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Structure of embryo sac, fertilization and development of embryo in Swedish clover (*Trifolium hybridum* L.) plants with reduced leaves

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Abstract

The causes of poor fertility and female infertility of Swedish clover plants with reduced leaves were investigated. Embryological analyses demonstrated that in these plants deviations are more frequent in megasporo- and megagametogenesis than in plants with normal leaves. The ovaries of plants with reduced leaves are frequently not coalesced and the ovules hang out beyond the ovary on long funiculus. It is supposed that the hanging out of ovules on the long funiculus often makes fertilization difficult or impossible. Therefore, plants with reduced leaves exhibit low fertility or are sterile.

INTRODUCTION

Swedish clover plants with reduced leaves, when crossed with normal ones seldom produce single seeds (Kazimierski and Kazimierska, 1979). The low fertility of these plants may be caused by deviations in the ovary, ovule and embryo sac structures or in the process of fertilization and development of the embryo. The present paper gives a description of the structure of the above named organs and an analysis of the processes of fertilization and embryo development in plants with reduced and with normal leaves.

MATERIAL AND METHODS

The plants of Swedish clover with reduced leaves used for study were the F_2 generation of a cross between plant with reduced leaves and a plant having normal leaves. Material for analysis of the ovary and ovule

structure, in the form of buds and flowers of various size was fixed in Nawashin's solution. After fixing and dehydration thorugh a series of alcohols, the ovaries were embedded in paraffin and cut with a microtome into 8—12 μ sections, stained with iron haematoxylin, counterstained with fast green and sealed in Canada balsam.

Material for analysis of fertilization and development of the embryo was prepared similarly. Pollinated ovaries were fixed beginning with 20 h to 5 days after pollination. The plants used as female partners were earlier pollinated with pollen from other plants. On the basis of analysis of seed setting two plants were selected for further investigation, namely 55/1 and 53/2. The first had 51.3 per cent of ovaries coalesced and from the 60 flowers pollinated on it 11 seeds were collected. From the second plant the per cent of coalesced ovaries was 9.1, and from the 60 flowers pollinated 2 seeds were obtained.

For comparison of the ovary, ovule and embryo sac structure and of the embryo development in plants with reduced leaves, flowers and pollinated ovaries from normal plants were also fixed.

RESULTS

In most of the examined ovules from plants with reduced leaves the embryo sac developed normally. The megaspore tetrad showed a linear arrangement, the embryo sac developed from the chalazal megaspore (Fig. 1a), from which, after 3 successive divisions, an 8-nucleate embryo sac arose. In the micropyle and of the mature embryo sac the egg apparatus was present, and the secondary nucleus of the embryo sac adhered to the apical end of the egg cell (Fig. 1b). The antipodals disappeared in the course of the process of 8-nucleate embryo sac differentiation and formation of the mature embryo sac. The mature embryo sac filled the entire nucellus and was surrounded by an adhering layer of integumental tapetum cells.

Beside the ovules in which the embryo sac developed normally, there were some in which two uninucleate embryo sacs differentiated from one megaspore tetrad (Fig. 1c). Moreover, one of the embryo sacs developed from the chalazal megaspore and the other from the megaspore lying close to the apical end of the tetrad. Two megaspores of this tetrad were crushed (Fig. 1c). Both the differentiating uninucleate embryo sacs were vacuolised and seemed to be further degenerating.

In part of the ovules — 12.1 per cent of those analysed — the embryo sac wasted away at various stages of development. Deviations were also observed in development. In a typical twonucleate embryo sac the nuclei surrounded with cytoplasm lie on the opposite poles of the embryo sac. Between them there is a large central vacuole (Fig. 1d). In twonucleate untypical embryo sacs both the nuclei surrounded with cytoplasm lay in

the central part of the embryo sac. The cytoplasm of the embryo sac was separated from its wall by a vacuole (Fig. 1e). Some twonucleate embryo sacs were found with a central vacuole and nuclei of different sizes (Fig. 1f).

