J. Appl. Genet. 38(2), 1997, pp. 131-141

Embryo development after interspecific hybridization of *Lupinus albus* L., *L. mutabilis* Sweet. and *L. angustifolius* L.

Jerzy A. PRZYBOROWSKI, Danuta PACKA

Department of Plant Breeding and Seed Production, University of Agriculture and Technology, Olsztyn

Abstract. Embryo development of *Lupinus albus* L. and *L. mutabilis* Sweet. after pollination with *L. angustifolius* L. was studied. Observations of ovules revealed non-typical embryo development. From the 5th day after pollination, high number of degenerated ovules was found; the highest number was observed on 15 and 20 days after pollination. The period between the 10th and 15th day after pollination was the most suitable for isolation of embryos in all the examined combinations, with exception *L. albus* × *L. angustifolius* where optimal isolation time was between 15th and 20 th day after pollination. Embryos were obtained in each cross-pollination. Some of them developed in vitro into plantlets and grew in an artificial medium for some time, but most of them died due to non-typical development of the root system, stem, or both. The hybrid character of the survived plants requires confirmation.

Key words: *Lupinus albus*, *Lupinus angustifolius*, *Lupinus mutabilis*, interspecific hybridization, embryo development, in vitro culture.

Introduction

The need for protein production, both for humans and animals, is continuously growing. Leguminous plants are characterized by the highest protein content, and among them the seeds of soybean and lupin contain the largest amounts of protein. Lupin protein is characterized by a well-balanced amino acid composition which may be compared with that of soybean protein. Another

Received: September 1996.
Correspondence: J.A. PRZYBOROWSKI, Department of Plant Breeding and Seed Production, University of Agriculture and Technology, Plac Łódzki 3, 10-724 Olsztyn, Poland.

advantage of *Lupinus* plants is that lupin cultivation is inexpensive. Like other legumes, lupin – through symbiosis with *Rhizobium* bacteria – constitutes a high-quality green manure and feed for many animal species, and may also be used in human nutrition.

Despite recent progress in lupin breeding, breeders are still trying to improve qualities of this crop and to produce its new, attractive forms. They are looking for possibilities to extend genetic variation. Wide hybridization is one of such possibilities. Research on different lupin species shows that experiments aimed at obtaining hybrid plants with desirable features seem to be advisable (JARANOWSKI 1962a,b, WILLIAMS et al. 1980, SCHÄFER-MENUHR et al. 1988, BUSMANN-LOOCK et al. 1992, PRZYBOROWSKI et al. 1996). New World lupins are characterized by a great genic potential; *Lupinus mutabilis* Sweet. is one of them. Its seeds contain ca. 40% of protein with a high content of exogenic amino-acids, and ca. 20% of high-quality fat (GROSS 1988). Moreover, it tolerates considerable soil acidification and is more resistant to frost and drought than Old World species, and since its low-alkaloid forms were obtained, this species has become even more attractive (BAER, GROSS 1983, BAER, BAER 1988). A very important feature of *L. mutabilis* is the lack of pod cracking and dropping. However, despite these numerous desirable characters, it has a serious disadvantage connected with late blooming and late, irregular ripening. The growing season of this species lasts for 5 to 11 months. Another feature that requires improvement is the ratio between leaf mass and shoot mass, which is low in comparison to that of other lupin species. For that reason *L. mutabilis* produces a smaller number of seeds than it might be expected from its large vegetative mass. Another late ripening species, but cultivated in Europe, is white lupin (*L. albus* L.). This is a large-seeded plant, with a high protein content. It influences the soil in a positive way and is of great worth as far as its nutritive values are concerned. Nevertheless, late ripening of white lupin limits its cultivation, especially in the north and north-east parts of Poland.

Reciprocal crossing was carried out between white, Andean and the early ripening blue lupin to shorten the growing season and advance the ripening period of the first two species.

The present study has aimed at: (a) examination of embryo development after reciprocal pollination of *L. albus* and *L. mutabilis* with *L. angustifolius*; (b) determination of the optimum moment for embryo isolation; (c) in vitro culture of immature embryos.

Material and methods

In the experiments the following lupin species were used: white lupin (*L. albus* L.) cv. Wat (Plant Breeding Station at Wiatrowo), blue lupin (*L. an-*

gustifolius L.) cv. Polonez (PBS at Wiatrowo) and Andean lupin (*L. mutabilis* Sweet.), population no. 098903 (Institute of Plant Breeding and Acclimatization at Radzików). The plants were cultivated in pots in a greenhouse. The seeds were sown in five rounds, from the beginning of April till the beginning of May.

Experiment I

The investigations concentrated on embryo development after reciprocal pollination between *L. albus* and *L. angustifolius* and between *L. mutabilis* and *L. angustifolius*, as well as after intraspecific pollination was studied.

