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Leghaemoglobin and Ureide Biosynthesis in *Pueraria Phaseoloides* and *Mimosa Invisa*: Implications for Symbiotic Nitrogen Fixation

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Abstract: This study investigates the relationship between leghaemoglobin concentration and ureide biosynthesis in root nodules of two leguminous cover crops, *Pueraria phaseoloides* and *Mimosa invisa*, collected from different locations in Kerala, India. Leghaemoglobin concentrations ranged from 0.0040 mM to 0.0232 mM in *Mimosa invisa* and 0.0063 mM to 0.0217 mM in *Pueraria phaseoloides*. Ureide content varied from 183 µg to 656 µg in *Mimosa invisa* and 291.85 µg to 523.84 µg in *Pueraria phaseoloides*. Correlation analysis revealed a positive correlation between leghaemoglobin concentration and ureide synthesis (γ = +0.83 for *Pueraria phaseoloides s* and γ = +0.38 for *Mimosa invisa*), indicating leghaemoglobin's crucial role in symbiotic nitrogen fixation. *Pueraria phaseoloides* exhibited higher average ureide synthesis, potentially contributing to its biomass accumulation. Results suggest leghaemoglobin concentration is directly related to nitrogen fixation ability, supporting findings that plant haemoglobins are vital for symbiotic nitrogen fixation. The study underscores the importance of leghaemoglobin in nitrogen metabolism and highlights species-specific differences in ureide production and nitrogen fixation capacities.
Keywords: Leghaemoglobin, Ureide Biosynthesis, Pueraria phaseoloides, Mimosa invisa, Nitrogen Fixation.

INTRODUCTION

Leghaemoglobin is a haemoprotein predominantly found in the root nodules of leguminous plants. They play a critical role in facilitating symbiotic nitrogen fixation by maintaining a low oxygen environment essential for the functioning of the oxygen-sensitive nitrogenase enzyme complex (Ott *et al.,* 2005). This process is vital for the conversion of atmospheric nitrogen (N_2) into ammonia (NH_3), thereby contributing significantly to the nitrogen economy of agricultural systems (Udvardi and Day, 2007). The importance of leghaemoglobin in biological nitrogen fixation (BNF) underscores its relevance in legume-based cropping systems aiming to reduce dependency on synthetic nitrogen fertilizers (Graham and Vance, 2003).

Leguminous cover crops such as *Pueraria phasioloides* and *Mimosa invisa* are valued for their ability to fix nitrogen, improve soil structure, suppress weeds, and contribute to nutrient cycling, making them integral components of conservation agriculture and crop rotation systems (Snapp *et al.,* 2005; Strock *et al.,* 2004). These cover crops not only enhance soil fertility but also provide ecosystem services including biodiversity conservation and soil carbon sequestration (Drinkwater and Snapp, 2007). Ureides are nitrogenous compounds such as allantoin and allantoic acid. They are prominent in certain legumes like soybean (Glycine max) and serve as key transport forms of fixed nitrogen from nodules to other plant parts (Todd *et al.,* 2006; Alamillo *et al.,* 2010). The biosynthesis of ureides involves enzymatic steps catalyzed by uricase and allantoinase (Werner *et al.,* 2008). Relative ureide production and metabolism can be indicative of the efficiency of nitrogen fixation and the metabolic status of the legume, offering insights into the physiological state of the symbiosis (King and Purcell, 2005).

Research has highlighted the interplay between leghaemoglobin expression, nitrogen fixation activity, and environmental factors such as water availability and temperature (Serraj *et al.,* 2001; Marino *et al.,* 2007). Leghaemoglobin concentration is closely associated with the effectiveness of nitrogen fixation in legume nodules (Marino *et al.,* 2007). Variations in its expression can reflect differences in symbiotic efficiency among legume species (Ott *et al.,* 2005). Studies comparing different legume species have shown variability in nitrogen fixation capacities, ureide production, and adaptability to environmental conditions, underscoring the need for species-specific understanding and management (Vance, 2001; Graham and Vance, 2003). The role of leghaemoglobin in creating a microaerobic environment necessary for nitrogenase activity is well established, and its interaction with nodule metabolism, including ureide biosynthesis, points to the intricate regulation of nitrogen fixation processes (Udvardi and Day, 2007). *Pueraria phasioloides* and *Mimosa invisa*, as cover

crops, present opportunities for improving soil health and nitrogen availability in cropping systems (Desbrosse and Stougaard, 2011). Their performance can be influenced by factors affecting leghaemoglobin expression and ureide metabolism (Ranells and Wagger, 1996; Strock *et al.,* 2004).

