Studies on the Radiation Breeding in the genus *Mentha*

(XIII) Effects of Irradiation on Pollen

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Introduction

Pollen grains of flowering plants are binucleate or trinucleate according to the time of division of the generative nucleus. Microspore formation which follows meiosis results in a generative cell and a vegetative cell, both included within the wall of the pollen grain. At pollen maturity the generative cell of binucleate pollen grains is arrested at prophase of the second mitotic division and the two sperms are formed in the pollen tube. In most species, e. g., *Lilium*, the prophasic organization and condensation of chromatin threads in the generative nucleus may be readily observed. Binucleate pollen grains of those plants germinate with ease in culture. When the division of the generative cell is not delayed the pollen grains are trinucleate, containing a vegetative nucleus and two densely staining male gametes or sperm cells (MAHESHWARI; BREWBAKER).

Quantitative irradiation effects on pollen germination and tube growth have been examined by several researchers.

A relatively great radio-resistance of tube elongation may be seen by comparing high lethal doses for pollen grain germination (order of 80 KR) with low doses favorable for observation of pollen-tube cytology (less than 1 KR). Also the effects of pollen irradiation on the production and viablity of seeds were examined by many researchers.

The present authors have investigated the effects of pollen irradiation on pollen germination, tube growth and production of seeds (IWANAMI). In this first paper of the series the irradiation effects of γ-rays on pollentube mitosis are reported.

Material and Methods

The materials used in this study were pollen grains of *M. rotundifolia* ($2n=24$), *M. spicata* ($2n=48$), *M. arvensis* ($2n=72$) and *M. arvensis* L. var. *piperascens* ($2n=96$). The pollen grains were collected from naturally dehisced anthers for investigation in August.

Gamma-rays were applied with ^{60}Co in the Institute of Radiation Breeding, Ministry of Agriculture and Forestry, at a dose rate 65/hr. The doses applied ranged from 1 to 80 KR.

As artificial culture medium for pollen grains served sugar agar plates with 6% sucrose and 1.5% agar. No inorganic substance or hormone was added. A piece of sugar agar plates 0.8 mm thick was put on a slide glass and the pollen grains were placed on thc medium in a straight row. Thus sown they sent their tubes always at right angles to the row.

The materials were fixed and the nucleus was stained with aceto-carmine. Agar of the sugar agar plates was dissolved by heating and the cover glass was fixed to the slide glass after excess agar was removed by blotting paper, in order to make

a semi-permanent preparation. Since the nucleus of the pollen tube becomes well stained during this procedure, pollen-tube mitosis at various stages can be easily observed.

Results

1. Preliminary experiment with normal pollen grains : Binucleate pollen grains undergo the second mitotic division during pollen-tube development. Radiation-induced inhibition of pollen-tube mitosis can be observed in two ways, namely 1) in the decrease in the frequency of pollen tubes showing the mitotic division and 2) in its delay. The results of preliminary experiments are shown schematically in Fig. 1.

