Europ.J.Hort.Sci., **70** (2). S. 96–103, 2005, ISSN 1611-4426. © Verlag Eugen Ulmer GmbH & Co. Stuttgart

Reproductive Biology of Chinese Chestnut (*Castanea mollissima* Blume)

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Summary

In Chinese Chestnut the reproductive biology is of great importance for nut development and production. In commercial chestnut growing the main problem is the high percentage of empty cupulae or nuts, resulting in not satisfactory yields. About the causes of this phenomenon, which has to be seen in the reproductive processes, not much is known. To elucidate this problem, investigations on the fertization biology in the early ripening cultivar 'Zaodali' and to some extent in the late ripening 'Jiujiazhong' and different pollinizers were undertaken in two experimental orchards in the province Yunnan, South China.

The most important results are: The relation of male and female flowers and catkins was almost identical in the 3 experimental years, i.e. 4–5 male catkins to one female or 1000 male flowers to one female. Per bisexual catkin only one involucrum (female flower) was evident.

'Hand' and 'open pollination' resulted in a fruit set of about 95 % at the time, when the isolation bags were removed and about 90 % shortly before harvest. Under conditions of 'open pollination' the percentage of empty cupulae was lowest with 17,6 to 38 %, and more than 50 % of cupulae with one or two nuts were formed.

In this investigation, pollen tube growth *in vivo* of Chinese Chestnut was first described at all. In almost all flowers less than 6 stigmas with pollen tubes were evident. Normally, one to two tubes could penetrate into the ovary containing 12–18 ovules, and only one tube per flower did enter the ovule. Pollen tube growth from the stigma to the ovule lasted for about one month. In about 20 % of the female flowers with pollen tubes being evident in the ovules, no fertilization occurred. In these investigations important problems on fertilization biology of the cultivar 'Zaodali' and other could be elucidated:

1. missing fertilization,
2. degeneration of zygote, proembryo and young embryo,
3. delayed differentiation of ovules and female gametophyte, and
4. abnormal embryo sacs.

Missing fertilisation has to be seen as the most important problem for nut formation. By hand pollination the percentage of empty cupulae or nuts could be reduced and the number of cupulae with 3 nuts

Zusammenfassung

Reproduktionsbiologie der Chinesischen Esskastanie (*Castanea mollissima* Blume). Bei der Chinesischen Esskastanie ist die Reproduktionsbiologie für die Nussbildung und damit für die Erträge von großer Bedeutung. Ein wichtiges Problem beim Anbau dieser Obstart in China ist der hohe Anteil leerer Cupulae bzw. Nüsse, die zu einer unbefriedigenden Erntemenge führen. Die Ausbildung von leeren (tauben) Nüssen wird als ein Problem der Reproduktionsbiologie angesehen. Zur Aufklärung dieser Fragen wurden befruchtungsbiologische Untersuchungen überwiegend bei der frühreifenden Sorte 'Zaodali', teilweise auch bei der spätreifenden 'Jiujiazhong' und verschiedenen Bestäubersorten in 2 Versuchsanlagen in der südchinesischen Provinz Yunnan durchgeführt.

Das Verhältnis von weiblichen und männlichen Blüten bzw. der weiblichen und männlichen Kätzchen war in den 3 Versuchsjahren (1999–2001) praktisch gleich. So kam bei 'Zaodali' auf 1000 männliche Blüten eine weibliche und auf 4–5 männliche Blütenkätzchen ein weibliches bzw. zweigeschlechtliches. Je zweigeschlechtlichem Blütenkätzchen war in der Regel nur ein Involucrum (weibliche Blüte) vorhanden.

Bei allen Bestäubungsvarianten mit 'Zaodali' waren nach Entfernen der Isoliertüten Fruchtansätze von über 95 % und vor der Ernte noch von über 90 % in allen 3 Versuchsjahren festzustellen. Zwischen den verschiedenen Vatersorten gab es im Fruchtansatz keine signifikanten Unterschiede. Bei Freier Abblüte fiel der Anteil leerer Cupulae am niedrigsten aus, und er lag zwischen 17,6 und 38 %. Bei den Varianten 'ohne Bestäubung' und 'Selbstung' war der prozentuale Anteil leerer Cupulae höher. Bei Freier Abblüte enthielten über 50 % der Cupulae eine oder zwei Nüsse, und insgesamt war ungefähr die Hälfte der Cupulae leer.

