ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. 61, nr 3–4: 307-318
1992

Morphometric characteristics of bacteroidal tissue in yellow lupine (*Lupinus luteus* L.) nodules

WŁADYSŁAW GOLINOWSKI, JOANNA KOPCIŃSKA,
WOJCIECH BORUCKI

Department of Botany, Institute of Plant Biology,
Agricultural University, Rakowiecka 26/30,
02-528 Warsaw, Poland

(Received: October 10, 1990. Accepted: November 27, 1991)

Abstract

Morphometric methods were used to analyse the bacteroidal tissue in yellow lupine nodules. Volume fraction — *Vv*, surface area — *Sv* and ratio of surface area to its volume — *R*, were calculated from electron micrographs for some selected cell structures in 9, 13, 15, 20, 29 and 60 day-old nodules. The rate at which bacteroid *Vv* increased varied in time. Between days 13 and 20 of nodule development, bacteroid *Vv* increased in geometrical progression. Rough endoplasmic reticulum was active in nodule development and/or protein manufacturing necessary for the functioning of the system whereas the role of mitochondria was apparently limited.

Key words: bacteroid, nodule, Lupinus luteus L., Bradyrhizobium lupini, bacteroidal tissue, morphometry

INTRODUCTION

In order to initiate nodule formation in the lupine root, cell divisions occur in the outer layers of the root cortex. At first, both the bacteroidal tissue and the

---

Explanation of the abbreviations used in this paper:
Vv — volume fraction: the ratio of volume of a particular structure to the measured volume [$\mu m^3/\mu m^3$],
Sv — surface area of structural components per unit test volume [$\mu m,^2/\mu m,^3$],
R — the ratio of surface area of a particular structure to its volume [$\mu m^2/\mu m^3$],
t_d — the time in which bacteroids' *Vv* doubles,
RER — rough endoplasmic reticulum.

cortical tissue are meristematic and the nodule expands equally in all directions. As the nodule develops, the meristematic activity is retained only at its edges and as a result of the activity of the two lateral meristems the nodule expands laterally and spreads around the root (Golinowski et al. 1987). Because of the longevity of these meristems whose activity was found even in two month-old nodules, lupine nodules are "indeterminate nodules" (Morrison et al. in press).

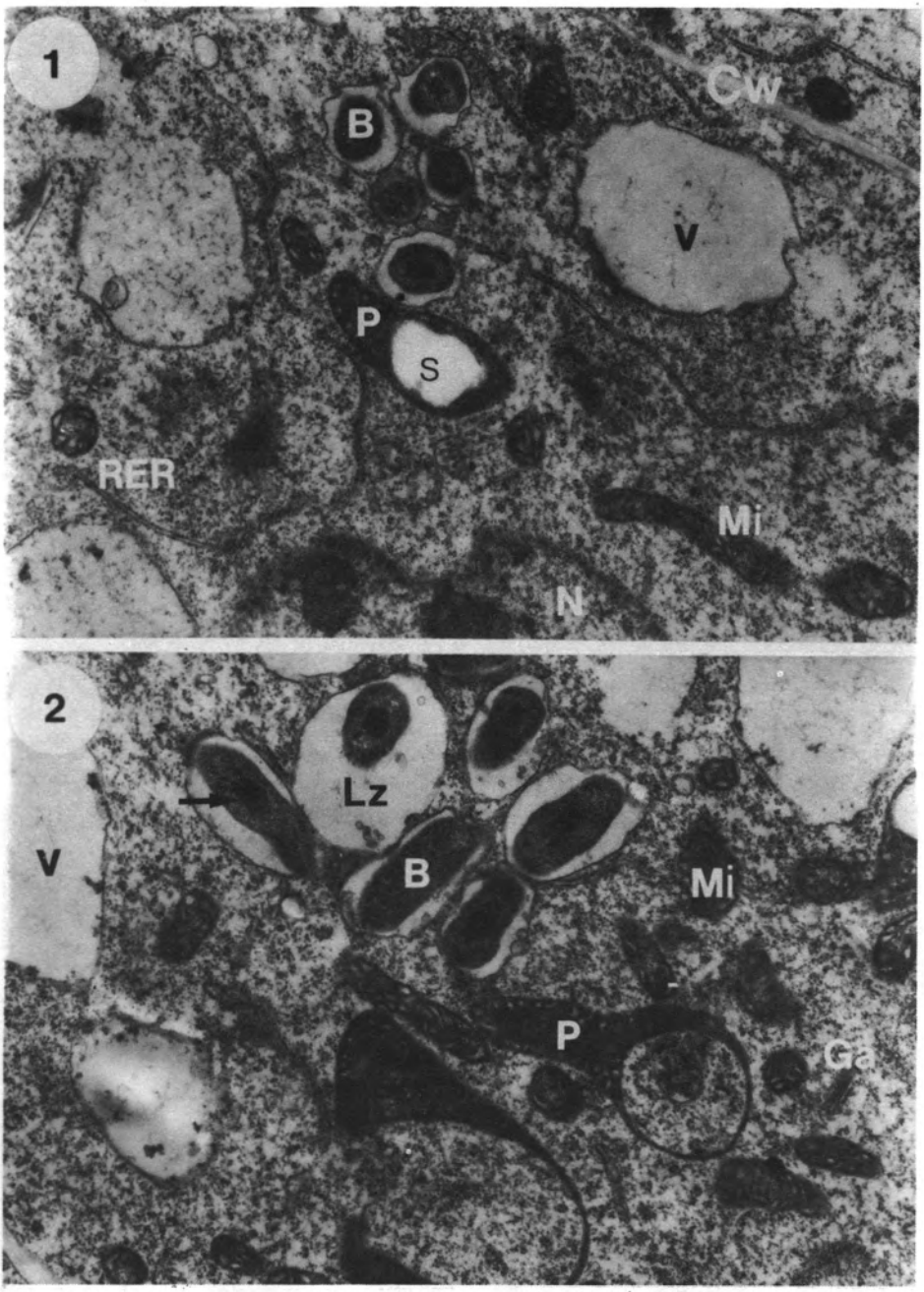
Classical work on the morphogenesis of the nodule has been based on a subjective evaluation of the changes occurring as revealed by light and electron microscopy (Mosse 1964, Goodchild and Bergersen 1966, Libbenga and Harkes 1973, Newcomb 1976, Newcomb et al. 1979, Golinowski et al. 1987). Visual observations, however, do not ensure an objective description of nodule development. In order to present more objective evaluation of the development of the lupine nodule we have attempt to quantify development using morphometric methods. These methods were used successfully in nodule studies in alfalfa (Hirsch et al. 1983), lotus (Wood et al. 1985) and soybean (Selker and Newcomb 1985, Lin et al. 1988)