In typical 4-nucleate embryo sacs the pairs of nuclei at the opposite poles were separated by a central vacuole. Sometimes, beside the central vacuole, an additional one was formed between two sister nuclei lying at one pole (Fig. 1g).

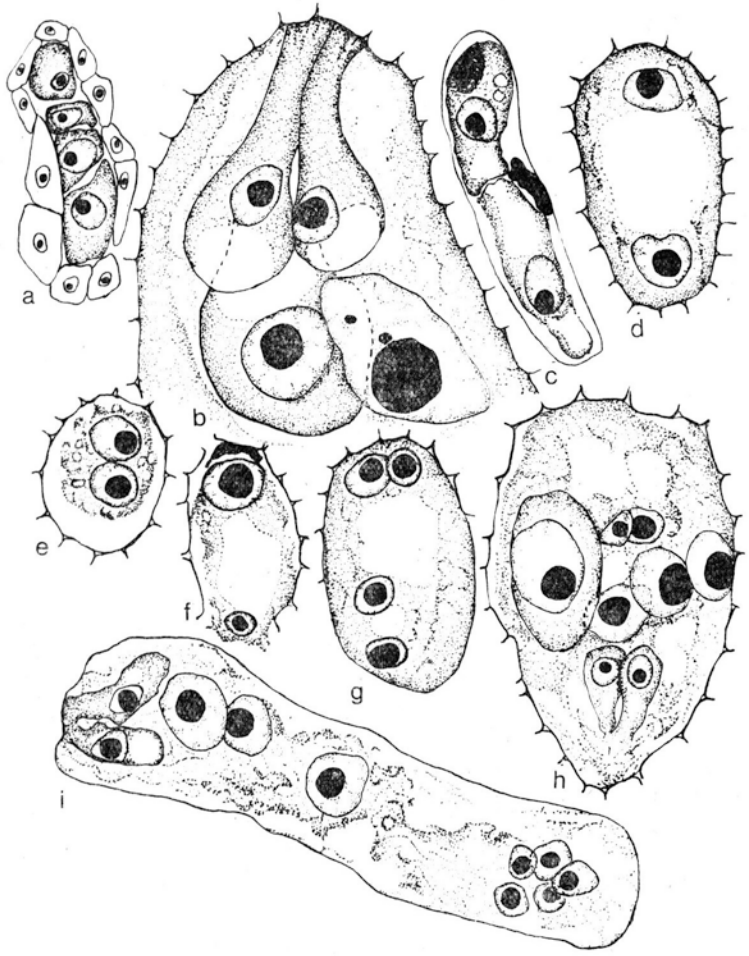


Fig. 1. Megaspore tetrad and embryo sac in Swedish clover:

a — megaspore tetrad, *b* — mature embryo sac, *c* — megaspore tetrad in which two megaspores have transformed to a uninucleate embryo sac, *d* — typical twonucleate embryo sac, *e* — twonucleate embryo sac, nuclei shifted towards mycropylar part, between them there is no vacuole; *f* — twonucleate embryo sac with nuclei of different diametre; *g* — four-nucleate embryo sac with additional vacuole; *h* — eight-nucleate embryo sac, unpolarized, nuclei of different diametre; *i* — maturating embryo sac, in chalazal end 5 nuclei. × 1000

In one of the small embryo sacs the nuclei differing in size lay in its central part (Fig. 1h), showing no noticeable polarisation. In this embryo sac the large cell with its nucleus at the left wall is probably the differentiating egg cell; the two large nuclei at the right wall are probably the polar ones; in the chalazal end of the embryo sac there are two cells resembling in shape synergids; the three following nuclei differing in size, may perhaps be considered as antipodal nuclei. The embryo sac in point is also characterized by advanced vacuolisation.

