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Effect of Polyamines on Dicarboxylate and Oxygen Uptake by Symbiosomes and Free Bacteroids from *Galega Orientalis* Nodules

VALYA VASSILEVA[1*] and GEORGE IGNATOV[2]
[1]*Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria, Tel. +359-2-979-3673, E-mail. vassilev@bio25.bas.bg; and* [2]*Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria, Tel. +359-2-979-3677, E-mail. ignato@bio25.bas.bg*

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Abstract

In this paper we report the presence of high concentrations of polyamines (PA) in the 40-day-old *Galega orientalis* plants and examine their effect on the permeability of symbiotic membranes to dicarboxylates and O_2. Intact symbiosomes and free bacteroids were isolated from *Galega* nodules and supplied with malate as carbon substrate in the presence of various PA. The results show that permeability of the symbiotic membranes to malate was altered substantially by the addition of PA to the reaction medium. The increase of malate utilization was observed in the presence of 10 and 50 μM of various PA. However, at 100 μM the diamines, putrescine (Put) and cadaverine (Cad) severely reduced malate uptake. Oxygen consumption of symbiosomes and bacteroids changed in proportion to the rates of malate utilization. Uptake studies also indicated that diamines affected to a higher extent permeability of the symbiotic membranes than the PA spermidine (Spd) and spermine (Spm). The possible mechanisms for PA mediated alteration of malate and O_2 uptake into symbiosomes and bacteroids from *G. orientalis* nodules are discussed.

Keywords: Bacteroids, dicarboxylate transport, *Galega orientalis*, oxygen consumption, polyamines, *Rhizobium* galegae, symbiosomes

*The author to whom correspondence should be sent.

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Abbreviations

PA = polyamine; Put = putrescine; Cad = cadaverine; Spd = spermidine; Spm = spermine; PBM = peribacteroid membrane; BM = bacteroid membrane.

1. Introduction

Polyamines are widely recognized to have multifunctional effects at the cellular level in various biological systems (Galston and Sawhney, 1990). It has been reported that they play an important role in the control of membrane functions (Altman et al., 1977; Roberts et al., 1986). Polyamines bind strongly *in vitro* to negatively charged acidic phospholipids and many types of proteins, including numerous enzymes whose activities are directly modulated by polyamine (PA) binding (Fink et al., 1987; Igarashi et al., 1989).

In the root nodules of legumes, the nitrogen-fixing bacteroids are separated from the host cell cytoplasm by a membrane of plant origin called the peribacteroid membrane (PBM). This membrane is not only a structural barrier but is also a functional barrier because it regulates the transport of various compounds between the symbiotic partners (Rosendahl and Jochimsen, 1995; Udvardi and Day, 1997; Whitehead et al., 1995), thereby controlling nitrogen fixation.

Established root nodules of leguminous plants contain high concentration of PA (Fujihara et al., 1994). The same authors found that PA levels in nodules were five to ten times higher than those in the other plant organs such as leaf, stem or root. Ozawa and Tsuji (1993) hypothesized that nodule PA might have a regulatory role on the proliferation of bacteroids within soybean root nodules. However, the precise role of PA in nodule metabolism is yet to be determined.

In the present study we provide data demonstrating that exogenous PA alter permeability of symbiotic membranes from *Galega orientalis* nodules to dicarboxylates and to O_2 that may serve as the basis for revealing the potential physiological significance of PA in the root nodules.

2. Materials and Methods

Plant material and bacterial strain

Goat's rue (*Galega orientalis*) seeds were scarified, surface sterilized with 70% ethanol (v/v) and germinated for 5 days on buffered water agar plates (pH 7.0) at 22°C. Sterile seedlings were transferred to plastic growth pots (2 l) and

cultivated in a naturally illuminated greenhouse as described by Vassileva et al. (1997). Inoculation was performed at sowing with *Rhizobium galegae* strain HAMBI 540 (HAMBI is the culture collection at the Department of Microbiology, University of Helsinki) using inoculant dose of 10^8 viable cells. Nodules were harvested 40 days after sowing and were used immediately.