In der vorliegenden Arbeit wurde das Pollenschlauchwachstum bei der Chinesischen Esskastanie zum ersten Mal detailliert untersucht. Etwa 3–6 Tage nach der Bestäubung konnten ca. 100 µm lange Pollenschläuche in der Narbenspitze beobachtet werden. Bei fast allen Blüten waren in weniger als 6 Narben, also etwa der Hälfte, Pollenschläuche vorhanden. In der Regel wuchs nur ein Pollenschlauch in eine Samenanlage ein, und je Blüte drangen nur ein oder zwei Pollenschläuche in Samenanlagen ein.

Die Dauer des Pollenschlauchwachstums von der Narbe bis zu den Samenanlagen betrug ungefähr ei-

increased. No apomictic embryo development was evident. Thus, to improve the production capacity adequate cross-pollination has to be assured by mixed plantings with other varieties and overlapping blooming times, since the effective pollination period in Chinese Chestnut is rather long.

Key words. *Castanea mollissima* – reproductive biology – pollination – fertilisation – embryo – nut development

Diese Untersuchungen haben wichtige befruchtungsbiologische Probleme bei der Sorte 'Zaodali' und anderen aufgedeckt:
1. fehlende Befruchtung,
2. Degeneration von Zygote, Proembryo und jungen Embryonen,
3. verzögerte Differenzierung von Samenanlagen und des weiblichen Gametophyten sowie
4. abnormal ausgebildete Embryosäcke.

Die fehlende Befruchtung ist als das Hauptproblem bei der Nussbildung anzusehen. Durch Handbestäubung konnte der Anteil leerer Cupulae bzw. Nüsse reduziert werden. Eine apomiktische Samenentwicklung konnte nicht festgestellt werden.

Um die Produktivität bei der Chinesischen Esskastanie zu erhöhen, ist es notwendig für eine ausreichende Fremdbestäubung durch Mischpflanzungen mit anderen Sorten und sich ausreichend überschneidenden Blühzeiten zu sorgen, zumal die effektive Bestäubungsperiode bei dieser Obstart relativ lang ist.

Introduction

The genus *Castanea* comprises 10–12 species (MOORE and BALLINGTON 1990), from which 3 are used for fruit production: The European Chestnut (*C. sativa*), the Japanese Chestnut (*C. crenata*) and the Chinese Chestnut (*C. mollissima*). The Chinese Chestnut is cultivated since millennia and used as food and timber. China is considered as a gene centre for the genus *Castanea* (VAVILOV 1951).

China has the highest production of chestnuts worldwide and in many hilly areas it can be the main source of income for many rural families. But in many cases productivity is not satisfactory, and thus efforts are undertaken to increase yield. One part of this programme is to illucidate the reproductive biology of the Chinese Chestnut, which is rather complicated, and our knowledge is compared with *C. sativa* rather poor (SOLTESZ et al. 2003). Since all chestnuts are monoecious or andromonoecious (MOORE and BALLINGTON 1990; BOTTA et al. 1995) cross-pollination is required for nut set. Thus, the following aspects were investigated:
1. Morphology and anatomy of male and female flowers,
2. pollination experiments,
3. pollen tube growth and
4. embryo and nut development.

For details of reproductive processes see BHOJWANI and SOH (2001).

Materials and Methods

In the province Yunnan, South China, the experimental orchards were selected in two important areas of Chinese Chestnut growing. (1) Experimental station in Yiliang, where most of the pollination experiments were done and the material for further processing (e.g. pollen tube growth, seed development etc.) was collected. (2) Yongren, were some pollination experiments were conducted.

Five cultivars were selected for the pollination experiments. 'Zaodali', 'Zaobanli', 'Bayon III', 'Jiujiazhong' and 'Chuxuhong'. The experimental period was from 1999 to 2001. For pollination trials the cultivar 'Zaodali' as mother was used in most cases. This cultivar is the most grown in Yunnan, and its fertilization biology is unknown. The other cultivars were taken as pollinizers. All cultivars are chance seedlings.

The flowers were isolated with paper or cotton bags. Sampling of catkins was done 2–3 days before pollination, and by drying the catkins the pollen grains were released. After pollination with a brush, the flowers were bagged again. After 3–4 weeks the bags were removed and fruit set counted, which was repeated shortly before harvesting. The treatments 'open pollination' and 'without pollination' were also included.