In the micropyle part of another maturating embryo sac there was an egg-apparatus and one polar nucleus. The second one lay in the central part of the embryo sac. In the chalazal end 5 nuclei were present, two of which were larger and 3 smaller (Fig. 1i). Probably the larger than normal number of antipodal nuclei arose by division of two of the three nuclei. The cytoplasm in the embryo sac showed a greatly advanced vacuolisation. The ovules in the ovaries from plants with reduced leaves were of structure similar to that in normal plants. They differed, however, by their position in the placenta. In plants with normal leaves the ovule was attached to the placenta by a short funiculus (Photo 1). In the plants with reduced leaves the latter was sometimes very long (Photo 2), so that the ovules frequently hung down outside (Fig. 2).

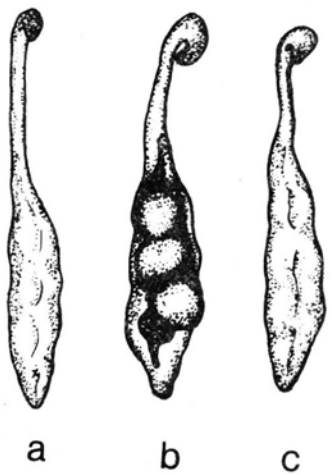
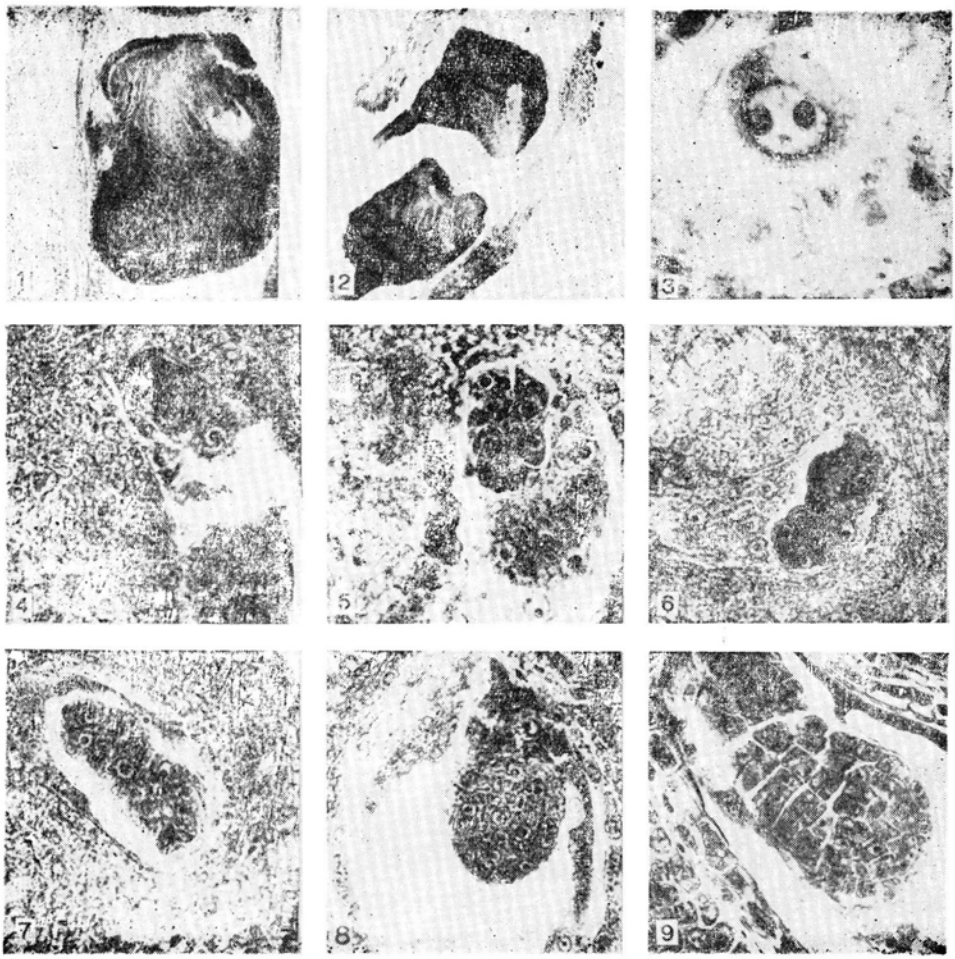