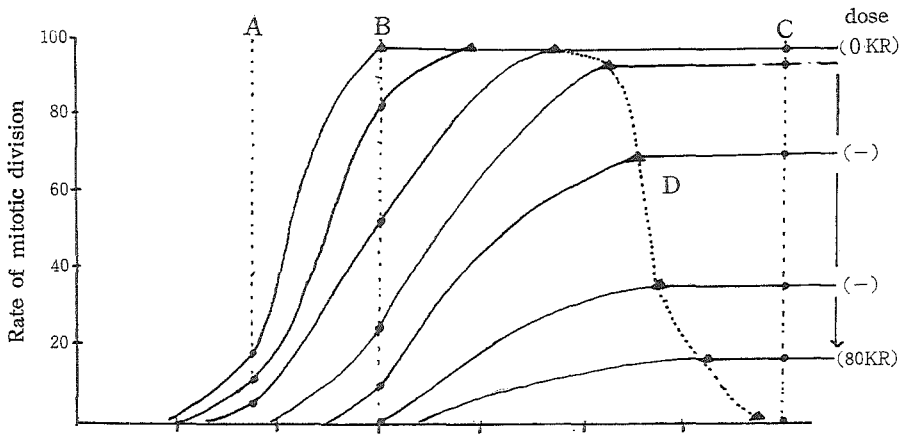

Fig. 1. Schematic curves of radiation induced inhibition of pollen-tube mitosis

The inhibition of mitosis increased with the increase of γ-ray dose; namely the higher was the dose, the more reduced was the frequency of the division and the more delayed was the time of its occurrence. In Fig. 1 observation time A was too early to see the whole of the experimental results and time D was suitable to ascertain accurately the number of inhibited nuclei, but the delay of the division could not be clearly observed. Thus observation at two different times, B and C, was more suitable.

The process of pollen-tube mitosis of the generative nucleus is divided into the following three stages : 1) interphase, 2) prophasic condensation of chromatin threads or chromosome, 3) formation of two sperm nuclei.

2. *M. rotundifolia* ($2n=24$) : The pollen tubes of *M. rotundifolia* become 5 mm long in the style, while their elongation is limited to only 3 mm on artificial culture media. Therefore, pollen-tube mitosis can be observed only in the earlier stages, A and B of Fig. 1.

At this point most of non-irradiated grain tubes have already two sperm nuclei. In irradiated pollen, the higher was the dose, the more delayed was the divison. At 5 KR the generative nucleus was mostly intact forming a chromosome mass without further division, and above 10 KR only in a few nuclei chromosomes could be distingished while most of them formed a chromatid mass.

The number of pollen-tubes with mitotic division along the three stages mentioned above is shown in Table 1. The percentage of nuclei forming chromosomes increased

Table 1. Frequency of pollen tubes in 3 mitotic stages 25 hours after γ-irradiation of grains in *M. rotundifolia*

Dose (KR)	Interphase	Prophasic chromosomes	Two sperms
1	3	24	2
5	8	42	0
10	40	5	1
20	39	6	0
40	42	0	0
80	52	0	0

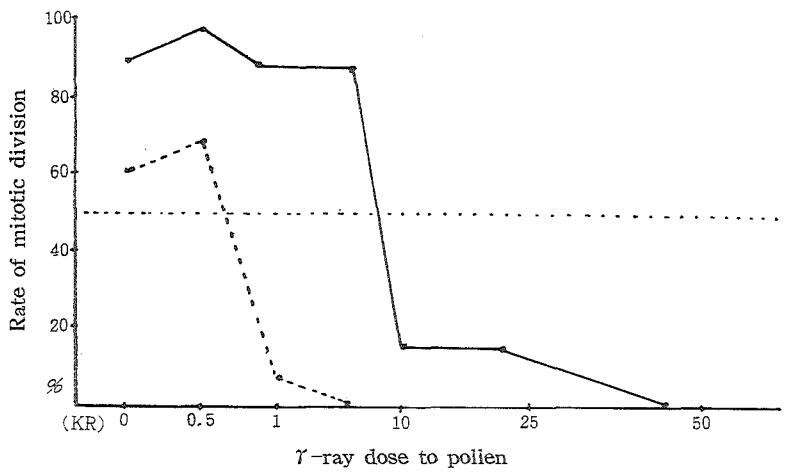

Fig. 2. Relation of radiation induced inhibition of pollen-tube mitosis to γ-ray doses in *M. rotundifolia*.
——: frecuency of generative nuclei in mitotic division;
····: frequency of the formation of two sperm nuclei.

to the maximum (100 %) at 1 KR and decreased gradually up to 5 KR and then fell suddenly to 10 % at 10 KR, as indicated by the solid line of Fig. 2. That of the pollen tubes with two sperm nuclei decreased promptly to 6 % at 1KR from 65 % at 1 KR, as shown by the broken line of Fig. 2.

