Fertilization and In Vitro Growth of Ovules in Japanese Chestnut (*Castanea crenata* Sieb. et Zucc.)

Masahiro Nakamura

Miyagi Agricultural College, Taihaku, Sendai 982–0215

Summary

Ovules were removed from ovaries from mid-July to early August and cultured in vitro. As a result, all ovules which were sampled and cultured by July 19 in the central female flowers and by July 22 in the lateral female flowers died before early September. However, in ovules sampled and cultured on and after July 22 and 26 in the central and lateral female flowers, respectively, nearly one ovule per ovary survived and developed on the culture medium until mid-September. The development and enlargement of the embryos within the ovules were also observed. Differences in the weight between the largest ovule per ovary and the other ovules began to become clear after July 22 in the central and lateral female flowers. The differences increased rapidly on and after July 29. The longest pollen tubes which penetrated into the ovaries reached the nucelli on July 19–22 and July 22–26 in the central and lateral female flowers, respectively. The pollen tubes which reached the zone where the nucelli were located were observed on July 15 and 19 in the central and lateral female flowers; their numbers increased rapidly during July 15–19 and July 19–22, respectively. Subsequently, their numbers gradually decreased. In the central female flowers, the ovules with a single pollen tube per ovary numbered 5, 6 and 5 on July 22, 26 and 29, respectively. A pollen tube, which penetrated into the micropyle, reached the nucellus in only one ovule in the ovary during July 22–29. The pollen tubes which had penetrated into the micropyles of the other ovules stopped growing at the same length, about half of the length of the entire micropyle, on July 29 in particular. It was suggested that when one ovule had already been fertilized, the other ovules, into which pollen tubes had penetrated, could not be fertilized owing to the cessation of the pollen tube growth.

Key Words: fertilization, ovule culture, ovule development, pollen tube growth.

Introduction

In Japanese chestnut, Nakamura (1986) observed that only one of 16–22 ovules within an ovary develops rapidly after fertilization, while the other ovules cease to grow, and then degenerate. Nakamura (1991, 1994) presumed that this degeneration of the ovules might be caused by the lack of fertilization. Furthermore, Nakamura (2001) showed that the time of fertilization corresponded with the period of rapid increase in the number of pollen tubes around the ovules, and that their number decreased after fertilization. Further detailed investigation on the behavior of pollen tubes around the ovules is necessary in order to confirm this lack of fertilization, and to clarify the cause of this lack. In this report, the relation between ovule culture and pollen tube growth was investigated, and whether the ovule degeneration is caused by the lack of fertilization was determined.

Materials and Methods

Female flowers of Japanese chestnut ‘Yamato-Wase’ (*Castanea crenata* Sieb. et Zucc.) grown in the orchard

of Miyagi Agricultural College were labeled just before the emergence of the milky-white and needle-shaped styles from involucre and allowed to be open-pollinated.

Culture of ovules

From July 15 to August 9, four burs were collected twice weekly in 3–4 day intervals. Ten to 17 ovules per ovary were excised from the central and lateral ovaries and cultured in 20 ml vials, containing 3 ml of Murashige and Skoog medium (pH 5.7) (Murashige and Skoog, 1962) under room light conditions at 25 °C. The cultures were replicated 4 times. The number of living ovules per vial was counted at 3–4 day intervals until September 13. The ovules were determined to be dead when the entire ovule turned brown.

Growth of ovules

Three burs were collected at 3–4 day intervals from July 19 to August 9. Fourteen to 18 ovules were excised from each of three ovaries in the central and lateral female flowers under a stereoscopic microscope, and weighed individually. The largest ovule was separated from the other ovules in the ovary.

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Fig. 1. Effect of time of in vitro culture on the longevity of ovules excised from ovaries of the central (●) and lateral (○) female flowers in Japanese chestnut 'Yamato-Wase'.