Fig. 2. Ovaries: a — coalesced from plant with normal leaves; *b*, *c* — from plants with reduced leaves; *b* — uncoalesced, ovules outside ovary, *c* — coalesced

The ovules from ovaries pollinated for analysis of fertilization and embryo development from plants with reduced and normal leaves were divided into 3 groups:

ovules with normally developed embryo sac,
„ with degenerating egg-apparatus,

PLATE I



Ovules, fertilization and embryos from plants with normal and reduced leaves
Photos *1*, *5*, *7* and *9* — plants with normal leaves
Photos *2*, *3*, *4*, *6* and *8* — plants with reduced leaves

1 — ovule with short funiculus; *2* — ovules with long funiculus; *3* — male nucleus in egg cell; *4* and *5* — embryo after 2 days; *6* and *7* — embryo after 3 days; *8* and *9* — embryo after 5 days

ovules in which the embryo sac wasted away in the course of development (Table 1).

The percentage of fertilized ovules was calculated in reference to those in which the embryo sac was normally developed.

Most numerous fertilized ovules were found in the ovaries of plants with normal leaves (Table 1). In the ovaries of plants with reduced leaves the per cent of fertilized ovules was: 45.0 on plant 55/1 and 1.3 in plant 53/2 (Table 2). Ovules with degenerated egg-apparatus and wasted away embryo sac in the course of development were most numerous on plant 53/2 less so on plant 55/1 and on plants with normal leaves the per cent of such ovules did not exceed 3.0 (Table 1).

Table 1

Number of ovule analysed and per cent of fertilized ones on Swedish clover plants with normal and reduced leaves

Leaves	No. of ovules examined	Ovules:				
		with normal embryo sac	fertilized		with degenerated embryo sac	with embryo sac wasted off in ontogenesis
			no.	%		
Normal	118	110	107	93,2	—	3
Reduced (55/1)	120	111	50	45,0	5	4
Reduced (53/2)	123	78	1	1,3	28	17

The male nucleus was found in the egg cell and at the secondary nucleus of the embryo sac 20 h after pollination (Table 2, Photo 3) in both plant groups. Thus, the pollen tube of plants with normal and reduced leaves penetrated the style within the same time. There were, however, certain differences between the plants with normal and those with reduced leaves. In the former the zygote was observed up to 20 and 24 h after pollination, while in the latter it was still visible after 48 h (Table 2).

In the fertilized embryo sac the primary endosperm nucleus divided before that of the zygota (Table 2). A 2-cell embryo was found in the plants analysed 30 h after pollination (Figs 3a, b). In plants with normal leaves the endosperm nuclei were more numerous in the embryo sac with 2-cell embryo than in plants with reduced leaves. Further differentiation of the embryo and increase of the number of endosperm nuclei occurred faster in the embryo sacs of plants with normal leaves that in those of plants with reduced leaves (Table 2, Photos 4—7). After 5 days, the embryo, hanging on a massive suspensor in the embryo sacs of plants with normal leaves was club-shaped (Photo 8), while in the ovules of plants with reduced leaves the embryo was at this time somewhat

smaller (Photo 9). The number of endosperm nuclei in the embryo sacs of plants with normal leaves at the same time after pollination was always higher than in plants with reduced leaves (Table 2).