For morphological and anatomical studies, pollen tube growth and embryo / seed development material was sampled at different dates and fixed in FPA (GERLACH 1984). Pollen tubes were demonstrated by their fluorescence, after staining with aniline blue. Sectioning of the material was done after plastic embedding. For details of sampling dates and methods of processing of the material see SHI (2003). The pollination experiments were conducted over 3 years and repeated 3–4 times.

Results

Structure of flowers

The male flowers are grouped in dichasia with 5–7 individual flowers; the inflorescences (catkins) are about

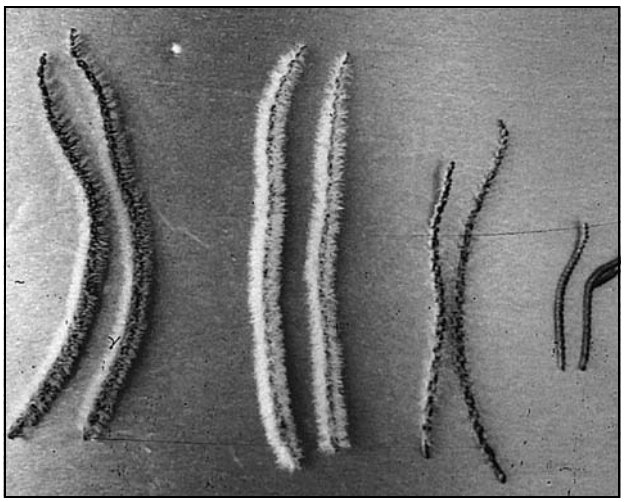


Fig. 1. Male catkins of the cultivars 'Zaodali' (left), 'Chuxuhong', 'Banyou III' and 'Jiujiazhong'.

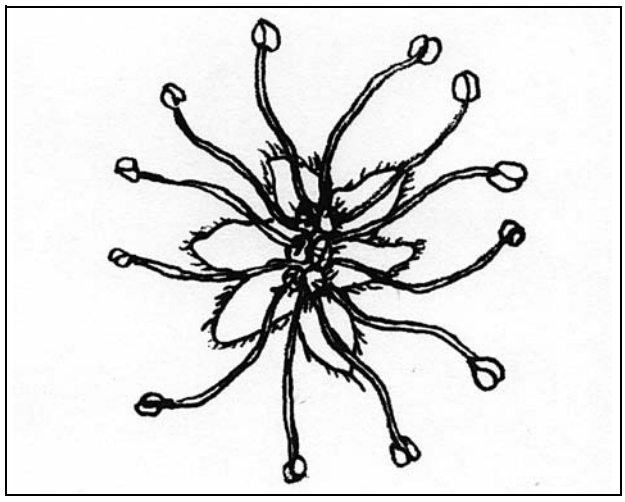


Fig. 2. Schematic drawing of a male flower.

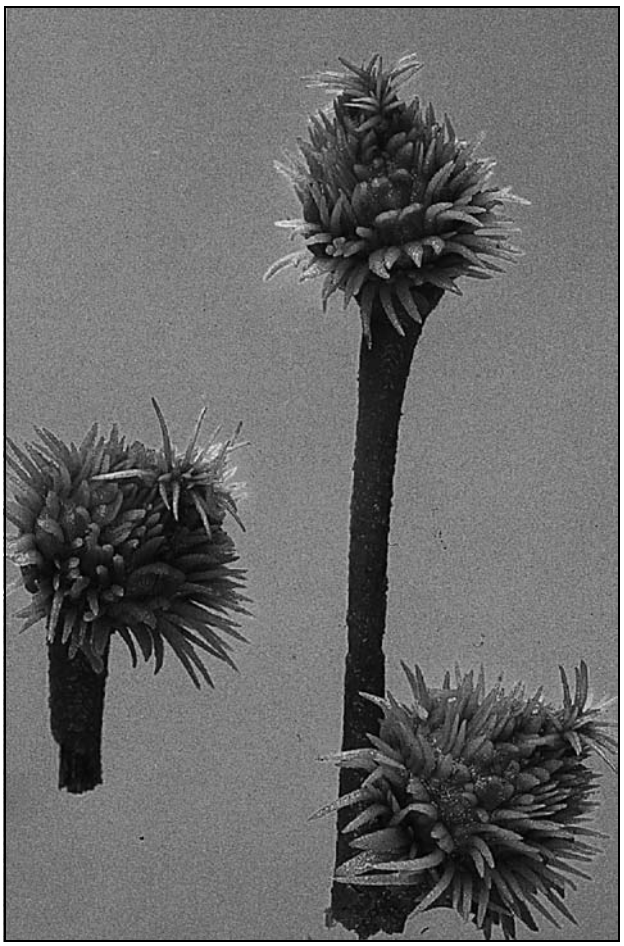


Fig. 3. One and two cupulae on a catkin.