MATERIALS AND METHODS

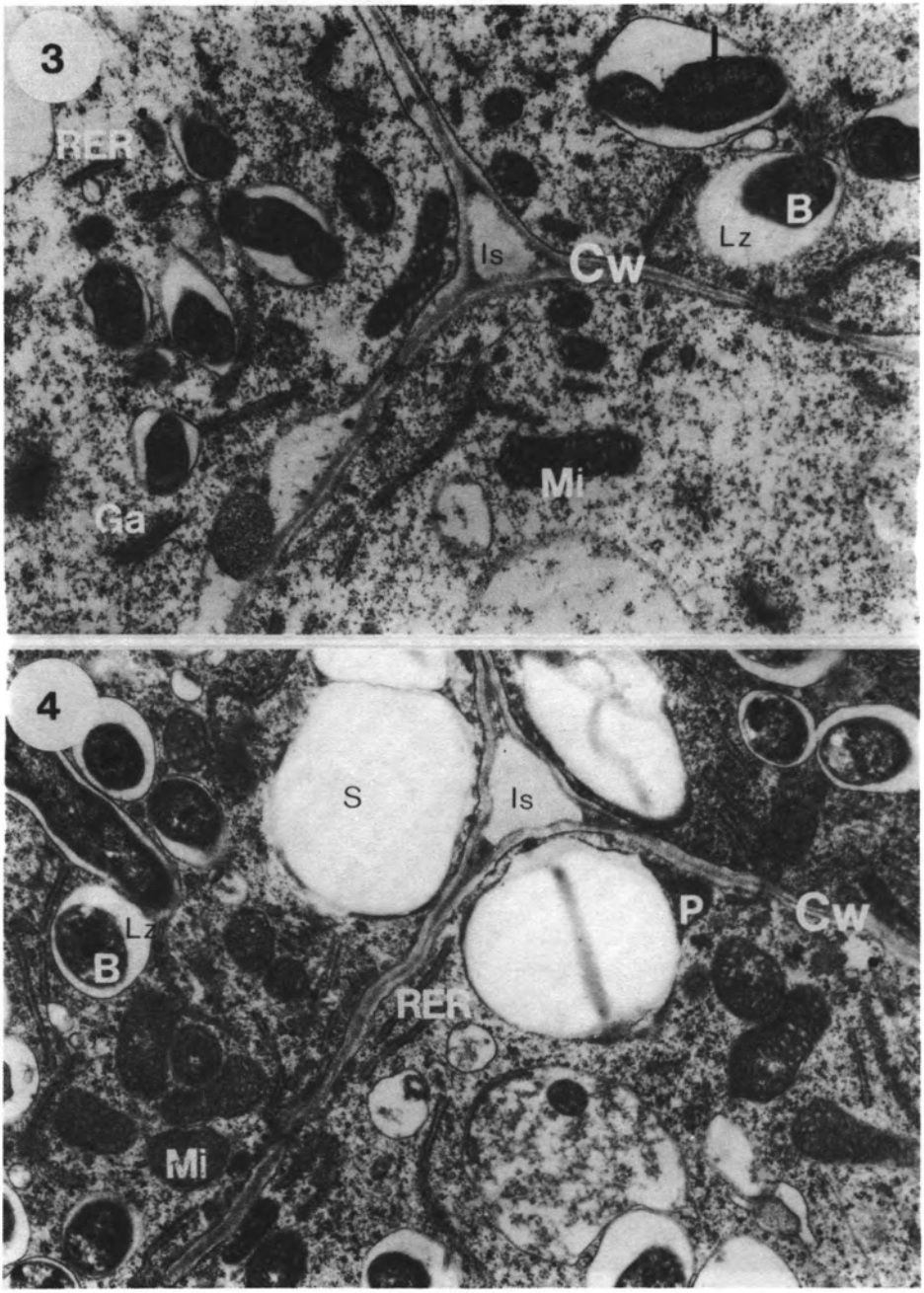
Yellow lupine (*Lupinus luteus* L. cv. Ventus) seeds were sterilized in a 3.5% calcium hypochlorite solution, then germinated at 24°C on filter paper moistened with distilled water. When roots were about 1 cm long, the seeds were put into pots filled with sterile perlite and pots were inoculated with a suspension of *Bradyrhizobium lupini* (strain 3045 USDA *Rhizobium* Culture Collection, Beltsville, Md.). During the first seven days, the plants were watered with distilled water, then with a medium lacking combined forms of nitrogen (Walsh and Layzell 1986). The plants were grown at 24°C with a photoperiod of 16 hrs of light (7000 lux) and 8 hrs of darkness. Under this conditions flowering began 60 days after planting when the lower flowers in a raceme were in full bloom.

