STRUCTURAL BASES FOR EFFECTIVITY OF BACTEROIDAL TISSUE IN PEA ROOT NODULES FORMED BY EFFECTIVE OR INEFFECTIVE BACTERIA STRAIN

WOJCIECH BORUCKI

Department of Botany, Faculty of Agriculture,
Warsaw Agricultural University SGGW,
Rakowiecka 26/30, 02-528 Warsaw

(Received: December 20, 1996. Accepted: April 24, 1997)

ABSTRACT

The performed comparison included changes in volume and surface area of the chosen cellular structures during the development of the effective and ineffective bacteroidal tissue of pea root nodules. It has been noted that the increase of the volume of the infected cell in effective nodules is associated with the increase of the volume of vacuome, symbiosomes and cystol while in ineffective bacteroidal tissue with the increase of the starch volume and to a lesser degree symbiosomes and cytosol volumes. In turn, the volume increase of uninfected cells in effective bacteroidal tissue is associated with the volume increase of vacuome and cystol and in case of ineffective nodules with the increase of the volumes of cystol, starch, and to a lesser degree, vacuome. This data point to the disturbances in the vacuolation process of infected and uninfected cells in ineffective bacteroidal tissue.

In effective symbiosis the volume of vacuome and peribacteroidal spaces in the infected cells change in the dependent way that stresses common features of these two compartments.

It has also been observed that the surface area and/or volume of such infected cell compartments as plastids, mitochondria, symbiosomes and cystol change in a more co-ordinated way during the development of the effective than ineffective bacteroidal tissue.

All these facts create a basis for discussion about the role of particular compartments in the functioning of bacteroidal tissue.

KEY WORDS:: morphometry, nitrogen fixation, bacteroidal tissue, cell differentiation

INTRODUCTION

Root nodule in *Fabaceae* is a totally new organ formed as a result of interaction between the plant and bacterium genome the function of which is nitrogen fixation. The degree of effectiveness of those nodules directly affects the rate of growth of plants vegetating on soil short of mineral nitrogen.

Root nodule is built of meristem, cortex and bacteroidal tissue. The bacteroidal tissue contains infected cells that are usually accompanied by uninfected cells (Brown and Walsh 1994). The characteristic feature of the infected cell cytoplasm is the presence of symbiosomes that can be regarded as a new lytic compartment of infected cells (Mellor 1989). Symbiosomes are separated from the host cytoplasm with the peribacteroidal membrane (Mellor and Werner 1987). An intensive exchange of metabolites between the plant and bacteroids is carried out through that membrane (Yang et al. 1990).

The function of nodule is to create proper conditions for symbiosomes to fix nitrogen which consists mainly in a signi-

ficant decrease of the free oxygen concentration in the infected cell cytoplasm (Tjepkema and Yocum 1974) with its simultaneous, quick diffusion due to leghemoglobin present in the cytosol (Appleby 1984).

The functioning of nodules has its structural bases resulting from proper cortex structure, spatial organisation of bacteroidal tissue, structure of infected and uninfected cells. Although, undoubtedly, the functioning of root nodules results from many complicated interactions between plant and bacterium at the genetic and biochemical levels, the different nodule effectiveness can have a simple structural base. It has been proved that the effectiveness in *Lotus* or soybean nodules depends on the degree of bacteria (bacteroids) multiplication in them (Wood et al. 1985, Lin et al. 1988). What is more, Wood et al. (1985) connected 1) smaller size of peribacteroidal space and 2) bigger part of cystol in the infected cell with more effective symbioses.

During the cell differentiation in the effective bacteroidal tissue of the root nodule there dominate such processes as cell vacuolation (not always!) and the volume increase of cystol,

symbiosomes and of starch. So far, there have been no quantitative data describing the structural changes characteristic for the development of the effective and ineffective bacteroid tissue in root nodules. That is why the morphometric methods were applied which allowed to obtain the data on the changes of volume and surface area of the chosen structures as the basis for correlative investigations in which the connection between the cell structure and the effectiveness of bacteroid tissue was analysed.

MATERIAL AND METHODS

Plant culture.

Pea seeds (*Pisum sativum* L. cv. Six Week) were surface sterilised with calcium hypochloride suspension for 10 min, then rinsed a few times with distilled water and then for three days germinated at room temperature on the Petri dishes lined with sterile blotting paper wetted with distilled water. Germinated seeds, the roots of which reached the length of 2 cm, were transferred to pots (five seeds per pot) of 1 liter volume filled with sterile perlite and inoculated with a proper bacterial strain. The plants grew in the photoperiod of 16h light and 8h dark at the temperature of 20-22°C during the day and 14-16°C at night. The plants were lighted with sodium lamp of the WLS400W type by POLAMP (600 mE/m²/s photosynthetically active radiation). They were watered every three day with nitrogen-free medium according to Fahraeus (1957) in the amount of 100 ml per pot and with distilled water on the remaining days.

Inoculation.

Pea plants were inoculated with *Rhizobium leguminosarum* bv. *viciae* effective strain CIAM 1026 (wild type – nod+ fix+) or ineffective CIAM 1064 (nod+ fix-) which were obtained from the collection of the All Russia Research Institute for Agricultural Microbiology in St. Petersburg. Previously, strain 1026 had been signed 250a (Borucki 1996). For 72 h bacteria were multiplied at room temperature on liquid medium at pH = 7, which contained in 1 liter: 0.5 g K₂HPO₄, 0.3 g MgSO₄, 0.1 g NaCl, 10 g saccharose and 0.5 g yeast extract shaken all the time. Inoculation was performed using 20 ml bacterial suspension per pot.

Collecting and fixing plant material.

Material for the morphometric investigations was collected 3 weeks after inoculation. It comprised specimens taken from developing or mature parts of the bacteroid tissue randomly selected nodules. The collected material was fixed according to Karnovsky (1965) at room temperature for 4 hours and post-fixed in 1% OsO₄ at 4°C for 2 hours, dehydrated in the increasing concentrations of ethanol, acetone and propylene dioxide and embedded in Epon 812 (Luft 1961).

Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in electron microscope JEM 100C.

Plant growth and nodulation.

At 7 day intervals after inoculation, 10 plants of each symbiosis were selected at random. Plant shoots were dried (100°C for 24h) and weighed as well as total nitrogen content was measured using Kjel-Foss-Automatic.

Morphometry

Light microscopy.

In order to minimize variability of bacteroid tissue resulting from its zonation, sections for light microscopy (1 µm

thick) were cut perpendicular to long nodule axis. At least 12 randomly chosen views of bacteroid tissue per one semithin section (each from different nodule) were analysed to quantify the relative areas (= volume fractions) occupied by nuclei, cytoplasm, vacuole in the infected cells and uninfected cells. It was done with the help of the ocular double lattice test grid of 391 points embracing 9409 m² of the bacteroid tissue surface (objective lens 100x). The number of cells of a given type was determined according to a formula $N_V = K/\beta N_A^3 / V_V$ where N_V means the number of infected or uninfected cells per unit volume, K – the coefficient of the cell size distribution, N_A – the number of particular cell profiles per unit area, V_V – the volume fraction of the particular cell type. The β coefficient was measured from the standard curve determining first the mean ratio of the length to the width of the cell profile if their shape is close to the ellipsoid obtained by revolving an ellipse around its long axis. The coefficient K was rejected because for the particle size distribution with the standard deviation of less than 20% its value varying from 1 to 1.05 and thus producing the error in the N_V determination of less than 5%. Then using the formula $V = V_V / N_V$ the mean volume of the infected and uninfected cell in the bacteroid tissue was calculated (Weibel et al. 1966, Weibel 1974, 1979, 1980).

Electron microscopy.

Twelve randomly chosen views within each thin section of bacteroid tissue prepared from the same nodule examined under the light microscope were recorded photographically at a final magnification of 7430 times (negative 3300x). The pictures were used to quantify the volume fractions and surface density of bacteroids, peribacteroid spaces, peribacteroid membranes and RER (only surface density), mitochondria, plastids and starch granules in the cytoplasm of infected and uninfected cells separately. The Weibel's test grid with 252 points and 126 segments was used (Weibel et al. 1966). It embraced 395 µm² area of the bacteroid tissue section. Also the point lettuce of 576 points was used which allowed more exact calculation of the volume fractions of below 5% value.

In order to obtain random choice of the photographed places the microphotographs were taken in such a way that the microscopic field in the electron microscope touched the left upper corner of the supporting lettuce on which the ultrathin sections were randomly arranged (Weibel et al. 1966).

The number of microphotographs (10-12 for each sample) necessary for counting the volume fraction at the 3% level with the error not exceeding 10% with 5% error risk was established in accordance to the procedure presented by Weibel (1979). In case of poor representation of uninfected cells in the analysed fragment of the bacteroid tissue the number of pictures was increased to 16.

Statistical analysis of results

While determining statistical significance of the determined Pearson correlation coefficient r the significance level of 0.01 was accepted. The $r \geq 0.72$ was accepted as the low limit of the tight association that gives the value of the determination coefficient $R^2 > 50\%$. Statistical data analysis and presentation were done according to computer programmes SPSS/PC+™ Base System version 5.0 and Microsoft EXCEL version 5.0.

Fig. 1. Dry weight accumulation (DW) and total nitrogen content (TN) in pea shoots of effective (fix+) and ineffective (fix-) symbioses. Values are presented as mean ($n = 10$) $\pm$ SD.

RESULTS

Plant growth

Dry weight content calculated per plant was strongly limited in ineffective symbiosis moreover the amount of total nitrogen in the stem did not increase (Fig. 1). Plants in both symbioses started to bloom 3 weeks after inoculation. The period of blooming comprised 1 week in case of effective symbiosis and it was prolonged to 3 weeks for ineffective symbiosis.

Comparison of cell structure in effective and ineffective bacteroidal tissue – qualitative analysis

Nodules on pea roots induced by ineffective bacteria strain, 1064, were similar to nodules formed by effective strain 1026 in having indeterminate habit with characteristic zonation of infected zone although without fully developed mature zone (see classification Vasse et al. 1990). Symbioses were found to differ in vacuolation and starch accumulation within infected cells (Fig. 2A and C). Besides vacuolation and starch, qualitative differences were noted in symbiosome development, with less distinct and irregular in shape peribacteroid space as well as greater bacteroid cytoplasm heterogeneity found in ineffective symbiosis (Fig. 2B and D). For more details see previous article of the author (Borucki, 1996).

Infected cell – relationship between the volumes of cell structures.

Nearly all coefficients r of the Pearson correlation between the volumes of infected cell structures in effective symbiosis are statistically significant and usually have larger values than in ineffective symbiosis (Table 1). Their values often reflect a strong or very strong positive linear association between the volumes of the investigated structures. The increase of the infected cell volume in effective symbiosis is most strongly associated with the volume increase of vacuome, symbiosomes and cystol, while in ineffective symbiosis with the volume increase of symbiosomes and starch (Tables 1, 3; Figs 3A, 4A, 5A, 6). During infected cell enlargement its vacuolation (expressed in % of total cell volume) increases in case of effective

symbioses and decreases in ineffective symbiosis. In infected cells of effective symbiosis vacuome level reaches 30% (calculated from Fig. 3A). Small and large infected cells in both symbioses contain the same amount of cytosol – about 35% (calculated from Fig. 4A). Starch reserves expressed as % of total cell volume decrease during infected cell enlargement in effective and increase in ineffective symbiosis. Maximal level of starch in infected cells can reach more than 30% in ineffective symbiosis. In case of effective symbioses the level of starch is much lower and comprises maximally 10% of cell volume (calculated from Fig. 5A). Infected cells in ineffective bacteroidal tissue can reach only $10^5 \mu\text{m}^3$ while in effective bacteroidal tissue they can be even 4 times larger (calculated from Fig. 3A).