Table 2. Frequency of pollen tubes in 3 mitotic stages 12 and 24 hours after γ-irradiation of grains in *M. spicata*

Dose (KR)	After 12 hrs.			After 24 hrs.		
	Interphase	Prophasic chromosomes	Two sperms	Interphase	Prophasic chromosomes	Two sperms
1	4	52	42	0	2	86
5	3	44	42	1	0	92
10	2	66	28	2	11	72
25	62	5	4	20	12	44
50	75	7	0	34	8	22
80	71	3	0	72	11	0

3. *M. spicata* ($2n=48$) : The pollen grains of *M. spicata* were cultured at 20°C in August and the effects of γ-rays were observed. The frequency and the percentage of mitotic division in various stages 12 and 24 hours after placing the grains on the agar are shown in Table 2, respectively. At the observation iime B (after 12 hours) the generative nucleous began to divide even at 10 KR, like in the non-irradiated lot. At 25 KR or more the division was clearly delayed, and was inhibited even at time C (after 24 hours). A similar result was observed in *M. arvensis*, as shown in Table 3.

Table 3. Frequency of pollen tubes in 3 mitotic stages 12 and 24 hours after γ-irradiation of grains in *M. arvensis*

Dose (KR)	After 12 hrs.			After 24 hrs.		
	Interphase	Prophasic chromosomes	Two sperms	Interphase	Prophasic chromosome	Two sperms
1	4	33	38	4	33	38
5	16	38	21	16	38	21
10	20	46	16	20	46	16
25	30	18	1	30	18	1
50	32	0	0	63	0	0
80	63	0	0	63	0	0

4. *M. arvensis* : The pollen grains of *M. arvensis* L. var, *piperascens* ($2n=96$) are rather small and have a high viability. Even on the culture media they could well elongate their pollen tubes, like in the style. They germinated quickly 2～3 minutes after sowing on the media and the generative nucleus began to divide 55 minutes after sowing. Therefore, they are a very suitable material for such studies.

The pollen grains were sown on the media immediately after γ-irradiation. The frequencies of the three stages 60 and 100 minutes after sowing are shown in Table 4. The percentages at observation times B and C are plotted with increasing dose in Fig. 3. In *M. arvensis* a marked retardation of the nuclear division is observed from the curves of Fig. 3, compared with *M. spicata*.

Table 4. Frequency of pollen tubes in 3 mitotic stages 60 and 100 minutes after γ-irradiation of grains in *M. arvensis* L. var. *piperascens*

Dose (KR)	After 60 min.			After 100 min.		
	Interphase	Prophasic chromosomes	Two sperms	Interphase	Prophasic chromosomes	Two sperms
1	20	67	3	2	0	82
3	42	15	2	0	0	91
5	49	31	4	0	2	87
20	52	21	5	4	0	91
40	45	30	0	0	0	72
80	62	11	0	41	42	20

In order to examine the retardation and the inhibition in detail, pollen-tube mitosis was observed from 10 to 80 minutes after sowing the grains at 10 minute intervals. Fig. 4 shows the percentages of generative nuclei with chromosomes or two sperms.

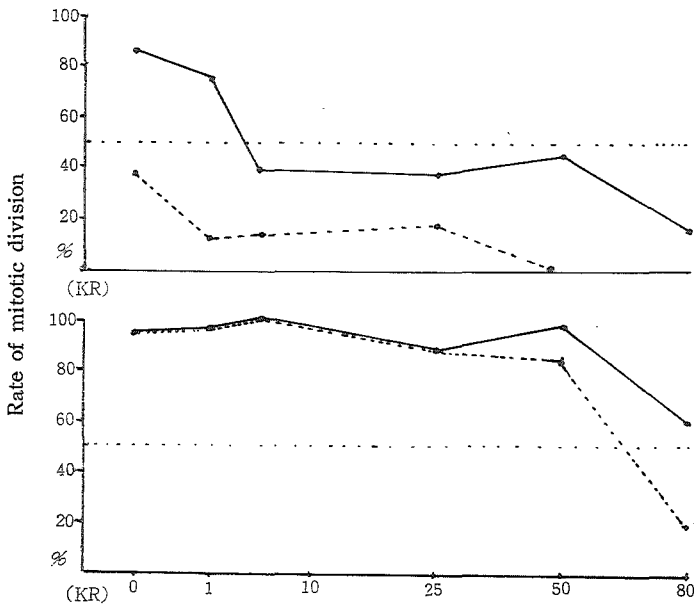

Fig. 3. Relation of radiation-induced inhibition of pollen-tube mitosis to γ-ray doses in *M. arvensis* L. var. *piperascens* 60 minutes and 100 minutes after placing the grains on culturemedium.
—— : frequency of generative nuclei in mitotic division.
···· : frequency of the formation of two sperm nuclei.

