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Research Article

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Ensifer meliloti from *Medicago denticulata* has a positive effect on the growth of *Phaseolus vulgaris* with 50% NH_4NO_3 avoid N_2O releasing

Abstract

Healthy growth of *Phaseolus vulgaris*, or common bean, requires nitrogen fertilizer in the form of NH_4NO_3. Excessive application of ammonium nitrate causes rapid loss of soil organic matter, leading to decreased fertility, non-uptake NH_4NO_3 also contaminated surface water and aquifers, and generates nitrous oxide (N_2O), a greenhouse gas that contributes to global warming. An alternative is to inoculate the seed with infective and effective *Rhizobium* spp bacteria from wild legumes such as *Medicago dendiculata*, which in its nodules contains *Rhizobium meliloti* (*Enfiser* or *Sinorhizobium meliloti*) bacteria from infective and effective groups, using a 50% dose of NH_4NO_3. Therefore, the objectives of this work were: i) to isolate *E. meliloti* from native soil effective nodules of *M. denticulata* (wild alfalfa) in Morelia, Michoacán, México; ii) to evaluate the response of different varieties of *P. vulgaris* to *E. meliloti* with 50% NH_4NO_3; and iii) to biochemically identify this type of *E. meliloti*. To this end, *E. meliloti* isolated from effective nodules of *M. denticulata* was inoculated into *P. vulgaris* sown in the Leonard Jar hydroponic system, based on the variables/response of biomass: fresh weight, dry weight, and phenology: plant height, root length, number and color of leaves. The results demonstrate the existence of a native population of *E. meliloti* in nodules of *M. denticulata* that is infective and effective for all varieties of *P. vulgaris*, proving that these *E. meliloti* isolates are indeed infective and effective for the healthy growth of *P. vulgaris* with 50% NH_4NO_3. The biochemical identification of *E. meliloti* isolates supports their membership in a cross-inoculation group. *E. meliloti* is a positive alternative for reducing and optimizing NH_4NO_3 at 50% for the healthy growth of *P. vulgaris*, preventing the loss of organic matter, the decrease in soil fertility, surface water contamination, and specifically the generation of N_2O, thus mitigating global warming in the agricultural production of *P. vulgaris*.

Keywords: soil, wild legume, nodule, promiscuity, *Enfiser* or *Sinorhizobium,* competition, nitrogen fertilizer, global warming.

Volume 14 Issue 1 - 2026

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Received: January 16, 2026 | **Published:** February 2, 2026

Introduction

The high percentage of Mexican soils deficient in mineral nitrogen is a limiting factor for agricultural productivity, especially for *Phaseolus vulgaris*.[1-3] Therefore, nitrogen fertilizer such as NH_4NO_3 is applied to *P. vulgaris* cropping as other domestic legumes.[2,3] A beneficial alternative is to reduce and optimize the applied NH_4NO_3 by selecting *Rhizobium meliloti* or *Enfiser* (*Sinorhizobium) meliloti* isolates from the cross-inoculation group with high infectivity and effectiveness in inducing a symbiosis that allows for maximum NH_4NO_3 uptake[4-6] *Enfiser* isolated from wild legumes such as *Medicago dendiculat*a are an option because this legumes are adapted to diverse and adverse nutritional and environmental conditions that hinder the healthy growth of *P. vulgaris*.[7-9] Inoculation of *P. vulgaris* seeds with this uniques types of *Rhizobium* prevents soil productivity loss and contamination of surface and groundwater due to excess NH_4NO_3,[8,10] which is not uptaken by the roots of *P. vulgaris*. This leads to the generation of N_2O, a greenhouse gas responsible for global warming, which causes other environmental problems that critically affect human life.[6,11,12] In these problems, plant-associated microorganisms, especially beneficial ones, are fundamental for short- and long-term solutions in agricultural and urban áreas.[10,13,14] Therefore, the objectives of this work were: i) to isolate *E. meliloti* from native soil effective nodules of *M. denticulata* (wild alfalfa) in Morelia, Michoacán, México; ii) to evaluate the positive response of different varieties of *P. vulgaris* to *E. meliloti* with 50% NH_4NO_3; and iii) to biochemically identify this type of *E. meliloti.*

Material and methods

Origen of isolates *E. meliloti* from *M. dendiculata*

The *E. meliloti* isolates for *P. vulgaris*, from *M. denticulata* from Morelia, Mich, México; collection and transport of root nodules from plants through a directed sampling, 20 *M. denticulata* plants were selected, previously taxonomically identified, that were in a defloration state, had greater foliage and did not shown symptoms and/or signs of any disease; from those the complete root systems with evidence of active nodules were extracted.[4-6] The nodules with part of the root or culm were collected and wrapped in aluminum foil and placed in bottles to be transported.[1,3]

