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Quantification of Leghemoglobin Extracted from Roots of Five Different Alfalfa (Medicago sativa) Cultivars: An Experimental Study

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ABSTRACT

Background: Leghemoglobin is an oxygen transporter and a hemoprotein found in nodule roots of nitrogen fixator plants from *Leguminosae* family. Leghemoglobin is produced in response to presence of bacteria belonging to *Rhizobia* family. Structurally, leghemoglobin is similar to hemoglobin and has a kind of heme iron structure. Study of leghemoglobin as a heme iron containing agent can open up a new way for treatment and natural prevention of iron deficiency anemia and substitution with mineral iron salts. **Methods:** Five different cultivars of alfalfa plants including Esfahani, Bammi, Yazdy, Nik shahri, and Hamedani were cultivated in gardening farm of the faculty of agriculture in Shahid Chamran University in a linear form. Cultivation was done with biological fertilizer containing *Sinorhizobium meliloti* for test groups and without fertilizer for sham groups. After 60 days, the samples were harvested, and the amount of leghemoglobin produced in nodules was quantified using LaRue and Child fluorometric method. **Results:** The results from five cultivars of alfalfa planted with biological fertilizer showed a significant increase in viscosity of leghemoglobin in nodules of the test group in comparison with sham group (P<0.0001). Moreover, the high viscosity of leghemoglobin in nodules of Bammi cultivar indicated the capacity of this plant to produce leghemoglobin in Ahvaz. **Conclusion:** According to the findings of this study, Bammi cultivar of alfalfa is a good option for future studies and even production of high amounts of leghemoglobin in Khuzestan Province.

Keywords: *Leghemoglobin; Alfalfa; Iron; Anemia; Nodule*

Introduction

Alfalfa (*Medicago sativa L.*) is a member of Leguminosae family. With approximately 32 million hectares all over the world, alfalfa is the most cultivated forage legume in the world. The cultivated varieties are synthetic cultivars, which are usually obtained through several generations of panmictic reproduction from a set of several parents (Flajoulot *et al.*, 2005). Legumes are important agricultural crops, which are characterized by root nodules formed as a result of symbiotic relationship with nitrogen-fixing rhizobia (Huhman and Sumner, 2002).

Bacteria belonging to Rhizobium, Bradyrhizobium, and Azorhizobium genera

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grow as free-living organisms in the soil; they can also live as nitrogen-fixing symbionts inside root nodule cells of plants belonging to the family of leguminosae. The symbiosis between *Rhizobium meliloti* and its host alfalfa begins when bacteria growing in the soil are attracted toward host root hairs to bind them. In root hairs, the bacteria respond to compounds secreted by the plant through transcribing nod genes, which direct the synthesis of a lipooligosaccharide signal molecule, namely the nod factor that initiates many developmental changes in the root early in the nodulation process (Andrews and Andrews, 2017, Datta *et al.*, 2015, Udvardi and Poole, 2013).

Leghemoglobin is a hemeprotein found in millimolar quantities in bacteroid-containing cells from central tissue of legume root nodules. This 16-kDa hemeprotein is an essential component for N_2 fixation by legume nodules, and it has been widely accepted that a function of leghemoglobin in nodules is to facilitate diffusion of O_2 to bacteroids. leghemoglobin is composed of a heme group (protoporphyrin IX) and a single polypeptide (globin). The amino acid sequence of the globin moiety depends on the legume species. Heme moiety is always present in ferrous state in vivo. When leghemoglobin is extracted from the nodules, a small amount of this ferrous state is oxidized to ferric state (Garrocho-Villegas *et al.*, 2007, Singh and Varma, 2017). Similar to hemoglobin, leghemoglobin is red, and there are close chemical and structure similarities between them. Leghemoglobin is believed to be a product of both plant and bacterium where the Apo protein and heme are produced by plant and bacterium, respectively (Hidayati *et al.*, 2008).

Iron in food is available in two forms: Heme iron in myoglobin of muscles and hemoglobin and non-heme iron in vegetables, cereals and meat. Heme iron accounts for only 10-20% of dietary iron in industrialized countries but can contribute a disproportionate 30% of absorbed iron because of higher bioavailability. Non-heme involves 80% of dietary iron in industrialized countries and an even greater proportion worldwide or among vegetarians (Skolmowska and Głąbska, 2019).