Controlled reciprocal pollination was performed between blue and white lupins and between blue lupin and Andean lupin. Controlled pollination with pollen of the same species was carried out as well. The ovaries or young seeds were collected 5, 10, 15 and 20 days after pollination. They were fixed for 24 h in FAA and, after rinsing, stored in 70% ethyl alcohol. The ovaries, or young seeds, were embeded in paraffin and then cut crosswise into 10-μm thick pieces. Iron hematoxylin solution, according to the method of Heidenhain, was used for staining of permanent preparations.

Experiment II

Like in experiment I, controlled interspecific pollination was conducted. The ovaries were collected 12-15 days after pollination. They were then sterilized in 3.5% solution of sodium hypochlorite for 20 minutes. After rinsing in 70% ethyl alcohol, the material was rinsed three times in sterile distilled water. Ovules were excised from the ovaries. Immature embryos were isolated and transferred on MS medium (MURASHIGE, SKOOG 1962). Embryo culture was carried out at the temperature of 19°C, with 16-hour light. The development of embryos and plantlets was observed.

Results

Embryo development

Observations of ovules after lupin interspecific hybridization revealed non-typical embryo development, in comparison to the control combinations. From the 5th day after pollination, a relatively high number of degenerated ovules was found in all interspecific combinations; their number reached its maximum 15 and 20 days after pollination.

Some delay in embryo development was observed in the combination *L. albus* × *L. angustifolius*, as compared to that of *L. albus* × *L. albus* (Table 1). Embryonic structures of irregular shapes with several nuclei were observed

Table 1. Embryo development after crossing of *L. albus* with *L. angustifolius* and in the control combination *L. albus* × *L. albus*

Days after pollination	*L. albus* × *L. albus*	*L. albus* × *L. angustifolius*
5	An embryo built of several cells with a suspensor in between the micropylar and chalazal poles, nuclear endosperm.	Structures of irregular shap in the micropylar part, with several nuclei, lack of endosperm.
10	An embryo built of tens of cells in the chalazal part, nuclear endosperm.	Structures with numerous nuclei in the micropylar part, lack of endosperm.
15	A bigger globular embryo in the chalazal part, nuclear endosperm.	Embryos consisting of several cells in the micropylar part, nuclear endosperm.
20	Globular and heart-shaped embryos, cellular endosperm around the embryo in the micropylar part, nuclear endosperm.	Globular embryos built of tens of cells in the chalazal part, nuclear endosperm. Numerous degenerated embryo sacs and whole ovules.

in the ovules examined 5-10 days after pollination. However, they did not contain endosperm nuclei. After 15 days, some cells of embryos and nuclear endosperm were found; in the case of the control combinations, baton-shaped embryos consisting of tens of cells and fully-developed nuclear endosperm were noted (Fig. 1 a, b, c). Larger embryos were observed in the combination *L. albus* × *L. angustifolius* 20 days after pollination (Fig. 1d), but they were accompanied by nuclear endosperm (in the control combination, cellular endosperm surrounded better developed heart-shaped embryos). Numerous degenerated embryo sacs and whole ovules were found afterwards.

Delayed embryo development, as compared to the control, was observed in the ovules obtained from the reciprocal crosses between *L. angustifolius* and *L. albus* (Table 2). Some delay and non-typical development were especially marked in the case of ovules examined 10 days after pollination, with oval or elongated embryos of no more than a dozen of cells, accompanied by relatively poorly-developed nuclear endosperm (Fig. 2). After 15 and 20 days, only degenerated ovules or embryo sacs with degenerated nuclear endosperm were found in that combination.

A similar result was observed in the combination *L. angustifolius* × *L. mutabilis* (Table 2). It should be emphasized, however, that 10 days after pollination the presence of a suspensor characteristic of *L. mutabilis* (the suspensor of *L. mutabilis* consists of large cells with huge nuclei) was noted in one of the examined ovules (Fig. 3a). Moreover, baton-shaped embryos consisting of dozens of cells were found as well (Fig. 3b, c). After 15 and 20 days, like

