilotus albus* and inoculation with *Rhizobium meliloti* (*Sinorhizobium meliloti*) is reported with a similar N_2 content, both in meadows under cutting and under grazing⁽⁴⁸⁾. They also point out that the density of plants modifies the amount of fixation, being greater in cutting since grazing reduces the persistence of the legume in the meadow. These authors report a nitrogenase activity between 1.83 and 1.36 nmol of C_2H_4 produced $\text{plant}^{-1} \text{ h}^{-1}$, respectively. Al Sherif⁽⁶⁾ points out that *M. indicus* is a species with a high percentage of nodulation (68-95 %) and high nitrogenase activity, 1.81 mmol C_2H_4 $\text{plant}^{-1} \text{ h}^{-1}$ on average, and high protein content (21-30 %); he concludes that the high percentage of nodulation and nitrogenous activity recorded in *M. indicus* plants gives the species economic importance as it can be used to improve soil fertility. The rhizobium-legume symbiotic system requires that there be no mineral limitations, either by excess or defect. High concentrations of nitrates inhibit the infection process, the development of nodules, and the expression of nitrogenase activity. The greater the presence of N in the soil, the fewer possibilities there are for Biological Nitrogen Fixation (BNF), and conversely, the lower the presence of N in the soil, the more

N from BNF. The presence of combined forms of nitrogen limits BNF. Fertile soils with moderate or high availability of inorganic forms of N at the time of sowing or high rates of mineralization during the crop cycle affect the establishment of symbiosis since they delay the beginning of nodulation or inhibit the functioning of the fixative system.

Ways of nitrogen incorporation

a) Incorporation as green manure. Using green manures is a practice that counteracts the negative effects of improper soil management. Some authors⁽⁴⁹⁾ incorporated melilots for a period of four successive years, finding improvements in the soil, such as an increase in organic matter (OM), which went from 0.32 to 0.69 %. Likewise, Fontana *et al*⁽⁵⁰⁾ incorporated whole plants or remnants of *Melilotus albus* combined with rye as green manure, with the latter being the control; then the nitrate content was determined, which behaved as follows: at the year of incorporation, the NO₃ values in ppm were 38.0, 39.0 and 44.0 for treatments of rye, rye + *Melilotus* remnant and rye + whole *Melilotus* plant, respectively. For the second year, the NO₃ values were as follows: 17.7, 26.0 and 47.4 ppm for the same sequence of treatments, respectively. It was observed that only the treatment that includes the whole melilot plant achieved consecutive increases in NO₃. These data show that at the end of year two, a difference of 30 ppm of NO₃ was found between the treatment of incorporation with *Melilotus* and rye.

b) Growth and symbiosis with nitrifying bacteria. A common feature of microorganisms involved in biological nitrogen fixation is the arrangement of nitrogenase enzymes, which reduce atmospheric nitrogen to NH₄⁺ ions, which are the assimilable form. This enzymatic activity is highly susceptible to oxygen concentration in the environment, so microorganisms have adapted the necessary adaptation mechanisms such as respiratory protection, conformational protection, and cellular compartmentalization⁽⁵¹⁾. These processes involve a group of families of specialized activating proteins, which interact with RNA polymerase (RNAP) containing the transcription factor (sigma σ 54), called enhancer-binding proteins (EBPs), in order to activate transcription from upstream sites through the DNA Loop. These proteins interact with upstream activating sequences similar to the enhancer proteins through a C-terminal DNA-binding domain and the conserved central domain belonging to the AAA+ family, which couples hydrolyzed ATP until activation of transcription by σ 54-RNAP. The activity of EBPs is highly regulated in response to environmental signals through amino-terminal regulatory modules and, in some cases, through interactions with other regulatory proteins^(52,53).

c) Soil improvement. Fontana *et al*⁽⁵⁰⁾ found positive effects of incorporating green manure from *M. albus* on the production of forage and CP of rye crops in subsequent cultivation cycles (two years). In addition, they mention that there was a remnant of nitrates in the soil, so they infer that the increased fertility did not fully translate into production.