Polyamine analysis

The PA in the nodules of *Galega* plants were extracted and detected by the method of direct dansylation (Smith and Best, 1977) using precoated Silica gel 60 TLC plates with concentrating zone and ethylacetate:cyclohexane (3:2, v/v) as the solvent. Fluorescence spots were scraped from the plates and extracted with 2 ml acetone. Fluorescence was measured with a spectrofluorimeter (excitation 360 nm, emission 505.5 nm). Quantification was performed by comparison with dansylated standards chromatographed under the same conditions.

Preparation of symbiosomes and bacteroids

Intact symbiosomes were isolated from *Galega* nodules using a modification of the technique for isolating soybean symbiosomes (Day et al., 1989). Eight to 10 g of nodules were crushed with a mortar and pestle in 30 ml of cold homogenisation buffer (450 mM mannitol, 10 mM EGTA, 10 mM $MgSO_4$, 5 mM dithiothreitol, 1% (w/v) BSA, 1% PVP-40, 20 mM ascorbate, and 25 mM MES-KOH buffer, pH 7.0). The homogenate was filtered and centrifuged on a Percoll step gradient (30, 60 and 80%) in a swing-out rotor at 4000 · g for 30 min at 4°C. The symbiosome fraction was located at the interface between 30 and 60% Percoll and a small pellet of free bacteroids was obtained at the bottom of the tube. The symbiosome fraction was collected with a Pasteur pipette, diluted with wash buffer (450 mM mannitol, 25 mM MES buffer, 3 mM $MgSO_4$, titrated to pH 7.0 with 1 M 1,3-bis[tris(hydroxymethyl) methylamino]propane), and pelleted at 500 · g (5 min, 4°C) to exclude Percoll. Symbiosomes were then resuspended in wash buffer to the desired concentration.

The integrity of the symbiosomes was routinely checked by light microscopy (Day et al., 1989). No contaminating plant organelles could be seen by electron microscopy and no stimulation of O_2 uptake was seen upon addition of NADH which is readily oxidized by nodule mitochondria (Day et al., 1986).

Free bacteroids were obtained by vigorously vortexing symbiosomes for 4 min to rupture PBM. Centrifugation of ruptured symbiosomes at 15 000 · g for 30 min pellets the bacteroids, leaving a mixture of PBM vesicles and peribacteroid space content in the supernatant.

Electron microscopy

Isolated symbiosomes were prepared essentially as described by Price et al. (1987). The symbiosome pellet was fixed with 2.5% glutaraldehyde in 30 mM PIPES-KOH buffer (pH 7.2) for 2 hours at room temperature, washed twice in 50 mM PIPES-KOH buffer (pH 6.8) for 30 min and postfixed in the same buffer with 1% OsO_4 for 2 hours at 4°C. Then pellet was washed several times and stained in 1% uranyl acetate (pH 5.5) overnight at 4°C, followed by dehydratation through an ethanol series (10–100%). The pellet was further dehydrated in propylene oxide, infiltrated in a mixture of Spurr's resin and propylene oxide (1/1, v/v) overnight; embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by electron microscope (JEM 100B; JEOL, Japan) at 80 kV.

Transport studies

The silicone oil filtration technique (Palmieri and Klingenberg, 1979) was used to measure the uptake of [^{14}C] labeled compounds into symbiosomes and bacteroids. The reaction mixture contained isolated organelles in wash buffer (see above), [^{14}C] malate, [^{14}C] sucrose or [^{14}C] glucose, and where indicated, the PA, Put, Cad, Spd or Spm (hydrochloride form) at concentration of 10, 50 or 100 μM. AR-200 silicone oil (density 1.04 g/l) was used undiluted. Reactions were carried out at a temperature of 24°C and pH 7.0.

Oxygen uptake measurements

Oxygen uptake was measured using a Clark-type electrode (DW 1, Hansatech, England) in 2 ml of wash buffer (see above) with about 2 mg symbiosome or bacteroid protein at 25°C. The pH was 7.2.

Protein concentration was determined according to Lowry et al. (1951) using BSA as a standard.