Pollen tube growth within ovary

Three ovaries from each of the central and lateral female flowers were collected at 3-4 day intervals from July 1 to August 9, and fixed in formalin-acetic acid alcohol (FAA). The ovaries, collected after the pollen tubes had reached the base of the styles, were embedded in paraffin after dehydration, sectioned longitudinally at 10 μ m with a rotary microtome, and then stained with 0.1% aniline blue (0.1N K_3PO_4). The length of the pollen tubes from the distal end of the ovary and the distance (D) from the distal end to the tips of the nucelli were measured by an ocular micrometer for 9-11 ovules per ovary under fluorescence microscopy. The degree of approach of the pollen tubes to the nucelli was calculated as (the pollen tube length) $\times$ 100/D, and the number of ovules and pollen tubes which were located within a distance of 1 mm each from the top of the ovary was counted. Ovules with a pollen tube which penetrated into the micropyle were investigated using 7-10 ovules per ovary in the central female flowers. The lengths of the pollen tube within the micropyle were measured by an ocular micrometer.

Results

Culture of ovules excised from ovary

The ovules, which were excised from ovaries sampled every 3-4 days from July 15 to August 9, were explanted in a vial, with one ovary per vial. As a result, in

flowers sampled from July 15 to July 26 in the central female flowers and up to July 29 in the lateral female flowers, there were few dead (browning) ovules for 14 days from the beginning of culture, but afterwards the number increased rapidly (Fig. 1). However, the number of dead ovules increased rapidly from the beginning of culture in the ovules which were sampled on and after July 29 and August 2 in the central and lateral female flowers, respectively.

Meanwhile, in the ovules sampled from July 22 in the central female flowers and from July 26 in the lateral female flowers, one or two ovules per vial (ovary) survived during the culture. All ovules sampled before that time died from mid-August to early September. The number of living ovules per vial (ovary) in the central and lateral female flowers was almost one, in which a growing embryo was observed.

Growth of ovules in ovary

The weight of the largest ovule per ovary increased rapidly after July 22, when one notably enlarged ovule per ovary began to appear, in both the central and lateral female flowers (Fig. 2.). At that time, the weight of the other ovules increased very little and then began to decrease after July 26.

Pollen tube growth

The longest pollen tubes which penetrated into the ovaries of the central and lateral female flowers reached the nucelli on July 19-22 and July 22-26, respectively

Fig. 2. Growth of ovules in the ovaries of the central and lateral female flowers in Japanese chestnut 'Yamato-Wase'. ● : the largest ovule, ○ : other ovules.

Fig. 3. Percent growth of pollen tubes for ovules within the ovary in Japanese chestnut 'Yamato-Wase'. — and ---- indicate percent distances from top of ovary to tips of pollen tubes and nucelli of ovules, respectively. ● : the longest, ○ : mean.

(Fig. 3). There was only a small increase in the average length of the pollen tubes after July 22 and 26 in the central and lateral female flowers, respectively.

Pollen tube growth and location of ovules in ovary

Pollen tubes of the central female flowers entered the upper part of the ovaries on July 5, whereas the same occurred for the lateral female flowers on July 8. The distance from the tips of the pollen tubes to the nucelli was over 2 mm (Fig. 4). The pollen tubes entered the zone where the nucelli were located on July 15 and 19 in the central and lateral female flowers, respectively. After that, in the central female flowers, 3, 11, 9, 6 and 5 pollen tubes entered the nucellar zone on July 15, 19, 22,

26 and 29, respectively, showing a rapid increase in the number from July 15 to July 19, and subsequently a gradual decrease. In the lateral female flowers, there were 8, 18, 11, 9 and 4 pollen tubes in the nucellar zone on July 19, 22, 26, 29 and August 2, respectively, showing a rapid increase in the number from July 19 to July 22, and afterwards a gradual decrease.

Pollen tube growth within ovules

In the central female flowers sampled on July 22, 26 and 29, ovules with one pollen tube were found. The ovules with a single pollen tube per ovary numbered 5, 6 and 5 on July 22, 26 and 29, respectively (Fig. 5). In the central female flower sampled on July 22, a pollen tube

Fig. 4. Pollen tube growth and location of ovules in the ovary in Japanese chestnut 'Yamato-Wase'. Upper: central ovary, lower: lateral ovary. ■ : pollen tubes, □ : ovules.