Isolation and phenotypic identification of *Ensifer spp*. The collected nodules were rehydrated to be later disinfected with 70% (v/v) ethanol and 1% (v/v) sodium hypochlorite. An active nodule was selected and crushed under aseptic conditions, to then be cultured by streaking on yeast extract mannitol agar (YEMA) with Congo red.[10,15] The cultures were incubated in the dark at 28°C for 7 days. The typical colonies: circular, convex, opaque, sometimes translucent and mucilaginous of the cultures that grew, were seeded again in YEMA with Congo red, Gram staining was performed to check the

purity of Gram-negative cells.[3,5,7] Biochemical tests described in Bergey's Manual of Determinative Bacteriology such as; catalase, oxidase, glucose, as well as characteristics such as mobility, growth in the Luria Bertani medium and in 2% mannitol (YEMB) yeast liquid medium and 2% NaCl.[15] In addition, antibiotic resistance against nalidixic acid was evaluated.[3,15] From root nodules of 50 *M. dendiculata* plants (Fabaceae) collected in agricultural areas of Morelia, Michoacán México, were selected 10 different isolates of the *Ensifer* genus that were, identified as *E. meliloti* whose differential phenotypic characteristics are described in Table 1 according to the macro and micromorphological characteristics observed, bacterial cultures isolated from *M. dendiculata* nodules; the presence in this legume is due to symbiotic interaction and the formation of nodules in the root system due to the specificity of the *Nod* and *Nif* factors secreted by *E. meliloti* in response to the flavonoids released by the plant.[3,5,8] Besides what was observed agrees with what is described in Bergey's Manual of Determinative Bacteriology, this genus as short Gram-negative rods, that were, seeded yeast extract mannitol agar (YEMA) with Congo red, form circular, convex, opaque, sometimes translucent, mucilaginous colonies that do not absorb Congo red, a distinctive characteristic. from the Rhizobiaceae family;[15-20] both isolates were assigned as a M-1 and M-2, were cultivated in; Congo red yeast extract mannitol agar (CRYEMA) whose composition was g/L: K_2HPO_4, 0.1; $MgSO_4$, 0.4; KCl, 0.02; mannitol, 2.0; yeast extract, 2.0; agar, 4.0; and Congo Red 2.0ml at pH 7.0 for 200mL of water. The response variables considered in *P. vulgaris* were: germination percentage, number and color of the nodules, fresh and dry total weight, plant height, root length, number of leaves, leaf color and the same for the relative and absolute control. For the isolation and purification of *E. meliloti*, successive reseedings were carried out in CRYEMA. *E. meliloti* in *P. vulgaris* seeds were washed with 96% alcohol/3 min, then with sterile water for 6-8 min, then with 0.1% (w/v) $HgCL_2$/min, with sterile water for 6 -8 times and nodules were left to rest for 30 min in sterile water, this nodules were sown in Leonard jars[13,15,17] unsterilized soil at 121ºC/2h. *P.vulgaris* were inoculated with 1mL of a 24h culture of *E. meliloti,* to check their infectivity and effectiveness the jars were covered with paper to avoid light when the seeds germinated, then were covered with sterile sawdust, that prevents external microbial contamination in the *P. vulgaris* (8-|0). Mineral solution for feeding the *P. vulgaris*, White's mineral solution was used, whose composition was (g/L): NH_4NO_3, 5.0; K_2HPO_4, 2.0; KH_2PO_4, 2.0; $MgSO_4$, 2.5; KCl, 1.91 dissolved into 1000 mL of water with a solution of microelements: $FeSO_4$, 0.1; $NaMoO_4$, 0.01; $MnSO_4$, 0.01; $CuSO_4$, 0.01; which was added in a proportion of 1mL/L of White mineral solution[2,4,5,13] Biochemical identification of *E. meliloti*. The identification of *E. meliloti* from *M. dendiculata* was made by biochemical tests such as: gelatin hydrolysis, casein, citrate utilization, 3-ketolactose formation, H_2S production, Congo red absorption, growth in Glucose-Peptone-Agar, growth in ºC pH 4.5, 9.5 and with 2% NaCl.[16,17] For the *P. vulgaris* used as relative control, 15 Leonard jars sown with 5 seeds uninoculated with *E. meliloti* from *M. dendiculata* were used, that were fed with a complete White solution, with 100% NH_4NO_3. *P. vulgaris* irrigates and fed only with water was used as absolute control; were used 15 Leonard jars with 5 seeds for each Jar, uninoculated with *E. meliloti*. The third treatment was with 15 Leonard jars sown with 3 seeds that were inoculated with *E. meliloti* in *P. vulgaris* fed with a white solution at 50% of the NH_4NO_3 recommended for this legume.[16-19] The experimental design that was used and measured the response variables: percent and day to germination, biomass: total fresh and dry weight of aerial and radical part of the plant and phenology: plant height, radical length, number of leaves, color of leaves Tuckey's statistical test was applied to verify similarities and/or differences between treatments.[15]