During infected cell enlargement in effective bacteroidal tissue there is an increase in symbiosomes to cytosol volumes ratio (Fig. 7). A strong positive linear association between the volumes of mitochondria and plastid stroma was observed, especially in effective symbiosis (Tables 1, 3). The volume of the effective symbiosis bacteroids is strongly associated with the volume of peribacteroid space and cystol while in ineffective symbiosis with the volume of starch (Table 1). In the infected cells of effective bacteroidal tissue there is a tight association between the volumes of symbiosomes and cytosol, vacuome and peribacteroid spaces (Tables 1, 3; Figs 7, 8), vacuome and cytosol (Table 1). Symbiosomes volume in effective bacteroidal tissue is tightly associated with the volume of cell, cytoplasm, cytosol, host cytoplasm, vacuome, mitochondria and plastid stroma while in ineffective bacteroidal tissue with the volume of cell, cytoplasm, host cytoplasm and starch (Tables 1, 3).

Infected cell – relationship between the surface areas of cell structures.

In infected cell in effective symbiosis strong positive associations are observed between the surface areas of such structures as: mitochondria and peribacteroid membranes (Fig. 9A), plastids and mitochondria (Fig. 9B), starch and mitochondria, mitochondria and bacteroids which are not observed in case of infected cell in ineffective symbiosis (Tables 4, 6). On the other hand, in infected cell of ineffective symbiosis opposite to effective symbiosis strong positive associations are observed between the surface areas of plastids and peribacteroid membranes, plastids and bacteroids, mitochondria and RER (Tables 4, 6). In case of ineffective symbiosis the level of RER surface in cytosol of infected cells is usually much higher than in effective symbiosis (Fig. 10A).

Uninfected cell – relationship between the volumes of cell structures.

The volume of uninfected cell in effective symbiosis most strongly depends on the volume increase of vacuome and cystol and in ineffective symbiosis on the volume of cytoplasm including cystol and especially starch (Tables 2, 3; Figs 3B, 5B). In uninfected cells of effective and ineffective bacteroidal tissue vacuome level can reach about 60 and 30% respectively (calculated from data in Fig. 4B). Starch reserves can comprise 20 and 35% of uninfected cell volume in effective and ineffective bacteroidal tissue (calculated from data in Fig. 5B). In the uninfected cells in ineffective symbiosis there is a whole network of tight associations between the volumes of such compartments as mitochondria, plastid stroma, starch, and cystol – not observed in case of effective symbiosis (Table 2).

Fig. 2. A and C. Light micrographs. Bars = 10 μ m. B and D. Transmission electron micrographs. Bars = 2 μ m. A and B. The portions of effective bacteroidal tissue taken from mature zone of 3 weeks old nodules show prominent vacuolation of infected cells (IC) and uninfected cells (UC). Amyloplasts within infected cells filled in irregularly shaped starch granules (S) are located close to intercellular spaces (IS). Bacteroids (B) are surrounded by regular in shape peribacteroidal spaces (ps). C and D. The portions of the ineffective bacteroidal tissue taken from mature zone of 3 weeks old nodules show slightly vacuolated infected cells (IC) with abundant starch granules (S) and large infection threads (IT). Peribacteroidal spaces (ps) are visible occasionally. Mitochondria (m) in both effective and in ineffective bacteroidal tissues within infected cells are located close to amyloplasts. Note: N – nucleus, rer – rough endoplasmic reticulum, V – vacuome.

Uninfected cell – relationship between the surface areas of cell structures.

In the uninfected cells in effective symbiosis only the surface areas of plastids and starch are tightly associated and in ineffective symbiosis the association between the surface areas of all the investigated structures are strong (Tab. 5). Cytosol of uninfected cells exhibits usually much higher level of

RER surface in ineffective symbiosis than in effective symbiosis (Fig. 10B).

DISCUSSION

Considering significant differences in the degree of development of the bacteroidal tissue resulting from its zonation in

Fig. 3. The relationship between cell volume and vacuome volume. A. Infected cell. B. Uninfected cell. Linear regressions through the raw data for effective (fix+) and ineffective (fix-) symbioses are presented (see Tables 1, 2 for significance and Tab. 3 for statistical comparisons of relationships). Note: arrow separates young (lower values on X-axis) and mature (higher values on X-axis) bacteroidal tissue.

Fig. 4. The relationship between cell volume and cytosol volume. A. Infected cell. B. Uninfected cell. Linear regressions through the raw data for effective (fix+) and ineffective (fix-) symbioses are presented (see Tables 1, 2 for significance and Tab. 3 for statistical comparisons of relationships). arrow – see Fig. 3.

Fig. 5. The relationship between cell volume and starch volume. A. Infected cell. B. Uninfected cell. Linear regressions through the raw data for effective (fix+) and ineffective (fix-) symbioses are presented (see Tables 1, 2 for significance and Tab. 3 for statistical comparisons of relationships). arrow – see Fig. 3.

Fig. 6. The relationship between infected cell volume and symbiosomes volume. Linear regressions through the raw data for effective (fix+) and ineffective (fix-) symbioses are presented (see Table 1 for significance and Table 3 for statistical comparisons of relationships). arrow – see Fig. 3.

Fig. 7. The relationship between cytosol volume and symbiosomes volume. Linear regressions through the raw data (calculated per cell) for effective (fix+) and ineffective (fix-) symbioses are presented (see Table 1 for significance and Tab. 3 for statistical comparisons of relationships). arrow – see Fig. 3.

TABLE 1. The relationships between volumes of infected cells and their structures in effective and ineffective bacteroidal tissue – the Pearson correlation coefficients.