Table 2

Embryo and endosperm development in Swedish clover plants with normal and reduced leaves

Leaves	Hours or days after pollination	Embryo	Endosperm
		No. of cells	No. of nuclei
Normal	20	male nucleus in nucleus of egg cell; zygote	male nucleus in secondary nucleus of embryo sac; primary endosperm nucleus; two endosperm nuclei
	24	zygote	2—4
	30	2	4—8
	36	2—6	12—22
	42	4—8	20—28
	2	6—14	42—56
	3	12—36	64—122
	5	club-shaped	abundant, nucleate
Reduced (55/1)	20	male nucleus in nucleus of egg cell; zygote	primary endosperm nucleus
	24	zygote	2
	30	zygote; 2	2—4
	36	2—6	4—8
	42	2	6
	2	zygote; 8	6—28
	3	6—16	28—46
	5	spherical	abundant, nucleate

In the two plants with reduced leaves which were examined, fertilization took place relatively often, and the embryos developed as described above in plant 55/1. The per cent of coalesced ovaries in this plant exceeded 50.0, and the per cent of flowers which produced pods with ripe seeds was 23.3. In the second plant the percent of ovaries which had not coalesced exceeded 90.0 and that of pods with seeds was 2.2.

Analysis of the frequency of ovule fertilization according to their position in the ovary — from the style towards the ovary — demonstrated that in plants with reduced leaves the ovule lying closest to the style was least frequently fertilized, most frequent to be fertilized was the third ovule from the style (Table 3). The difference between the per cent of fertilized ovules occupying in the ovary positions 1 and 3 amounts to 18.8 per cent. Ovules 2 and 4 counting from the style, the latter being closest to the ovary bottom, do not differ in the frequency of their fertilization. In plants with normal leaves the thrid ovule from the style was most frequently fertilized, and the one closest to the ovary bottom

the least frequently (Table 3). In general in plants with normal leaves the per cent of fertilized ovules occupying a given position in the ovary proved by 40.0 to 60.0 per cent higher than in the plants with reduced leaves.

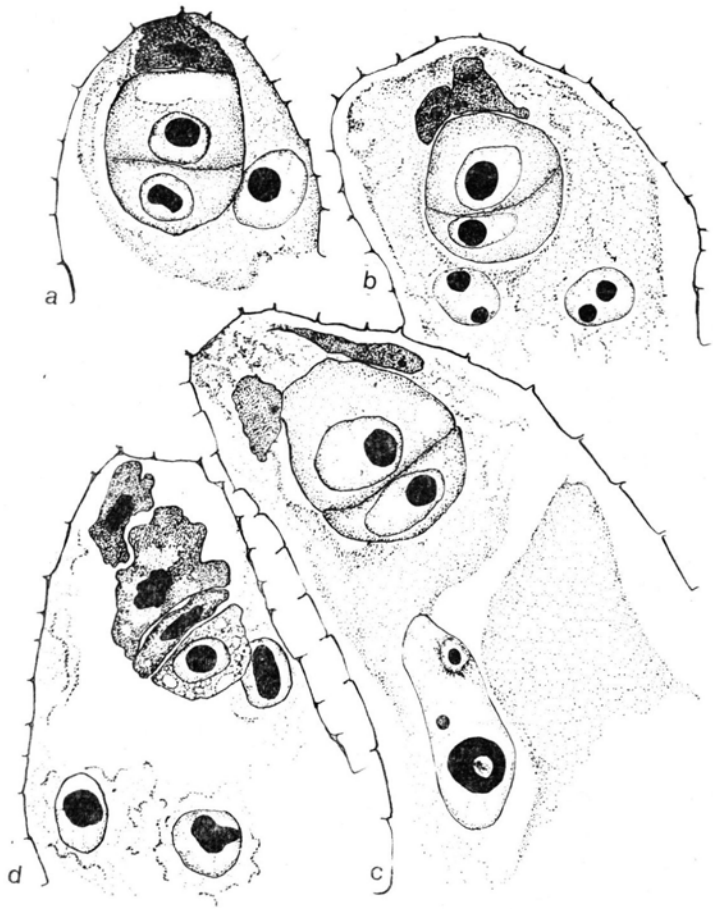


Fig. 3. Micropylar part of embryo sac with embryo and endosperm:

a, *b* — two-cell embryo: *a* — from plant with normal leaves, *b* — from plant with reduced leaves; *c* — two-cell embryo from plant with normal leaves and secondary nucleus of embryo sac; *d* — 4-cell degenerated embryo from plant with normal leaves. × 1000