Reagents

Silicone oil (AR-200) was purchased from Wacker Chemie (Munich, Germany). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Statistical analyses

Data calculation and statistical analyses including ANOVA were conducted using SYSTAT software (SYSTAT, Inc.).

3. Results

The root nodules of *G. orientalis* plants contained high concentration of PA (Table 1). These nodules were rich in diamines Put and Cad, making up over 80% of the total PA. Tetraamine Spm was found to be a minor PA in *Galega* nodules (only 1.7% of the total PA).

Electron microscopy studies indicated that bacteroids in the central zone of *G. orientalis* nodules were surrounded singly by PBM, which closely reflected the

Table 1. Endogenous content of polyamines in the nodules from 40-day-old *Galega orientalis* plants. Data are means of triplicate experiments.

Polyamine	Nodule polyamine concentration (µmol/g dry wt)
Put	23.5±1.82
Cad	13.9±1.10
Spd	8.5±0.92
Spm	0.8±0.06

Table 2. Oxygen consumption by symbiosomes and bacteroids isolated from *Galega orientalis* nodules in the presence of polyamines at concentration of 50 and 100 µM. External malate concentration was 1 mM. Endogenous O_2 uptake rates: symbiosomes, 6.3±0.3 nmol/min/mg protein; bacteroids, 7.8±0.4 nmol/min/mg protein. Data are means of triplicate experiments.

Treatments	O_2 consumption (nmol/min/mg protein)	
	Symbiosomes	Bacteroids
Malate (control)	12.1	14.2
Malate + 50 µM Put	31.3 **	31.2 **
Malate + 100 µM Put	4.83 **	7.9 **
Malate + 50 µM Cad	29.3 **	28.9 **
Malate + 100 µM Cad	6.2 **	8.9 **
Malate + 50 µM Spd	17.6 **	18.6 **
Malate + 100 µM Spd	10.1 *	12.4 **
Malate + 50 µM Spm	16.3 **	17.0 **
Malate + 100 µM Spm	11.6 ns	13.2 ns

*Significant at 5% level, according to ANOVA; **significant at 1% level, according to ANOVA; ns = not significant.