Sampling was done 6, 9, 13, 15, 20, 29 and 60 days after inoculation. Nodules were collected from the basal part of the main root and fixed according to Karnovsky (1965) for 4 hrs at room temperature, then postfixed in 1% OsO_4 for 2 hrs at 4°C, dehydrated in increasing concentrations of ethyl alcohol, acetone and propylene oxide and embedded in Epon 812 (Luft 1961). Semithin sections for light microscopic observations were stained with methylene blue and azure A and ultrathin sections were stained with uranyl acetate and lead citrate and analysed with a JEM 100C electron microscope.

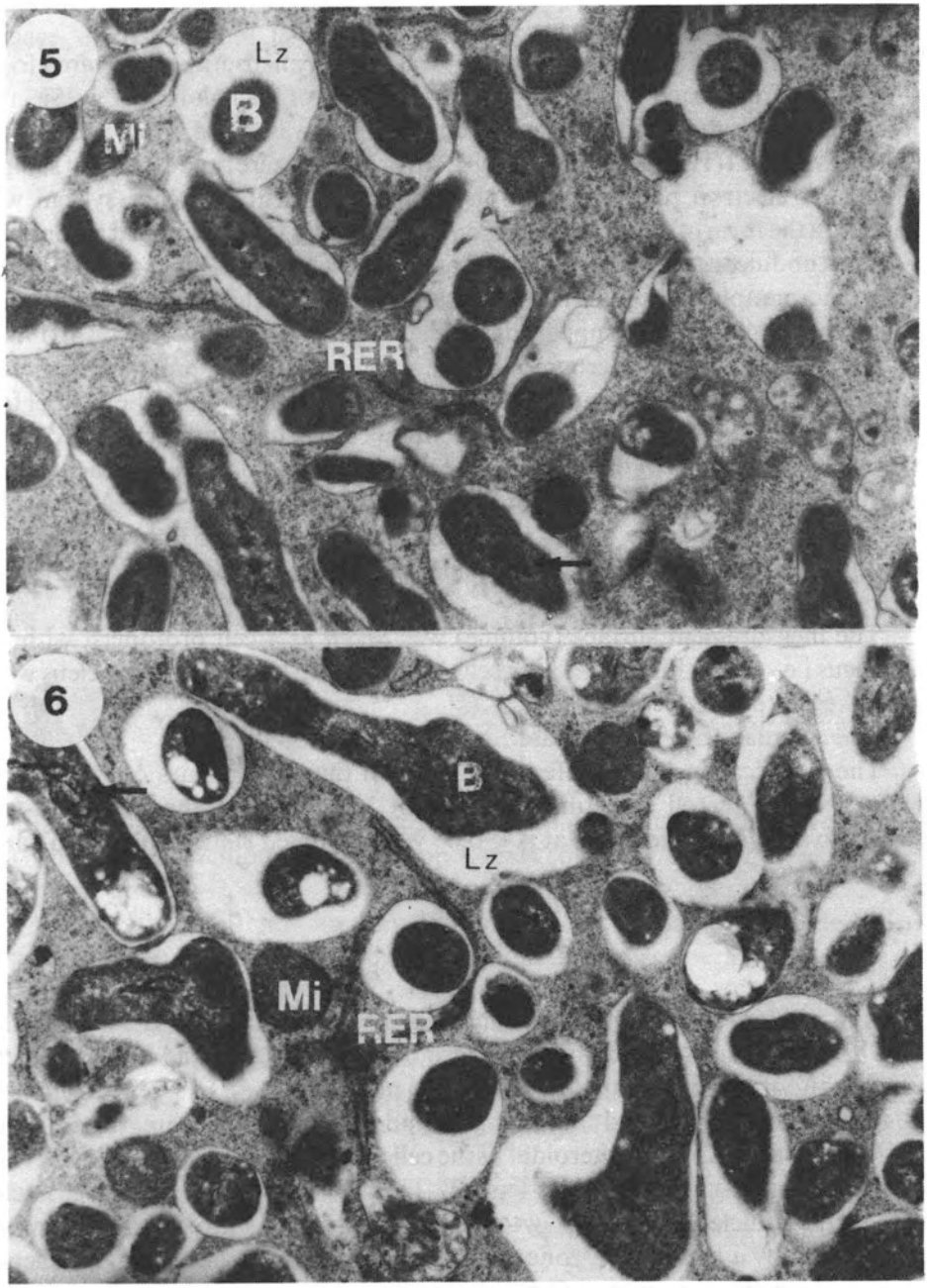
In the morphometric study 9, 13, 15, 20, 29 and 60 day-old nodules were analysed. In the older nodules, where the meristematic and mature central parts could be distinguished, only the latter were taken into consideration. In the case



Figs. 1, 2. Fragments of bacteroidal tissue at different stages of development of yellow lupine nodules. 1 — a 9 day-old nodule, 2 — a 13 day-old nodule. B — bacteroid, Cw — cell wall, Ga — Golgi apparatus, Lz — light zone around the bacteroid (= peribacteroid space), Mi — mitochondrium, N — nucleus, P — plastid, RER — rough endoplasmic reticulum, S — starch, V — vacuole, arrow — nucleoid zone with osmophilic fibrillar structures. × 18 260



Figs. 3, 4. Fragments of bacteroid tissue at different stages of development of yellow lupine nodules. 3 — a 15 day-old nodule, 4 — a 20 day-old nodule. Is — intercellular space. Other symbols as described in Figs. 1, 2. × 18 260



Figs. 5, 6. Fragments of bacteroidal tissue at different stages of development of yellow lupine nodules. 5 — a 29 day-old nodule, 6 — a 60 day-old nodule. Symbols as described in Figs 1, 2. × 18 260