Results and discussion

Table 1 shows the positive effect of *E.meliloti* isolates M-1 and M-2 recovered from *M.dendiculata* nodules on *P. vulgaris* var. *Negro* in that it was evident that this class of non-homologous native *Ensifer* caused an effect beneficial in the growth of the legume based on: the values of total dry weight of 1.2g and 46 nodules of red color, in addition to the positive response in the variables of the phenology of the plant: with a height of 41.2cm, one color dark green in the 19 leaves, a root length of 24.3 cm according to the literature[1,6,17] it is reported that in nature there is *E.meliloti* not specific to nodulate a cultivated plant and that *E.meliloti* can be useful in the inoculation of a domestic legume such as *P. vulgaris*, this *E.meliloti* is a symbiont of *M.dendiculata* that grows in Morelia soil from that competitive and effective *E.meliloti* for *P. vulgaris* var *Negro*; better than *R. etli* of commercial inoculant,[9-11] that do not have a positive effect on *P. vulgaris* production in certain regions of México such as Michoacán, State that is why we investigate, isolated and selected native *E.meliloti,* Morelia from nodules of a legume genetically related to *P. vulgaris* such as wild *M. dendiculata* since the M-1 and M-2 isolates that were competitive in nodulating *P. vulgaris* var *Negro* and effective in fixing nitrogen (N_2) shown in the results of each inoculation experiment.[6,9-13] In common *P.vulgaris* there is a problem that few *E. meliloti* are infective and effective in supporting their healthy growth and profitable yield, hence the alternative was to select a *E. meliloti* from *M.dendiculata*, that positively and effectively infected the roots of *P. vulgaris* to fix atmospheric N_2,*M.dendiculata* was chosen because it grows successfully in various soils of Morelia, despite the lack of enough mineral nitrogen,[5,8,11,12] this induces the existence of a native and promiscuous *Rhizobium* or *Enfiser* spp that, as part of their adaptation to the environment, live in a symbiotic association with *M.dendiculata*, which is useful to be used as a inoculant for enhance health growth of *P.vulgaris* var *Negro*, since according to experimental data *E. meliloti* from *M. dendiculata* had no problem for nodulate the roots of *P. vulgaris* and exert a positive effect on their growth, which is supported by the evidence[13,14] shown in Table 1.

Table 1 Effect of *Ensifer meliloti* M-1 and M-2 from *Medicago denticulata* on *Phaseolus vulgaris* var *Bayo* at 50% NH_4NO_3 at flowering stage

*Parameter/Isolated *E. meliloti*	Total, fresh weight (g)	Total, dry weight(g)	Plant height (cm)	Radical length (cm)	Leaves number	Leaves color	Nodule number	Nodule color
M-1 NH_4NO_3 at 50%	19.7c**	1.07a	38.1b	22.1a	17a	Dark green	15b	Dark brown
M-2 NH_4NO_3 at 50%	22.9a	2.1a	41.2a	24.3a	19a	Dark green	46a	red
Relative control NH_4NO_3 at 100%	20.2b	1.1b	41.1a	20.0b	17a	Dark green	-	-
Absolute control irrigated water	13d	0.7c	19.1b	16.2c	12b	Light green	7c	white

*The average values of the variables in each experiment were: n= 15**The same letters with no difference according to Tukey (P<0.05).

In general, the two isolates of *E. meliloti* caused a positive action similar to or greater than that observed in *P. vulgaris* var *Negro*, fertilized with the complete dose of NH_4NO_3 in the mineral solution in balance, uninoculated *P. vulgaris* was used as a relative control.[13,15] This indicates that in Morelia soils there is a population of highly infective and effective *E. meliloti* in *M. sativa* nodules that are used to benefit a domestic legume such as *P. vulgaris* var *Negro*.[17-22] This represents a high potential opportunity for *P. vulgaris* inoculation at reduced doses of NH_4NO_3 without affecting its healthily growth.[2-4] It was evident that there was a difference in the infectivity and effectiveness of the isolates of *E. meliloti* M-1 and M-2 symbionts of *M. dendiculta* in *P. vulgaris*, except when both were inoculated, for this reason it is advisable to select a *Ensifer* from an alternative legume such as *M. dendiculata* that grows on virgin and/or agricultural soil in Morelia.[4,6,9,10] When *E. meliloti* of *P. vulgaris* does not work in its counterpart as in the case of the *P. vulgaris* var *Negro*.[20,22] The evidence shown in Table 1 derived from a correct selection of *E.meliloti* from *M.dendiculata* would solve the problem of sustainable *P.vulgaris* production, avoiding the excessive application of NH_4NO_3 or other nitrogen fertilizers,[4,5,13,14] the above supports why *E.meliloti* M-2 was effective in *P. vulgaris* var *Negro*, causing a dry weight of 2.1g, in contrast to M-1, that caused a dry weight of 1.07g; both values were statistically higher than the same variable in the *P. vulgaris* used as relative control and that was fed with a mineral solution with the recommended NH_4NO_3 concentration. While the *P. vulgaris* used as relative control showed statistically different and lower dry weight and phenology values: height, root length, number and color of the leaves, to the *P. vulgaris* with *E. meliloti* M-2 compared to the *P. vulgaris* that was not it was inoculated with *E. meliloti* from *M. dendiculata*.[3-6] The nodules on its roots were not effective, which proves that this class of *E. meliloti* in the soil of Morelia, is not effective in fixing N_2.[11,13,16] This fact supports the obligatory selection of native *E. meliloti* from an alternative legume to inoculate common *P. vulgaris*.[8-12] Under this premise, *E. meliloti* was isolated from *M. dendiculata* for *P. vulgaris* since this class of *E. meliloti* from another legume may be compatible with *P. vulgaris*.[10,18-21]