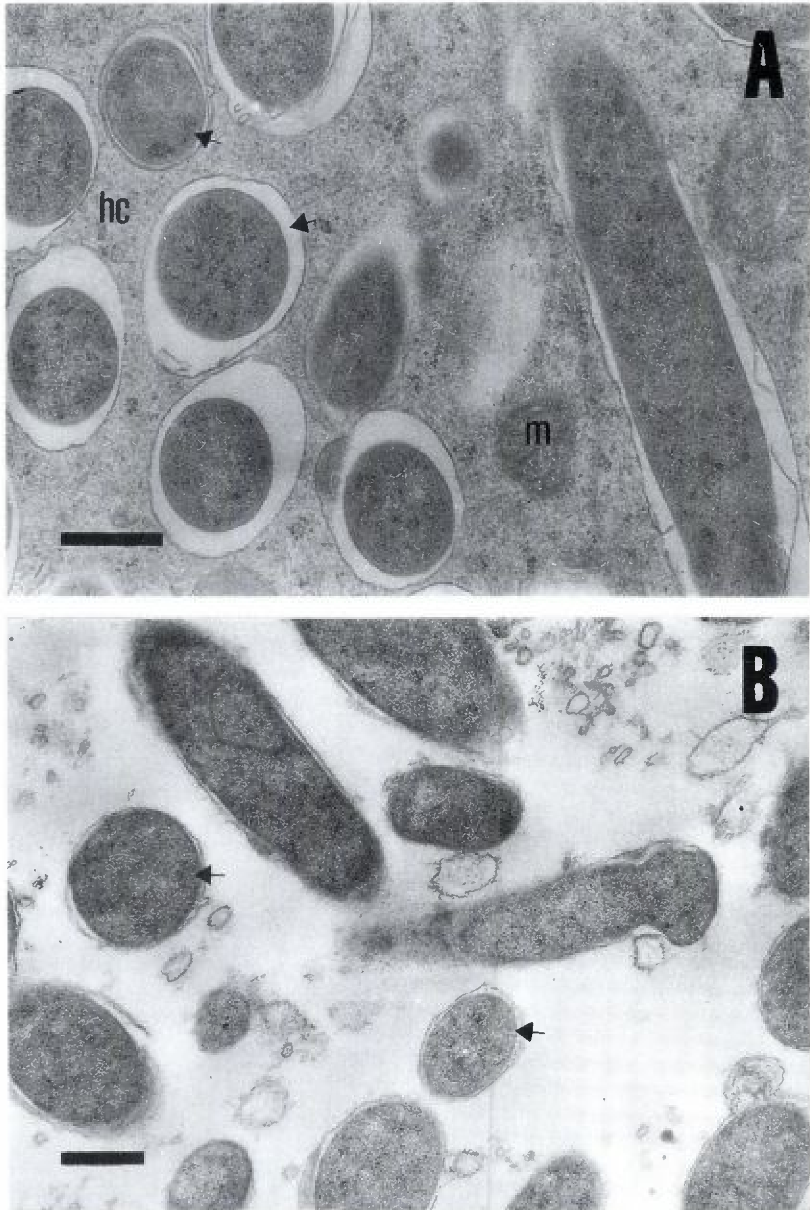

Figure 1. Transmission electron micrographs of thin sections of (A) nodule from a 40-day-old *Galega orientalis* plants showing the prominent symbiosomes (arrows) in the infected cells and (B) symbiosome preparation (arrows) from nodules of a compatible age to that in Fig. 1A. m = mitochondria; hc = host cytosol. Bar = 0.5 μm.

contours of the enclosed bacteroids (Fig. 1A). Intact symbiosomes were isolated from the nodule homogenate using a Percoll gradient centrifugation. In general, the isolated symbiosomes resemble those seen in sections of intact nodules (Fig. 1 A,B), which attests to the minimal damage the symbiosomes undergo during isolation. The degree of purity of symbiosomes was routinely checked by electron microscopy of fixed and embedded material (Fig. 1B). Contamination of symbiosome preparations by free bacteroids and mitochondria was less than 2.5%. Mitochondrial contamination can be ruled out because we observe no measurable stimulation of oxygen uptake upon addition of NADH to symbiosome preparations while such NADH oxidation is very rapid in isolated nodule mitochondria (Day et al., 1986).

Uptake experiments show that both *Galega* symbiosomes and bacteroids can rapidly accumulate [^{14}C] malate (Fig. 2). Moreover, malate utilization by symbiosomes was about 35% slower than that by free bacteroids. Rates of uptake of [^{14}C] sucrose and [^{14}C] glucose by symbiosomes and bacteroids were extremely slow (Fig. 2).

Fig. 3 indicated the effect of different PA concentrations on the uptake of [^{14}C] labeled malate by isolated organelles. Malate most readily penetrated the symbiotic membranes in the presence of 10 and 50 μM Put and Cad. However, the 100 μM Put or Cad treatment resulted in a significant decrease of the malate transport across the PBM and BM. The addition of various PA to symbiosome and bacteroid suspensions did not affect sugar uptake (data not shown).

The time course studies, presented in Figs. 4 and 5, showed significant differences in the rates of dicarboxylate transport across the symbiotic membranes after treatment with various concentrations of PA. An increase of malate uptake ranging from 2.0- to 4.0-fold was observed after incubation of isolated symbiosomes with 50 μM PA concentration (Fig. 4A). At 100 μM, Put and Cad reduced this uptake significantly (Fig. 5A). Adding PA at 50 μM to bacteroid suspension resulted in an increase in the radioactivity by 1.3- to 2.8-fold (Fig. 4B). As with symbiosomes, malate uptake by bacteroids was inhibited after incubation with 100 μM diamine concentration.