Iron deficiency anemia (IDA) is defined as a condition in which there are no mobilizable iron stores, and the signs of a compromised supply of iron appear in tissues (World Health Organization, 2001). Iron deficiency is one of the most common nutritional deficiencies in the world as well as a primary cause of anemia (World Health Organization, 2008). Oral iron is the first-line treatment option for IDA during pregnancy. The most frequent oral iron preparations are ferrous [Fe (II)] sulfate, ferrous fumarate, ferrous glycine sulfate, and ferrous gluconate (Toblli *et al.*, 2012). 60–120 mg doses of elemental iron per day have been widely recommended in adults, although the daily dosage of iron should be determined according to the severity of anemia. Most oral iron supplements are associated with erosive mucosal injury in upper gastrointestinal tract, nausea, vomiting, and epigastric discomfort along with other gastric adverse effects such as diarrhea or constipation. A direct relationship has been observed between the severity of adverse effects and the dose of iron. Adverse effects of this type may harmfully affect treatment compliance and possibly lead to withdrawing from treatment (Cancelo-Hidalgo *et al.*, 2013). The underlying mechanisms and the type of organisms responsible for these clinical and epidemiological observations remain unclear. Recent field studies suggest that oral iron supplementation in children augments susceptibility to bacterial infections, particularly diarrhea, changes gut microbiota, and increases the virulence of many common bacterial enteropathogens (Cross *et al.*, 2015). Oral iron supplements have been shown to disrupt microbiota with disturbances in bacterial phylotypes and associated aberrations in fecal metabolites compared with IV treatment (Mahalhal *et al.*, 2018).

Previous studies have revealed that heme iron is taken up by receptor mediated endocytosis which is distinct from non-heme iron receptor. Heme iron within a vesicle directly enters into the cytoplasm, and none of its elemental iron is released in lumen (West and Oates, 2008). Therefore, further studies on heme iron are required to use this type of iron as a supplement. As the production of herbal

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substances is highly dependent on local culture conditions, in this study, leghemoglobin as a heme iron substance produced in legume root nodules was assessed in five different cultivars of alfalfa infected by rhizobium bacteria. Our aim was to choose the best cultivar to achieve a high amount of this substance in Khuzestan Province.

Materials and Methods

Five alfalfa cultivar seeds, including Bammi, Isfahani, Nick shahri, Hamedani and Yazdi, were purchased from Pakan bazr© Company and authorized by a botanist. To determine the maximum germination potential of seeds, 30 seeds of each cultivar were placed between two layers of filter papers in a Petri dish with enough amount of moisture; they were incubated in darkness of lab cabinet for three days. After that, the number of germinated seeds were counted to ensure their health and growth ability.

A biological fertilizer containing *Rhizobium meliloti* spores in 10^8 CFU/ml concentration was prepared from Mehr Asia Biotechnology© Company, and its microbial load was authorized by nutrient agar streaking culture. After 48 hours of incubation in 37°C and suitable moisture, colonies in diluted areas were studied to ensure suitable microbial load of fertilizer.

In the last days of fall, 12 square meters of gardening farm in Faculty of Agriculture at Shahid Chamran University of Ahvaz were prepared and divided into four sections. Sections 1 to 3 were devoted to three cultivations of all five cultivars with biological fertilizer as test groups 1 to 3, and one section without any fertilizer was considered as sham group. Five deep-set lines were created with trowel in each section, the seeds were placed in lines, and fertilizer liquid was spilled on them. After covering seeds with a thin layer of soil, irrigation with tap or rain water started and continued for 60 days. After 60 days, the plants were uprooted with spade and their roots were collected.

According to "LaRue and Child" fluorometric assay (LaRue and Child, 1979), the alfalfa roots were rinsed free of vermiculite, and 100 mg nodules from each sample were weighed and ground with pestle and mortar. The obtained paste was added to 4 ml of solution containing 0.02% (w/v) potassium ferricyanide and 0.1% sodium bicarbonate. The samples were centrifuged at a speed of 3500 rpm for 10 minutes. Then, 0.4 ml of transparent supernatant was added to three screw-capped tubes, and 4 ml saturated oxalic acid were added to each of them. Two tubes were sealed and heated for 30 min at 120 °C in an autoclave and then cooled to room temperature; the third one was kept out of autoclave as the blank tube. The fluorescence of solutions was measured with a spectrophotometer equipped with a mercury-xenon lamp. The excitation wavelength was 405 nm and the emission spectrum setting was 550-650 nm. The difference in fluorescence between heated and unheated samples was proportional to heme protein concentration (LaRue and Child, 1979).