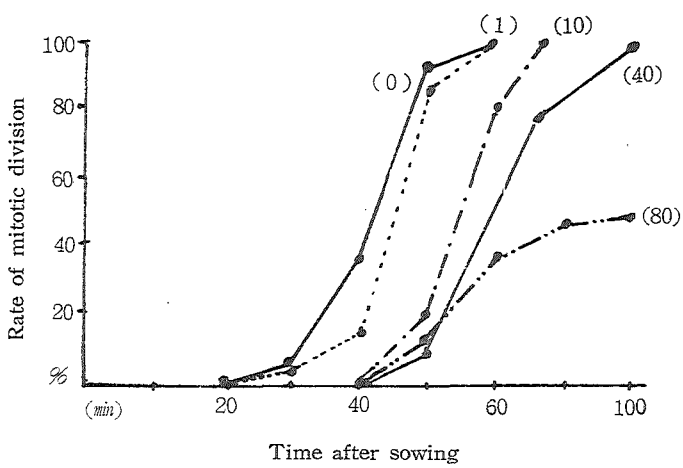

Fig. 4. Relation of radiation induced inhibition and retardation of pollen-tube mitosis to γ-ray doses in *M. arvensis* 20～120 minutes after placing the pollen grains on culture medium. Figures in ( ) indicate γ-ray doses in KR.

There was no marked difference between the non-irradiated and a lot irradiated at 1 KR, but lots irradiated at 5 KR and more showed a clear effects in retarding and inhibiting mitosis, especially those at 80 KR.

Discussion

Pollen germination and tube growth generally are inhibited only after massive radiation doses, with LD_{50}'s ranging up to 550 KR. The relatively great radio-resistance of tube elongation may be seen when medium lethal doses of 250 KR for germination are compared with the medium doses favorable for cytological studies of pollen tubes. Similar results were observed again by the senior author. In the present study, division of the generative nucleus was delayed at small doses, namely at 1KR in *M. rotundifolia* and 1 KR in *M. spicata* and *M. arvensis*. But the division was greatly impaired after doses of 10 KR in *M. rotundifolia*, 25 KR in *M. arvensis*, 50 KR in *M. spicata* and 80 KR in *M. arvensis* L. var. *piperascens*.

In the inhibition of pollen-tube mitosis is compered among 4 species used in the present study, taking non-irradiated pollen as 100 for each species. Doses estimated as inhibiting pollen-tube mitosis by 50 percent are presented in Table 5 for the four

Table 5. LD_{50}'s doses for pollen tube mitosis from γ-irradiated pollen grains of 4 species

Species	Formation of chromosomes	Formation of two sperm nuclei
M. arvensis L. var. *piperascens*	80 ~ 90	60 ~ 70
M. arvensis	50 ~ 55	26 ~ 30
M. rotundifolia	11 ~ 25	5
M. spicata	6 ~ 9	0.5~ 1

species. These deta were obtained from artificial culture media. Pollen tubes of *M. rotundifolia*, for instance, do not smoothly grow on the media. Therefore, the LD_{50}'s dose of *M. rotundifolia* pollen may be larger in natural condition than on the media.

In their earliest study of irradiation-induced inhibition of pollen germination, HASKINS and MOORE irradiated dry pollen of several *M. rotundifolia* species with X-rays. Irradiation with soft X-rays (37 kV, 20 mA) and hard X-rays (200 kV, 30 mA) produced essentially comparable results, significantly depressing germination above 150 KR with LD_{50}'s around 225KR. PRICE treated turgid pollen grains of mature anthers of *Lilium superbum* with 100 kV X-rays, nothing briefly that germination was greatly depressed at 200 KR and evidently inhibited at 210 KR. In contrast, studies of *L. longiflorum* by BREWBAKER and EMERY showed LD_{50}=250KR for desiccated pollen, a figure comparable to that observed by HASKINS and MOORE, while an LD_{50}=500 KR was obtained for fresh pollen.