of 60 day-old nodules, the portions in which advanced stages of bacteroidal degradation were observed were ignored.

The morphometric measurements were based on micrographs prepared from 4 nodules from each age group. The manner of calculating *Vv*, *Sv* and *R* (explanations above) for particular cell structures was based on W e i b e l et al. (1966), W e i b e l (1969) and T o t h (1982). In order to assure randomness of sample choice for electron photomicrography, an independent reference system was applied in the form of a supporting net on which sections were arranged at random. For each nodule segment embedded in Epon 812, which represented one nodule, 15 photomicrographs were taken in such a manner that the field of view in the electron microscope touched the left upper edge of the supporting net (W e i b e l et al. 1966).

For measurements, micrographs were used, whose total magnification was × 18260 (initial magnification, × 8300) (Figs. 1-6), and on which the Weibel test net was used. The size of the net was 120 mm × 121.2 mm and it consisted of 84 lines each of 10 mm length. The net covered an area of 43.6 μm and each of 168 points corresponding to an area of 0.26 μm (W e i b e l et al. 1966). A point net was also used with a size of 120 mm × 120 mm, comprising 576 test points and enabling therefore a more accurate calculation of the volume fraction with values of less than 2%.

The measurement surface (volume) was taken as a cell protoplast without a nucleus i.e., points hitting the intercellular spaces, cell wall and the nucleus were not taken into consideration.