Fig. 5. Growth of pollen tube within the micropyle of the ovule in the ovary of the central female flower in Japanese chestnut 'Yamato-Wase'.

had already reached the nucellus in one ovule per ovary, and the distance from the tip of the micropyle was 0.92 mm. In the other 4 ovules, the lengths of the pollen tubes from the tips of the micropyles ranged from 0.342 to 0.171 mm. Within the micropyles, the pollen tube which reached the nucellus was over three times longer than the other pollen tubes which did not reach the nucelli. In the ovary sampled on July 26, a pollen tube had already reached the nucellus in one ovule, and the length of that pollen tube from the tip of the micropyle was 1.123 mm, while the lengths of the pollen tubes within the micropyles in the other 5 ovules ranged from

0.635 to 0.383 mm, except the shortest one (0.049 mm). The longest pollen tube was a little less than twice as long as the other pollen tubes. In the ovary sampled on July 29, one pollen tube had reached the nucellus and its length from the tip of the micropyle was 1.083 mm. In the other 4 ovules with pollen tubes within the micropyles, the lengths of the pollen tubes from the tips of the micropyles ranged from 0.594 to 0.464 mm. Within the micropyles, the pollen tube reached the nucellus was about two times longer than the other pollen tubes which did not reach the nucelli.

The longest pollen tube within the micropyle, which

reached the nucellus on July 22, grew slightly up to July 26, but then stopped growing. Although the other pollen tubes, except for the longest one within the micropyles, grew two times from July 22 to July 26, they grew little after that, and ceased to grow when they were halfway in the micropyle. In all of the growth patterns of the pollen tubes within the micropyles on July 22, 26 and 29, only the length of the longest pollen tube was markedly larger than that of the other pollen tubes. Also, the lengths of the other pollen tubes were almost uniform, especially in the pollen tubes on July 29.

Discussion

For ovules/embryos to grow and develop, they need to be fertilized and nourished. In this experiment, sufficient nutrients were supplied by the culture medium. Therefore, it was considered that the browning (abortive) ovules were caused by non-fertilization. Almost one ovule per ovary survived during cultures on and after July 22 and 26 in the central and lateral female flowers, respectively. The number was almost one per ovary. The developing embryos were observed within all ovules which survived, showing that the ovules which survived continuously within the vials were fertilized. In the chestnut, therefore, the result of the previous report (Nakamura, 2001), in which only one ovule was fertilized and developed, while the others degenerated by non-fertilization, was confirmed. It is assumed from the data on the sampling dates of the surviving ovules that fertilization occurred between July 19 and 22 in the central female flowers, and between July 22 and 26 in the lateral female flowers. Furthermore, in all cultures of ovules sampled before and after the fertilization stage, rapid death of the ovules began to occur from July 29 to early August, showing that the death of these ovules was caused by non-fertilization.

In the ovary of *Quercus*, there are six ovules originally, and typically five of these abort very early, resulting in a one-seeded acorn at maturity (Mogensen, 1975). Mogensen (1975) reported that the exact mechanism whereby one ovule develops ahead of the others and eventually becomes the only seed in the mature acorn remains unknown. McKay (1942) said that in *Castanea mollissima* each ovule might be fertilized and be capable of producing a seed, but generally only one developed to maturity in each ovary to form the one-seeded fruit or nut within a single shell. Botta (1995) reported that in *Castanea sativa* Mill. only one ovule per ovary developed in most of the observed nuts and that bearing single-seeded nuts seems to be related in part to the high occurrence of anomalies of the embryo sac. However, it seems unreasonable to attribute the cause of bearing one-seeded nuts to the anomalies of ovules, because the ovary in *Castanea sativa* Mill. contains 10 to 16 ovules (Botta, 1995).

In this experiment, a difference in weight between the largest ovule and the other ovules began to arise after

July 22 in the central and lateral female flowers. As judged by the rate of proximity of the longest pollen tube within the ovary to the ovule, the longest pollen tube reached the location in which the ovules existed during July 19–22 in the central ovaries, and July 22–26 in the lateral ones, respectively. Therefore, the time of fertilization, which was presumed from the ovule culture, corresponded to the result of the growth of the largest ovule and of the longest pollen tube within the ovary.