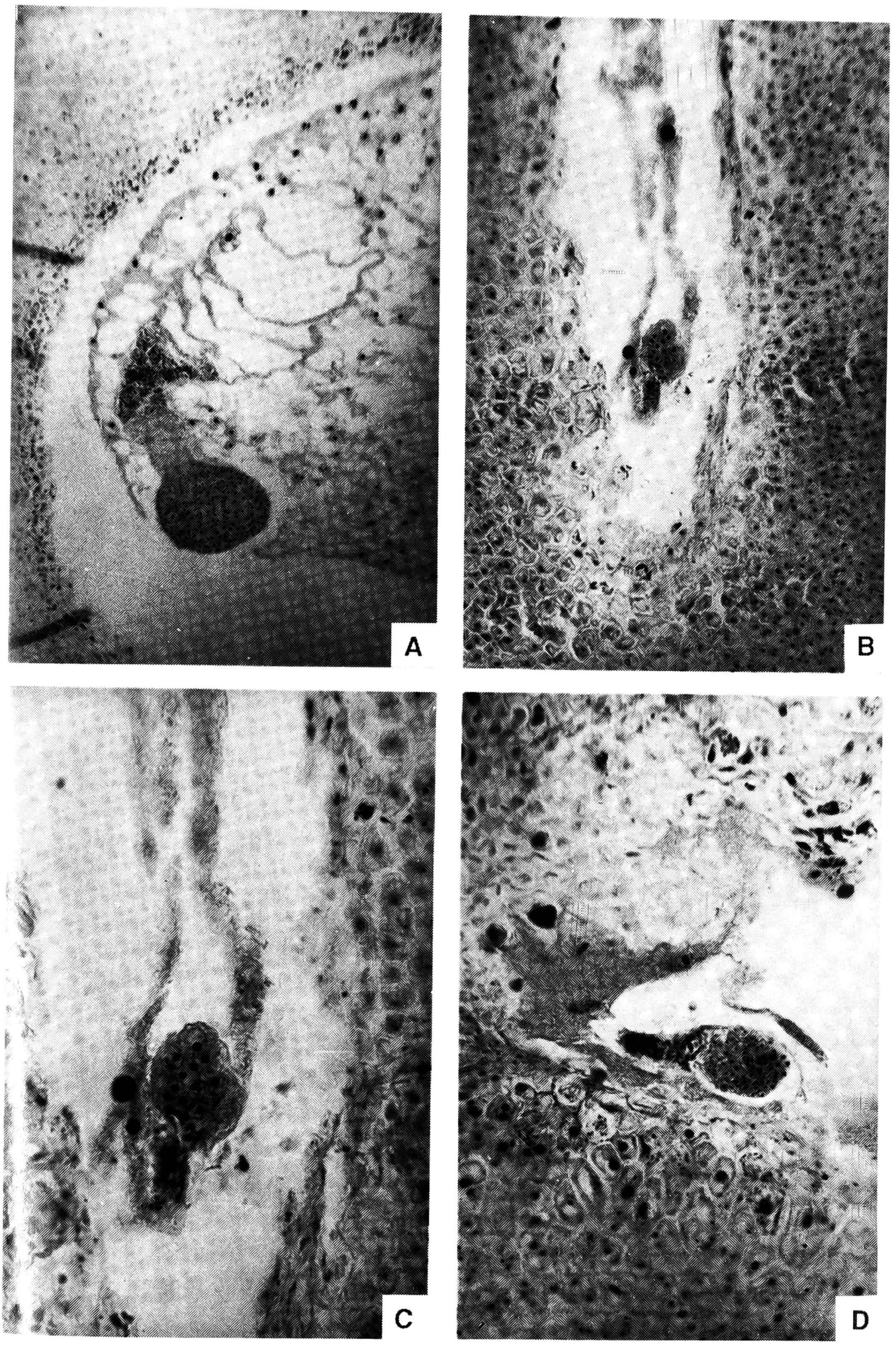

Fig. 1. Ovule cross-sections with: (a) *L. albus* × *L. albus* embryo 20 days after pollination, ca. 130×; (b) and (c) *L. albus* × *L. angustifolius* embryo 15 days after pollination, ca. 260× and 500×; (d) *L. albus* × *L. angustifolius* embryo 20 days after pollination, ca. 500×