Purified symbiosome and bacteroid suspensions were also assayed for O_2 consumption after PA treatment (Table 2). The low endogenous rates of O_2 uptake were strongly enhanced after addition of 1 mM malate as respiratory substrate. Table 2 shows that, similar to the results of the [^{14}C] malate uptake experiments (Figs. 3–5), PA altered O_2 consumption in both *Galega* symbiosomes and bacteroids. Exposure of isolated organelles to 50 μM of various PA caused an increase in O_2 uptake ranging from 1.35- to 2.6-fold for the symbiosomes and from 1.2- to 2.2-fold for the bacteroids. However, in the presence of 100 μM Put and Cad, their O_2 consumption was reduced significantly.

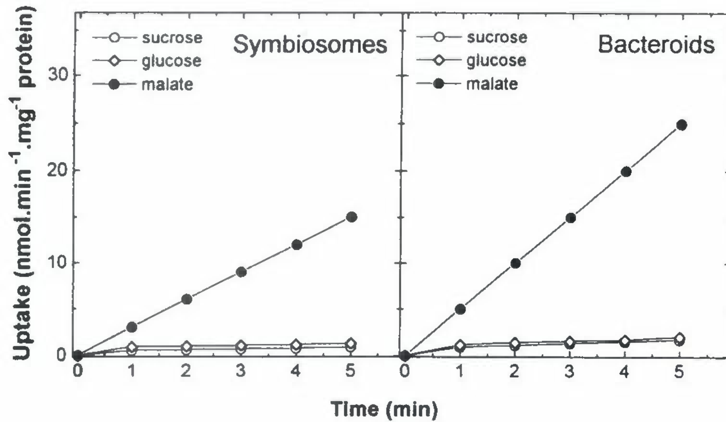

Figure 2. Time-dependent uptake of [14C] malate and [14C] sugars by symbiosomes and bacteroids isolated from *Galega orientalis* nodules. A final concentration of 1 mM was used for each substrate. Data points are means of triplicate experiments and standard errors did not exceed 5%.

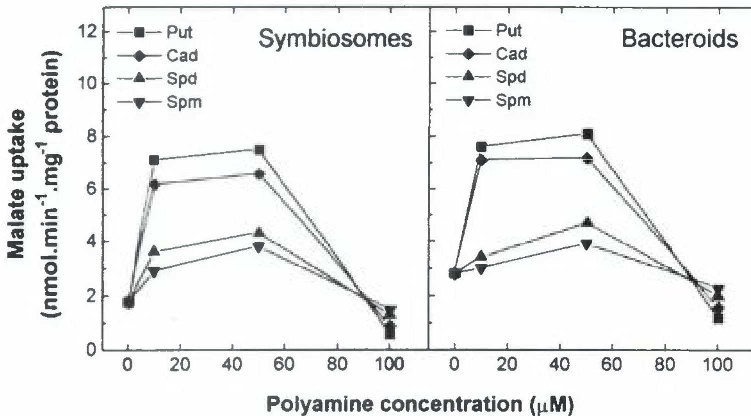

Figure 3. Effect of different concentration of polyamines on [14C] malate uptake by symbiosomes and bacteroids isolated from *Galega orientalis* nodules. External malate concentration was 1 mM. Uptake was measured for 30 s and terminated by centrifugation through silicone oil into perchloric acid. Polyamines were added prior to malate. Data points are means of triplicate experiments and standard errors did not exceed 5%.

4. Discussion

In the present study a high level of PA (46.7 μmol per g nodule dry weight) was detected in the nodules from 40-day-old *G. orientalis* plants. Putrescine was the most abundant PA followed by Cad, Spd and Spm. Fudjihara et al.