In one of the 4 ovules in the ovaries from plants with normal leaves there was a 2-cell embryo (Fig. 3c) and a secondary nucleus of the embryo sac which did not occupy the position typical for the mature embryo sac (Fig. 1b), lying in its central part. In the embryo sacs of the three remaining ovules of the ovary there were 2- and 4-cell embryos with 12 and 20 endosperm nuclei, respectively. In the above mentioned

Table 3

Relation between position of ovule in ovary and fertilization in Swedish clover plants with normal and reduced leaves

Leaves	Position of ovule in ovary	No. of ovules			fertilized, %
		examined	including		
			fertilized	unfertilized	
Normal	1	32	29	3	90.6
	2	32	28	4	86.8
	3	32	30	2	93.7
	4	19	15	4	78.9
Reduced (55/l)	1	32	10	22	31.2
	2	32	13	19	40.6
	3	32	16	16	50.0
	4	24	10	14	41.6

embryo sac with 2-cell embryo and secondary nucleus the egg cell was fertilized. The second male nucleus, perhaps defective, had degenerated.

After fertilization the embryos died in some of the embryo sacs (Fig. 3d) and the endosperm degenerated. Embryo sacs with a dying embryo were usually narrow with a small amount of cytoplasm. The embryos died more frequently (in 5 of 50 fertilized ovules, 10.0%) in ovaries of plants with reduced leaves. The per cent of dead embryos in the ovaries of normal plants was 2.7.

DISCUSSION

Genetically conditioned female sterility seems to be in plants not less frequent than male sterility. The descriptions of this phenomenon are rare, probably because specimens without seeds are usually noticed in the period of maturity and harvest, that is when it is too late for a cyto-embryological analysis. Besides, the lack of seeds in plants may be also due to nongenetic factors such as diseases (Kazimierski et al., 1973), poor pollination by insects and a number of others. Another cause of the paucity of papers devoted to female sterility may be the fact that this phenomenon has not been taken into account in breeding.

Among the so far published papers devoted to genetically conditioned female sterility in plants may be quoted those of Hsiung et al. (1967), Dhesi (1966), Filutowicz (1957) and Jassem (1967). Hsiung Wan and Mann (1967) performed their studies on tobacco and demonstrated that spontaneous female sterility in this species depends on one or two recessive genes. The flowers of female-sterile plants had pistils deprived of stigmata and style, were without open tube down the center and were shorter than in flowers of fertile plants. In the cotton plant (Dhesi, 1965) and the sugar beet (Filutowicz, 1957; Jassem,

1967) female sterility was detected in the progeny of plants growing from seeds exposed to X-rays. Ovules of female-sterile plants of the two mentioned species had a untypical structure and did not contain embryo sacs.

The low female fertility — frequently equivalent to sterility — of Swedish clover, as shown in the present analyses, resembles but little that described in tobacco, sugar beet and cotton. Three elements contribute to the low female fertility of Swedish clover: the structure of the ovary, the development of the sporophyte and female gametophyte and fertilization. Part of the megasporocytes and megagametophytes waste away in the process of differentiation and maturation. In undeveloped flowers the per cent of degenerated ovules with sporogenic cells and deviations in the development of the embryo sac was 12.1. In fully developed flowers the per cent of degenerated embryo sacs or wasted away in the course of ontogenesis was on the average 23.4. The increased percentage of ovules with degenerated embryo sac indicates that the process of wasting away is enhanced as the flower develops.