The analysis of the type of native *E. meliloti* nodule from Morelia soil showed that M-2 was competitive and effective due to the red color in the roots of the *P. vulgaris* var *Negro*, that confirms the presence of leghemoglobin and ensured vigorous plant growth that was dependent on biological N_2 fixation at the recommended 50% NH_4NO_3 dose.[16,18,22] These data suggest that this *E. meliloti* meets the characteristics to be used as an inoculant for *P. vulgaris* because it is competitive with the native microbiota of Morelia soils,[23-27] in the same way the positive effect of *E. meliloti* M-1 was observed. in *P. vulgaris* it was observed in the dry weight value with 1.07g, statistically higher than the value of *P. vulgaris* uninoculated, fertilized with a mineral solution with the NH_4N0_3 recommended for this crop and used as a relative control.[23,28,29] In the phenology of *P. vulgaris* inoculated with M-1, a favorable effect was also detected with a height of 38.1cm, a root length of 22.1cm, with 15 dark brown nodules that indicated the presence of leghemoglobin, being effective in fixing N_2.[11,12,25,28] For this reason, the selection of *E. meliloti* isolates is necessary to achieve a favorable response in *P. vulgaris* at reduced doses NH_4NO_3 (22-26), in contrast to *P. vulgaris* uninoculated with *E. meliloti* fed with NH_4NO_3 recommend dose registered as relative control, either with poor mineral feeding the plant suffered from nutritional stress, consequently[4,8,15,23] it had an abnormal development registered in this *P. vulgaris* used a as absolute control.

Table 2 shows the effect of *E. meliloti* M-1 and M-2 of *M. dendiculata* on *P. vulgaris* var *Peruvian* growth, in this trial the infectivity and effectiveness of this type of *E. meliloti* was verified spp, that supports a way to solve the problem of N_2 fixation effectiveness between *P. vulgaris* and its symbiosis with *E. meliloti*.[25-28] It is the alternative use of a *M. dendiculata* as a source of *E. meliloti* isolation for common *P. vulgaris*, thus the combination of *E. meliloti* M-1 and M-2 established a positive relationship with this legume.[3,9,10,13] The results of the inoculation effect of *E.meliloti* on the plant showed that *E. meliloti* M-2 caused an infection and was effective in fixing N_2 since the *P. vulgaris* reached the maximum value in dry weight with 1.7g with a fresh weight of 29.9g.[3,10,12,15] While *E. meliloti* M-1 caused a dry weight of 0.72g, a fresh one of 18.8g, in contrast to the phenology of the *P.vulgaris* inoculated with M-2, that produced in the plant the values that reflect a favorable action of *E. meliloti* with a height of 36.1cm 21.4 cm of root length, a green color in the leaves, 46 red nodules that supports that it is a competitive, positive and effective *E. meliloti* in fixing N_2, that ensured the healthy growth of *P. vulgaris*.[14,15,17,22]

Table 2 Effect of *Ensifer meliloti* M-1 and M-2 from *Medicago denticulata* on *P. vulgaris* var *Peruvian* at 50% NH_4NO_3 at flowering stage

*Parameter/Isolated *E. meliloti*	Total, fresh weight (g)	Total, dry weight(g)	Plant height (cm)	Radical length (cm)	Leaves number	Leaves color	Nodule number	Nodule color
M-1 NH_4NO_3 at 50%	18.8^c	0.72^b	34.8^c	19.8^b	17^b	Dark green	15^b	Dark brown
M-2 NH_4NO_3 at 50%	21.9^a	1.7^a	36.1^b	21.4^a	19^a	Dark green	46^a	red
Relative control NH_4NO_3 at 100%	19.0^c	0.86^b	37.0^a	17.1^c	17^b	Dark green	-	-
Absolute control irrigated water	9.5^b	0.3^c	17.0^d	13.5^d	12^c	Light green	7^c	white

*The average values of the variables in each experiment were: n= 15

**The same letters have no difference according to Tukey ($P < 0.05$).

Table 3 shows the positive effect of *E.meliloti* isolates M-1 and M-2 on *P. vulgaris* var. *Negro* in that it was registered that *E.meliloti* isolates exerted the best favorable effect on the growth of the *P. vulgaris* especially *E.meliloti* M-2 that caused the maximum increase in the dry weight with 2.0 g, of the fresh one with 22.5g, there the maximum number of 46 red nodules were detected in the roots of *P. vulgaris*, consistent with the phenology of this legume of a healthy and vigorous plant[20,22,23] *E.meliloti* M-2 caused 39.9cm in height, a root length of 24.3cm, with a maximum number of 19 dark green leaves, the foregoing supports that *M.dendiculata* is a source of *E.meliloti* infective positive and effective for common *P. vulgaris* that supports the idea that in nature there are *E.meliloti* of *M.dendiculata* that can be selected,[22-25] to be inoculated in domestic plants such as *P.vulgaris* that do not respond positively to inoculation with *E.meliloti* obtained from varieties of the same legume according to what the literature points out[17,21,26] and reaffirms the option of selecting *M.dendiculata*; *E.meliloti* infective and effective in the preparation of inoculants for legumes in agricultural areas where the *P. vulgaris*