The calculation of t_d was based on M o n o d (1949).

The significance of the differences between the mean values of *Vv*, *Sv* and *R* for particular cell structures was tested by means of the Student t-test (O k t a b a 1976). Differences at $p < 0.05$ were assumed statistically significant.

RESULTS

As a result of division and differentiation processes, between the 9th and the 13th days of nodule development, bacteroidal tissue (Figs. 1 and 2) and cortical tissue without bacteroids are formed (G o l i n o w s k i et al. 1987). Morphometric measurements indicated that in this period a considerable increase of *Vv* and *Sv-RER* occurred in the bacteroidal tissue cells, whereas the increase of bacteroid *Vv* was rather slow (Fig. 7, Tables 1 and 2). The cells of that tissue had large nuclei with distinct nucleoli and dense cytoplasm. Bacteroids occurred singly and were surrounded by a translucent zone containing structureless material. A nucleoid zone in which osmiophilic fibrillar structures are seen (Fig. 2) could be distinguished inside each bacteroid.

Between day 13 and 20 of nodule development, bacteroidal tissue differentiated into the meristematic and mature parts (G o l i n o w s k i et al. 1987). In

the latter, a logarithmic phase of bacteroidal *Vv* increase took place. In this phase, the bacteroidal *Vv* doubled with a t_d of 70 hrs (Fig. 7).

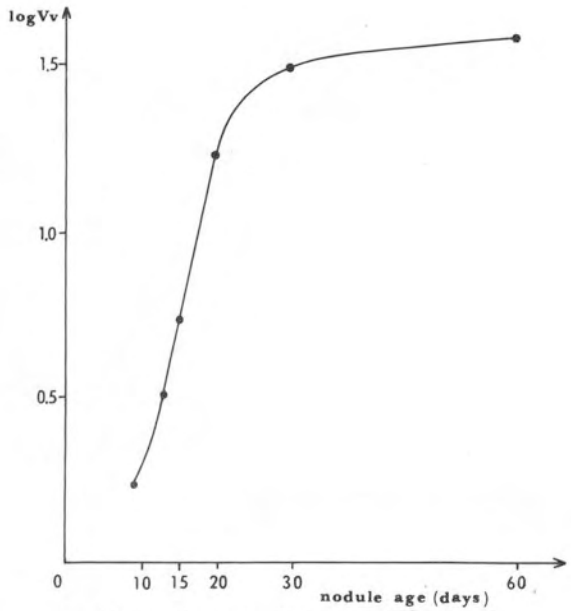


Fig. 7. The rate of bacteroid *Vv* increase

Mitochondrial *Vv* and *Sv* values did not change between the 9th and 20th days of nodule development, but dropped markedly in 29 and 60 day-old nodules (Tables 1 and 2).

In the clearly differentiated mature zone of the 20 day-old nodule, stored starch could be seen in the amyloplasts located in the peripheral parts of the cell (Fig. 4).

After 20 days of nodule development the rate of increase of bacteroid *Vv* and *Sv* slowed gradually (Tables 1 and 2).

In a 60 day-old nodule (Fig. 6) bacteroid *Vv* and the light zone *Vv* reached maximum values, and at the same time a decrease of mitochondrial *Vv* and *RER Vv* was noticed.

The changes in the light zone *Vv* and the changes in amyloplasts *Vv* followed a similar pattern, especially between the 9th and 20th days of nodule development.

Although the changes in the light zone *Vv* and bacteroid *Vv* had different dynamics, the dynamics of *Sv* changes in this structures were very similar (Tables 1 and 2).

Between days 9 and 29 of nodule development no important differences

Table 1

Volume fraction of cell structures — Vv [%]

Nodule age [days]	Bacteroids	Light zone	Mitochondria	RER	Amyloplasts[x]	Starch	Vacuoles
9	1.70[a]	0.96[a]	3.70[a]	0.15[a]	1.70[a]	0.17[a]	18.00[a]
S =	0.50	0.28	0.40	0.02	0.35	0.16	4.20
13	3.15[a]	3.42[b]	4.12[a]	0.65[b]	3.25[b]	0.17[a]	12.30[a]
S =	1.35	0.32	0.19	0.13	0.80	0.02	2.80
15	5.35[a]	2.42[c]	3.22[a]	0.67[b]	0.98[c]	0.17[a]	11.50[a]
S =	2.27	0.43	0.60	0.06	0.18	0.11	2.80
20	16.60[b]	8.60[c]	3.64[a]	1.00[b]	7.00[bd]	5.82[b]	6.20[b]
S =	3.70	4.60	0.92	0.22	3.35	3.03	1.70
29	29.45[c]	12.20[c]	0.93[b]	0.83[b]	7.05[d]	5.40[b]	0.00
S =	1.25	4.40	0.21	0.06	1.95	1.80	0.00
60	38.35[c]	23.80[d]	0.77[b]	0.37[c]	2.25[abc]	2.00[ab]	0.00
S =	5.25	4.70	0.41	0.12	1.95	1.70	0.00

S = standard error of mean values, [x] including starch.
Means of 4 replications within a column followed by the same letter are not significantly different from each other.