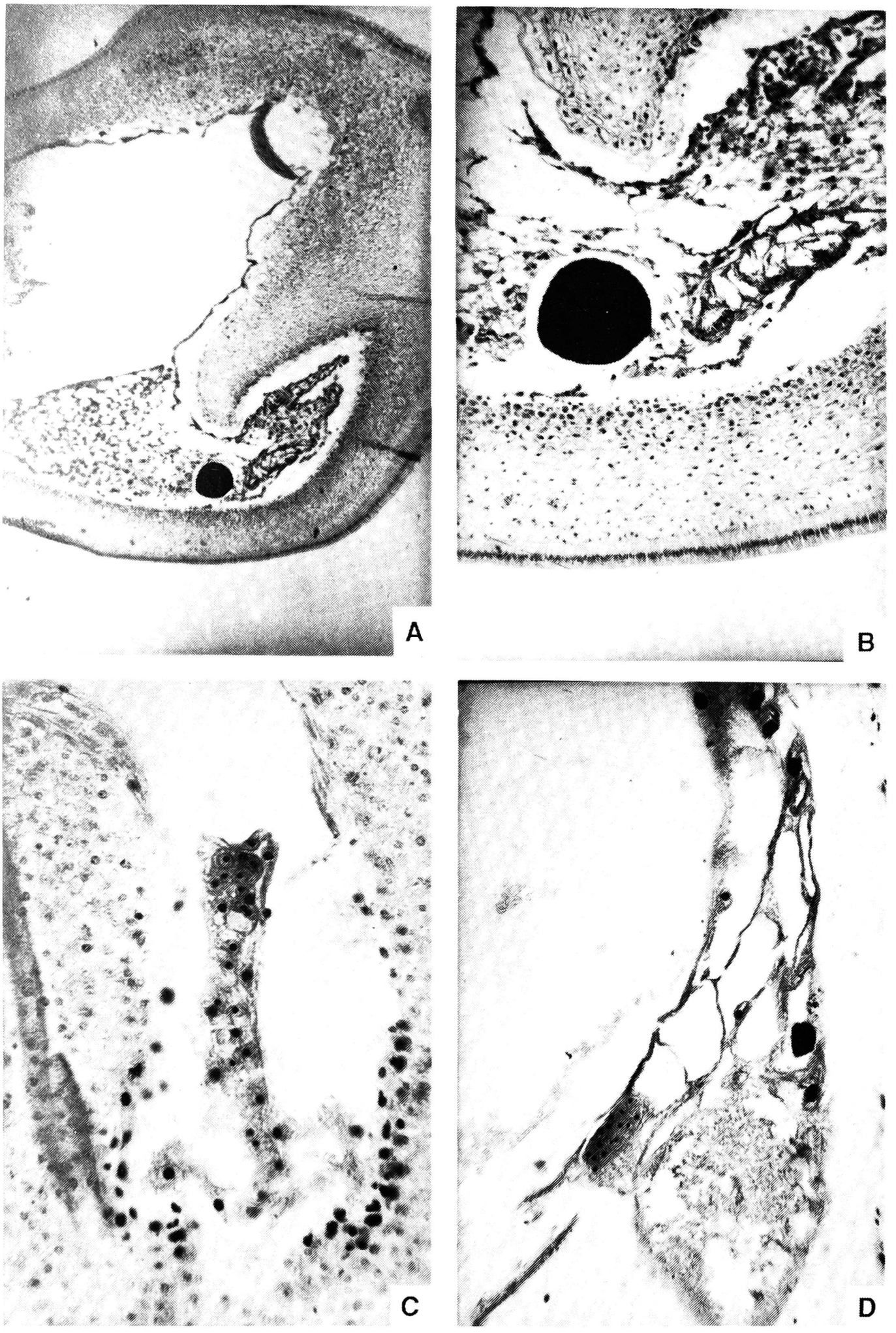

Fig. 2. Ovule cross-sections with: (a) and (b) *L. angustifolius* × *L. angustifolius* embryo 10 days after pollination, ca. 40× and 130×; (c) *L. angustifolius* × *L. albus* embryo 5 days after pollination, ca. 500×; (d) *L. angustifolius* × *L. albus* embryo 10 days after pollination, ca. 500×