	B	PS	SYM	M	SP	S	N	CL	HC	CP	V	IC
B	1.0000**	.9518**	.9938**	.8128**	.7883**	.4595*	.5607**	.8960**	.8944**	.9768**	.8518**	.9622**
PS	<u>.3879</u>	1.0000**	.9801**	.7852**	.7501**	.3909	.5487**	.8839**	.8786**	.9609**	.8656**	.9574**
SYM	<u>.9641**</u>	<u>.4803</u>	1.0000**	.8119**	.7833**	.4398*	.5627**	.9017**	.8988**	.9821**	.8664**	.9713**
M	<u>.6316**</u>	<u>-.0835</u>	<u>.5544*</u>	1.0000**	.9142**	.5145**	.5129**	.8144**	.8515**	.8520**	.6593**	.8055**
SP	<u>.6430**</u>	<u>-.0869</u>	<u>.5315*</u>	<u>.8326**</u>	1.0000**	.5239**	.5010**	.7522**	.7999**	.8120**	.6449**	.7745**
S	<u>.7968**</u>	<u>.4712</u>	<u>.7144*</u>	<u>.4310</u>	<u>.6802**</u>	1.0000**	.2825	.4551*	.5760**	.5158**	.2906	.4443*
N	<u>-.0705</u>	<u>-.3576</u>	<u>-.1321</u>	<u>.4562</u>	<u>.2570</u>	<u>-.1797</u>	1.0000**	.6913**	.7017**	.6221**	.4836*	.5981**
CL	<u>.3569</u>	<u>-.2341</u>	<u>.2882</u>	<u>.7768**</u>	<u>.4159</u>	<u>-.0043</u>	<u>.5604*</u>	1.0000**	.9853**	.9601**	.8123**	.9378**
HC	<u>.8330**</u>	<u>.1739</u>	<u>.7256**</u>	<u>.8536**</u>	<u>.8265**</u>	<u>.7645**</u>	<u>.2748</u>	<u>.6355**</u>	1.0000**	.9639**	.7856**	.9298**
CP	<u>.9260**</u>	<u>.3348</u>	<u>.8462**</u>	<u>.7796**</u>	<u>.7722**</u>	<u>.8345**</u>	<u>.1073</u>	<u>.5252*</u>	<u>.9737**</u>	1.0000**	.8527**	.9779**
V	<u>-.1545</u>	<u>.2676</u>	<u>-.0113</u>	<u>-.4069</u>	<u>.4624</u>	<u>-.2401</u>	<u>-.1219</u>	<u>-.3935</u>	<u>-.4351</u>	<u>-.3415</u>	1.0000**	.9428**
IC	<u>.9311**</u>	<u>.3696</u>	<u>.8679**</u>	<u>.7684**</u>	<u>.7408**</u>	<u>.8165**</u>	<u>.1475</u>	<u>.5119</u>	<u>.9550**</u>	<u>.9879**</u>	<u>-.2008</u>	1.0000**

N of cases: 41 – effective symbiosis, 23 – ineffective symbiosis
2 – tailed Signif: * – .01 ** – .001
Note: B – bacteroids, PS – peribacteroidal spaces, SYM – symbiosomes, M – mitochondria, SP – plastid stroma, S – starch granules, N – nuclei, CL – host cytosol, HC – host cytoplasm, CP – host cytoplasm including symbiosomes, V – vacuome, IC – infected cell, underline-ineffective symbiosis.

TABLE 2. The relationships between volumes of uninfected cells and their structures in effective and ineffective bacteroidal tissue – the Pearson correlation coefficients.

	M	SP	S	N	CL	HC	V	UC
M	1.0000**	.4183*	.4473*	.4085*	.6404**	.6251**	.4102*	.5880**
SP	<u>.8169**</u>	1.0000**	.5374**	.1950	.6068**	.6528**	.5386**	.6923**
S	<u>.8046**</u>	<u>.9238**</u>	1.0000**	.1096	.6674**	.8895**	.1102	.4956**
N	<u>.4760</u>	<u>.6290**</u>	<u>.6302**</u>	1.0000**	.1808	.2005	.2974	.3068
CL	<u>.8210**</u>	<u>.7813**</u>	<u>.7632**</u>	<u>.6691**</u>	1.0000**	.9321**	.5318**	.8186**
HC	<u>.8673**</u>	<u>.9241**</u>	<u>.9554**</u>	<u>.6997**</u>	<u>.9192**</u>	1.0000**	.3938	.7512**
V	<u>.4577</u>	<u>.4386</u>	<u>.4874</u>	<u>.1209</u>	<u>.4070</u>	<u>.4769</u>	1.0000**	.9025**
UC	<u>.8255**</u>	<u>.8588**</u>	<u>.9017**</u>	<u>.5615*</u>	<u>.8412**</u>	<u>.9301**</u>	<u>.7665**</u>	1.0000**

N of cases: 41 – effective symbiosis, 23 – ineffective symbiosis
2 – tailed Signif: * – .01 ** – .001
Note: UC – uninfected cell, underline – ineffective symbiosis. For more abbreviations see Table 1.

TABLE 3. R², y intercept (b₀) and slope (b₁) from linear regression of cell structure volume vs cell volume or another cell structure volume using raw data for effective (fix+) or ineffective (fix-) symbioses. For effective symbiosis n = 41, for ineffective symbiosis n = 23. y = b₀ + b₁x.