Table 2

The surface of cell structures per unit test volume — Sv $\mu m^2/\mu m^3$

Nodule age [days]	Bacteroids	Light zone	Mitochondria	RER	Amyloplasts[x]	Starch	Vacuoles
9	0.124[a]	0.154[a]	0.310[a]	0.085[a]	0.110[ac]	0.014[a]	0.480[a]
S =	0.040	0.040	0.021	0.018	0.026	0.013	0.160
13	0.240[ab]	0.360[ab]	0.370[a]	0.310[b]	0.170[a]	0.030[a]	0.190[b]
S =	0.120	0.140	0.070	0.090	0.070	0.004	0.060
15	0.380[b]	0.460[b]	0.310[a]	0.330[b]	0.065[b]	0.016[a]	0.190[b]
S =	0.140	0.150	0.040	0.094	0.015	0.011	0.025
20	1.170[c]	1.370[c]	0.360[a]	0.540[b]	0.150[ab]	0.150[b]	0.205[b]
S =	0.150	0.220	0.110	0.220	0.070	0.100	0.050
29	1.970[d]	2.150[d]	0.090[b]	0.330[b]	0.098[abc]	0.162[b]	0.00
S =	0.120	0.300	0.024	0.150	0.016	0.065	0.00
60	2.000[d]	2.090[d]	0.080[b]	0.180[ab]	0.045[bc]	0.053[ab]	0.00
S =	0.310	0.370	0.050	0.087	0.042	0.047	0.00

S = standard error of mean values, [x] including starch.
Means of 4 replications are compared as in Table 1.

Table 3
Surface to volume ratio of cell structures — $R = Sv/Vv$

Nodule age [days]	Bacteroids	Bacteroids + Light zone	Mitochondria	RER	Amyloplasts*	Vacuoles
9	7.3[a]	5.8[a]	8.5[a]	58.4[a]	6.5[a]	2.6[a]
S=	0.6	0.3	1.5	6.1	0.3	0.3
13	7.6[a]	5.5[a]	9.0[a]	47.6[a]	4.9[a]	1.6[b]
S=	1.3	0.9	1.6	14.2	1.4	0.3
15	7.3[a]	5.9[a]	9.7[a]	49.2[a]	6.7[a]	1.7[b]
S=	0.5	0.2	0.7	14.1	2.4	0.2
20	7.2[a]	5.2[a]	9.8[a]	53.2[a]	2.1[b]	3.2[a]
S=	0.8	0.6	1.8	16.8	0.2	1.2
29	6.7[a]	4.9[a]	9.7[a]	38.9[a]	1.4[c]	—
S=	0.3	0.8	2.1	14.7	0.2	—
60	5.0[b]	3.2[b]	10.6[a]	49.8[a]	2.2[b]	—
S=	0.3	0.3	7.3	23.6	0.2	—

S= standard error of mean values, * including starch. Means of 4 replications are compared as in Table 1.

occurred in the R values for such structures as bacteroids, bacteroids together with the light zone, mitochondria and *RER* (Table 3). However, the 60 day-old nodule could be characterized by the decrease of the R value for both bacteroids and bacteroids together with their light zones.

DISCUSSION

The initial phase of nodule formation in lupine, i.e. the induction of cell division in the outer parts of the root cortex, is analogous to that described for soybean (Newcomb et. al. 1979) and bean (Dart 1975). Differentiation of the so-formed mother tissue leads to the separation of the bacteroidal tissue and the nodule cortical tissue. As the nodule begins to develop, all the cells of the bacteroidal tissue divide. In the period between days 9 and 13 a fourfold increase in *RER Vv* and *Sv* was noticed in the cells of this tissue and the increased amount of *RER* remained on a higher level between days 13 and 29 (Tables 1 and 2). This corresponds with the observations made by Newcomb (1976) for *Pisum sativum*. We suggest that this cell component is active in the development of the host plant-*Rhizobium* symbiotic system. The role of this cell component may be to provide the membrane for the growing surface of the light zone surrounding the bacteroids and/or the production of proteins necessary for this process. The increase of the amount of *RER* was also observed during the differentiation of pea root cells by Chaly and Setterfield (1975) and in bean cotyledons (Briarty 1980). It is therefore difficult to speak of the specificity of this increase

of *Vv* and *RER Sv* in the differentiating cells of the lupine nodule bacteroidal tissue. At the same time, the increase of bacteroidal *Vv* between the 9th and 13th day is slow, which may imply that in the initial period of nodule development the nutrients which are supplied are used, to a great extent, for building those structures of the nodule that would enable active nitrogen fixation.