Aspects such as forage:

It is mentioned that both *M. albus* and *M. indicus* are forage plants used for animal feed due to their abundant source of protein⁽⁵⁴⁾; however, their use should apply only to major animals (cattle, horses, goats, and sheep), who consume them as a whole plant in a mixed form: (grazing and forage), since they are toxic to smaller species, especially the species *M. indicus*⁽⁵⁵⁾. Although *Melilotus* species have a high content of coumarins and derivatives thereof, some authors⁽⁵⁶⁾ report that, depending on the part analyzed, the quantification of coumarin may vary; for example, it is quantified in a greater quantity in flowers, followed by stems and leaves in plants before regrowth; they also observed that after several phenological cycles, these compounds decrease both in leaves and stems (after 2 and 4 cycles, respectively). In this sense, melilots are a reliable and cost-effective source of protein in ruminants and non-ruminants because they are independent of soil nitrogen. In addition, melilots are an excellent source of minerals, and intakes of melilots are generally higher than those of grasses with equal digestibility⁽⁵⁵⁾.

In order to know the behavior of sowing density (SD) and the effect of cut age (CA), researchers⁽⁵⁶⁾ developed a study in which they reported significant differences for SD and CA with respect to the production of *M. albus*. They observed an increase of more than 100 % when SD went from 500 to 1,500 seeds (from 333.34 to 736.62 plants after emergence per m², respectively), which represented a higher production of fresh (FM) and dry matter (DM), with values between 1.66 and 2.29 kg m⁻² of FM and values from 0.37 to 0.52 kg m⁻² of DM. The cut age (before bud break [A], bud break [B] and full flowering [C]) had behaviors similar to SDs; FM was observed with values between 1.11 and 3.06 kg m⁻² and, for DM, values between 0.18 and 0.80 kg m⁻²; however, a decrease in the percentage of leaves was observed as the cut age increased (40 [A] to 19 % [C]); it is worth mentioning that in an evaluation period, no differences were found between years for these variables. Regarding the variables of total protein (TP), crude fat (CF), fiber (F), and ash (As), there were no differences between the SD or in the years evaluated, but differences were observed concerning harvest age; for TP it was 21.72, 17.08 and 14.81 in the A-B-C stages, for CF it was 2.41, 2.08, and 1.71 in A-B-C, for F it was 34.55, 40.27, and 42.82 for A-B-C, respectively.

Quero *et al*⁽⁵⁷⁾ point out that the agronomic characterization of species with high forage productivity for the purpose of cultivar development and seed production would be one of the tools to improve the productivity and adaptability of pastures to the environment. One

of the most important species for restrictive environments is *Melilotus albus* Medik as it has high productivity, wide genetic variability, and extensive environmental adaptation⁽⁵⁸⁾.

Medicinal aspects:

Since time immemorial, medicinal plants have been consumed by man all over the world to treat various ailments or disorders in their health or that of their domestic animals, in acute ailments, and as adjuvants in chronic problems because they produce hundreds of substances of very different types, and some with negative effects. In this sense, the coumarin produced by *Melilotus* species has negative effects due to the hemorrhages caused in calves fed with this plant; however, from the medicinal point of view, it was identified as an anticoagulant, and reports indicate that cattle suffered severe bleeding disorders after having ingested sweet clover (*Melilotus albus*) stored in silos⁽⁵⁹⁾. It is also mentioned⁽⁶⁰⁾ that *Melilotus* has potential for the management of side effects in the management of diabetics since *Melilotus officinalis* can be used in herbal medicine; previous studies have shown that it is effective in reducing skin aging, induces microvascularization, and has anti-inflammatory effects^(61,62).

With all the above, from an ecological, agricultural, and livestock point of view, melilots are an opportunity point for the improvement and reclamation of overexploited or unproductive soils, forage production, and substantive changes in soil fertility, as well as for favoring the diversity of microbial species in the environment and broad utility in medicine.

Conclusions

The sweet clover or melilot is a plant that manages to grow as a weed in a great diversity of crops, where its presence is not pleasant due to the characteristic smell that the plant generates when it develops, especially in grasses, which are usually used to produce flours or pasta. However, its characteristics of growth and agroecological development make it a plant desirable for the improvement or remediation of soils poor in organic matter, salty or alkaline, in climates of temperate to very cold temperatures where its association with symbiotic nitrogen-fixing bacteria of the *Sinorhizobium meliloti* type is detected, with which it associates to obtain nitrogen, which is favorable for improving the nutritional quality of the soil where it grows.