response to inoculation is negative as reported in the literature.[27-29] While *E. meliloti* M-1 from *M. dendiculata* nodules also caused a positive effect on the dry weight of *P. vulgaris* of 1.1g, from 19.7g fresh, with 15 dark brown root nodules, that explain why the plant it showed a healthy appearance with a dark green color in its leaves, with a height of 39.6cm and a root length of 21.7cm.[22,24,28] In general, this type of *P. vulgaris* response is only observed when it is inoculated with highly infective and effective *E. meliloti.*[20,23] In the biological fixation of N_2 it is considered that after the selection and inoculation of *M.dendiculata R. etli* in *P. vulgaris*, those that establish a positive relationship with legumes were isolated, as a strategy to solve the problem of the lack of positive response in cultivars to *P. vulgaris* varieties with *E.meliloti* from Morelia soils, based on the literature in this fact,[10-15] this reports that it is possible to take advantage of the genetic compatibility between the genera of legume families, to establish a symbiotic relationship with their hosts that nodulate and cause a beneficial effect on the biological fixation of N_2 for a specific type of legume,[16-26] with respect to *P. vulgaris* that was used as relative control where a total dry weight of *P.vulgaris* was observed equivalent to the positive effect of M-2 according to the statistical analysis there was no statically difference between the response of both legumes, that implies that the inoculation of *P. vulgaris* with *E.meliloti* from *M.dendiculata* since its effect was similar to that of *P. vulgaris* treated with the recommended NO_4NO_3 dose for healthy growth[20,22,24] used as relative control and that inoculated with *E.meliloti*, that showed a dry weight of 1.3g, a fresh one of 20.1g, a height of 40cm, a root length of 20.3cm and 17 dark green leaves,[28-30] in evident contrast with the poor response and health of *P. vulgaris* used as relative control uninoculated with *E. meliloti* fed with complete mineral solution with recommend dose of NH_4NO_3.[1-3]

Table 3 Effect of *Ensifer meliloti* M-1 and M-2 from *M. denticulata* on *Phaseolus vulgaris* var *Negro* at 50% NH4NO3 at flowering stage

*Parameter/Isolated *E. meliloti*	Total, fresh weight (g)	Total, dry weight(g)	Plant height (cm)	Radical length (cm)	Leaves number	Leaves color	Nodule number	Nodule color
M-1 NH_4NO_3 at 50%	19.7c**	1.1^b	39.6^b	21.7^b	17^b	Dark green	15^b	Dark brown
M-2 NH_4NO_3 at 50%	22.5^a	2.0^a	39.9^a	24.3^a	19^a	Dark green	46^a	red
Relative control NH_4NO_3 at 100%	20.1^b	1.3^b	40.0^a	20.3^c	17^b	Dark green	-	-
Absolute control irrigated water	12.5^d	0.9^c	19.1^b	16.2^d	12^c	Light green	7^c	white

*The average values of the variables in each experiment were: n= 15

**The same letters with no difference according to Tukey ($P < 0.05$).

Table 4 shows the effect of both *E. meliloti* isolated M-1 and M-2 from *M. dendiculata*, on *P. vulgaris* var *Pinto* that registered that M-2 caused an increase in the dry weight of *P. vulgaris* with 2.7g per day. the same as the increase in the fresh one of 24.3g, in agreement with the number of nodules detected in the roots of 46 of red color that indicates that nodules were positive and effective to fix N_2 given the type of native *E. meliloti*.[6,15,22,24] In addition, in response of *P. vulgaris* reached a length root 27.3cm, with 19 dark green leaves characteristic of a legume infected by a *E. meliloti* that fixed N_2, consequently of *P. vulgaris* showed healthy growth. *E. meliloti* M-1 caused a positive effect on this *P. vulgaris,* with a statistically increase in the dry weight of 1.4g a fresh one of 20.7g, with 15 dark brown nodules, an evident sign that *E. meliloti* originates from *M.dendiculata* that had no problem infecting and fixing N_2, in *P. vulgaris* var *Pinto* that supports that this is a *E. meliloti* that belongs to a group of cross-inoculation, because legumes contain organic compounds in their roots that serve as recognition for, interacting with different species of *Rhizobium* or *Enfiser* in the cross-inoculation group, between *P. vulgaris* and wild legumes,[1,3,7,10,13] which the literature indicates has the genes to interact in symbiosis, with more than one genus and species of legumes[25-29] favorable, that represents an alternative for when a commercial inoculant based on *R. etli* does not work with common *P. vulgaris.*[10,13-15] Regarding the *P. vulgaris* used as a relative control, it was registered that it reached a dry weight of 2.3g, a fresh one of 21.6g, a height of 43.7cm and a root length of 22.8cm, with 17 intense green leaves, values equivalent to the positive response of *P. vulgaris* with *E. meliloti* isolated from *P. vulgaris* such as *M. dendiculata* as reported in the literature.[2,4,16,20,25]