Understanding the physiological and biochemical aspects of leghaemoglobin and ureide biosynthesis in cover crops like *Pueraria phasioloides* and *Mimosa invisa* can contribute to optimizing their utilization in agricultural systems for better nitrogen management and sustainability (Drinkwater and Snapp, 2007). Comparative studies on these aspects can provide insights into the differential capacities of these crops for nitrogen fixation and their responses to environmental conditions.

METHODOLOGY

- **Collection of the plant material:** Root nodules of the two cover crops *Pueraria phasioloides* and *Mimosa invisa* were used for the present investigation. The plants were collected from 10 different locations of the Vallicode Grama Panchayat of Pathanamthitta District, Kerala state, India. *Pueraria phasioloides* were collected from immature rubber plantations of 2-3 years of growth. *Mimosa invisa* was collected from the bare lands.
- **Estimation of Leghaemoglobin (Ott *et al.,* 2005; Appleby, 1984):** 5g of fresh root nodules were grinded with 5 mL of potassium phosphate buffer of pH 7.4 with a pestle and mortar and filtered through two layers of cheesecloth. Then centrifuged at 10,000 rpm for 10-30 minutes and supernatant was collected. To 3mL of the extract equal volume of alkaline pyridine reagent was added and mixed well. The solution became greenish – yellow due to the formation of ferric hemochrome. The hemochrome is divided equally. To one portion few crystals of sodium dithionite is added to reduce the hemochrome and stirred without aeration and optical density was measured at 556nm after 2-5 minutes against a reagent blank. To the other portion a few crystals of potassium hexacynoferrate is added to oxidize the hemochrome and optical density was measured at 539nm.
  Leghaemoglobin (Lb) concentration (mM) = A539 -A 539 x 2D x 23.4
  Where D is the initial dilution
- **Estimation of Ureides (Ott *et al.,* 2005; Appleby, 1984):** Fresh root tissue was grinded with 10mL of 0.05M Potassium phosphate buffer (pH 7.4) and centrifuged for 5minutes to collect the supernatant. 1 mL of the supernatant is pipetted out into a test tube and diluted to 2.5mL with distilled water. 0.5mL of 0.5N sodium hydroxide was added and placed the tubes in vigorously boiling water for 7 minutes. Then the tubes were brought to room temperature by placing in a water bath. 0.5mL of 0.65N hydrochloric acid and then 0.5mL of phenylhydrazine solutions were added and tubes were placed in a boiling water bath for 2 minutes. Then the tubes were plunged into an ice bath and chilled it for 20 minutes. To the tubes removed from the bath 2mL of already chilled 10N hydrochloric acid and 0.5mL of pottassium ferricyanide solution were added and mixed the contents thoroughly. After 30 minutes absorbency was measured at 520nm in a spectrophotometer. And standard graph is prepared with 0 to 40µg concentration of allantoin.
- **Correlation Analysis;** Correlation analysis of the results was conducted using the SPSS software 16.0.

RESULTS AND DISCUSSION

The concentration of leghaemoglobin in the root nodules of *Mimsa invisa* found to limit from 0.0040 mM to 0.0232mM (Table-1). In *Pueraria phaseoloides* concentration of leghaemoglobin varies from 0.0063 mM to 0.0217 mM (Table-2). The abundance of root nodules (No. / Cm of root length) is very high in *Mimosa invisa* but their size is comparatively very small to that *Pueraria phaseoloides*. The quantity of ureides in the root tissues ranges from 183µg to 656 µg in *Mimosa invisa* (Table -3) and that of *Pueraria phaseoloides* varies from 291.85 µg to 523.84µg (Table -4). The correlation of studies of concentration of leghaemoglobin and biosynthesized ureides of plant root tissues elucidated that the concentration of leghaemoglobin (LHb) in the root nodules shows perfect positive correlation with the concentration of estimated ureides; $\gamma = +0.83$ for *Pueraria phaseoloides* (Figure 1) and $\gamma = +0.38$ for *Mimosa invisa* (Figure 2). As the concentration of leghaemoglobin increases a corresponding increase in the concentration of ureides was observed. In specifically the concentration of leghaemoglobin in the root nodules have some major controlling effect on the synthesis of ureides; this data indicates leghaemoglobins are crucial for symbiotic nitrogen fixation (Ott, *et al.,* 2005).