Acknowledgments and conflicts of interest

The authors declare no conflicts of interest.

Literature cited:

1. Zamora NJF, Zapata HI, Villalvazo HA. Fijación biológica del nitrógeno en tres especies silvestres del género *Lupinus* (Leguminosae, Papilionoideae) en México. Act Bot Mex 2019;(126):e1543. <https://doi.org/10.21829/abm126.2019.1543>.
2. Córdova-Sánchez S, Castelán-Estrada M, Salgado-García S, Palma-López JD, Vera-Núñez JA, Peña-Cabriales JJ, *et al.* Biological nitrogen fixation by three fabaceas (Leguminosae) in acid soil of Tabasco, México. Avances en Investigación Agropecuaria 2011;15(1):31-50.
3. Castro-Rincon E, Mojica-Rodríguez JE, Carulla-Fornaguera JE, Lascano-Aguilar CE. Abonos verdes de leguminosas: integración en sistemas agrícolas y ganaderas del trópico. Agron Mesoam 2018;29(3):711-729.
4. Salas ME. La simbiosis fijadora de nitrógeno *Sinorhizobium meliloti*-alfalfa: aproximaciones ómicas aplicadas a la identificación y caracterización de determinantes genéticos del rizobio asociados a la colonización temprana de la raíz de alfalfa (*Medicago sativa*) [tesis posgrado]. Argentina: Instituto de Biotecnología y Biología Molecular; 2015. <https://doi.org/10.35537/10915/46558>.
5. Mondragón PJ, Vibrans H. Manual de malezas de México. CONABIO; 2009. <http://www.conabio.gob.mx/malezasdemexico/fabaceae/melilotus-indica/fichas/ficha.htm>.
6. Al Sherif EA. *Melilotus indicus* (L.) All., a salt-tolerant wild leguminous herb with high potential for use as a forage crop in salt-affected soils. Flora: Morphol Distrib Funct Ecol Plants 2009;204(10):737-746.
7. Toll VJR. Los tréboles de olor como recurso forrajero. 1ª ed. Argentina: Universidad Nacional de Tucumán; 2018. ISBN 978-987-754-136-6.
8. Tropicos. Tropicos.org. Missouri Botanical Garden. Consultado 28 Jun, 2023. <https://tropicos.org/name/13035799>. 2022.
9. López C, Odorizzi A, Basigalup DH, Arolfo V, Martínez MJ. El trébol de olor blanco y su uso en la provincia de Córdoba. 1ª ed. Buenos Aires, Argentina: Ediciones INTA; 2016. ISBN 978-987-521-716-4.

10. Aboel-Atta AMI. Isozymes, RAPD and ISSR variation in *Melilotus indica* (L.) All. and *M. siculus* (Turra) BG Jacks. (Leguminosae). Acad J Plant Sci 2009;2(2):113-118.
11. Villaseñor JL, Espinosa GFJ. Catálogo de malezas de México. Universidad Nacional Autónoma de México. Consejo Nacional Consultivo Fitosanitario. México, D.F. Fondo de Cultura Económica; 1998.
12. Mesa D. Obtención de plantas resistentes a la salinidad para los suelos salinos cubanos. Rev Cubana Cienc Agr 2003;(37):217-226.
13. Floraiberica. *Melilotus*. LXXXVIII. LEGUMINOSAE – TRIFOLIEAE. 2020. http://www.floraiberica.es/floraiberica/texto/pdfs/07_39%20Melilotus.pdf. Consultada 28 Jun, 2023.
14. Wu F, Zhang D, Ma J, Luo K, Di H, Liu Z, Zhang J, Yanrong Wang Y. Analysis of genetic diversity and population structure in accessions of the genus *Melilotus*. Ind Crop Prod 2016;(85):84-92. <https://doi.org/10.1016/j.indcrop.2016.02.055>.
15. Zabala JM, Marinoni L, Ribero G, Sánchez R, Del Valle E. Rev Fave Secc Cienc Agrar 2016;15(1):14.
16. Martínez PJJ. *Melilotus indicus* (L.). Herbario nacional de México (MEXU) Plantas vasculares, UNAM; 2012. <https://datosabiertos.unam.mx/IBUNAM:MEXU:656408>.
17. Nair R, Whittall A, Hughes S, Craig A, Revell D, Miller S, *et al.* Variation in coumarin content of *Melilotus* species grown in South Australia. NZ J Agric Res 2010;53(3):201-213. <https://doi.org/10.1080/00288233.2010.495743>.
18. Flórez DDF. La alfalfa (*Medicago sativa*): origen, manejo y producción. Conexión Agropecuaria JDC 2015;5(1):27-43. <https://revista.jdc.edu.co/index.php/conexagro/article/view/520>.
19. Nichols PGH, Loi A, Nutt B, Evans PM, Craig AD, Pengelly BC *et al.* New annual and short-lived perennial pasture legumes for Australian agriculture 15 years of revolution. Field Crop Res 2007;104(1-3):10-23. <https://doi.org/10.1016/j.fcr.2007.03.016>.
20. Dear BS, Ewing MA. The search for new pasture plants to achieve more sustainable production systems in southern Australia. Aust J Exp Agric 2008;48(4):387-396. <https://doi.org/10.1071/EA07105>.
21. Evans PM, Kearney GA. *Melilotus albus* (Medik.) is productive and regenerates well on saline soils of neutral to alkaline reaction in the high rainfall zone of south-western Victoria. Aust J Exp Agric 2003;43(4):349–355. <https://doi.org/10.1071/EA02079>.

