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A Functional Relationship Between Leghaemoglobin and Nitrogenase Based on Novel Measurements of the Two Proteins in Legume Root Nodules

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A combination of physiological and structural measurements made on nodulated cowpea and soybean plants cultured with roots in different pO_2 permitted the expression of data in various ways. Values of leghemoglobin concentration and nitrogenase activity from the two legumes were expressed conventionally either on a per plant or per gram nodule fresh weight basis, and where microscopy was done, on the basis of nitrogenase-containing, N_2-fixing units (i.e. per bacteroid, per infected cell, or per gram infected tissue). In both legumes, acetylene reduction, N fixed and ureide content expressed on the basis of whole plants or per nitrogenase-containing units were very significantly correlated with values of leghaemoglobin concentrations expressed in a similar manner. The use of mathematical correlations in this study involving leghaemoglobin concentrations and various indices of N_2 fixation indicated a strong functional relationship between the two proteins in symbiotic legumes. These findings confirm previous suggestions that leghaemoglobin and the nitrogenase complex are two proteins closely associated with N_2-fixing efficiency in legume root nodules.

INTRODUCTION

Leghaemoglobin (Lb) is a hemoprotein in N_2-fixing nodules which facilitates O_2 diffusion to respiring bacteroids in infected cells (Appleby, 1984). The ATP produced from bacteroid respiration via oxidative phosphorylation is used to reduce N_2 to form NH_3. This capacity of Lb molecules to supply O_2 to support bacteroid respiration and ATP production for nitrogenase activity functionally relates the hemoprotein to N_2 fixation. The physiological relationship between Lb concentration and N_2-fixing efficiency in legume root nodules was initially suggested by Virtanen *et al.* (1947). Bergersen and Goodchild (1973) later observed concurrent changes in both nitrogenase activity and Lb concentration in soybean nodules. A recent study (Dakora, 1990) of cowpea and soybean symbioses also showed increased nitrogenase activity where Lb concentrations were high.

In an earlier study (Graham and Parker 1961) a highly significant correlation was obtained between the Lb concentration in lupin nodules and total N in plant tops. However a later study by Nash and Schulman (1976) found that, in soybean, young nodules had quite high nitrogenase activity even though only low levels of Lb were detected; and in older nodules, Lb content declined after a fall in nitrogenase activity. That report is rather unusual because the reverse has commonly been observed, i.e. a reduction in nodule Lb concentration from oxidation reactions often leads to a decline in nitrogenase activity due to Lb's inability to support high levels of bacteroid respiration (Appleby, 1984). In fact, it has been demonstrated that with high Lb concentration in nodules, a high level of nitrogenase activity was maintained; however as Lb concentration declined from oxidation by excess O_2, nitrogenase activity also declined (Dakora, Appleby and Atkins, 1991).

This study reports on a novel technique for relating N_2 fixation to Lb content in legumes, and demonstrates through correlation analyses that a functional relationship exists between Lb and nitrogenase proteins in N_2-fixing nodules. To my knowledge, this is the first report that uses simple mathematical correlations at the level of bacteroids and infected cells to establish a functional relationship between Lb and N_2 fixation in symbiotic legumes.

MATERIALS AND METHODS

Plant culture and data collection

Data used in this study come from experiments that were initially conducted to test the effects of pO_2 on nodule development (Dakora and Atkins, 1990; Dakora and Atkins, 1991; Dakora *et al.*, 1991). Symbiotic cowpea (*Vigna unguiculata* L. Walp. cv. Vita 3 infected with *Bradyrhizobium* strain CB 756) and soybean (*Glycine max* L. Merr. cv. White Eye infected with *Bradyrhizobium* strain CB 1809) were cultured as described by Dakora and Atkins (1990). Cowpea and soybean plants were assayed for nitrogenase activity by acetylene reduction in a flow-through assay system and harvested at 28 d after planting. Some nodule samples were weighed and stored in liquid N_2 prior to analysis for Lb (Dakora *et al.*, 1991), while the remaining ones were processed for microscopy (Dakora and Atkins, 1990). Nitrogen fixation was estimated based on plant dry weights and N content determined by Kjeldahl digestion, while ureides were measured in collected xylem fluids as the