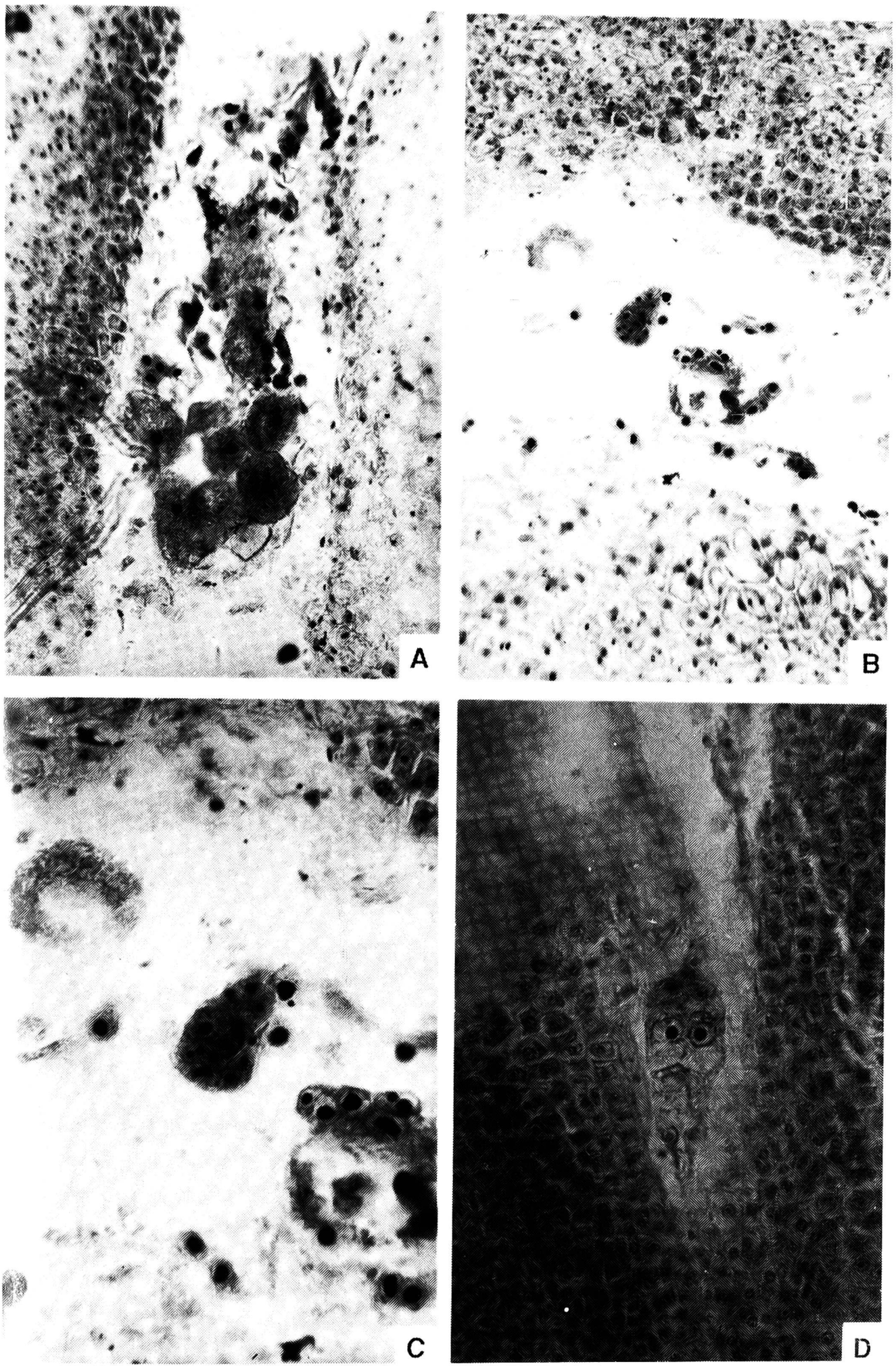

Fig. 3. Ovule cross-sections with: (a) a suspensor characteristic of *L. mutabilis* 10 days after pollination of *L. angustifolius* flowers with *L. mutabilis* pollen, ca. 260×; (b) and (c) *L. angustifolius* × *L. mutabilis* embryo 10 days after pollination, ca. 260× and 500×; (d) *L. mutabilis* × *L. angustifolius* embryo 5 days after pollination, ca. 500×

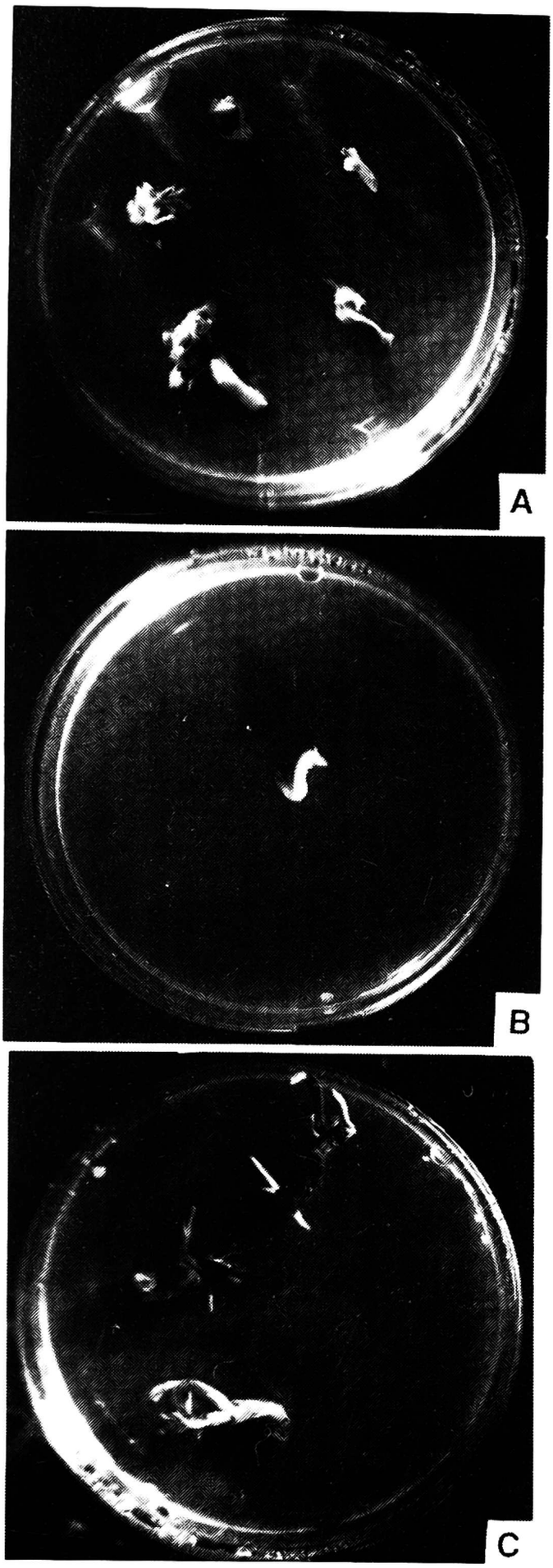

Fig. 4. In vitro developing embryos: (a) *L. albus* × *L. angustifolius*; (b) and (c) *L. angustifolius* × *L. albus*