Normal development and formation of the embryo sac, however, were not decisive for fertilization. In plants with normal leaves the per cent of fertilized ovules was 93.2, and in the plants with reduced leaves it was on the average 28.1, being higher in one plant — 45.0, while in the other it was lower — 1.3. A wide difference in the percentage of fertilized ovules between the plants with reduced leaves may be attributed to the structure of the ovaries in these plants. In the former the ovaries were coalesced in 51.3 per cent and in the latter in 9.1 per cent of cases. In the uncoalesced ovaries the ovules were connected with the placenta by a long funiculus and frequently they lay beyond the ovary. It would seem that the low female fertility — frequently equivalent to sterility — in plants with reduced leaves is largely due to the lack of coalescence of the carpel connected by the greatly elongated funiculus and to disturbances in the processes of megasporo- and megagametogenesis.

The above discussed differences in the structure of the ovary, the embryo sac, fertilization and embryonal development in plants with normal and reduced leaves indicate that the gene causing leaf reduction (*reductivus*) in Swedish clover influences also on the development of the ovary, the female gametophyte, the fertilization and embryonal development processes. This factor reduces drastically fertility in all the individuals, and in extreme cases causes female sterility. The trait of reduced leaves is recessive and monomerically heritable (Kazimierski, Kazimierska, 1979). Among the plants with reduced leaves, there were, however, female-sterile specimens and others which produced seeds, though in a small amount. These differences may be conditioned by the incomplete homozygosity of the plants with reduced

leaves, particularly in the loci of the *reductivus* factor conditioning the development of female generative organs and cells. Or else it may be due to an incomplete linkage between the *reductivus* factor and the gene(s) responsible for the female reproductive ability of the plant. Both suppositions are possible and require further elucidation.

The above described low fertility — female sterility — in Swedish clover, where in part of the ovules a normally developed female gametophyte is present, may be termed female structural sterility.

CONCLUSIONS

1. The embryo and endosperm in plants with reduced leaves develop normally, however, somewhat slower than in normal plants. The percentage of dead embryos proved to be higher in plants with reduced leaves. Thus, the factor producing leaf reduction affects the development of the female gametophyte, fertilization and development of the embryo.

2. The differences between the plants with reduced leaves, some being female sterile and others giving single seeds, may be due to incomplete homozygosity of the plants or to incomplete linkage between the *reductivus* factor and the gene(s) responsible for the female reproductive ability of the plant.

3. The established low fertility — female sterility — in Swedish clover, where part of the female gametophytes develop normally, may be termed female structural sterility.

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Budowa woreczka zalążkowego, zapłodnienie i rozwój zarodka u roślin o listkach zredukowanych koniczyny szwedzkiej (Trifolium hybridum L.)

Streszczenie

Rośliny koniczyny szwedzkiej o zredukowanych listkach rzadko dawały nasiona. Przypuszczano, że niska płodność i bezpłodność takich roślin może być powodowana jakimiś odchyleniami w budowie zalążni, zalążka, woreczka zalążkowego, albo zaburzeniami w procesie zapłodnienia i rozwoju zarodka. Badania embriologiczne wykazały, że u takich roślin zalążki w zalążniach były podwieszone na bardzo długich funiculusach. Gametofit żeński przeważnie rozwijał się normalnie, odchylenia stwierdzono w około 10,0% analizowanych zalążków. Odsetek zapłodnionych zalążków u roślin o listkach normalnych był większy niż u roślin o listkach zredukowanych, wśród których znajdowano także osobniki bezpłodne. Zarodek i bielmo u roślin o listkach zredukowanych rozwijały się nieco wolniej niż u roślin o listkach normalnych. Zatem czynnik wywołujący redukcję listków wpływa na rozwój zalążni, gametofitu żeńskiego, zapłodnienie i rozwój zarodka. Opisaną dziedziczną słabą płodność — bezpłodność u koniczyny szwedzkiej, gdzie spora część żeńskich komórek rozrodczych osiąga normalną dojrzałość, można nazwać żeńską bezpłodnością strukturalną.