The fluorometric assay was standardized using standard bovine hemoglobin. 0.1 mg of bovine hemoglobin powder prepared from Sigma Aldrich© Company was added to 100 ml of solution containing 0.02% (w/v) potassium ferricyanide and 0.1% sodium bicarbonate to obtain 10 ppm hemoglobin stock solution. Afterwards, four solutions of stock were prepared at 0.05, 0.1, 0.15, and 0.2 ppm dilutions. The fluorescence of solutions was measured exactly like that of nodules solution (LaRue and Child, 1979).

Data analysis: Statistical analysis of sample concentrations was performed by ANOVA. Data was shown as mean ± standard deviation (SD), and $P \leq 0.05$ was considered statistically significant

Results

The maximum germination potential of all cultivar seeds was 30/30 (100%) after 3 days, which indicated their perfect health and growth capacity. The biological fertilizer streaking culture showed white and wide colonies on culture media.

All nodule fluorometric assays were standardized, and test and sham groups showed a specific emission peak at 601 nm after excitation in 405 nm wavelength. Data indicated that there was a significant difference between all test groups and sham groups ($P<0.0001$). The sham groups,

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cultivated without any biological fertilizer, had approximately 12.75-16 transmittance spectra in diluted sample which was close to blank tube with a 10 rate of transmittance.

According to **Table 1**, Bammi cultivar showed maximum transmittance and a significant difference in comparison with other cultivars (P<0.0001). There was no significant difference between Isfahani, Nik shahri and Yazdi cultivars (P>0.06). However, Hamedani cultivar demonstrated a low rate of transmittance and a specific difference in comparison with other test groups (P<0.02).

All test groups demonstrated a significant increase (P<0.0001) in comparison with sham group. Moreover, Bammi group showed a high concentration of leghemoglobin in comparison with other groups.

All test groups showed a significant increase (P<0.0001) in comparison with sham group. In addition, Bammi group showed a high concentration of leghemoglobin in comparison with other groups.

All four dilutions of standard powder for fluorometric assay of bovine hemoglobin suggested similar results in nodule samples after excitation in 405 nm wavelength and emission peak at about 601 nm. Transmittance numbers mentioned in **Table 2** were analyzed in Microsoft® Excel software to achieve linear equation and slope of standard curve.

Table 1. Transmittance numbers and leghemoglobin concentration of all the test and sham groups in 100 mg nodule samples after excitation in 405 nm wavelength and emission peak of 601 nm.

Samples / Cultivars	Hamedani	Nik shahri	Yazdi	Bammi	Isfahani
No 1	29.50	35.30	30.00	56.10	40.00
No 2	25.50	35.70	32.20	54.90	34.10
No 3	26.00	35.50	30.80	55.50	35.00
No 4	28.00	31.00	29.00	57.00	39.00
No 5	25.40	36.00	31.50	56.00	36.00
No 6	27.60	37.00	30.00	55.50	36.90
Average	27.00	35.08	30.58	55.83	37.00
Average after blank tube transmittance was subtracted	16.00	34.08	19.58	44.83	26.00
Leghemoglobin concentration in ppm	0.07	0.12	0.08	0.14	0.09
Groups without fertilizer (sham groups)					
No 1	14.12	12.00	13.00	16.09	13.00
No 2	13.92	13.50	14.00	15.95	12.50
Average	14.04	12.75	13.50	16.04	12.75
Average after blank tube transmittance was subtracted	3.0	2.5	3.5	5.0	3.0
Leghemoglobin concentration in ppm	0.037	0.036	0.038	0.042	0.037

Table 2. Transmittance numbers of all standard dilutions after excitation in 405 nm wavelength and emission peak of 601 nm

Standard concentrations (ppm)	Transmittance	Transmittance after blank tube was subtracted
0.05	19.00	5.00
0.10	43.45	29.45
0.15	60.15	46.15
0.20	76.48	62.48