Table 2. Embryo development after crossing of *L. angustifolius* with *L. albus* and *L. mutabilis*, and in the control combination *L. angustifolius* × *L. angustifolius*

Days after pollination	*L. angustifolius* × *L. angustifolius*	*L. angustifolius* × *L. albus*	*L. angustifolius* × *L. mutabilis*
5	Pear-shaped or globular embryos, consisting of several cells or bigger, in the chalazal part of the sac; nuclear endosperm; in the micropylar part small size endosperm nuclei and huge in the chalazal part.	Some embryos consisting of several cells in between the micropylar and chalazal poles, nuclear endosperm.	Some embryos of irregular shapes, consisting of several cells, poorly-developed nuclear endosperm in the micropylar part.
10	Heart-shaped embryos, cellular and nuclear endosperm.	Some oval or elongated embryos in the micropylar part or between the micropylar and chalazal parts, poorly-developed or degenerated nuclear endosperm.	Some embryos consisting of several or a dozen cells, nuclear endosperm. A suspensor characteristic of L. mutabilis was found in one of the ovules.
15	Big embryos, with clearly differentiated radicular and cotyledonar parts. Cellular endosperm and some remains of nuclear endosperm.	Lack of normal embryo structures. Ovules with degenerated nuclear endosperm.	Degenerated structures.
20	An embryo filling the whole seed. Some remains of cellular endosperm.	Degenerated structures.	Degenerated structures.

in the cross *L. angustifolius* × *L. albus*, only degenerated structures were observed inside the ovules.

In the cross *L. mutabilis* × *L. angustifolius*, degenerated cellular structures of the embryo sac were noted in most of the examined ovules (Table 3). Both in five-day-old ovules and in the control, only an egg apparatus was found (no embryo was observed in the examined control ovules), and an embryo-like structure consisting of several cells was noted in one case (Fig. 3d). Ten days after pollination, single ovules with globular embryos and poorly-developed nuclear endosperm were observed among numerous degenerated ovules.

Embryo culture

1349 plants were pollinated according to the following scheme (Table 4):
L. albus × *L. angustifolius* – 439,
L. angustifolius × *L. albus* – 377,

Table 3. Embryo development after crossing of *L. mutabilis* with *L. angustifolius*, and in the control combination *L. mutabilis* × *L. mutabilis*

Days after pollination	*L. mutabilis* × *L. mutabilis*	*L. mutabilis* × *L. angustifolius*
5	Only egg apparatus in the micropylar part (an egg cell and two synergids), a central cell (nucleus in the chalazal part).	Egg apparatus (an egg cell and a synergid.); one case of an embryo-like structure, consisting of several cells, in the micropylar part, with dividing cells.
10	A pear-shaped embryo, consisting of tens of cell; nuclear endosperm; large suspensor cells.	Mostly degenerated ovules; few globular embryos with poorly-developed nuclear endosperm.
15	Embryo with differentiated radicular and cotyledonar part; cellular endosperm around the embryo, and from the side of micropyle – nuclear chalazas; big, characteristic suspensor cells with huge nuclei.	Degenerated embryo sacs and whole ovules.
20	An embryo much bigger than after 15 days, with a large primary root; almost the whole seed filled with the embryo; some remains of cellular endosperm in the micropylar and chalazal parts.	Degenerated embryo sacs and whole ovules.

L. mutabilis × *L. angustifolius* – 361,
L. angustifolius × *L. mutabilis* – 172.

The largest number of set pods was found in the combinations where *L. angustifolius* was a maternal form. In combinations involving *L. albus* as a paternal form, the percentage of set pods was equal to 25.5 and was over threefold higher than in the case where *L. mutabilis* was a paternal form (Table 4). In the remaining combinations, with *L. angustifolius* as a paternal form, set pods constituted ca. 2%.

In the combination *L. albus* × *L. angustifolius*, 35 ovules were excised from set pods, i.e. 3.89 ovules per pod on average. 32 embryos were isolated from these ovules, i.e. 3.56 embryos per set pod on average. 43.8% of 32 isolated embryos developed into plants (Fig. 4a).

In the combination *L. angustifolius* × *L. albus* 278 ovules were found in set pods (2.9 ovules per pod). 30 embryos were isolated from them. In that combination, there were, on average, 0.31 of embryo per set pod, and 60% of embryos developed into plants (Fig. 4b, c).