cell type	effectivity	dependent variable (y)	independent variable (x)	R ²	b ₀	(P)	b ₁	(P)
IC	fix+	vacuome	cell	0.889	-18.59	0.001	0.381	0.001
IC	fix-	vacuome	cell	-0.040 ^{ns}	8.37	0.001	-0.032	0.348
IC	fix+	cytosol	cell	0.880	11.19	0.001	0.220	0.001
IC	fix-	cytosol	cell	0.263	12.45	0.029	0.225	0.010
IC	fix+	starch	cell	0.197	2.84	0.016	0.020	0.004
IC	fix-	starch	cell	0.667	-17.28	0.002	0.486	0.001
IC	fix+	symbiosomes	cell	0.943	3.04	0.204	0.350	0.001
IC	fix-	symbiosomes	cell	0.753	-4.37	0.041	0.251	0.001
IC	fix+	symbiosomes	cytosol	0.813	-5.82	0.263	1.38	0.001
IC	fix-	symbiosomes	cytosol	0.083 ^{ns}	6.12	0.122	0.190	0.172
IC	fix+	symbiosomes	mitochondria	0.659	14.08	0.015	22.00	0.001
IC	fix-	symbiosomes	mitochondria	0.307	1.19	0.730	7.68	0.005
IC	fix+	symbiosomes	plastid stroma	0.614	23.46	0.001	20.13	0.001
IC	fix-	symbiosomes	plastid stroma	0.282	6.05	0.009	4.38	0.007
IC	fix+	mitochondria	plastid stroma	0.836	0.50	0.002	0.867	0.001
IC	fix-	mitochondria	plastid stroma	0.693	0.72	0.001	0.495	0.001
IC	fix+	perib. space	vacuome	0.749	6.01	0.001	0.281	0.001
IC	fix-	perib. space	vacuome	0.072 ^{ns}	0.82	0.001	0.145	0.206
UC	fix+	vacuome	cell	0.815	-3.47	0.096	0.648	0.001
UC	fix-	vacuome	cell	0.756	5.06	0.001	0.274	0.001
UC	fix+	cytosol	cell	0.670	1.68	0.112	0.223	0.001
UC	fix-	cytosol	cell	0.766	-0.834	0.396	0.276	0.001
UC	fix+	starch	cell	0.246	1.34	0.301	0.110	0.001
UC	fix-	starch	cell	0.910	-4.05	0.001	0.392	0.001

Note: IC – infected cell, UC – uninfected cell, P – probability, not marked R² values are statistically significant (P), ns – not significant (P 0.05). Slopes compared in pairs (fix+ vs fix-) were different (P 0.05) except cytosol/cell relationship in infected cells.

Fig. 8. The relationship between vacuome volume and peribacteroid spaces volume. Linear regressions through the raw data (calculated per cell) for effective (fix+) and ineffective (fix-) symbioses are presented (see Table 1 for significance and Tab. 3 for statistical comparisons of relationships). arrow – see Fig. 3.

Fig. 9. The relationships between A. mitochondria and peribacteroid membranes, B. plastids and mitochondria surface areas within infected cells. Linear regressions through the raw data (calculated per cell) for effective (fix+) and ineffective (fix-) symbioses are presented (see Table 4 for significance and Table 6 for statistical comparisons of relationships). arrow – see Fig. 3.

Fig. 10. The relationship between cytosol volume and RER surface. A. Infected cell. B. Uninfected cell. Linear regressions through the raw data (calculated per cell) for effective (fix+) and ineffective (fix-) symbioses are presented (see Tables 4 and 5 for significance and Table 6 for statistical comparisons of relationships). arrow – see Fig. 3.

TABLE 4. The relationships between cell structure surface areas vs another cell structure surface area or cytosol volume, or infected cell volume in effective and ineffective bacteroidal tissue – the Pearson correlation coefficients.

	B	PM	M	RER	P	S	CL	IC
B	1.0000**	.9955**	.7903**	.2806	.6916**	.5997**	.9157**	.9658**
PM	<u>.9896**</u>	1.0000**	.8000**	.2799	.7012**	.6083**	.9213**	.9737**
M	<u>.6648**</u>	<u>.6415**</u>	1.0000**	.4132*	.8964**	.7505**	.8243**	.8003**
RER	<u>.4583</u>	<u>.3985</u>	<u>.7129**</u>	1.0000**	.4779*	.5960**	.2889	.2274
P	<u>.7820**</u>	<u>.7948**</u>	<u>.6232*</u>	<u>.3104</u>	1.0000**	.8477**	.7062**	.7072**
S	<u>.6545**</u>	<u>.6942**</u>	<u>.3057</u>	<u>-.0005</u>	<u>.8854**</u>	1.0000**	.6020**	.5988**
CL	<u>.5370*</u>	<u>.4745</u>	<u>.8598**</u>	<u>.8341**</u>	<u>.4108</u>	<u>.0700</u>	1.0000**	.9378**
IC	<u>.8882**</u>	<u>.8873**</u>	<u>.6655**</u>	<u>.4036</u>	<u>.9062**</u>	<u>.8454**</u>	<u>.5119</u>	1.0000**

N of cases: 41 – effective symbiosis, 23 – ineffective symbiosis

2 – tailed Signif: * – .01 ** – .001

Note: surface area of bacteroids (B), peribacteroidal membranes (PM), mitochondria (M), rough endoplasmic reticulum (RER), plastids (P), starch granules (S), volume of host cell cytosol (CL), volume of infected cell (IC); underline-ineffective symbiosis.

TABLE 5. The relationships between cell structure surface areas vs another cell structure surface area or cytosol, or uninfected cell volume in effective and ineffective bacteroidal tissue – the Pearson correlation coefficients.

	M	RER	P	S	CL	UC
M	1.0000**	.3279	.4086*	.3264	.5343**	.5219**
RER	<u>.8814**</u>	1.0000**	.5089**	.5346**	.2734	.3642
P	<u>.9052**</u>	<u>.8063**</u>	1.0000**	.9083**	.6625**	.5509**
S	<u>.8890**</u>	<u>.7572**</u>	<u>.9867**</u>	1.0000**	.6182**	.5206**
CL	<u>.8754**</u>	<u>.9326**</u>	<u>.8719**</u>	<u>.8474**</u>	1.0000**	.8049**
UC	<u>.8798**</u>	<u>.7203**</u>	<u>.8938**</u>	<u>.9110**</u>	<u>.8419**</u>	1.0000**

N of cases: 41 – effective symbiosis, 23 – ineffective symbiosis

2 – tailed Signif: * – .01 ** – .001

Note: UC – volume of uninfected cell. For more abbreviations see Table 4.

pea root nodules no mean values regarding the volume or surface area of the investigated structures are presented here as being loaded with a considerable error. Thus it has been accepted as more reasonable and useful to look for correlations between the changes of volume and surface area of the intracellular structures against the volume changes of infected and uninfected cells. It should also be stressed that uninfected cells in the bacteroidal tissue are not randomly distributed similarly as plastids and mitochondria in the infected cells (Fig. 2. B and D). Thus a large number of nodules and large area of bacteroidal tissue had to be analysed. It should be remembered that during bacteroidal tissue development infected and uninfected cell volume enlarges but it depends also on the nodule size (data not presented). So, arrows on all figures mark an average value for cell or cell compartments giving only a crude separation between young and mature bacteroidal tissue.