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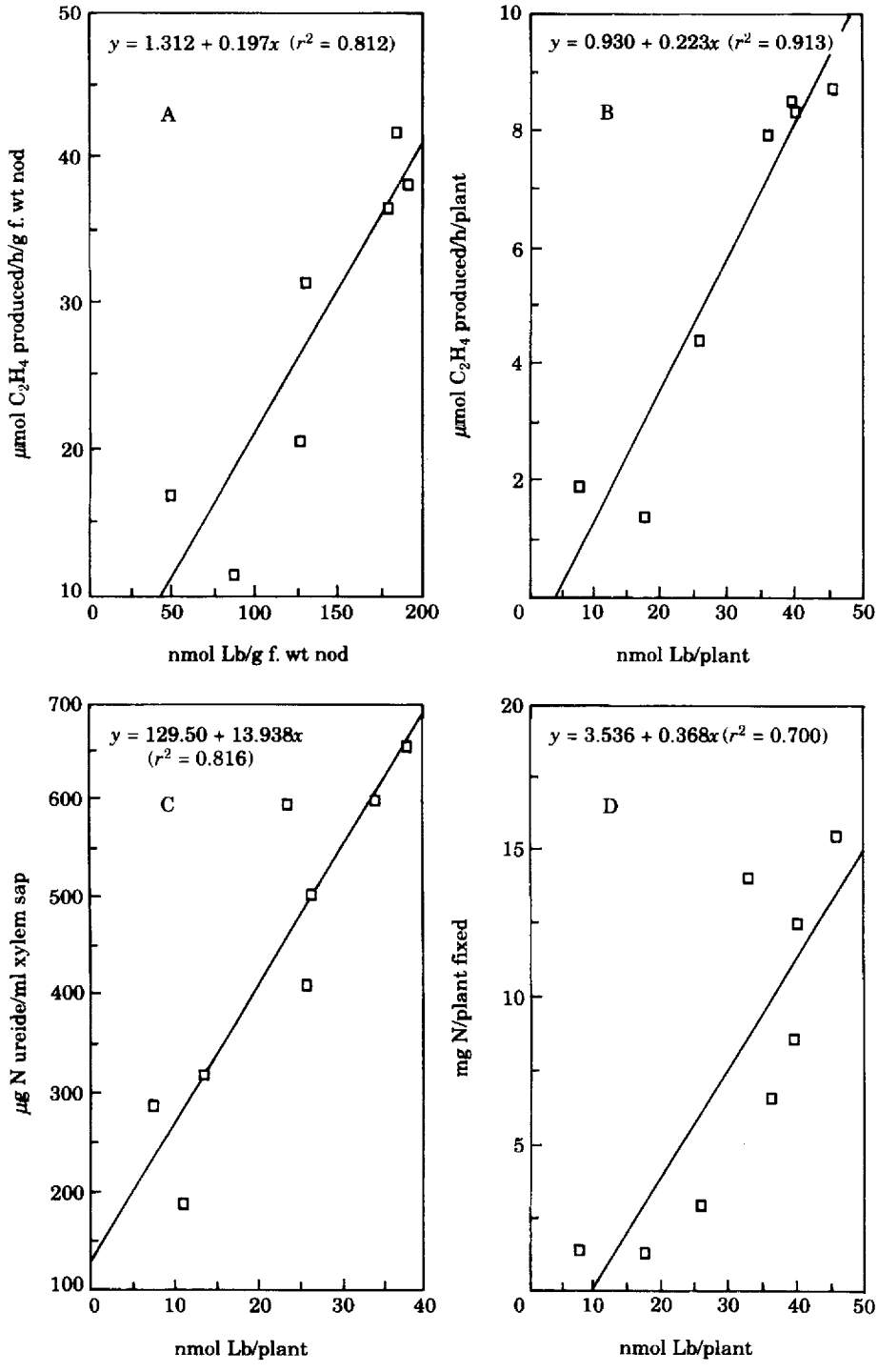