Between days 13 and 20 a logarithmic phase of increase of bacteroid *Vv* occurs (Fig. 7). The increase takes place in the central part of the nodule in which cell division ceases. The time necessary for doubling bacteroid *Vv* is about 70 hrs which is much longer than the generation time of slow-growing species of *Rhizobium* (6 to 8 hrs — R a w s t h o r n e et al. 1980, P a u et al. 1977).

It should be remembered that the term volume fraction (*Vv*) does not imply the absolute volume of particular structures. Calculating t_d for bacteroids on the basis of changes in their *Vv* during the logarithmic phase with no regard for the changes in the cell volume, does not allow us to interpret the obtained value precisely in relation to the generation time. It seems that this value may be a good indicator of the rate at which the bacteroid population expands in the nodule under different experimental conditions.

Between the 15th and 20th day of nodule development, that is, still during the phase of intensive bacteroid *Vv* increase, storage of starch begins which could support the glycolytic processes and the tricarboxylic acid cycle in bacteroids and the plant cytosol during the night (M i n c h i n and P a t e 1973, R a w s t h o r n e et al. 1980).

It is striking that during the logarithmic increase of bacteroid *Vv*, the *Vv* of the light zone surrounding the bacteroids decreases (15 day-old nodule). Perhaps this provides support for the assumption that the substance stored in the light zone is used up by the bacteroids. It has been suggested by B e r g e r s o n (1982) that the volume of the light zone may be an important factor in controlling the flux of oxygen and other substances to and from the bacteroids. V a n c e and J o h n s o n (1983) also observed the correlation between the size of the light zone and nodule effectiveness in alfalfa. In the case of ineffective symbiosis these authors noted an increase in the size of the light zone. Similarly, W o o d et al. (1985) found the smaller light zones in lotus to be correlated with higher symbiotic effectiveness. However, at the same time the *Vv* of mitochondria and amyloplasts also decreases. Therefore it cannot be ruled out that the decrease of *Vv* of these structures is caused by an increase of the basic cytoplasm volume after 13 days of nodule development, not accompanied by a proportional growth of the valume of these organelles. The more "dilution" of the studied structures there is, the more the calculated t_d value would exceed that which we would observed if we knew the absolute values of the bacteroid volume in the whole nodule. Since we did not measure the cell volumes we cannot give an explicit answer to this problem.

Assuming that a fivefold increase of the bacteroid *Vv* occurs in the logarithmic phase (Table 1), there is no simultaneous increase in the mitochond-

ria *Vv*, which suggests that the role of this organelle as a source of ATP for fixing notrogen is rather small. A decrease in the mitochondrial *Vv* after 20 days of nodule development, when the rate of nitrogen fixation presumably is high, supports this suggestion. However, according to Gunning and Steer (1975) neither volume nor morphology of mitochondria can be taken as reliable indicators of their activity.

The ratio of the surface of a particular organelle to its volume (*R*) may serve as an indicator of its activity. The greater the *R* value, the greater is the surface of the membrane surrounding the organelle per unit volume. Therefore the metabolite exchange between the organelle and the cytoplasm is more efficient, which enables it to retain its metabolism at a high level. So, a decrease of *R* for the bacteroids found in 60 day-old nodules (Table 3) could suggest their lower activity at that time.

Acknowledgment

The study was conducted on grants from the Polish Research Program CPBP 04.12.

REFERENCES

Bergersen F.J., 1982. Root nodules of legumes: structure and functions. John Wiley and Sons Ltd., Chichester, United Kingdom.

Briarty L.G., 1980. Stereological analysis of cotyledon cell development in *Phaseolus*. II. The developing cotyledon. J. Exp. Bot. 31: 1387-1398.

Chaly N.M., Setterfield G., 1975. Organization of the nucleus, nucleolus and protein-synthesizing apparatus in relation to cell development in roots of *Pisum sativum*. Can. J. Bot. 53: 200-218.

Dart P.J., 1975. Legume root nodule initiation and development. In: The development and function of roots. Torrey J.G., Clarkson D.T. (eds.). Academic Press, London-New York-San Francisco, pp. 467-506.

Golinowski W., Kopcińska J., Borucki W., 1987. The morphogenesis of lupine root nodules during infection by *Rhizobium lupini*. Acta Soc. Bot. Pol. 56: 687-703.