According to BREWBAKER and EMERY, the radio-sensitivity of fresh pollen for 18 species span a wide range from 35 KR to 55 KR. An apparent correlation is indicated with pollen size; namely large grains generally more sensitive. In the present study the smaller pollen grains of *M. arvensis* L. var. *piperascens* were most radio-resistant. A striking exception represents *Lilium* according to the data of BREWBAKER and EMERY and our results. Nuclear volumes have been observed by SPARROW and EVANS to be highly correlated with radio-sensitivity to chronic γ-irradiation. Volumes of generative nuclei were estimated for the species above mentioned, and no correlation was apparent either with radio-sensitivity or with pollen volume.

The doses shown in Table 5 are still unexpectedly high, compared with those for meiotic division of PMC's and mitotic one of root tips. Such high tolerance of pollen grains and tube mitosis might be due to the following causes :

1) In pollen-tube mitosis DNA synthesis is completed and the chromosomes are

effectively doubled in the generative nucleus.

2) Mature pollen grains have all elements, except water, which are necessary for germination.

3) The pollen grains before absorbing water are physiologically in a quite different state from that of other tissues or cells.

Summary

The effects of pollen grain irradiation on pollen-tube mitosis were investigated. The data were obtained from artificial culture. For irradiation gamma-rays were applied with ^{60}Co at a dose rate 65 KR/hr. The doses ranged from 1 to 80 KR.

1) Inhibition of mitosis increased with increasing dose; namely the higher the dose, the more reduced was the frequency of nuclear division and the more delayed was the time of its occurrence.

2) In *M. rotundifolia* the generative nucleus remained mostly undiveded in showing chromosome elements even at 5 KR, while at 20 KR a few nuclei consisted of chromatin masses or chromosomes. The pollen-tubes of *M. rotundifolia* did not grow smoothly on the media. In natural condition, therefore, their pollen-tube mitosis may be more radio-resistant.

3) At 80 KR irradiation of *M. spicata* pollen the mitotic division of the generative nuoleus was almost completely inhibited.

4) In *M. arvensis* the generative nucleus did not divide into 2 sperm nuclei above 25 KR, while relatively many generative nuclei with chromosomes were observed at 50 KR.

5) The pollen grains of *M. arvensis* L. var. *piperascens* had the highest radio-resistance. At 50～80 KR the generative nucleus underwent complete mitotic division.

6) Radio-sensitivity of pollen grains varied widely according to the species. In general, their pollen-tube mitosis was much more radio-resistant than the meiosis in PMC's and mitosis in root tips.

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放射線によるハッカ属植物の育種学的基礎研究
（第13報）ハッカの花粉におよぼす放射線の影響

小　野　清　六

ハッカの4種の花粉にγ線（^{60}Co・65/hr.）を照射してから，これらの花粉を人工培養して，生殖核の核分裂におよぼすγ線の影響をみた．

1）　γ線の線量の増加にともなって，生殖核は分裂の速度を減じ，やがて分裂能力を完全に失った．

2）　*M. spicata* の花粉において，生殖核が完全に2つの精核に分れるのは1～5KRまでであった．ただし20KRの照射のときも，染色体にまでは変るものがみられた．

3）　*M. rotudifolia* の花粉の核は50KRの照射によって，約半数のものが分裂能力を失い，80KRにおいて完全に分裂しなくなった．

4）　*M. arvensis* の花粉は25～50KRの照射で分裂能力を失った．

5）　*M. arvensis* L. var. *piperascens* の花粉は極めて放射線に対して抵抗性が強く，80KRの照射によっても，約半数の花粉の核が分裂能力をもっていた．

6）　以上のように，花粉が花粉母細胞や生長点と比較にならないほど放射線に対して強い抵抗性を有しているのは，やくからでる花粉が特殊な状態にあるからと考えられる．