22. Nichols PGH, Craig AD, Rogers ME, Albertsen TO, Miller SM, McClements DR, *et al.* Production and persistence of annual pasture legumes at five saline sites in southern Australia. *Aust J Exp Agric* 2008;48(4):518-535. <https://doi.org/10.1071/EA07167>.
23. Rogers MJ, Colmer TD, Frost K, Henry D, Cornwall D, Hulm E, *et al.* Diversity in the genus *Melilotus* for tolerance to salinity and waterlogging. *Plant Soil* 2008;(304):89-101. <https://doi.org/10.1007/s11104-007-9523-y>.
24. Yañez AA. Recuperación de rizobacterias del cultivo de frijol (*Phaseolus vulgaris*) de San Andrés Tlalamac, Estado de México [tesis Licenciatura]. México: Universidad Autónoma Agraria Antonio Narro; 2017.
25. Young JPW, Haukka KE. Diversity and phylogeny of Rhizobia. *New Phytol* 1996;(136):87-94.
26. Aizawa S-I. 2014. *Sinorhizobium meliloti* — Nitrogen–fixer in the grassland. *The Flagellar World* 2014;(1):82-83. <https://doi.org/10.1016/B978-0-12-417234-0.00026-8>.
27. Lagares A. La simbiosis fijadora de nitrógeno *Sinorhizobium meliloti* - alfalfa (*Medicago sativa*) caracterización del rol biológico del ARN pequeño sm8 en la vida libre y simbiótica de los rizobios [tesis doctorado]. Argentina: Universidad Nacional de La Plata; 2015. <http://sedici.unlp.edu.ar/handle/10915/66185>.
28. Cerdeño GGA. Tolerancia a estrés hídrico y promoción del crecimiento en alfalfa (*Medicago sativa*) inoculada con bacterias de la rizósfera [tesis doctorado]. Chile: Universidad de Concepción; 2018. <http://repositorio.udec.cl/jspui/handle/11594/3363>.
29. Ormeño OE, Vinuesa P, Zúñiga-Dávila D, Martínez-Romero E. Molecular diversity of native bradyrhizobia isolated from lima bean (*Phaseolus lunatus* L.) in Peru. *Sys Appl Microbiol* 2006;29(3):253–262. <https://doi.org/10.1016/j.syapm.2005.09.002>.
30. De Lajudie P, Willems A, Pot B, Dewettinck D, Maestrojuan G, Neyra M, *et al.* Polyphasic Taxonomy of Rhizobia: Emendation of the Genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb, nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. *Int J Syst Evol Microbiol* 1994;44(4):715-733. <https://doi.org/10.1099/00207713-44-4-715>.
31. Soto-Urzu L, Baca BE. Mecanismos de protección de la nitrogenasa a la inactivación por oxígeno. *Rev Latinoam Microbiol* 2001;(43):37-49.