Plant growth

Plants inoculated with the ineffective strain 1064 *R. leguminosarum* suffered from acute nitrogen deficiency manifesting itself with a prolonged blooming period, the lack of nitrogen content increase in the shoot and resulting from it the limitations of the dry weigh increase (Fig. 1).

Infected cell

In effective symbiosis together with the volume increase of the infected cell, the volume of most cell structures also increases which may prove the high degree of co-ordination of the cell differentiation processes. For example, if the volume of an infected cell is known, one can predict, with a high precision, the volume of vacuome or symbiosomes. It is either totally impossible in case of ineffective symbiosis or it is loaded with a considerable error (Figs 3A, 6).

Attention is called by the fact that the volume increase of the infected cell of effective symbiosis is accompanied by the

TABLE 6. R^2 , y intercept (b_0) and slope (b_1) from linear regression of cell structure surface area vs another cell structure surface area or cytosol volume using raw data for infected or uninfected cells of effective (fix+) or ineffective (fix-) symbioses. For effective symbiosis $n = 41$, for ineffective symbiosis $n = 23$.

$$y = b_0 + b_1x$$

cell type	effectivity	dependent variable (y)	independent variable (x)	R^2	b_0	(P)	b_1	(P)
IC	fix+	PM	mitochondria	0.640	58.91	0.001	6.07	0.001
IC	fix-	PM	mitochondria	0.411	6.96	0.466	2.69	0.001
IC	fix+	PM	plastids	0.492	84.72	0.001	4.89	0.001
IC	fix-	PM	plastids	0.632	9.71	0.109	2.10	0.001
IC	fix+	mitochondria	plastids	0.803	3.93	0.008	0.824	0.001
IC	fix-	mitochondria	plastids	0.388	6.83	0.001	0.393	0.001
IC	fix+	RER	cytosol	0.083 ^{ns}	16.90	0.001	0.142	0.067
IC	fix-	RER	cytosol	0.696	-8.86	0.102	1.30	0.001
UC	fix+	RER	cytosol	0.075 ^{ns}	1.89	0.052	0.147	0.088
UC	fix-	RER	cytosol	0.870	-0.181	0.731	0.830	0.001

Note: IC – infected cell, PM – peribacteroid membranes, RER – rough endoplasmic reticulum, P – probability, ns – not significant ($P > 0.05$), not marked R^2 values are statistically significant ($P < 0.01$). Slopes compared in pairs (fix+ vs fix-) were different ($P < 0.05$).

vacuome and cytosol volume enlargement (Figs 3A, 4A). A vacuole plays an important role in the plant cell in solute storage, regulation of cytoplasmic volume, turgor generation, cytosolic ion regulation and compartmentation of hydrolases (Marty et al. 1980, Raven 1987). What is more, a vacuole and cytoplasm have similar buffer capacities although in view of considerable differences in the volume of these compartments it is questionable whether they cooperate in the regulation of their acidity (Kurkdjan and Guern 1989). However, in case of infected cells in effective symbiosis vacuome and cytosol (the largest part of host cytoplasm) have similar volumes at the time of the bacteroid tissue development (compare Figs 3A, 4A). Thus they could interact in regulating their pH without dramatic changes in acidification in one of them.

Significant energy costs are connected with the increase of the plant cell volume even when mainly the vacuome volume increases. The most energy-consuming is the synthesis of the cell wall components, cell membrane and the maintenance of the enlarged vacuome (Raven 1985). In case of infected cells additional costs result from the increase of the cystol and symbiosome volume especially in effective symbiosis. Possibly the infected cells of ineffective symbiosis cannot bear these costs rather because of the limitation of protein synthesis resulting from nitrogen deficiency than because of the deficiency of energy substrates as these cells accumulate more starch than the infected cells of effective symbiosis (Table 3, Fig. 5A). It has also been demonstrated that the inhibition of protein synthesis limits the vacuolation of cells (Davies et al. 1996). It suggests that the nitrogen fixation by bacteroids allows the synthesis of proteins necessary for the growth of infected cells including also their vacuolation.

It is also interesting to observe a strong relationship between the volume of peribacteroid spaces and vacuome in effective symbiosis (Tabs. 1, 3; Fig. 8). That fact confirms a thesis about the "affinity" of those compartments that results from the common traits characterising the peribacteroid membrane and tonoplast (Johnson et al. 1990) and the presence of hydrolytic enzymes in these compartments (Mellor 1989, Brewin 1991). Thus it is possible that osmotic pressure

in the peribacteroid space and vacuome of the effective symbiosis change in the same way regulating and adjusting the volumes of both these compartments. Verma (1992) postulated the necessity of close regulation of osmotic pressure value in the peribacteroid space as this compartment may take up the bigger part of the cell volume than the cytoplasm. At the same time the width of the peribacteroid space can be regulated in the same way and according to Sheehy et al. (1985) the rate of oxygen diffusion from cystol to bacteroids depends on it. The disturbances in the osmotic pressure regulation observed in the infected cells of ineffective nodules in soybean manifest themselves by being crushed by the uninfected cells in full turgor (Werner et al. 1980). Thus, the adjustment of volumes of particular compartments by proper changes of osmotic pressure can be a deciding factor in the differentiation of infected cells and their functioning in effective symbiosis.