16 ovules were excised from 8 pods set after pollination of *L. mutabilis* with the pollen of *L. angustifolius*. 12 embryos were isolated from them (1.5 embryos per set pod), and they all developed into plants. In the case

Table 4. Results of interspecific crossings between *L. albus*, *L. mutabilis* and *L. angustifolius*

Cross	No. of pollinated flowers	Set pods		No. of ovules		No. of embryos		Plants developed from embryos			
								after 6 weeks		after 6 months	
		No.	%[1)]	total	per pod	total	per pod	No.	%[2)]	No.	%[2)]
L. albus × *L. angustifolius*	439	9	2.1	35	3.89	32	3.56	14	43.8	3	21.4
L. angustifolius × *L. albus*	377	96	25.5	278	2.90	30	0.31	18	60.0	18	60.0
L. mutabilis × *L. angustifolius*	361	8	2.2	16	2.00	12	1.50	12	100.0	12	100.0
L. angustifolius × *L. mutabilis*	172	12	7.0	34	2.83	5	0.42	5	100.0	1	20.0

[1)] of the number of pollination
[2)] of the total number of embryos

of the reciprocal cross, 34 ovules were obtained from 12 set pods (2.83 ovules per pod), but embryos were observed only in 5 of them (0.42 of embryo per set pod). All the embryos cultured in vitro developed into plants.

Some of plants developed from embryos in individual cross combinations were characterized by non-typical development of the stem, root system or both. Due to that, after several weeks of in vitro culture the non-typical plants died.

Discussion

Studies concerning the possibilities of obtaining interspecific hybrids within the genus *Lupinus* were started at the beginning of our century. Different Mediterranean species were crossed with one another (GOLLMICK 1937, JARANOWSKI 1962b, KAZIMIERSKI, KAZIMIERSKA 1965, TOMASZEWSKI et al. 1971), and with African species (ROY, GLADSTONES 1985, 1988). Some attempts to obtain hybrids of Old and New World species were made as well (GOLLMICK 1937, VUILLAUME, HOFF 1986, BUSMANN-LOOCK et al. 1992). Numerous authors indicated different barriers that make obtaining hybrids difficult or impossible. The lack of cross-compatibility may result not only from nucleus and cytoplasm interaction, but also from differences between genomes of the crossed species. Differences in the number of chromosomes may play a less important role, as the crossing between *L. atlanticus* (2n=38), *L. pilosus* (2n=42) and *L. cosentini* (2n=32) permited to obtain partly fertile hybrids (ROY, GLADSTONES 1988).

The results presented in this paper seem to confirm the above-mentioned suggestions. Observations of ovules produced as a result of both autogamy and reciprocal crossing generally remain in agreement with the results of similar investigations conducted by GOLLMICK (1937) on white and yellow lupins, by JARANOWSKI (1962a, b) on white and blue lupins, and by GOLLMICK (1937) and BUSMANN-LOOCK et al. (1991, 1992) on Andean lupin. Non-typical development of embryos and endosperm, as compared to the control, as well as numerous cases of degeneration were observed in all interspecific combinations. JARANOWSKI (1962b) reported that the effect of crossing barriers is visible not earlier than after the fusion of parental gametes. Similarly, WILLIAMS et al. (1980) and PRZYBOROWSKI et al. (1996), who examined the efficiency of interspecific pollination between *L. albus, L. mutabilis* and *L. angustifolius*, found a delay in alien pollen germination and a slower pollen tube growth. They, however, observed pollen tubes reaching the ovary. The proposed thesis is supported by the fact that developing hybrid embroys

used to die at different stages due to certain disturbances in the development of the proembryo, suspensor and endosperm. The process of dying depended also on the direction of crossing. This finds confirmation in the results of histological research on hybrid embryo formation after the crossing of *L. hartwegii* and *L. mutabilis* carried out by BUSMANN-LOOCK et al. (1992).

Due to the fact that incompatibility is revealed at early stages of hybrid embryo development, contrary to reports of GOLLMICK (1937) that there is no hope of a successful embryo culture on media, many researchers see some chances for obtaining hybrids using in vitro culture of embryos isolated at a proper moment (TOMASZEWSKI et al. 1971, WILLIAMS et al. 1980, SCHÄFER-MENUHR et al. 1988). The results of the presented observations suggest that the period between the 10th and 15th day after pollination is the best time for isolation of hybrid embryos in all the examined interspecific combinations, except for the cross *L. albus* × *L. angustifolius*, in the case of which the time between the 15th and 20th day after pollination is most suitable.