32. Spaink HP. Root nodulation and infection factors produced by rhizobial bacteria. *Annu Rev Microbiol* 2000;(54):257-288. http://arquivo.ufv.br/dbv/pgfvg/BVE684/htms/pdfs_revisao/estresse/infectionfactors.pdf.
33. Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Prome JC. *et al.* Symbiotic host specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature* 1990;(344):781-784. <https://doi.org/10.1038/344781a0>.
34. Geurts R, Fedorova E, Bisseling T. Nod factor signaling genes and their function in the early stages of *Rhizobium* infection. *Curr Opin Plant Biol* 2005;8(4):346-352. <https://doi.org/10.1016/j.pbi.2005.05.013>.
35. Fernández-Luqueño F, Espinosa-Victoria D. Bioquímica, fisiología y morfología de la senescencia nodular: una revisión crítica. *Terra Latinoam* 2008;26(2):133-144.
36. Franche C, Lindstrom K, Elmerich C. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. *Plants Soil* 2009;(321):35-59. <https://doi.org/10.1007/s11104-008-9833-8>.
37. Guzmán DD, Montero TJ. Interacción de bacterias y plantas en la fijación del nitrógeno. *RIIARn* 2021;8(2):87-101. <https://doi.org/10.53287/uyxf4027gf99eAguilar2004>.
38. Lodwig E, Poole P. Metabolism of *Rhizobium* Bacteroids. *Crit Rev Plant Sci* 2003;(22):37-78. <https://doi.org/10.1080/713610850>.
39. Amarelle LV. Elucidación de los sistemas de implicados en la captación y utilización de hemina como fuente de hierro nutricional en *Sinorhizobium meliloti* 1021. [tesis doctorado]. Uruguay: Universidad de la República; 2016. <https://www.colibri.udelar.edu.uy/jspui/bitstream/20.500.12008/8853/1/uy2418174>.
40. Graham PH, Draeger KJ, Ferrey ML, Conroy MJ, Hammer BE, Martínez E, *et al.* Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. *Can J Microbiol* 1994;(40):198-207. <https://doi.org/10.1139/m94-03>.
41. Batanony NHE, Castellano-Hinojosa A, Mamdouh A, Ashraf N, Bedmar EJ. Agronomical parameters of host and non-host legumes inoculated with *Melilotus indicus*-isolated rhizobial strains in desert unreclaimed soil. *Arch Microbiol* 2020;202(7):1929-1938. doi.10.1007/s00203-020-01907-x.

42. Kuykendall D, Young J, Martínez E, Kerr A, Sawada H. *Rhizobium* (Frank 1889), 338. In: Bergey's Manual of Systematic Bacteriology. Editorial Springer US; 2005.
43. Delgado MJ, Casella S, Bedmar EJ. Denitrification in Rhizobia-legume symbiosis. *Biology of the nitrogen cycle* 2007;(1)83-91. <https://doi.org/10.1016/B978-044452857-5.50007-2>.
44. Moreno RA, García MV, Reyes CJL, Vásquez AJ, Cano RP. Plant growth promoting rhizobacterias: a biofertilization alternative for sustainable agriculture. *Rev Colombi Biotecnol* 2016;20(1):68-83.
45. Velasco-Jiménez A, Castellanos-Hernández O, Acevedo-Hernández G, Aarland RC, Rodríguez-Sahagún A. Bacterias rizosféricas con beneficios potenciales en la agricultura. *Terra Latinoam* 2020;38(2):333-45. <https://doi.org/10.28940/terra.v38i2.470>.
46. Antonio Del AM. Aislamiento e identificación *Sinorhizobium meliloti* de nódulos de plantas de alfalfa [tesis licenciatura]. México: Universidad Autónoma Agraria Antonio Narro; 2021. <http://repositorio.uaaan.mx:8080/xmlui/bitstream/handle/123456789/48036/K%2067227%20Antonio%20del%20c%3%81ngel%2c%20Maricela.pdf?sequence=6&isAllowed=y>.
47. Bolger TP, Pate JS, Unkovich MJ, Turner NC. Estimates of seasonal nitrogen fixation of annual subterranean clover-based pastures using the ¹⁵N natural abundance technique. *Plant Soil* 1995;175:57-66. <https://doi.org/10.1007/BF02413010>.
48. Delgadillo MJ, Ferrera-Cerrato R, Galvis-Spínola A, Hernández-Garay A, Cobos-Peralta MA. Fijación biológica de nitrógeno en una pradera de trébol hubba/ballico de corte o de pastoreo. *Terra Latinoamericana* 2005;23(1):73-79.
49. Fontana LMC. Efectos de la alfalfa y del melilotus usados como forraje y abono verde, sobre la producción de pasturas y cultivos [tesis licenciatura]. Argentina: Universidad Nacional de Córdoba; 2014.
50. Fontana LMC, Juan NA, Ruiz MA, Babinec FJ. Utilización de trébol de olor blanco (*Melilotus albus* Medik.) como abono verde, efecto sobre las condiciones del suelo y la productividad del cultivo subsiguiente. *Semiárida* 2018;28(2)25-33. [http://dx.doi.org/10.19137/semiarida.2018\(02\).25-33](http://dx.doi.org/10.19137/semiarida.2018(02).25-33).
51. Mayz-Figueroa, J. Fijación biológica de nitrógeno. *Rev Científ UDO Agríc* 2004;4:1-20. <https://dialnet.unirioja.es/servlet/articulo?codigo=2221548>.