One of the routes of supplying bacteroids energetic substrates and carbon skeletons to bacteroids lead through plastids and mitochondria (Yang et al. 1990). Mitochondria produce organic acids that are the best source of carbon for bacteroids (Bryce and Day 1990). Metabolic connections of mitochondria and plastids are expressed by the presence of these organelle next to each other (Figs 2B, 2D), associations between the volume of mitochondria and plastid stroma (Tables 1, 3) and between the surfaces of mitochondria and plastids (Fig. 9B). These dependencies are strong especially in the effective bacteroid tissue that stresses the importance of metabolite exchange between mitochondria and plastids in effective symbiosis. What is more, the adjustment of the surfaces of mitochondria and peribacteroid membranes observed in effective symbiosis (Fig. 9A) may be essential for exporting organic acids from mitochondria to symbiosomes that is done through the cystol. In turn, the volume of cystol in effective symbiosis is tightly connected with the surfaces of peribacteroid membranes, mitochondria and plastids (Tab. 4) which probably helps to supply symbiosomes with organic acids (e. g. malic acid) and at the same time regulates a proper level of metabolites (including organic acids) in cystol. It may also be

significant because malic acid and K^+ ions form a buffer system regulating the cytosol pH (Raven 1985).

On the other hand during infected cell development there is faster rate of peribacteroid membrane surface enlargement than the surface of mitochondria (Fig. 9A). The same situation occurs in lupine bacteroid tissue (Golinowski et al. 1992). From this point of view the importance of mitochondria in organic acids delivery can be questioned. It is also possible that mitochondria volume and surface do not fully characterise their activity. Biochemical data showed high oxidative and phosphorylative capacity of mitochondria isolated from soybean nodules (Suganuma and Yamamoto 1987) and operation of truncated form of the TCA cycle in soybean nodules (Bryce and Day 1990). Nodule mitochondria export ATP to the cytosol (Bryce and Day 1990) but under micro-aerobic conditions ATP production may be low (Rhawsthorpe and LaRue 1986). Data concerning internal structure of mitochondria during bacteroid tissue development could help in explanation of their role in bacteroid tissue functioning.

Uninfected cell

A complicated network of correlations between volumes and surfaces of particular compartments in uninfected cells of ineffective symbiosis and its lack in case of effective symbiosis (Tables 2, 5) shows that these cells differ considerably concerning their inner structure. In fact, one can expect that uninfected cells of effective symbiosis are more subordinate metabolically to infected cells than it is observed in ineffective symbiosis, where infected cells do not reduce N_2 . Thus one cannot expect structural differentiation between the cells in ineffective bacteroid tissue.

Uninfected cells of effective symbiosis are more vacuolated than in ineffective symbiosis (Fig. 3B). Thus similarly as in case of infected cells we deal with the disturbances in the vacuolation of these cells.

Infected and uninfected cells of ineffective symbiosis showed elevated level of RER in cytosol (Fig. 10. A and B). Also infected cells in ineffective alfalfa nodules contain more RER profiles as a response to nitrogen starvation or an abnormal synthesis and export of nodule-specific proteins (Hirsch et al. 1983). In case of infected cells of ineffective bacteroid tissue an additional explanation can include the restriction in peribacteroid membranes extension as a result of low level of symbiosomes (Fig. 6). RER and Golgi membranes participate in peribacteroid membrane extension (Verma 1992).

It is also interesting that in case of ineffective symbiosis the increase of the volume of infected and uninfected cells is strongly connected with the accumulation of starch (Fig. 5. A and B). High concentration of starch in ineffective bacteroid tissue reflects low energy demand for its functioning (lack of nitrogen fixation) and shows starch accumulation as a process independent on nitrogen fixation (Forrest et al. 1991). Uninfected and infected cells in effective bacteroid tissue show much higher diversity in starch content comparing to ineffective bacteroid tissue. Starch reserves calculated as per cent of cell volume decrease during effective bacteroid tissue differentiation (Fig. 5. A and B) what confirms data for effective alfalfa nodules (Hostak et al. 1987). The increase of starch reserves in ineffective bacteroid tissues found also in this study (Fig. 5. A and B) may be one of the main factors of cell enlargement. High starch concentration within cells can result in structural and biochemical (?) perturbances. Starch accumulation and metabolism under stress conditions should reveal new aspects about the role of this compound in nodule functioning.

CONCLUSIONS

1. Mechanisms adjusting volumes and surfaces of cell structures must operate in developing cells. One of them is probably a control of osmotic pressure within particular cell compartment.
2. Data presented in this paper point to disturbances in vacuolation of infected and uninfected cells in ineffective bacteroid tissue. One of possible explanations of this phenomenon is nitrogen deficiency.
3. Further investigations are necessary to establish common quantitative parameters characteristic for effective or ineffective symbioses including also nodule functioning under stress conditions as O_2 limitation, salinity or shading.

ACKNOWLEDGEMENTS

The author thanks Ms. E. Znojek and Mrs. M. Wasilewska-Gomułka for their excellent technical assistance.

LITERATURE CITED

- APPLEBY C.A., 1984. Leghemoglobin and rhizobium respiration. *Ann. Rev. Plant Physiol.* 35: 443-478.
- BORUCKI W., 1996. Some new aspects of the pea (*Pisum sativum* L.) root nodule ultrastructure. *Acta Societatis Botanicorum Poloniae*. 65: 221-233.
- BREWIN N.J., 1991. Development of the legume root nodule. *Ann. Rev. Cell Biol.* 7: 191-226.
- BROWN, S.M., WALSH K.B., 1994. Anatomy of the legume nodule cortex with respect to nodule permeability. *Aust. J. Plant Physiol.* 21: 49-68.
- BRYCE J.H., DAY D.A., 1990. Tricarboxylic acid cycle activity in mitochondria from soybean nodules and cotyledons. *J. Exp. Bot.* 41: 961-967.
- DAVIES T.G.E., STEELE S.H., WALKER D.J., LEIGH R.A., 1996. An analysis of vacuole development in oat aleurone protoplasts. *Planta* 198: 356-364.
- FAHRAEUS G., 1957. The infection of clover root hairs by nodule bacteria studied by a single glass slide technique. *J. Gen. Microbiol.* 16: 374-381.
- FORREST S.I., VERMA D.P.S., DHINDSA R.S., 1991. Starch content and activities of starch-metabolizing enzymes in effective and ineffective root nodules of soybean. *Can. J. Bot.* 69: 697-701.
- GOLINOWSKI W., KOPCIŃSKA J., BORUCKI W., 1992. Morphometric characteristics of the bacteroid tissue in yellow lupine (*Lupinus luteus* L.) nodules. *Acta Soc. Bot. Pol.* 61: 307-318.
- HIRSCH A.M., BANG M., AUSUBEL F.M., 1983. Ultrastructural analysis of ineffective alfalfa nodules formed by nif⁻ Tn 5 mutants of *Rhizobium meliloti*. *J. Bacteriol.* 155: 367-380.
- HOSTAK M.S., HENSON C.A., DUKE S.H., VAN DEN BOSCH K.A., 1987. Starch granule distribution between cell types of alfalfa nodules as affected by symbiotic development. *Can. J. Bot.* 65: 1108-1115.
- KARNOVSKY M.J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* 25: 137A.
- KURKDJIAN A., GUERN J., 1989. Intracellular pH: measurement and importance in cell activity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40: 271-303.
- LIN J., WALSH K.B., CONVIN D.T., LAYZELL D.B., 1988. Structural and physiological bases for effectivity of soybean nodules formed by fast-growing bacteria. *Can. J. Bot.* 66: 526-534.
- LUFT J.M., 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* 9: 409.