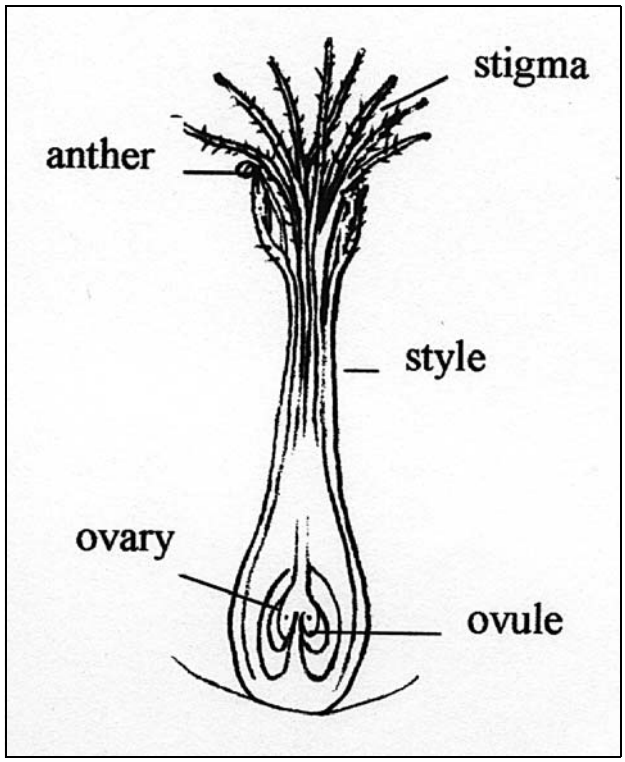


Fig. 4. Schematic drawing of a female or hermaphrodite flower.

10–20 cm long, on which about 100 dichasia can be found (Fig. 1). The individual male flower contains 5–6 sepals and 10–12 anthers (Fig. 2). Three female flowers are normally assembled in a spiny cupula, but in rare cases two or three cupulae stand in one group (Fig. 3).

The individual female flowers have the form of a green tricolpate bottle, the opening of which is surrounded by 6 hairy sepals. 12 weakly developed staminodiae with short filaments and anthers are conspicuous, in which pollen grains are differentiated. The female flower, also called hermaphrodite, has 6–9 free and conspicuous styles. The stigmatic surface is limited to the utmost top of the style (Fig. 4, 5).

Male catkins and the racemose inflorences are differentiated together in the same mixed bud. Male flowers are formed in the middle and top region of the cat-



Fig. 5. Female flowers.

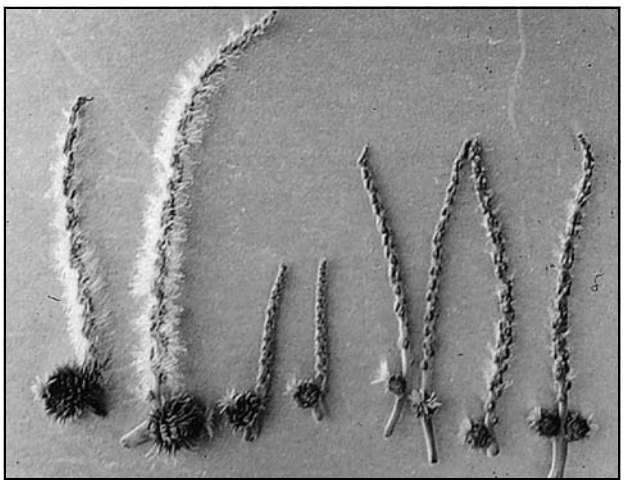


Fig. 6. Mixed or bisexual catkins in the cultivars 'Zaodali' (left), 'Jiujiazhong', 'Banyou III' and 'Chuxuhong'.



Fig. 7. Fruiting shoot showing male and mixed catkins.