52. Bush M, Dixon R. The role of bacterial enhancer binding proteins as specialized activators of σ^{54} -dependent transcription. *Microbiol Mol Biol Rev* 2012;76(3):497-529. doi: 10.1128/MMBR.00006-12.
53. Romero JL. Importancia del segundo mensajero c-di-GMP en la simbiosis rizobio-leguminosa [tesis doctorado]. España: Universidad de Granada; 2016.
54. Aguirre-Mendoza Z, Jaramillo-Diaz N, Quizhpe-Coronel W. Arvenses asociadas a cultivos y pastizales del Ecuador. Universidad Nacional de Loja. Ecuador. 2019. https://unl.edu.ec/sites/default/files/archivo/2019-12/ARVENSES%20ASOCIADOS%20A%20CULTIVOS%20Y%20PASTIZALES%20DEL%20ECUADOR_compressed.pdf.
55. Castañeda SR, Albán CJ, Gutiérrez PH, Cochachin GE, La Torre AMI. Plantas silvestres empleadas como alimento para animales en Pisha, Ancash. *Ecología Aplicada* 2014;13(2):153-168.
55. Sowa-Borowiec P, Jarecki W, Dzugan M. The effect of sowing density and different harvesting stage on yield and some forage quality characters of the white sweet clover (*Melilotus albus*). *Agriculture* 2022;12(5):575. <https://doi.org/10.3390/agriculture12050575>.
57. Quero CAR, Enríquez QJF, Miranda JL. Evaluación de especies forrajeras en América Tropical, avances o status quo. *Interciencia* 2007;32(8):566-571.
58. Mosca J. Evaluación agronómica en caracteres reproductivos de una colección de *Melilotus albus* Medik en Pergamino, Buenos Aires [tesis licenciatura]. Argentina: Universidad Nacional del Noroeste de la Provincia de Buenos Aires; 2019.
59. Waizel-Bucay J, Waizel-Haiat S, Revilla-Peñaloza F. Los productos herbolarios, la coagulación sanguínea y la cirugía otorrinolaringológica. *Otorrinolaringología* 2017;62(2):115-142.
60. Chorepsima S, Tentolouris K, Dimitroulis D, Tentolouris N. *Melilotus*: Contribution to wound healing in the diabetic foot. *J Herbal Med* 2013;3(3):81-86. <https://doi.org/10.1016/j.hermed.2013.04.005>.
61. Asres K, Eder U, Bucar F. Studies on the antiinflammatory activity of extracts and compounds from the leaves of *Melilotus elegans*. *Ethiopian Pharmaceutical J* 2000;18:15-24.
62. Pleșca-Manea L, Pârvu AE, Pârvu M, Taămaș M, Buia R, Puia M. Effects of *Melilotus officinalis* on acute inflammation. *Phyther Res* 2002;16(4):316-9. doi: 10.1002/ptr.875. PMID: 12112285.