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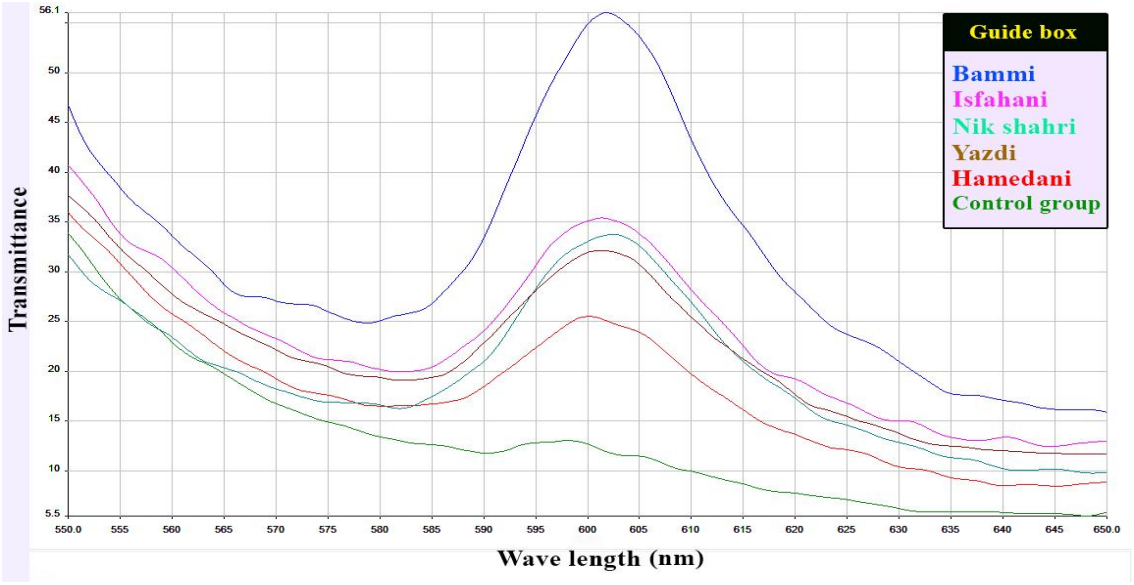

Figure 1. Fluorometric graph of emission spectrum showing test and sham groups in 100 mg nodule samples after excitation in 405 nm wavelength and emission peak of 601 nm

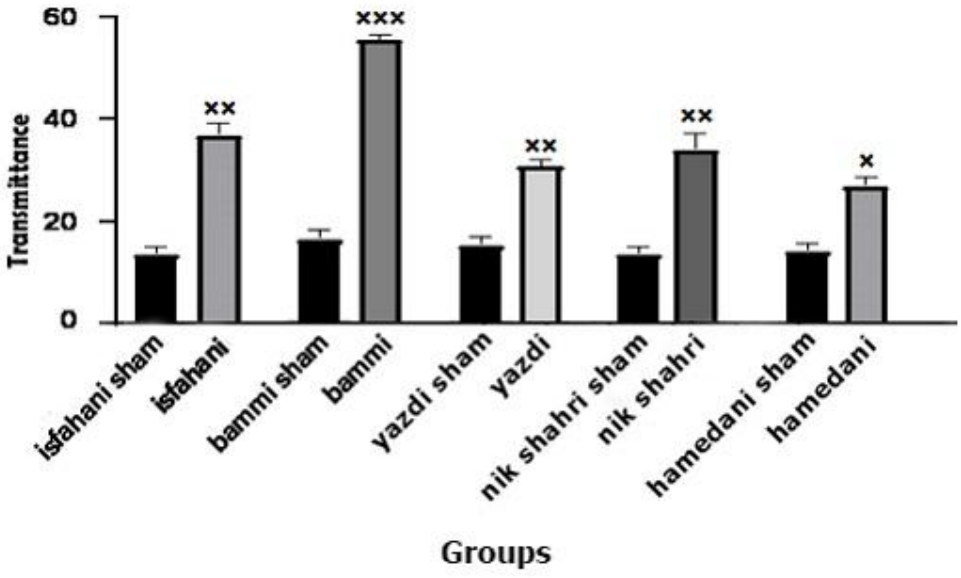

Figure 2. Transmittance graph of test and sham groups in 100 mg nodule samples after excitation in 405 nm wavelength and emission peak of 601 nm.

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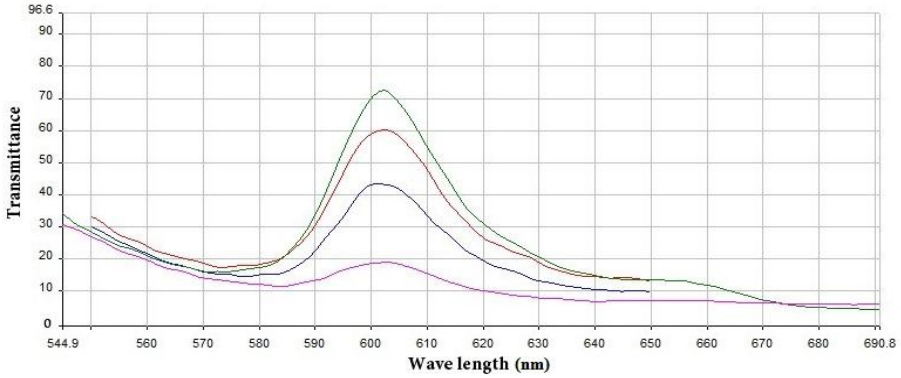

Figure 3. Emission spectrum for fluorometric graph related to all bovine hemoglobin standard dilutions after excitation in 405 nm wavelength and emission peak of 601 nm.