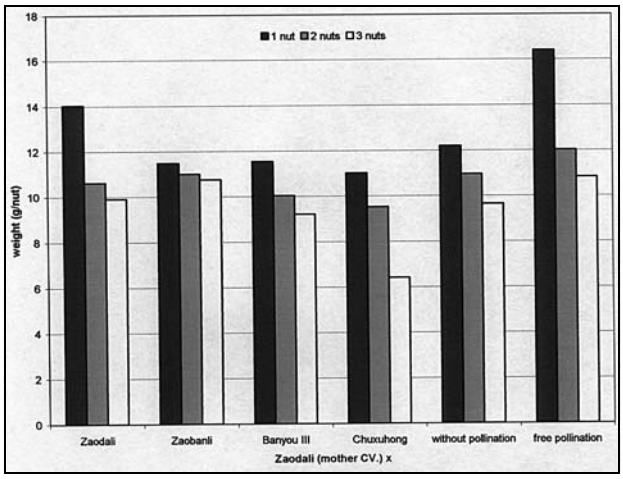


Fig. 8. Mean nut weight (g) with one, two or three nuts per cupula in 'Zaodali' as mother and different pollinizers in 1999.

kin. At the basis of the mixed catkin one female flower is evident (Fig. 6, 7). The number of male flowers is much higher than of female ones. The ratio in Chinese Chestnut was found to be about 1:1000 to 1:1500, depending on the variety; this means 4 to 5 male catkins to one mixed catkins. The ovary contains normally 6–9 loculi with 2 anatropous ovules in each, i.e. 12–18 ovules, mostly 16. From these ovules only one, in rare cases 2–3 ovules, develop to a fruit, which is a nut, and the involucrum forms the spiny cupula (Fig. 3). The mature ovule consists of integuments, a narrow nucellus and an embryo sac.

Pollination experiments

As shown in Table 1, in 1999 and 2000 fruit set was with over 90 % very high at both dates of counting. Highest set occurred in 'Zaodali' open pollinated. In 2001 fruit set was lower than in the 2 experimental years before, which may be due to the different isolation methods (Table 2). No significant differences were found between the pollinizers.

Despite high percentages of fruit set the number of empty cupulae was considerable. In 'Zaodali', especially 'selfing' and the treatment 'without pollination' resulted in a high percentage of empty cupulae. After pre-

Table 1. Fruit set (%) after removing of isolation bags (A) and at harvest (B) in 1999 (top) and 2000.

'Zaodali' (♀) X	'Zaodali' (♂)	'Zaobanli' (♂)	'Chuxuhong' (♂)	without pollination *	open pollination
A	98	98	100	98	100
B	96	96	91	96	98
A	96	98	98	96	98
B	94	96	94	92	94

* flowers were isolated by bags, but without hand pollination

Table 2. Fruit set (%) after removing of cotton cloth as isolation material (A) and at harvest (B) in 2001.

'Zaodali' (♀) X	'Zaodali' (♂)	'Jiujiazhong' (♂)	'Chuxuhong' (♂)	without pollination *	open pollination
A	87	86	88	86	96
B	85	85	86	84	95

* flowers were isolated by bags, but without hand pollination

Table 3. Number of nuts (%) in one cupula at harvest in 1999 (top), 2000 and 2001 (bottom).

'Zaodali' (♀) x	1 nut	2 nuts	3 nuts	empty cupulae
'Zaodali' (♂)	22.3	26.4	18.3	33.0
Zaobanli (♂)	27.0	43.0	23.0	7.0
Banyou III (♂)	39.5	29.0	21.0	10.5
'Chuxuhong' (♂)	27.0	34.0	23.0	16.0
without pollination *	20.0	8.6	11.4	60.0
open pollination	19.0	31.0	31.0	19.0
'Zaodali' (♂)	15.4	7.7	3.8	73.1
Zaobanli (♂)	36.6	10.0	6.7	46.7
'Chuxuhong' (♂)	22.2	11.1	1.4	65.3
without pollination *	11.4	4.5	2.3	81.8
open pollination	23.5	20.6	38.3	17.6
'Zaodali' (♂)	18.0	8.0	4.0	70.0
'Chuxuhong' (♂)	8.0	11.0	5.0	76.0
'Jiujiazhong' (♂)	10.0	4.0	2.0	84.0
without pollination *	0.0	0.0	0.0	100.0
open pollination	17.0	23.0	22.0	38.0

* fertilization prevented by removing the stigmas with a razor blade

vention of fertilization by cutting off the stigmas all cupulae were empty (Table 3), indicating that no apomixis occurred in 'Zaodali'. Open pollination showed the lowest figures of empty cupulae.

The three flowers in a cupula could develop into a nut, if fertilization took place and they normally developed. But in this investigation a high number of cupulae with only one or two nuts in all treatments was evident. Only 18,3 to 31 % of all cupulae contained three nuts (Table 3).