Table 4 Effect of *Ensifer meliloti* M-1 and M-2 from *Medicago denticulata* on *Phaseolus vulgaris* var *Pinto* at 50% NH_4NO_3 at flowering stage

*Parameter/Isolated *E. meliloti*	Total, fresh weight (g)	Total, dry weight(g)	Plant height (cm)	Radical length (cm)	Leaves number	Leaves color	Nodule number	Nodule color
M-1 NH_4NO_3 at 50%	20.7c**	1.4^c	45.3^a	24.8^b	17^b	Dark green	15^b	Dark brown
M-2 NH_4NO_3 at 50%	24.3^a	2.7^a	44.3^b	27.3^a	19^a	Dark green	46^a	red
Relative control NH_4NO_3 at 100%	21.6^b	2.3^b	43.7^c	22.8^c	17^b	Dark green	-	-
Absolute control irrigated water	17.0^d	1.5^c	21.4^d	19.1^d	12^c	Light green	7^c	white

*The average values of the variables in each experiment were: n= 15

**The same letters with no difference according to Tukey ($P < 0.05$).

Table 5 shows the effect of co-inoculation of *E. meliloti* M-1 and M-2 from *M. denticulat*a on *P. vulgaris* var *Bayo*, was registered that the mixture of *E. meliloti* in a 2:1 ratio it had a positive effect that was reflected in the dry weight of *P. vulgaris* var *Bayo* with 1.2g, value that was higher than the response of the same *P. vulgaris* with a 1:2 ratio. While the effect of *E.meliloti* on *P. vulgaris* with a 1:1 ratio was a dry weight of 0.97g, in contrast with 0.84g of dry weight with a 2:1 ratio, statistically higher than the mixture of M-1 and M-2, ratio 1: 1

that generated a dry weight of 0.97g, in contrast to the dry weight of 0.84g of *P. vulgaris* with *E.meliloti* ratio 1:2, while the 2:1 of The dry weight of the *P. vulgaris* was 0.84g, the lowest weight registered in *P. vulgaris* when combining the two isolates of *E.meliloti*. Regarding the number of nodules, the 1:1 ratio of *E. meliloti* caused the maximum number of nodules in *P. vulgaris* with 33, statistically higher than the 25 nodules in *P. vulgaris* with a 2:1 ratio of *E. meliloti.* In contrast to the statistically low value with 19 *P. vulgaris* root nodules, registered with a 1:2 out of 19 ratios, all legume nodules had a red color indicating the activity of leghemoglobin for N_2 fixation causing a positive effect on the growth of *P. vulgaris*.[5,10,11,22-25] Regardless of the proportion of the two isolates of *E.meliloti* from *M. dendiculata*, a positive response of the *P. vulgaris* was observed, in comparison to *P. vulgaris* used as absolute control, with a dry weight of 0.75g, the lowest registered in the experiment, as well as than other response variables in *P. vulgaris*: total fresh weight of 17.6g, a height of 20.8cm, root length of 18.3cm and a number of leaves of 17, light green in color, evidence of a nutritional deficiency associated with nitrogen, and iron,[2,13,20,23] while the 13 white nodules observed in the *P. vulgaris* root were the product of an infective but ineffective *E.meliloti*[15,22,29] In contrast, *P. vulgaris* used as a relative control reached a dry weight statistically similar to that registered in the *P. vulgaris* with the mixture of *E. meliloti* isolates in a 2:1 ratio with 1.1g of weight dry; the same as in the fresh weight of 19g, with a height of 36.5cm and a root length of 20.9cm, with leaves of an intense green color and without no nodule.[28,29] These results indicate that the density of infective and effective *E. meliloti* was fundamental in the positive effect on *P. vulgaris*, specifically with the 2:1 ratio. These properties of *E. meliloti* from *M. dendiculata* are key in the inoculation of legumes of nutritional interest and /or economical like *P. vulgaris.*[14-16]

Table 5 Effect of the combination of isolates from *E. meliloti* of *M. dendiculata* on the growth of *P. vulgaris* var. *Bayo* at flowering stage

Dose combination of the two isolates of *E. meliloti*/*P. vulgaris*	Total, fresh weight (g)	Total, dry weight(g)	Plant height (cm)	Radical length (cm)	Leaves number	Leaves color	Nodule number	Nodule color
M-1 (1ml). M-2 (1ml). NH_4NO_3 at 50%	19.0a**	0.84c	37.3a	20.3c	21a	Green dark	19c	Dark brown
M-1 (1ml). M-2 (1ml). NH_4NO_3 at 50%	19.1a	0.97b	35.9c	22.1a	22a	Green dark	33a	Dark brown
M-1 (1ml). M-2 (1ml). NH_4NO_3 at 50%	18.7c	1.2a	33.2d	20.1c	19b	Green dark	25b	red
Relative control NH4NO3 at 100%	19.2b	1.1a	36.5b	20.9b	21a	Green dark	-	-
Absolute control irrigated water	17.6d	0.70d	20.8e	18.3d	17c	Green Light	13d	white