The number of pollen tubes which reached the zone where the nucelli were located increased rapidly prior to the time of fertilization, and afterwards gradually decreased. This result was the same as that of the former report (Nakamura, 2001). There were some pollen tubes that reached the zone where the nucelli were located from July 15 and 19 in the central and lateral female flowers, respectively. However, fertilized ovules appeared around 7 days after that in the culture, when the largest number of pollen tubes reached the zone where the nucelli were located, showing that the fertilization occurred when the largest number of pollen tubes met with ovules.

Concerning the reason why only one ovule was fertilized out of 16–22 ovules within an ovary, the pollen tubes which penetrated into the micropyles of the ovules grew better in only one ovule, and grew only a little in the other ovules, in all ovaries sampled on July 22, 26 and 29, at the time of fertilization. The lengths of the pollen tubes within the micropyles were 1/3 and 1/2 of the longest tube in the ovules sampled on July 22 and 26, respectively. On July 29, no pollen tubes grew in any of the ovules. Also, all of the pollen tubes except for the longest one per ovary varied little in length within the micropyle. The difference in the length of the pollen tubes, except the longest one, became smaller with the passage of time.

As the average growth rate of pollen tubes per day in Japanese chestnut is 0.3 mm (Nakamura, 1994), 3–4 days are necessary for the pollen tubes to grow to 1 mm from the top of the micropyle to the nucellus. As there were 7 days between July 22 and July 29, the pollen tubes within the micropyles of the ovules, except for the fertilized tubes, seemed to cease growing on the way. Therefore, it is possible that once the fertilization in the ovule in which the longest pollen tube entered occurred, the pollen tubes which entered into the micropyles of the other ovules ceased to grow. Mogensen (1975) reported that the most common type of abortive ovule is one in which the embryo sac and all of its contents develop normally, yet fertilization does not take place. He proposed, as the most plausible explanation, however, that the functional ovule was fertilized before any of the others in a given ovary, and that this ovule subsequently suppressed the growth of the other fertilized ovules, or might be influential in preventing fertilization in some of the normally developed ovules. There have been no

detailed reports on pollen tube growth within the micropyles of ovules in the ovary, although there have been many reports in which pollen tubes were observed within ovules or micropyles. From this experiment, it was conclusively suggested that when one ovule had already been fertilized, the other ovules, into which pollen tubes had penetrated, could not be fertilized, owing to the cessation of pollen tube growth.

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ニホングリ (*Castanea crenata* Sieb. et Zucc.) の受精と in vitro での胚珠の生長

中村正博

宮城県農業短期大学 982-0215 仙台市太白区

摘 要

7月中旬から8月上旬にかけて子房から胚珠を取り出し、バイアルに移植して胚珠の培養を行った。その結果、中心子房で7月19日、側子房で同22日までに採取した胚珠では置床後9月上旬までにすべての胚珠が枯死したが、中心子房で7月22日以降、側子房で同26日以降に採取した胚珠ではいずれも子房当たりほぼ1個の胚珠が9月中旬まで生存し、内部に胚の生長・肥大が認められた。中心子房、側子房とも7月22日以降、子房当たり1個の最大胚珠とその他の胚珠とで重さに違いが生じ始め、7月29日以降その差は急速に拡大した。子房内に侵入した最長花粉管は、中心子房で7月19~22日、側子房で7月22~26日に胚珠の珠心位置に達した。胚珠珠心の位置する区分に達した花粉管は、中心子房、側子房そ

れぞれ7月15日と19日に認められ、中心子房では7月15~19日にかけて、側子房では同19~22日にかけてその数は急増し、その後は徐々に減少した。7月22日、26日、29日に採取した中心子房において、珠孔内に侵入した花粉管を持つ胚珠は子房当たりそれぞれ5, 6, 5個であった。珠孔内の花粉管は7月22~29日のいずれの子房においても1個の胚珠でのみ珠心に達していた。他の胚珠の珠孔内花粉管は珠孔内の侵入距離がおおむねそろっており、特に、7月29日のものではいずれも珠孔内の約1/2の距離で停止していた、以上のことから、子房内で一つの胚珠が受精すると、他のすでに花粉管が侵入した胚珠では、花粉管の伸長が停止するため、受精には至らないものと考えられた。