The weight of an individual nut was depending on the number of nuts in a cupula. The higher the number, the lower the nut weights (Fig. 8). No clear effects of pollinating cultivars on nut weight were found.

Pollen tube growth

The female flower shows on the average 8–9 styles and stigmas (Fig. 9a). The wet stigmatic surface is very small and has the form of a "crater". The number of

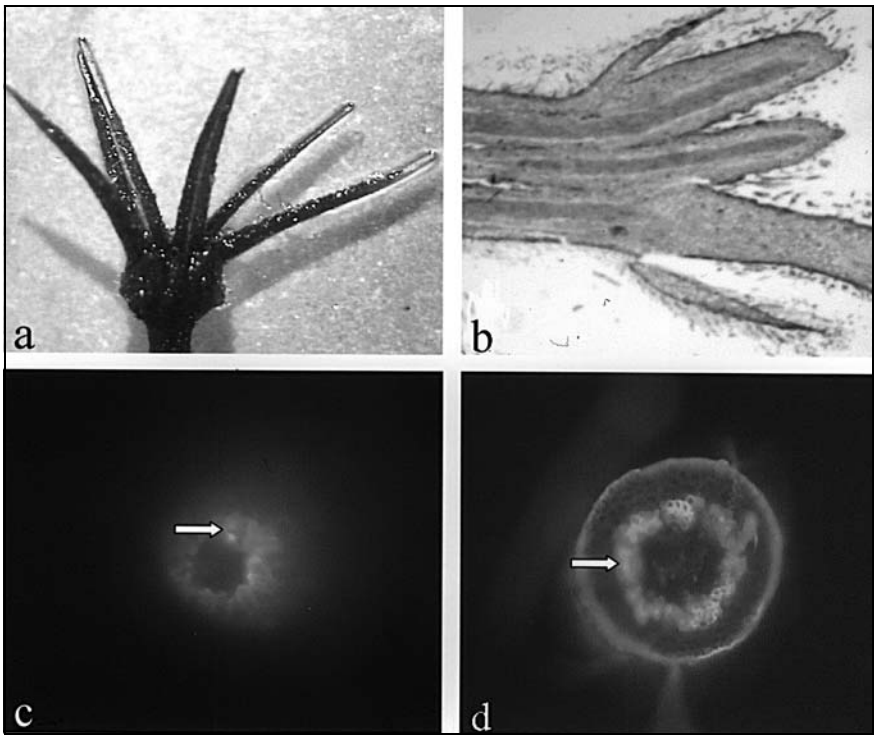


Fig. 9. Form and structure of stigma. a. stigmas showing a small functional tip, b. longitudinal section, c. crater-like tip with one pollen tube (arrow), d. cross-section of a stigma indicating xylem elements and conducting tissue in the centre.