Only few papers concerning this problem have been published so far. VUILLAUME and HOFF (1986) described results of studies on induction of *L. albus* × *L. mutabilis* hybrids, in the case of which in vitro culture of pods, ovules and isolated embryos was applied. The experiments did not result in obtaining hybrid plants, but made it possible to find that phytohormones had a negative effect on embryos cultured in vitro. SCHÄFER-MENUHR et al. (1988) obtained hybrid plants of *L. mutabilis* × *L. hartwegii* as a result of in vitro culture of embryos isolated from 18-day-old pods. This authors found also that phytohormones applied during the first few days of culture had a positive influence on embryo development and late, better effects were achieved with a medium containing no growth regulators. Morphological features and isoenzymatic analysis confirmed the hybridity of the plants. In the present paper, embryos were obtained in each of the examined cross-combinations. Some of them developed into plants whose hybrid character requires identification.

Conclusions

Observations of the ovules after interspecific pollination between lupin species revealed non-typical embryo development in comparison to the control combinations. From the 5th day after pollination, a relatively high number of degenerated ovules was found in all the interspecific cross combinations; it reached its maximum 15 and 20 days after pollination.

The period between the 10th and 15th day after pollination appeared the most suitable for isolation of embryos in all the examined combinations,

except for the cross *L. albus* × *L. angustifolius*, where optimal time was between the 15th and 20th day after pollination was suitable.

As a result of the performed crossings, embryos were obtained in each combination. Some of them developed in vitro into plants. However, most of them died due to non-typical development of the root system, stem or both. The hybrid character of the survived plants requires confirmation.

Acknowledgements. This work was supported by the State Committee for Scientific Research in Poland, Grant No. PB0064/S3/94/06.

REFERENCES

BAER E., GROSS R. (1983). Sweet strains of *Lupinus mutabilis*. Z. Pflanzenzüchtg. 91: 334-337.

BAER E., BAER D. (1988). *Lupinus mutabilis*: cultivation and breeding. Proc. V Int. Lupin Conf. Poznań, July 1988: 237-247.

BUSMANN-LOOCK A., DAMBROTH M., MENGE-HARTMANN U. (1991). Histologische Untersuchungen zur Embryo- und Endospermentwicklung von *Lupinus luteus, L. mutabilis* und *L. hartwegii*. Landbaufors. Völk. 41(3): 123-132.

BUSMANN-LOOCK A., DAMBROTH M., MENGE-HARTMANN U. (1992). Histological observations on interspecific crosses in the genus *Lupinus*. Plant Breeding 109: 82-85.

GOLLMICK F. (1937). Über Artkreuzungen bei Lupin. Der Züchter 9: 65-68.

GROSS R. (1988). Lupins in human nutrition. Proc. V Int. Lupin Conf. Poznań, July 1988: 51-64.

JARANOWSKI J. (1962a). Fertilization and embryo development in the genus *Lupinus*. Part I. Seed development in cases of autogamy. Genet. Pol. 2: 210-246.

JARANOWSKI J. (1962b). Fertilization and embryo development in the genus *Lupinus*. Part II. Fertilization and embryo development following reciprocal species hybridization. Genet. Pol. 3: 333-368.

KAZIMIERSKI T., KAZIMIERSKA E. (1965). Studies on a species hybrid *Lupinus rothmaleri* Klink. × *Lupinus luteus* L. Genet. Pol. 1-2: 125-140.

MURASHIGE T., SKOOG F.J. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

PRZYBOROWSKI J.A., SAMBORSKA-CIANIA A., WIWART M. (1996). Intra- and interspecific pollination between *Lupinus albus* L., *Lupinus mutabilis* Sweet. and *Lupinus angustifolius* L. J. Appl. Genet. 37: 261-275.

ROY N.N., GLADSTONES J.S. (1985). Prospects for interspecific hybridization *of Lupinus atlanticus* Gladst. with *Lupinus cosentini* Guss. Theor. Appl. Genet. 71: 238-241.

ROY N.N., GLADSTONES J.S. (1988). Further studies with interspecific hybridization among mediterranean/African lupin species. Theor. Appl. Genet. 75: 606-609.

SCHÄFER-MENUHR A., CZERWIŃSKI T., BRUSMANN A. (1988). Der Einsatz von Embryokultur zur Gewinung von Artbastarden aus der Kreuzung *Lupinus mutabilis* × *Lupinus hartwegii*. Landbaufors. Völk. 38, 3: 173-177.

TOMASZEWSKI Z., KUBOK I., WNUKOWSKA A. (1971). Badania embriologiczne nad tworzeniem się nasion przy krzyżowaniu *Lupinus luteus* × *Lupinus angustifolius*. Biul. Inst. Hodowli Aklim. Rośl. 1-2: 77-85.

VUILLAUME E., HOFF T. (1986). Development in vitro d'embryons immatures de *Lupinus albus* L. et de *Lupinus mutabilis* Sweet. par culture de gousses, d'ovules ou d'embryons isoles. Agronomie, 6 (10): 925-930.

WILLIAMS W., AKHTAR M.A., FALUYI M. (1980). Cross compatibility between European and American *Lupinus* species. Bot. J. Linn. Soc. 81: 225-232.