*The average values of the variables in each experiment were: n= 15

**The same letters with no difference according to Tukey (P < 0.05).

Table 6 shows the effect of *E. meliloti* of *M. dendiculta* on *P. vulgaris* var *Peruvian* where e 2:1 ratio was a positive effect in the dry weight of the legume with 1.4g, compared to *P. vulgaris* used as relative control with 1.6g of dry weight followed by the *P. vulgaris* with the mixture of *E.meliloti*, ratio 1:1 with 1.0g dry weight; then *P. vulgaris* with the same combination with a 1:2 ratio with a dry weight of 1.2g, higher than the dry weight of the *P. vulgaris* used as absolute control, that reached a dry weight of 0.8g. In all three cases, the combination of *E. meliloti* on *P. vulgaris* registered that the nodules formed were infective and effective, compared to those observed in *P. vulgaris* used as relative control this fact demonstrates that the natural beneficial activity of *Rhizobium* or *Enfiser* ensures optimal uptake of NH_4NO_3 at 50% compared, to *P. vulgaris* with NH_4NO_3 at 100% not inoculated with *Rhizobium* from wild legume.[3,5,10,13] In the three combinations of M-1 and M-2 in a 1:2 ratio; 1:1 and 2:1 infective and effective nodules were detected inside of nodules of *M. dendiculata,*[16,17] compared to *R. etli* with nodulation capacity but ineffective at fixing N_2, found in the *P. vulgaris* used as absolute control, that means that the *E. meliloti* selection worked and was viable and effective strategy for the inoculation of *P. vulgaris var. Peruvian* preserving organic matter, soil fertility and preventing N_2O releasing due to not uptake NH_4NO_3 by the root system of the *P.vulgaris.*[3,4,10,13]

Table 6 Effect of *Ensifer meliloti* M-1 and M-2 isolated from *M. denticulata* on *P. vulgaris* var *Peruvian* at flowering stage

Dose combination of the two isolates of *E. meliloti*/*P. vulgaris*	Total, fresh weight (g)	Total, dry weight(g)	Plant height (cm)	Radical length (cm)	Leaves number	Leaves color	Nodule number	Nodule color
M-1 (1ml). M-2 (1ml). NH_4NO_3 at 50%	19.3b**	1.2c	39.4a	22.8c	21b	Green dark	19c	Dark brown
M-1 (1ml). M-2 (1ml). NH_4NO_3 at 50%	19.6a	1.0d	39.0a	22.6c	20b	Green dark	33a	Dark brown
M-1 (1ml). M-2 (1ml). NH_4NO_3 at 50%	20a	1.4b	38.1b	25.1a	23a	Green dark	25b	Red
Relative control NH4NO3 at 100%	19.1c	1.6a	36.9c	23.2b	22a	-	-	-
Absolute control irrigated water	18.2d	0.8e	23.2d	20.0d	19c	Green Light	13d	white

*The average values of the variables in each experiment were: n= 15

**The same letters with no difference according to Tukey (P < 0.05).

Table 7 shows the effect of *E. meliloti* M-1 and M-2 on *P. vulgaris* var *Negro* where it was observed that depending on the relationship of both isolates, variable results were registered in terms of dry weight, in the number of nodules and in the color of the leaves, the combination of M-1 and M-2 of *E. meliloti*, in a 1:2 ratio, caused an increase in the dry weight of the *P. vulgaris* with 2.1g with 19 effective nodules; while the same 1:1 combination of *E. meliloti* in *P. vulgaris* var Negro caused a dry weight of 1.9g with 33 effective and positive nodules for N_2 fixation, this result in *P.vulgaris* was better than that generated by the *E. meliloti* 1 ratio 1:2 that caused a positive effect on *P.vulgaris*

var *Negro*, although less so when *E. meliloti* M-2 was inoculated in the same proportion with M-1, that was not infective and effective to improve the growth of *P. vulgaris* var *Negro*.[3,9,23,26] According to the literature, it is reported that between there are some *E. meliloti* species that are more than competitive, to colonize the roots of the genetically related *P. vulgaris* with *M. denticulata;* causing a positive effect on *P. vulgaris* var *Negro* where is inoculated,[6,10,11,13] while with the 2:1 ratio of *E. meliloti* M-1 and M -2, an effect similar to the 1:2 ratio was registered with a dry weight of 2.0g, with 25 nodules, that indicates that the selection of *E. meliloti,* due inoculation with a heterologous legume such as *M.dendiculata* on *P.vulgaris* confirms, that promiscuity of *E. meliloti* among domestic or wild legumes,[15,20,23,25] as a positive fact therefore the percentage of nodules in the host plant, is an ecological advantage for both since, it allows them a greater probability of survival, with successful reproduction in the roots of *P.vulgaris* and then to invade inside to cause effective nodules to fix N_2 with NH_4NO_3 at 50%, to ensure maximum uptake of NH_4NO_3, preventing loss of soil productivity, contamination of surface and groundwater, and reducing N_2O generation, which mitigates global warming.[10,12,14,23]

Table 7 Effect of *E. meliloti* M-1 and M-2 of *M. denticulata* on *P. vulgaris* var *Negro* at flowering stage