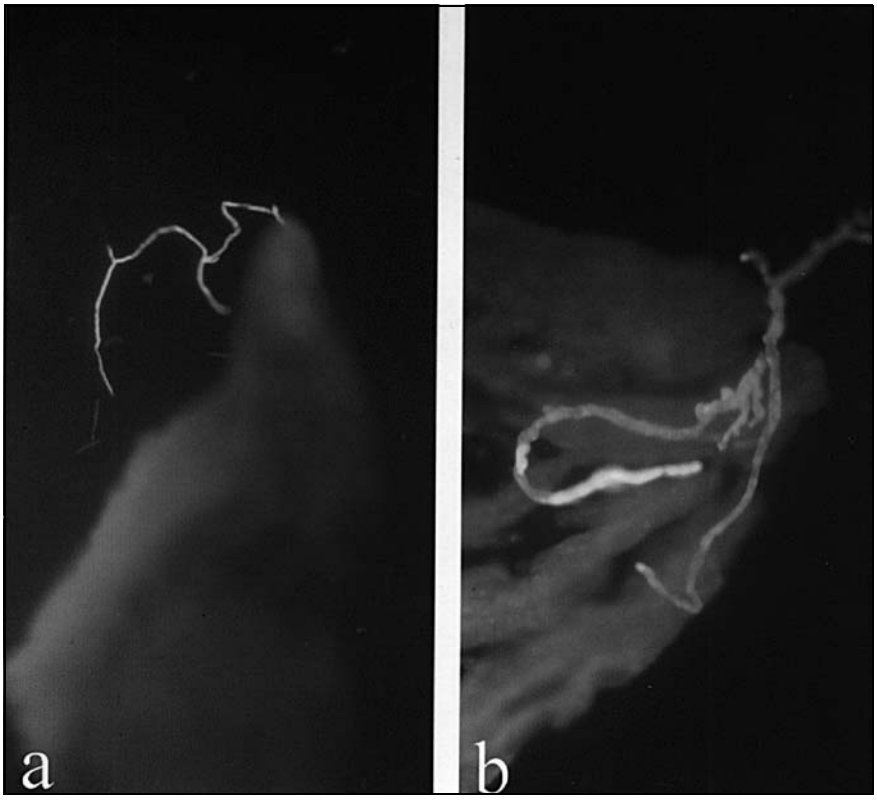


Fig. 10. a. Pollen tube entering the ovule, b. ramification of pollen tubes.

styles corresponds with the number of locules, i.e. each locule with two ovules has its own conducting tissue, which is located in the centre of the style (Fig. 9b) and is the pathway for the pollen tubes, growing from the stigma to the ovules.

In terms of pollen tube growth no differences between cultivars have been found. The number of pollen tubes in the stigma could differ considerably. Mostly, up to 6, sometimes up to 10 pollen tubes could be observed in the stigma (Fig. 9c). In 'open pollination' the number of flowers with pollen tubes in the stigma was highest, and the tubes could be found earlier than in 'cross-pollination'. In the flowers of the treatment 'without pollination' the pollen tubes appeared the latest.

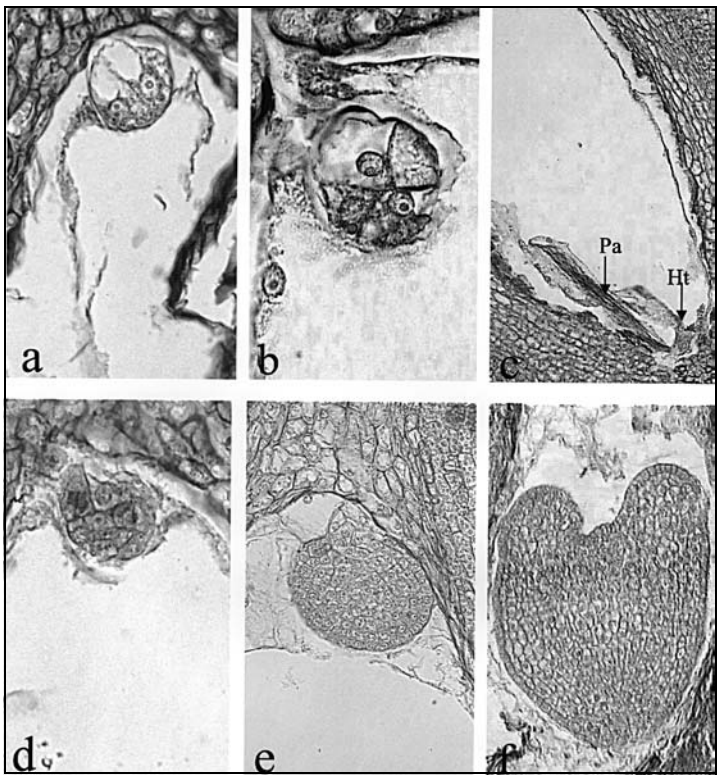


Fig. 11. Embryo development. a, b. proembryos, c. Haustorium (Ht) and postament (Pa) of ovule as shown in "b", d, e. globular embryos at different stages, f. beginning of cotyledon differentiation.

After germination of the pollen grains, it took the pollen tubes about 1 month to penetrate the style and the ovary wall to reach the ovules through the micropyle. Quite often pollen tubes were twisted and showed ramifications, and it is not clear, if they are able to fertilize the ovules (Fig. 10a, b).

Seed / embryo development

The embryo sac of *Castanea* belongs to Polygonum-type, i.e. it consists of 8 nuclei. From the fertilized egg cell the embryo and later the seed develops. In 'Zaodali' fertilization occurred at the end of May, and the first division of the zygote occurred at the beginning of June. The embryo sac extends almost to the chalaza, forming the haustorium, which finally resorbs the nucellus (Fig. 11c–d), with the exception of the central part, forming the 'column', which is also called 'postament' (BROWN and MOGENSEN 1972).

The endosperm develops from the 2 fused secondary endosperm nuclei after fertilization. It fills out the haustorium completely, first by division of the nuclei, and later cell walls are formed (nuclear vs. cellular endosperm).