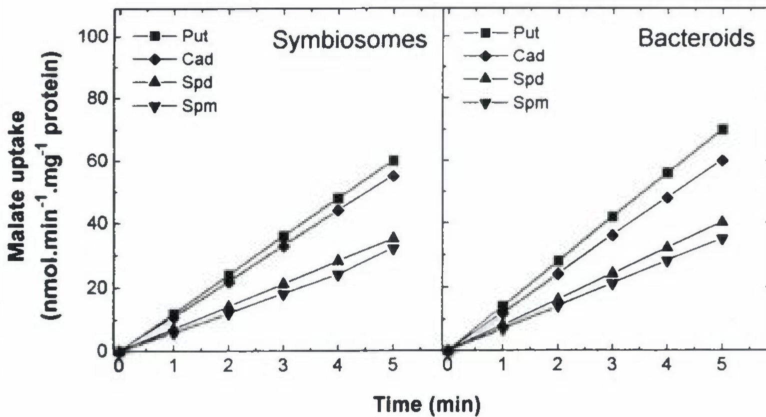

Figure 4. Time-dependent uptake of [^{14}C] malate by symbiosomes and bacteroids isolated from *Galega orientalis* nodules in the presence of various polyamines at concentration of 50 μM. External malate concentration was 1 mM. Uptake was measured by centrifugation through silicone oil into perchloric acid. Polyamines were added prior to malate. Data points are means of triplicate experiments and standard errors did not exceed 5%.

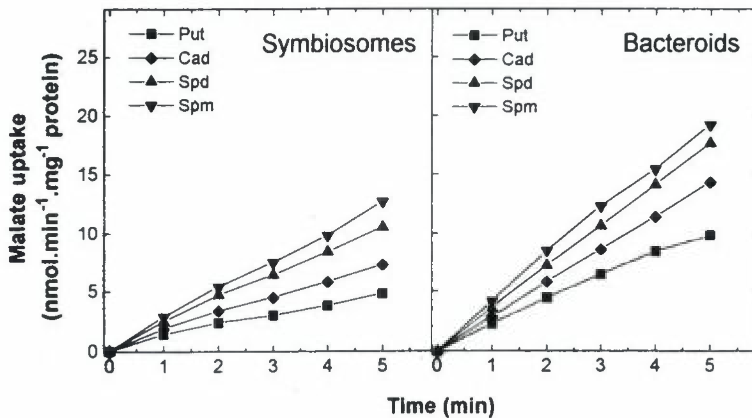

Figure 5. Time-dependent uptake of [^{14}C] malate by symbiosomes and bacteroids isolated from *Galega orientalis* nodules in the presence of various polyamines at concentration of 100 μM. Details as in Fig. 4.

(1994) also found relatively high PA concentration in root or stem nodules of many leguminous plants. Although some information is available regarding the PA metabolism during nodule development or senescence in leguminous plants (Chatterjee et al., 1983; Lahiri et al., 1992), little is known about the function of these substances within the root nodules. Ozawa and Tsuji (1993) supposed that

PA can play an important role in regulating the growth of the endosymbiont in the host plant since Put and Spd at concentration above 0.1 mM had a bacteriostatic effect on the bacteroids of *Bradyrhizobium japonicum*.

The results reported here serve as the basis for another hypothesis that the changes in the endogenous levels of PA could be involved in the regulation of the carbon supply to the nitrogen-fixing bacteroids. This role of PA within nodules may be related to an effect of PA on membrane permeability due to their polycationic nature at physiological pH (Naik and Srivastava, 1978).

Intact symbiosomes and free bacteroids were isolated for the first time from *G. orientalis* nodules and incubated with malate as carbon substrate in the presence of various PA. The results on the high activity of malate transport across the PBM and BM (Fig. 1), strongly support the general assumption that dicarboxylates are major effective substrates supplied to bacteroids *in vivo* in legume nodules (Udvardi and Day, 1997). In contrast to the rapid uptake of malate, the utilization of sugars was very slow, suggesting that the transport of these substances across the symbiotic membranes of *Galega* nodules is via passive diffusion.