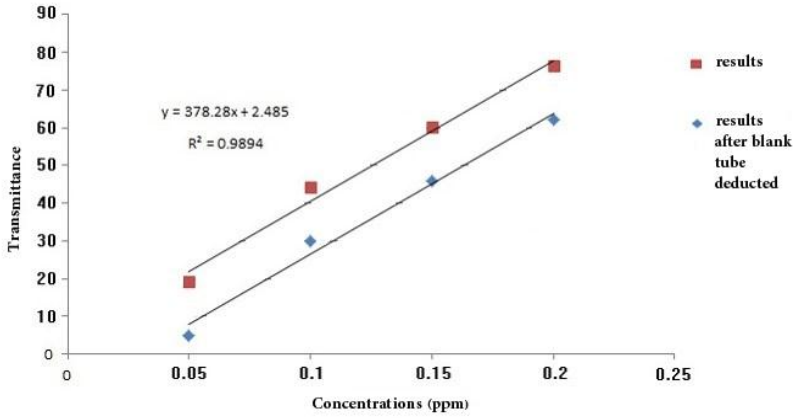

Figure 4. Standard curve of fluorometric results for all four bovine hemoglobin concentrations after blank tube was subtracted at 405 excitation wavelength and emission peak of 601 nm.

Discussion

The bioavailability and absorption of iron from daily diet are influenced by the type and quantity of iron in food as well as the presence of inhibitors and promoters of iron absorption in diet along with the individual's iron status (Duque *et al.*, 2014). Therefore, iron supplements have been widely used to overcome IDA. Due to the low cost, ferrous sulfate ($FeSO_4$) is the most frequently used iron supplement, but it could cause gastrointestinal side effects such as abdominal pain, nausea, and diarrhea (Mimura *et al.*, 2008). In order to solve this problem, ferrous lactate and sodium iron (ш) ethylenediaminetetraacetate (Ferrazone) have been developed, but the relatively low absorption of these salts in duodenal alkaline environment is still unable to meet the requirements (Ballot *et al.*, 1989).

As mentioned earlier, oral iron supplements have been shown to disturb the intestinal microbiota leading to gastrointestinal side effects (Mahalhal *et al.*, 2018). Heme iron within a vesicle directly enters cytoplasm and none of its elemental iron is released in lumen (West and Oates, 2008).

In previous studies on LB in alfalfa and soybean nodules, measuring process occurred after 30 to 600 days after culture and indicated that maximum level of LB had been found in 30 to 60 days. *Sinorhizobium meliloti* had been most rhizobium that used for alfalfa nodulation (Becana *et al.*, 1986a, Becana *et al.*, 1986b, Mohammadi and Karr, 2001).

Some of the previous studies were conducted in a greenhouse and some, like this study, in a farm.

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It seems that there was no particular difference between them (Becana *et al.*, 1986b, Mohammadi and Karr, 2001).

Conclusions

The same emission peak of bovine hemoglobin and nodule extracts at 601 nm confirms the presence of leghemoglobin in alfalfa nodules. In addition, this study demonstrated that the presence of Rhizobia such as *Sinorhizobium meliloti* is necessary for production of a high amount of leghemoglobin due to the decrease of leghemoglobin concentration in the sham group with regard to all other test groups. There was a significant difference between Bammi cultivar leghemoglobin concentration and all other cultivars; this indicates the unique potential of Bammi cultivar for production of high amounts of leghemoglobin in Khuzestan Province. Moreover, Hamedani cultivar demonstrated a significant decrease in leghemoglobin production in comparison with all other cultivars. These capacities may refer to genomic specifications and also adaptation with native environments where they were cultivated. Bammi cultivar belongs to the desert of Bam in Kerman Province which is similar to Khuzestan, and Hamedani cultivar belongs to mountainous area of Hamedan province, which is different from Khuzestan environment. According to these results, we can conclude that Bammi is the best among the five cultivars for leghemoglobin production in local Khuzestan environment.

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Conflict of interest

The authors declared no conflict of interest.

Authors' contributions

Siahpoosh A and Alaei S designed and collected data. Siahpoosh MR and Alaei S were involved in herbal and agricultural part of the study.

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