From mid June onwards, the zygote continued to divide, forming the proembryo and the globular stage of the embryo. Later primordia of the 2 cotyledons were evident (heart shaped embryo) and in early to mid July the embryo was fully developed after resorption of the endosperm (Fig. 11). The 2 integuments developed into the testa, thus forming the seed, which in *Castanea* is called nut. The nuts were harvested late July to early August. 'Zaodali' is an early ripening cultivar. In the late ripening cultivar 'Jiujiazhong' the different developmental stages were delayed by 2–4 weeks.

From this normal nut development numerous aberrations were found. In one ovary normally more than 16 ovules can be found. Only if one ovule is fertilized an embryo or nut can be formed. Normally, one embryo is formed in a nut, but also 2 embryos or rarely 3 embryos per seed could be found.

Empty cupulae occurred frequently, even after 'hand' or 'open pollination', indicating that many ovules in a flower or cupula degenerated (Table 3). In only 50 % of the ovules normal embryo sacs were found. The development of the ovules differed to a great extend; some could show already a globular embryo, others were still in the stage of integument development.

In ovules without fertilization first the embryo sacs degenerated, then the nucelli and finally the ovules collapsed. At the end of June empty nuts could be distinguished from normal ones by their smaller diameter. Formation of empty nuts is mainly caused by lack of fertilization. But after fertilization abnormalities could also be observed, e.g. degeneration of zygote, proembryo and globular embryo. After formation of cellular endosperm no degeneration of embryos was evident any longer.

Discussion

Anthesis and the phenology of flowering in *C. mollissima* can be determined only with difficulties by external criteria since different growth processes occur within internal flower tissues at anthesis. The female flowers develop from the involucrum before the appearance of the stigmas, and the male flowers develop from the appearance of the anthers until their wilting.

Related to the female gametogenesis, the phenological end of flowering of female flowers occurred before or shortly after the differentiation of the embryo sac mother cell. Therefore, pollination experiments should be performed according to phenological flowering stages of the male flowers, combined with the status of the stigmas.

Thus, the optimal pollination time should be according to NIENSTAEDT (1956) between the 10th and 13th day after beginning of anthesis of the male catkins. In other experiments, pollination was performed when the angle between the stigmas was 30–45 ° (XU et al. 1988). According to these criteria, the flowers were pollinated between the 10th and 15th day after beginning of anthesis of male catkins.

Concerning fruit set, different isolation methods were used, since the effective pollination period is rather long. So the isolation materials should be kept for at least 4 weeks, without damaging the blossoms, and thus pollen tube growth and fertilization. Different isolation bags, paper or cloth, and isolation of the stigmas with cotton showed no major differences in terms of fruit set.

Fruit set was counted as cupula set at 2 dates: (1) after removal of isolation bags and (2) shortly before harvest. At the first date cupulae with or without nuts could not be distinguished. In 'open' or 'cross-pollination' fruit set in 'Zaodali' was with over 90 % rather high, in contrast to 'self-pollination' and 'without pollination', indicating self-sterility of this variety as well as the lack of apomictic seed development.

The pollinizers did also effect nut set and the number of nuts per cupula. Again best results were achieved in 'open' or 'cross-pollination', worst ones with 'selfing' or 'without pollination'. The pollination experiments of MCKAY (1942) gave similar results. In this investigation no metaxenia have been found, in contrast to the results of MCKAY and CRAUE (1939).

Pollen tube growth *in vivo* was registered in 'Zaodali' after 'cross-' and 'open-pollination'. After 3–6 days pollen tubes in the stigma were visible. In order to follow pollen tube growth in a better way, the distance from the stigma to the ovules was divided into 4 parts: (1) from the surface of the stigma to the conducting tissue, (2) to the basis of the stigma, where all stigmas converge, (3) from the basis of stigma to basis of style, (4) and finally from the basis of the style to the ovary.

Velocity of pollen tube growth differed in the 3 experimental years, which may be due to temperature effects. It is well documented that growth of pollen tubes depends on the prevailing temperatures (SHI 2003). For example in 2000 in 'Zaodali' the first phase of pollen tube growth took 3–4 days, whereas in 2001 the duration was 6–7 days.

The causes of low yields of the Chinese Chestnut have to be seen in lack of fertilization, since no apomixis was found. Also quite often abortion of embryos in different stages of development is evident. Since dichogamy and self-sterility occur, cross-pollination with other cultivars and overlapping blooming times have to be provided to ensure satisfactory yields.

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Received February 25, 2004 / Accepted January 10, 2005

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