FIG. 1. Correlation of nitrogenase activity with leghemoglobin concentrations in cowpea, expressed on a per g nodule fresh weight basis (A), a per plant basis (B), or that between ureide level in xylem sap and amount of leghemoglobin per plant (C), or N fixed and leghemoglobin content per plant (D). See text for details on data used for these plots.

phenylhydrazone derivative of glyoxylate (Trijbels and Vogels, 1966).

Extracts of Lb were prepared anaerobically from frozen nodules grown in 1–80% O_2 (cowpea) or 1–20% O_2 (soybean); only in the case of cowpea was TEM used to analyse bacteroid numbers in nodules from sub-ambient pO_2 (Dakora and Atkins, 1990). With the availability of structural data, nitrogenase activity, N_2 fixation (total N) and Lb content could be expressed on the basis of different parameters. For example, acetylene reduction values were expressed as μmol C_2H_4 produced per h per plant, per g f. wt nodules, per g infected tissue, per infected cell, or per

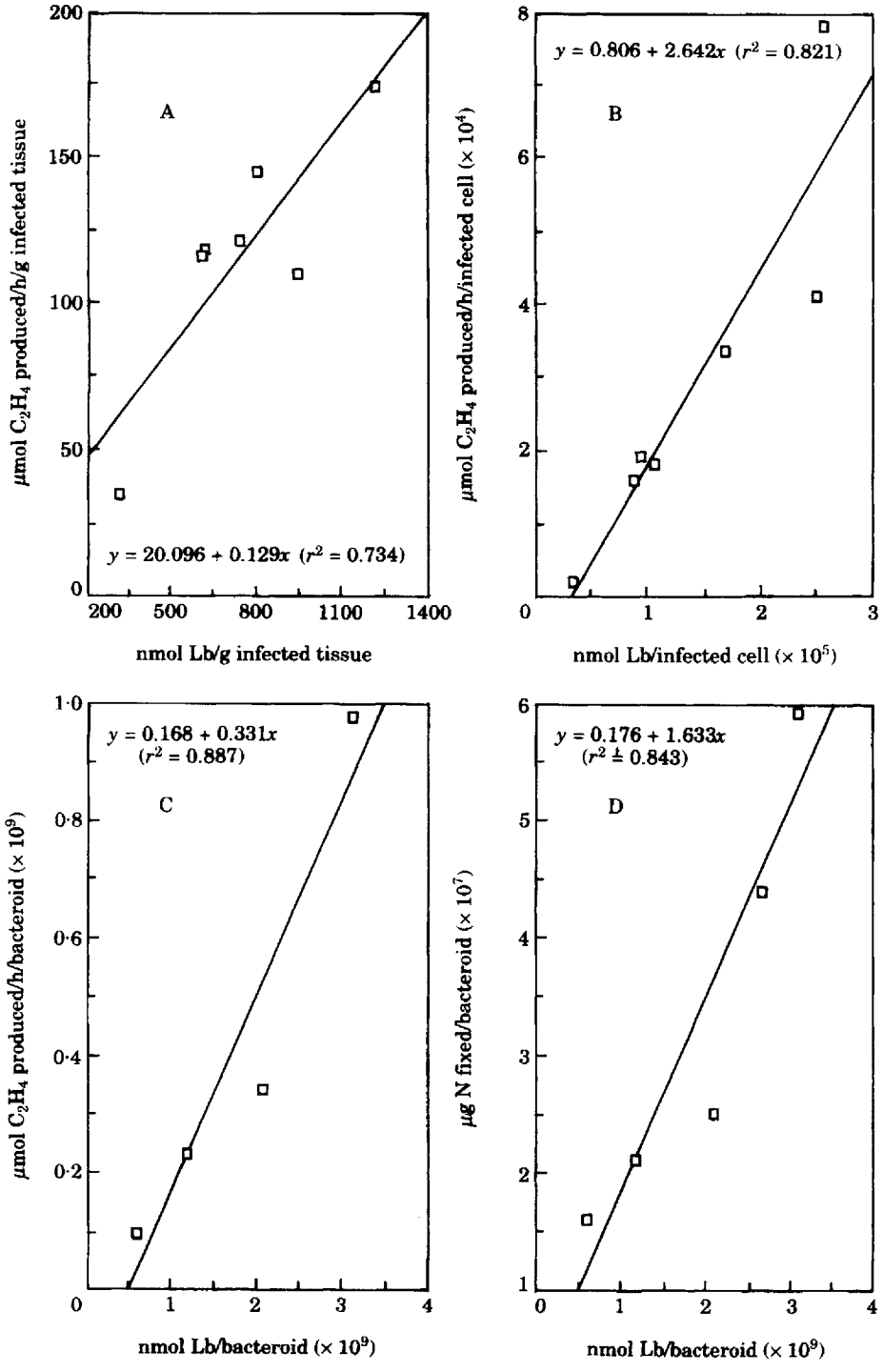

FIG. 2. Correlation of nitrogenase activity with leghemoglobin concentrations in cowpea nodules, expressed per g infected tissue (A), per infected cell (B), per bacteroid (C), or that between N fixed and leghemoglobin content per bacteroid (D). See text for details on data used for these plots.

bacteroid (Dakora and Atkins, 1990). The content of Lb, where possible, was similarly expressed (Dakora *et al.*, 1991). In both cowpea and soybean, data from the different ways of expressing nitrogenase activity and N_2 fixation rates were used in simple mathematical correlations with values of Lb concentrations.

Since the ureides allantoin and allantoic acid are products of recently fixed N (Atkins, 1982) and their content in the xylem stream of cowpea and soybean reflect N_2 fixation in these species (Pate *et al.*, 1980; Herridge, 1982), their measure was also used to correlate with Lb concentrations in nodules. The ureide data were obtained from Dakora and Atkins (1991) and Atkins, Dakora and Storer (1990).