Exposure of *Galega* symbiosomes and bacteroids to three different PA levels (10, 50 or 100 μM) significantly altered malate transport across the PBM and BM (Fig. 3). The prominent stimulatory effect was observed with 10 and 50 μM PA concentration, while treatment with 100 μM Put or Cad strongly inhibited permeability of the symbiotic membranes. Stimulation of malate consumption by the lower PA concentrations used (Fig. 4), as was indicated also by the oxygen uptake experiments (Table 2), will lead to a greater sink for the malate and thereby will stimulate uptake into both symbiosomes and free bacteroids. Rapid uptake by symbiosomes requires subsequent rapid transport into the bacteroids contained within them. Ou Yang et al. (1990) have shown that addition of ATP to a suspension of symbiosomes from soybean nodules resulted in a significant stimulation of malate uptake but KCN (poison of bacteroid respiration) severely inhibited malate consumption indicating that dicarboxylate transport across the PBM is driven primarily by uptake and subsequent metabolism in the bacteroids. *In vivo*, succinate and malate synthesized in the plant cytosol via phosphoenolpyruvate carboxylase are directly incorporated into bacteroids and respired (Vance and Heichel, 1991). Treatments that reduce dicarboxylate transport across symbiotic membranes from *Galega* nodules (Fig. 5) usually reduce O_2 consumption (Table 2). Adding 100 μM Put or Cad to *Galega* symbiosomes and bacteroids inhibited substantially malate uptake. Oxygen consumption by these organelles directly followed the use of malate, suggesting that uptake limited respiration of the bacteroids. It is difficult to explain the mechanism by which 100 μM Put and Cad concentration reduced malate consumption. In a previous study we established that Put at 100 μM decreased significantly (to 0.46 of control

values) nitrogenase activity of nodulated roots from *G. orientalis* plants (Vassileva and Ignatov, unpubl.). There is evidence that the rate of nitrogen fixation limits the rate of C_4-dicarboxylate transport in bacteroids, rather than vice versa (Jording et al., 1994). However, our experiments have been conducted under aerobic conditions where nitrogenase was not functional. Thus, the question for inhibition of malate uptake at 100 μM diamine concentration remains open to experimental verification.

It should also be noted that Put and Cad, which have two amino groups, altered to a higher extent permeability of symbiotic membranes than the PA Spd and Spm. The reason for this effect might be that the PA are more capable to rigidify the membranes then the diamines (Roberts et al., 1986) which would decrease their effect on dicarboxylate transport across the PBM and BM. On the other hand, the exogenous supplied PA have different compartmentation in the plant cell: while Put is localized mostly in the cytoplasmic soluble fraction, Spd and Spm are found mostly in the cell wall (Pistocchi et al., 1987). It may be suggested that the effect of diamines *in vivo*, is mainly related to the physiological role of their endogenous counterparts, while PA have nonspecific effect which occur when they act as polycations and bind noncovalently to the external surface of the cell.

In conclusion, the results presented in this report have pointed to a possible regulatory mechanism in symbiotic nitrogen fixation based on the changes in nodule PA concentration. The precise physiological role of high PA content in *Galega* nodules is unknown, but it might be partly associated with the control of permeability of symbiotic membranes to dicarboxylates.

Acknowledgments

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REFERENCES

Altman, A., Kaur-Sawhney, R., and Galston, A.W. 1977. Stabilization of oat leaf protoplasts through polyamine-mediated inhibition of senescence. *Plant Physiology* **60**: 570–574.

Chatterjee, S., Choudhuri, M.M., and Ghosh, B. 1983. Changes in polyamine content during root and nodule growth of *Phaseolus mungo*. *Phytochemistry* **22**: 1553–1556.

Day, D.A., Price, G.D., and Gresshoff, P.M. 1986. Isolation and oxidative properties of mitochondria and bacteroids from soybean root nodules. *Protoplasma* **134**: 121–129.

Day, D.A., Price, G.D., and Udvardi. M.K. 1989. Membrane interface of the *Bradyrhizobium japonicum-Glycine max* symbiosis: peribacteroid units from soybean nodules. *Australian Journal of Plant Physiology* **16**: 69–84.

Fink, J., Jeblick, W., Blaschek, W., and Krauss, H. 1987. Calcium ions and polyamines activate the plasma membrane-located 1,3-β-glucan synthase. *Planta* **171**: 130–136.

Fujihara, S., Abe, H., Minakawa, Y., Akao, S., and Yoneyama, T. 1994. Polyamines in nodules from various plant-microbe symbiotic associations. *Plant and Cell Physiology* **35**: 1127–1134.