Goodchild D.J., Bergersen F.J., 1966. Electron microscopy of the infection and subsequent development of soybean nodule cells. J. Bacteriol. 92: 204-213.

Gunning B.E.S., Steer M.W., 1975. Ultrastructure and the biology of plant cells. Edward Arnold, London.

Hirsch A.M., Bang M., Ausubel F.M., 1983. Ultrastructural analysis of ineffective alfalfa nodules formed by nif: Tn5 mutants of *Rhizobium meliloti*. J. Bacteriol. 155: 367-380.

Karnovsky M.J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 25: 137A.

Libbenga K.R., Harkes P.A.A., 1973. Initial proliferation of cortical cells in the formation of root nodules in *Pisum sativum* L. Planta 114: 17-28.

Lin J., Walsh K.B., Canvin D.T., Layzell D.B., 1988. Structural and physiological bases for effectivity of soybean nodules formed by fast-growing and slow-growing bacteria. Can. J. Bot. 66: 526-534.

Luft J.M., 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9: 409.

Minchin F.R., Pate J.S., 1973. The carbon balance of a legume and the functional economy of its root nodules. J. Exp. Bot. 24: 259–271.
Monod J., 1949. The growth of bacterial cultures. Ann. Rev. Microbiol. 3: 371-394.
Morrison N.A., Bisseling T., Verma D.P.S. Development and differentiation of the root nodules: involvement of plant and bacterial genes. Developmental biology: A comprehensive synthesis. Browder L.W. (ed.), Plenum Publishing Corp. (in press).
Mosse B., 1964. Electron-microscope studies of nodule development in some clover species. J. Gen. Microbiol. 36: 49-66.
Newcomb W., 1976. A correlated light and electron microscopic study of symbiotic growth and differentiation in *Pisum sativum* root nodules. Can. J. Bot. 54: 2163-2186.
Newcomb W., Sippell D., Peterson R.L., 1979. The early morphogenesis of *Glycine max* and *Pisum sativum* root nodules. Can. J. Bot. 57: 2603-2616.
Oktaba W., 1976. Elementy statystyki matematycznej i metodyka doświadczalnictwa. PWN, Warszawa.
Pau A.S., Cowles J.R., Oro J., 1977. Flowmicrofluorometric analysis of *Escherichia coli, Rhizobium meliloti* and *Rhizobium japonicum* at different stages of the growth cycle. Can. J. Microbiol. 23: 1165-1169.
Rawsthorne S., Minchin F.R., Summerfield R.J., Cookson C., Coombs J., 1980. Carbon and nitrogen metabolism in legume root nodules. Phytochemistry 19: 341-355.
Toth R., 1982. An introduction to morphometric cytology and its application to botanical research. Amer. J. Bot. 69: 1694-1706.
Selker J.M.L., Newcomb E.H., 1985. Spatial relationships between uninfected and infected cells in root nodules of soybean. Planta 165: 446-454.
Vance C.P., Johnson L.E.B., 1983. Plant determined ineffective nodules in alfalfa (*Medicago sativa*): structural and biochemical comparisons. Can. J. Bot. 61: 93-106.
Walsh K.B., Layzell D.B., 1986. Carbon and nitrogen assimilation and partitioning in soybean exposed to low root temperatures. Plant Physiol. 80: 249-255.
Weibel E.R., 1969. Stereological principles for morphometry in electron microscopic cytology. Int. Rev. Cytol. 26: 235-302.
Weibel E.R., Kistler G.S., Scherle W.F., 1966. Practical stereological methods for morphometric cytology. J. Cell Biol. 30: 23-36.
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.

Morfometryczna charakterystyka tkanki bakteroidalnej w brodawkach łubinu żółtego (Lupinus luteus L.)

Streszczenie

Tkankę bakteroidalną brodawek łubinu żółtego analizowano metodami morfometrycznymi. Obliczono wartości: frakcji objętościowej (*Vv*), pola powierzchni (*Sv*) i stosunek powierzchni do objętości (*R*) wybranych struktur komórkowych w brodawkach 9, 13, 15, 20, 29 i 60 dniowych. Tempo wzrostu *Vv* bakteroidów nie jest jednakowe w badanym okresie. Między 13 i 20 dniem rozwoju brodawki *Vv* bakteroidów wzrastała w postępie geometrycznym. Szorstkie endoplazmatyczne retikulum jest aktywne w rozwoju brodawki i/lub dostarcza białek niezbędnych dla funkcjonowania układu symbiotycznego, podczas gdy rola mitochondriów jest prawdopodobnie niewielka.