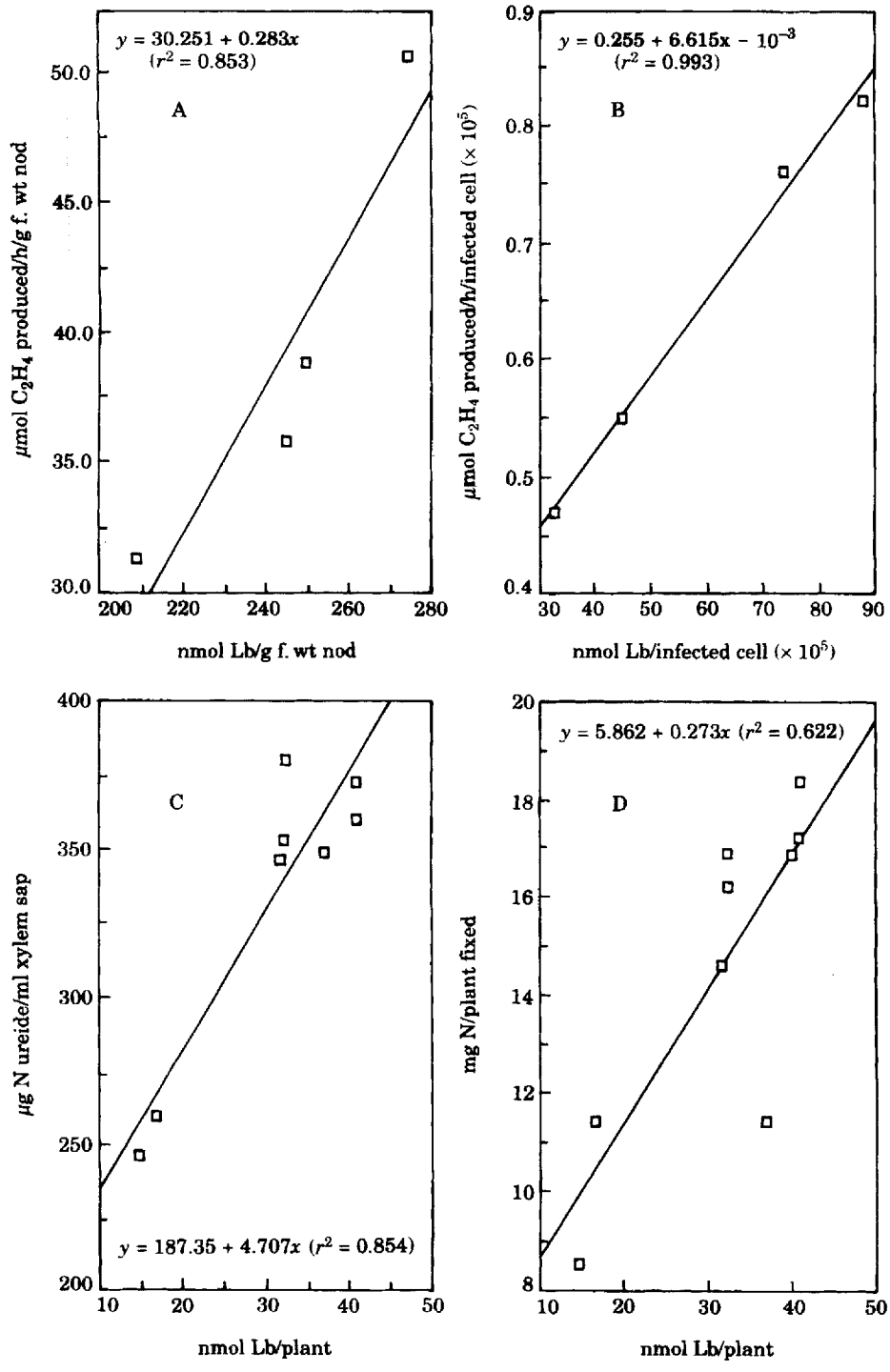

FIG. 3. Correlation of nitrogenase activity with leghemoglobin concentrations in soybean nodules, expressed on a per g nodule fresh weight basis (A), per infected cell (B), or that between ureide level in xylem sap and leghemoglobin content of plant (C), or N fixed and leghemoglobin per plant (D). See text for details on data used for these plots.

RESULTS AND DISCUSSION

Functional relationship between N_2-fixing activity and Lb content in nodules

Relating Lb content to various indices of N_2-fixing efficiency showed a strong and significant agreement between Lb and N_2 fixation in cowpea and soybean (Figs 1, 2 and 3). In cowpea, values of Lb correlated strongly and significantly (r^2 = 0·812) with measures of nitrogenase activity on the basis of nodule fresh weight (Fig. 1 A), and very significantly (r^2 = 0·913) when Lb concentration and nitrogenase activity were correlated on a per plant basis (Fig. 1 B). Ureide content of xylem sap (Fig. 1 C) correlated significantly (r^2 =

0·816) with Lb concentration in root nodules. As a result, the correlation between N fixed and Lb content was also significant (Fig. 1D; $r^2 = 0·700$).