Dose combination of the two isolates of *E. meliloti*/*P. vulgaris*	Total, fresh weight (g)	Total, dry weight(g)	Plant height (cm)	Radical length (cm)	Leaves number	Leaves color	Nodule number	Nodule color
M-1 (1ml). M-2 (1ml). NH_4NO_3 at 50%	21.7a**	2.1b	40.0b	28.5c	22b	Green dark	19c	Dark brown
M-1 (1ml). M-2 (1ml). NH_4NO_3 at 50%	21.5a	1.9c	40.8b	29.1b	22b	Green dark	33a	Dark brown
M-1 (1ml). M-2 (1ml). NH_4NO_3 at 50%	20.9b	2.0b	44.3a	30.0a	24a	Green dark	25b	Red
Relative control Uninoculated NH4NO3 at 100%	21.2a	2.6a	41.1b	28.9c	23a	-	-	-
Absolute control irrigated water	19.7c	1.2d	26.1c	22.3d	20c	Green Light	13d	white

*The average values of the variables in each experiment were: n= 15

**The same letters with no difference according to Tukey ($P < 0.05$).

Table 8 Biochemical profile of *Ensifer meliloti* M-1 and M-2 of *M. denticulata* nodules.

Table 8 Biochemical profile of *Ensifer meliloti* M-1 and M-2 of *M. denticulata* nodules from soil of Morelia, Michoacán, México

Biochemical test	Isolated M-1	Isolated M-2	*Ensifer meliloti* reference strain
Gelatin hydrolisis	-	-	-
Casein hydrolysis	-	+	-
Formation of 3 Ketolactose	-	-	-
H_2S production	-	-	-
Congo red absorption yeast extract mannitol agar (YEMA)	+	+	-
Growth in glucose peptone agar	+	+	+
Reaction on yeast extract mannitol bromothymol blue agar (YEMBBA)	-	+	-
2% growth of NaCl.	+	+	+
Growth in YEMA at pH 4.5	+	+	+
Growth in YEMA at pH 9.5	+	+	+

All results are the average of 3 repetitions, (+) positive response (-) negative response.

Table 8 shows the biochemical profile of *E. meliloti* isolated from *M. dendiculata*, indicating that these are *Rhizobium* strains biochemically associated with a type species of *E. meliloti*, with the exception of Congo red absorption.[16,19] In general, most of the responses of the two isolates M-1 and M-2 corresponding to a type of cross-inoculation *Rhizobium*, which supports why these isolates were positive and effective in nodulating the varieties of *P. vulgaris* tested. The differences in the profile indicate between M-1 and M-2, the genetic diversity generated when these isolates interact inside and outside, the nodule in the roots of the legumes as *P. vulgaris* different varieties, with which both of them are positively associated as well as *M. dendiculta* and also with other legumes.[21,25]

Table 9 Sensitivity pattern of *E. meliloti* M-1 and M-2 of *M. denticulata*.

Table 9 Sensitivity pattern of *E. meliloti* M-1 and M-2 of *M. denticulata*, from soil of Morelia, Michoacán, México

Antibiotic	M-1	M-2
Amikacin	-	-
Ampicillin	+	+
Chloramphenicol	+	-
Carbencillin	-	+
Cephalotonin	+	+
Cef-triaxone	-	+
Cefotaxime	-	+
Nitrofurantoin	-	-
Netilmicin	-	-
Pefloxacin	+	-
Gentamicin	-	-
Sulfamethoxazol	-	+

(-) =no growth (+) = growth, average 3 repeats of antibiotic sensitivity of *E. meliloti.*

Table 9 shows the sensitivity profile of the two *E. meliloti* isolates recovered from *M. dendiculata* that were positive, effective and infective in the different varieties of *P. vulgaris.* The fact that both isolates have the capacity to exchange genetic information in the roots and nodules of the legumes both M-1 and M-2, infect allows them to have varying degrees of resistance to a wide variety of antibiotics.[19,23,26] Consequently, it was found that these isolates are not exactly the same, which also explain the difference between them in positively affecting the growth of the *P. vulgaris* varieties.

Conclusion

This research demonstrates the potential of *M. dendiculata* as an important source of *Rhizobium genera* and species of the *Enfiser* type for the inoculation of *P. vulgaris*, a useful option in situations were *R. etli* is not infective and effective at a 50% dose of NH_4NO_3. This is especially true due to the adaptation of both *E. meliloti*, which are resistant to adverse environmental conditions where *R. etli,* does not survive long enough to be infective and effective. Additionally, other genera of plant growth promoters were isolated that could be useful for non-leguminous plants, making *M. dendiculata* an excellent source of plant growth-promoting microorganisms. Ongoing research could contribute further knowledge on this topic.

Acknowledgements

To the Coordinación de Investigación Científica de la UMSNH "Aislamiento y selección de microorganismos endófitos promotores de crecimiento vegetal para la agricultura y biorecuperación de suelos" from the Research Project (2026), Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México. To Phytonutrimentos de México and BIONUTRA S, A de CV, Maravatío, Michoacán, México for the *P. vulgaris* seeds and verification of greenhouse tests.

Conflicts of interest

The authors declare no conflicts of interest.

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