Table 1: Concentration (mM) of Leghaemoglobin (LHb) in root nodules of *Mimosa invisa.*

Sl.no.	Locality	Wt. of Root Nodules (mg).	A_{556}	A_{539}	Concentration of LHb (mM).
1	L1	1000	1.376	1.240	0.0117
2	L2	1000	1.452	1.403	0.0042
3	L3	1000	1.827	1.555	0.0232
4	L4	1000	1.525	1.368	0.0134
5	L5	1000	1.000	0.953	0.0040

Table 2: Concentration (mM) of Leghaemoglobin (LHb) in root nodules of *Pueraria phasioloides.*

Sl.no.	**Locality.**	**Wt. of the Nodule (mg).**	**A_{556}**	**A_{539}**	**Concentration of LHb (mM).**
1	L1	1000	1.890	1.810	0.0068
2	L2	1000	1.576	1.480	0.0082
3	L3	1000	1.140	0.885	0.0217
4	L4	1000	1.605	1.505	0.0085
5	L5	1000	0.770	0.696	0.0063

Table 3: Concentration of ureides(µg) in root tissues of *Mimosa invisa*.

Sl.no.	**Locality**	**Wt. of plant tissue (mg).**	**O.D of the test sample.**	**Corresponding ureide concentration(µg)**	**Ureide concentration (µg)/ 100mg plant tissue.**
1	L1	1.200	0.292	292	243.34
2	L2	1.000	0.189	189	189.00
3	L3	0.500	0.328	328	656.00
4	L4	1.250	0.344	344	275.00
5	L5	1.500	0.274	274	183.00

Table 4: Concentration of ureides(µg) in root tissues of *Pueraria phasioloides*.

Sl.no.	**Locality.**	**Wt. of plant tissue (mg).**	**O.D of the test sample.**	**Corresponding ureide concentration (µg).**	**Ureide concentration(µg)/ 100 mg plant tissue.**
1	L1	1.300	0.391	391	300.76
2	L2	1.600	0.667	667	416.87
3	L3	1.300	0.681	681	523.84
4	L4	1.600	0.693	693	433.12
5	L5	1.350	0.394	394	291.85

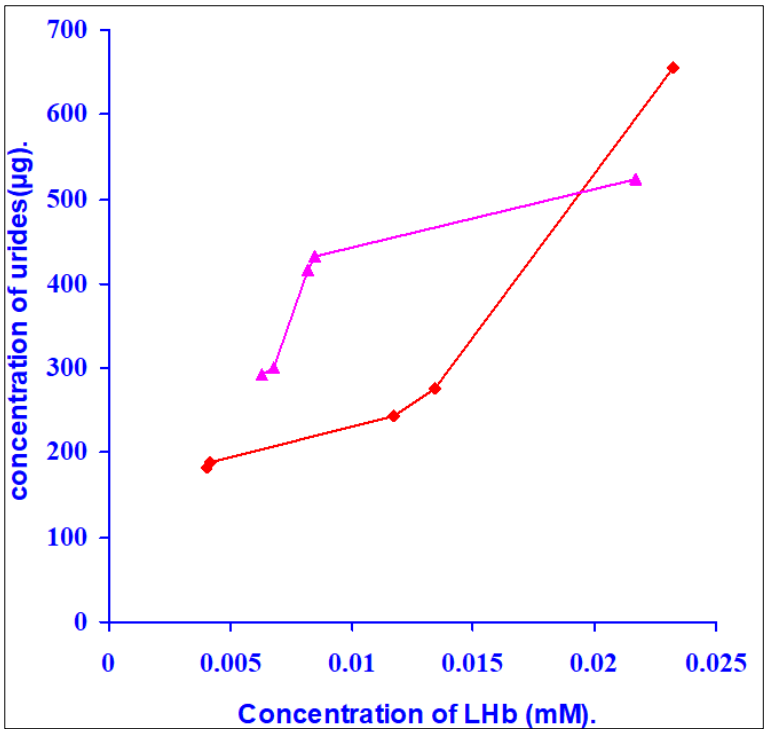

Figure 1: Graph showing relationship between concentrations of leghaemoglobin and concentration of biosynthesized ureides in *Mimosa invisa*.