Figure 2 shows the relationship between cowpea Lb and nitrogenase activity expressed on the basis of nitrogenase-containing, N_2-fixing units (bacteroids, infected cells, or gram infected tissue). Even at the cellular level, the agreement between nitrogenase and Lb content was strong and highly significant irrespective of whether the two proteins were expressed per g infected tissue (Fig. 2A; $r^2 = 0·734$), per infected cell (Fig. 2B; $r^2 = 0·821$), or per bacteroid (Fig. 2C; $r = 0·887$). The level of N fixed per bacteroid was also significantly ($r^2 = 0·843$) correlated with the amount of Lb serving each bacteroid (Fig. 2D). As with cowpea, the correlation between soybean Lb and nitrogenase activity expressed on nodule fresh weight basis (Fig. 3A) was strong and highly significant ($r^2 = 0·853$). However, this relationship was stronger and most significant ($r^2 = 0·993$) when both nitrogenase and Lb content were expressed per infected cell (Fig. 3B). There was also a significant ($r^2 = 0·854$) correlation between ureide level in transport fluids of xylem and Lb concentration in soybean nodules (Fig. 3C). Quite expectedly, N derived from symbiotic fixation in soybean showed significance ($r^2 = 0·622$) when correlated with Lb concentration (Fig. 3D).

The data presented here (Figs 1, 2 and 3) are based on Lb contents and nitrogenase activities. Acetylene reduction by nitrogenase requires a micro-aerobic environment. Thus, nitrogenase activity is dependent upon the presence of Lb to produce the desired environment for the functioning of the enzyme. However, because nitrogenase synthesis was not measured in this study, it is difficult to indicate whether biosynthesis of the enzyme is dependent upon the presence of Lb to produce a micro-aerophillic environment. The fact that the functioning of Lb and nitrogenase correlated on a per cell, tissue, organ or whole plant basis does not necessarily indicate a direct link between the two proteins. The Lb concentrations measured in this study represent total O_2-binding protein in nodules, while the acetylene reduction values utilized in the correlation analyses only indicate the presence of active nitrogenase, and not total nitrogenase protein present. Although it has already been demonstrated that Lb is not essential for the development of nitrogenase activity in free-living rhizobia (Kurz and LaRue, 1975; McComb, Elliott and Dilworth, 1975; Pagan *et al.*, 1975), it remains to be determined whether nitrogenase proteins can be synthesized in the absence of Lb in legume nodules. Further studies are thus needed to indicate whether the formation of nitrogenase and Lb are directly linked.

The findings from this study confirm previous suggestions (Virtanen *et al.*, 1947; Bergersen and Goodchild, 1973; Dakora, 1990) and the long-held view that the functioning of bacteroid nitrogenase is closely related to the content of Lb protein in N_2-fixing nodules. However from an ontogenic assessment of the ratio of Lb to nitrogenase activity in soybean nodules, Nash and Schulman (1976) suggested that Lb content was more closely correlated with nodule size rather than nitrogenase activity. Their suggestion was based on the detection of high nitrogenase activity in nodules with low Lb concentration, and a decline in Lb content following a fall in nitrogenase activity. Clearly, the lack of correlation obtained in that study, especially the low concentration of Lb found in nodules with high nitrogenase activity, suggests that the pyridine hemochrome method used by Nash and Schulman (1976) to measure Lb under-estimated the nodule concentration of the O_2-binding protein. Anaerobic extraction of Lb as done in this study (see also Dakora *et al.*, 1991) reduces oxidation and destruction of the protein, thus leading to higher yields.

The highly significant correlations obtained here between levels of the two proteins in both cowpea and soybean are indirectly consistent with an earlier suggestion by Appleby (1962) that the main function of Lb might be to support nitrogenase activity via the supply of adequate flux of O_2 to bacteroids. Although *in vitro* experiments (Wittenberg *et al.*, 1974) have since confirmed Appleby's (1962) concept of facilitated O_2 diffusion by Lb, the data from this study have provided yet further evidence for bacteroid nitrogenase being functionally related to the content and activity of Lb.

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