Galston, A.W. and Sawhney, R.K. 1990. Polyamines in plant physiology. *Plant Physiology* **94**: 406–410.

Igarashi, K., Kashiwagi, K., Kobayashi, H., Ohnishi, R., Kakegawa, T., Nagasu, A., and Hirose, S. 1989. Effect of polyamines on mitochondrial F1 ATPase-catalyzed reactions. *Journal of Biochemistry* **106**: 294–303.

Jording, D., Uhde, C., Schmidt, R., and Puhler, A. 1994. The C4-dicarboxylate transport-system of *Rhizobium meliloti* and its role in nitrogen fixation during symbiosis with alfalfa (*Medicago sativa*). *Experientia* **50**: 874–883.

Lahiri, K., Chattopadhyay, S., Chattopadhyay, S., and Ghosh, B. 1992. Polyamine metabolism in nodules of *Vigna mungo* during senescence. *Phytochemistry* **31**: 4087–4090.

Lipsanen, P. and Lindstrom, K. 1988. Infection and root nodule structure in the *Rhizobium galegae* sp. nov.-*Galega* sp. symbiosis. *Symbiosis* **6**: 81–96.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**: 265–275.

Naik, B.I. and Srivastava, S.K. 1978. Effect of polyamines on tissue permeability. *Phytochemistry* **17**: 1885–1887.

Ou Yang, L.-J., Udvardi, M.K., and Day, D.A. 1990. Specificity and regulation of the dicarboxylate carrier on the peribacteroid membrane of soybean nodules. *Planta* **182**: 437–444.

Ozawa, T. and Tsuji, T. 1993. A possible role for polyamines in the repression of growth of *Bradyrhizobium japonicum* bacteroids in soybean nodules. *Plant and Cell Physiology* **34**: 899–904.

Palmieri, F. and Klingenberg, M. 1979. Direct methods for measuring metabolite transport and distribution in mitochondria. *Methods of Enzymology* **56**: 279–301.

Pistocchi, R., Antognoni, F., Bagni, N., and Zannoni, D. 1990. Spermidine uptake by mitochondria from *Helianthus tuberosus*. *Plant Physiology* **92**: 690–695.

Pistocchi, R., Bagni, N., and Greus, J.A. 1987. Polyamine uptake in carrot cell cultures. *Plant Physiology* **84**: 374–380.

Price, G.D., Day, D.A., and Gresshoff, P.M. 1987. Rapid isolation of intact peribacteroid envelopes from soybean nodules and demonstration of selective permeability to metabolites. *Journal of Plant Physiology* **130**: 157–164.

Roberts, D.R., Dumbroff, E.B., and Thompson, J.E. 1986. Exogenous polyamines alter membrane fluidity in bean leaves – a basis for potential misinterpretation of their true physiological role. *Planta* **167**: 395–401.

Rosendahl, L. and Jochimsen, B.U. 1995. *In vitro* indole-3-acetic acid uptake in symbiosomes from soybean (*Glycine max* L.) root nodules. *Symbiosis* **19**: 99–110.

Smith, T.A. and Best, G.R. 1977. Polyamines in barley seedlings. *Phytochemistry* **16**: 841–843.

Udvardi, M.K. and Day, D.A. 1997. Metabolite transport across symbiotic membranes of legume nodules. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 493–523.

Vance, C.P. and Heichel, G.H. 1991. Carbon in N_2 fixation: Limitation or exquisite adaptation. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**: 373–392.

Vassileva, V., Milanov, G., Ignatov, G., and Nikolov, B. 1997. Effect of low pH on nitrogen fixation of common bean grown at various calcium and nitrate levels. *Journal of Plant Nutrition* **20**: 279–294.

Whitehead, L.F., Tyerman, S.D., Salom, C.L., and Day, D.A. 1995. Transport of fixed nitrogen across symbiotic membranes of legume nodules. *Symbiosis* **19**: 141–154.

Young, D.H. and Kauss, H. 1983. Release of calcium from suspension-cultured *Glycine max* cells by chitosan, other polycations, and polyamines in relation to effects on membrane permeability. *Plant Physiology* **73**: 698–702.