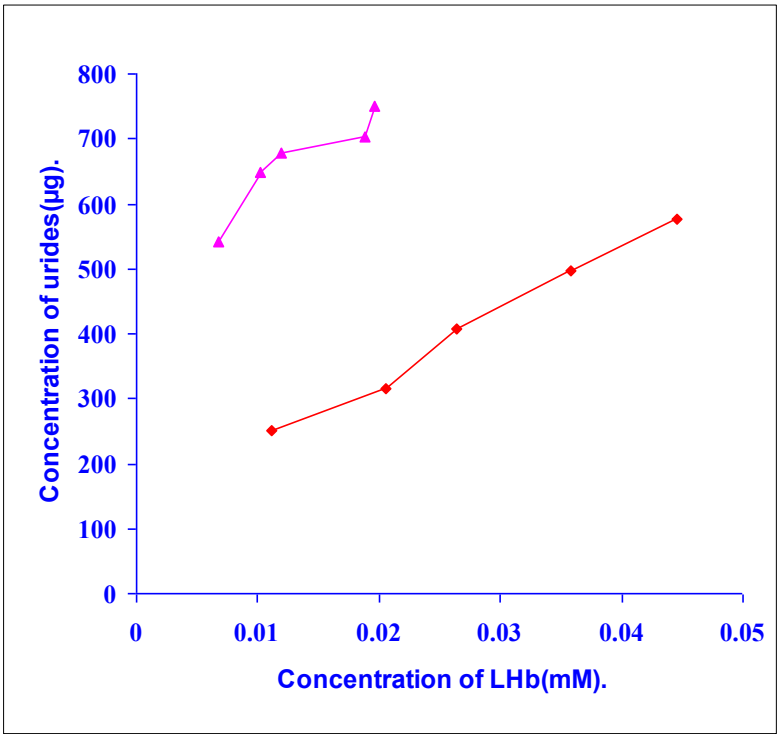

Figure-2: Graph showing relationship between concentrations of leghaemoglobin and concentration of biosynthesized ureides in *Pueraria phasioloides*.

The average ureide synthesis is greater in *Pueraria phaseoloides* than that of *Mimosa invisa* in the estimated range of leghaemoglobin concentration. This may be one of the reasons for high biomass accumulation of *Pueraria phaseoloides*. At higher concentrations of leghaemoglobin the ureide synthesizing ability of *Mimosa invisa* exceeds that of *Pueraria phaseoloides*. The quantity of synthesized ureides (allantoin and allantoic acid) at specific concentration of leghaemoglobin varies greatly in two species. This might be due to the environmental factors to they were subjected to. The dependence of plants on nitrogen fixation shows high correlation with the percentage of xylem sap nitrogen as ureides (Herridge

and Peoples, 2002). Being concentration of leghaemoglobin is positively correlated to the concentration of ureides of root tissues; the relative concentration of leghaemoglobin is thought to positively correlate with the ability of plants to fix atmospheric molecular nitrogen. The correlation studies revealed that the concentration of leghaemoglobin in the root nodules is directly proportional to the concentration of synthesized ureides in the root tissues. While correlating the obtained results with this it is evident that leghaemoglobin concentration and ability of biological nitrogen fixation are positively correlated, if other requirements are not limiting. The RNAi used studies (Gehring,C. and Vlek,P.L.G.,2004) in model plant *Lotus japonicaus* revealed that the leghaemoglobin is much important in oxygen transport and buffering and prove that plant haemoglobins are crucial for symbiotic nitrogen fixation (Valentine *et al.,* 2013).

CONCLUSION

The study demonstrates a positive correlation between leghaemoglobin concentration and ureide synthesis in *Pueraria phaseoloides* (γ = +0.83) and *Mimosa invisa* (γ = +0.38), highlighting leghaemoglobin's crucial role in symbiotic nitrogen fixation. *Pueraria phaseoloides* showed higher average ureide synthesis, potentially contributing to its biomass accumulation. Leghaemoglobin concentration is directly related to nitrogen fixation ability, underscoring its importance in sustainable agriculture. Species-specific differences in ureide production and nitrogen fixation capacities were observed, likely influenced by environmental factors. Findings support leghaemoglobin's vital role in maintaining a microaerobic environment for nitrogenase activity, essential for biological nitrogen fixation.

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