- MARTY F., BRANTON D., LEIGH R.A., 1980. Plant vacuoles. In: The Biochemistry of Plants, vol. 1 (Ed. by N. E. Tolbert), pp. 625-658. Academic Press, New York.
- MELLOR R.B., 1989. Bacteroids in the Rhizobium-Legume Symbiosis Inhabit a Plant Internal Lytic Compartment: Implications for other Microbial Endosymbioses. J. Exp. Bot. 40: 831-839.
- MELLOR R.B., WERNER D., 1987. Peribacteroidal membrane biogenesis in mature legume root nodules. Symbiosis 3: 75-100.
- RAVEN J.A., 1985. Regulation of pH and generation of osmolarity in vascular plants: a cost-benefit analysis in relation to efficiency of use of energy, nitrogen and water. New Phytol. 101: 25-77.
- RAVEN J.A., 1987. The role of vacuoles. New Phytol. 106: 357-422.
- RHAWSTHORNE S., LARUE T.A., 1986. Metabolism under microaerobic conditions of mitochondria from cowpea nodules. Plant Physiol. 81: 1097-1102.
- SUGANUMA N., YAMAMOTO Y., 1987. Respiratory metabolism of mitochondria in soybean root nodules. Soil Sci. Plant Nutr. 33: 93-101.
- TJEPKEMA J.D., YOCUM C.S., 1974. Measurement of oxygen partial pressure within soybean nodules by oxygen microelectrode. Planta 119: 351-360.
- WEIBEL E.R., 1974. Selection of the best method in stereology. J. Microscop. 100: 261-269.
- WEIBEL E.R., 1979. Stereological methods. Vol. 1. Practical methods for biological morphometry. Academic Press Inc., London.
- WEIBEL E.R., 1980. Stereological methods. Vol. 2. Theoretical Foundations, Academic Press Inc., London.
- WEIBEL E.R., KISTLER G.S., SCHERLE W.F., 1966. Practical stereological methods for morphometric cytology. J. Cell Biol. 30: 23-38.
- WOOD S.M., LAYZELL D.B., NEWCOMB W., PANKHURST C.E., 1985. A morphometric study of effective nodules induced by *Rhizobium loti* and *Bradyrhizobium* sp. (Lotus) on *Lotus pedunculatus*. Can. J. Bot. 63: 43-53.
- VASSE J., DE BILLY F., CAMUT S., TRUCHET G., 1990. Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J. Bacteriol. 172: 4295-4306.
- VERMA D.P.S., 1992. Signals in root nodule organogenesis and endocytosis of *Rhizobium*. The Plant Cell 4: 373-382.
- YANG L.-J. O., UDVARDI M.K., DAY D.A., 1990. Specificity and regulation of the dicarboxylate carrier on the peribacteroid membrane of soybean nodules. Planta 182: 437-444.

STRUKTURALNE PODSTAWY EFEKTYWNOŚCI TKANKI BAKTEROIDALNEJ BRODAWEK GROCHU UTWORZONYCH PRZEZ EFEKTYWNY LUB NIEEFEKTYWNY SZCZEP BAKTERII

STRESZCZENIE

Porównywano zmiany objętości i powierzchni wybranych struktur komórkowych podczas rozwoju efektywnej i nieefektywnej tkanki bakteroidalnej. Stwierdzono, że wzrost objętości komórki zainfekowanej w efektywnej tkance bakteroidalnej powiązany jest ze wzrostem objętości wakuoli, symbiosomów i cytosolu podczas, gdy w nieefektywnej tkance bakteroidalnej ze wzrostem objętości skrobi a w mniejszym stopniu symbiosomów i cytosolu. Z kolei wzrost objętości komórki niezainfekowanej efektywnej tkanki bakteroidalnej powiązany jest ze wzrostem objętości wakuoli i cytosolu a w przypadku brodawek nieefektywnych ze wzrostem objętości cytosolu i skrobi a w mniejszym stopniu wakuoli. Dane te wskazują na zaburzenia w procesie wakuolizacji komórek zainfekowanych i niezainfekowanych nieefektywnej tkanki bakteroidalnej.

W komórkach zainfekowanych efektywnej symbiozy objętości wakuoli i przestrzeni peribakteroidalnych są ściśle powiązane, co wskazuje na wspólne własności obu tych przedziałów.

Zaobserwowano także, że powierzchnia i/lub objętość takich przedziałów komórek zainfekowanych jak plastydy, mitochondria, symbiosomy i cytosol zmieniają się w sposób bardziej skoordynowany podczas rozwoju efektywnej niż nieefektywnej tkanki bakteroidalnej.

Uzyskane wyniki stworzyły podstawę do dyskusji o roli poszczególnych przedziałów komórkowych w funkcjonowaniu tkanki bakteroidalnej.

SŁOWA KLUCZOWE: morfometria, wiązanie azotu, tkanka bakteroidalna, różnicowanie się komórki.