Alpha-Particles and ^{60}Co γ-Rays Have Different Biological Effects on Upland Cotton (*Gossypium hirsutum* L.) Pollen Grains

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Received: August 8, 2011 Accepted: August 25, 2011 Online Published: December 29, 2011

doi:10.5539/jas.v4n3p145 URL: http://dx.doi.org/10.5539/jas.v4n3p145

This work was supported by the National Science Foundation of China (no. 10475041).

Abstract

To compared the irradiation effects by γ-rays and alpha-particles on the structure and function of upland cotton pollen grains during pollination, pollen grains from *Gossypium hirsutum* L. cultivar "Lumian 21" were irradiated by alpha-particles (1, 10, 30, 40 and 100 Gy) and γ-rays (20, 30 and 40 Gy). We looked for changes in the ultrastructure of the exine and interior walls of the pollen grains. After germinating the irradiated pollen grains in liquid medium, we looked for changes in germination rate, the number of pollen tubes in styles after pollination and F-actin distribution in the pollen tubes. Alpha-particles penetrated and etched the exine and interior walls of the pollen grains and also damaged the organelles in the pollen grains. More pollen grains were destroyed as the alpha-particles dose increased. γ-rays had no effects on the exine walls of the pollen grains but had stronger effects on the interior structure of the pollen grains than those induced by alpha-particle irradiation. The pollen grain germination rate, the number of pollen tubes in the styles and the average length of the pollen tubes in pollen grains treated with γ-ray irradiation were decreased and shortened with increased γ-rays irradiation dose, compared with that of control. However, under alpha-particle irradiation, these features initially increased at a low level of irradiation (1 Gy) and then decreased as the dose was increased. There was evident damage to F-actin structures in pollen tubes induced by the two kinds of mutagens. The mechanisms underlying the biological effects induced by alpha-particles and γ-rays were different. The function and structure of pollen tubes in pollination and fertilization may be affected by the ultrastructure of pollen grains.

Keywords: Upland cotton, Pollen grain, Biological effects, Alpha-particles, γ-rays, Irradiation

Abbreviations

SEM, scanning electron microscope; TEM, transmission electron microscope; TRITC, tetraethyl rhodamine isothiocyanate; PIPES, 1, 4-piperazinediethanesulfonic acid, piperazine-1, 4-bis (2-ethanesulfonic acid), piperazine-N, N′ - bis (2-ethanesulfonic acid); EGTA, ethylene glycol - bis (2 - aminoethylether) - N, N, N′, N′-tetraacetic acid; LSM, laser scanning confocal microscope; F-actin, actin filament; LSD, least significant difference

1. Introduction

Since the discovery by Muller (1927) in Drosophila and by Stadler (1928) in barley that X-rays can induce mutations, mutagenesis caused by radiation has been widely studied. A large number of mutagens are frequently used, such as UV-B, X-rays, β-rays, γ-rays, nitrogen ions, protons and alpha-particles. γ-ray irradiation is the most efficient method for creating mutations in plants, and it has the advantages of convenient operation, short cycle and high mutation frequency (Misra, *et al*., 2003; Datta, *et al.*, 2001; Venkateshwarlu, 2008; Sato, *et al*., 2006; Eroglu, *et al*., 2007; Selvi, *et al.*, 2007; Wu, *et al*., 2008; Srivastava and Singh, 2002). Alpha-particles, which are directly ionizing particles with a high linear energy transfer (LET), have a strong interaction with the target substance (Hu, *et al*., 2005; Nagasawa and Little, 1992). Alpha-particles have been widely used as a mutagenic source in mammalian cells (Zhou, *et al*, 2000). However, low doses of ionizing particle radiation have beneficial effects on mammalian cells (Calabrese and Linda, 2003; Kaiser, 2003; Feinendegen, 2005; Duport, 2003; Calabrese, 2004). Because plant cells have tough cell walls and because alpha-particles transfer energy to very limited regions, it is not known whether this type of radiation could be used as an effective mutagen in plant breeding.

Mature pollen grain, which have a nutritional nucleus and two sperm, are considered to be the most sensitive of all plant cells to mutagens. It is easier to obtain mutants with pollen grains as the mutation substrate than by using seeds. Treatment of pollen grains with different types of radiation has been done in many plants, usually with one of the following objectives: (a) to study the cytological and physiological effects resulting from irradiation; (b) to induce mutations; (c) to induce haploidy; (d) to overcome problems arising from self- and cross-incompatibility (Cheng, *et al*., 2001; Wu and Yu, 2001; Morishita, *et al*., 2003; Pfahler, 1967; Sanam' ian, 2003; Naito, *et al*., 2005; Koti, *et al*., 2004; Torabinejad, *et al*., 1998; Zu, *et al*., 2003; Vizir, *et al.*, 1994; Li, et al., 2005; Li and Li, 1997; Ren, *et al*., 2000; Huang, *et al*., 2001). Swaminathan and Murty (1959) reported that irradiation of mature pollen grains increased the frequency of hybrids in interspecies crosses of Nicotiana by enhancing fertilization capacity. Sanam' ian(2003) studied the M_2 karyotypes of morphologically abnormal cotton plants (*Gossypium hirsutum* L.) after pollination with pollen grains irradiated at various doses (from 10 to 25 Gy) and detected various genomic and chromosomal mutations in 57 M_2 families. Gao *et al*. conducted whole-genome radiation hybrid mapping of cotton genomes after the pollen grains were irradiated with γ-rays. Pollination with irradiated pollen grains directly transfers the mutation induced in the sperm to the offspring of the pollinated plants. However, the mechanistic details of the interaction between irradiation and the structure and function of pollen grains have yet to be described.

Pollen grain germination and pollen tube growth are essential processes that ensure the normal reproduction of flowering plants. Pollen tubes are highly elongated cells. The polarized growth of pollen tubes permits them to accomplish invasive growth within female pistils to deliver sperm cells for fertilization. This process is relatively fast and is dependent on intact F-actin within the pollen tube (Hepler, *et al*., 2001; Chen, et al., 2007). F-actin is composed of actin polymers and actin-binding proteins. The highly dynamic features of the actin skeleton are absolutely required for pollen germination and pollen tube growth after pollination (Pierson and Cresti, 1992; Taylor and Heler, 1997). To date, there are no studies on the ultrastructure of mature cotton pollen grains, F-actin and the development of pollen tubes.

In this study, we compared the effects of irradiation by γ-rays and alpha-particles on the structure and function of upland cotton pollen grains during pollination. We used mature pollen grains of upland cotton as irradiation material to observe changes in the following: the ultrastructure of the pollen exine and interior walls, the germination rate of the pollen grains in culture medium, the number of pollen tubes in the styles after pollination, and the F-actin distribution in the pollen tubes in medium. We found that alpha-particle and γ-ray irradiation have different biological effects on the structure and function of cotton pollen grains.

2. Materials and Methods

2.1 Plant materials and growth conditions

A field experiment was conducted using the upland cotton cultivar (*Gossypium hirsutum* L.) “Lumian 21” at the

Institute of Plasma Physics, Chinese Academy of Sciences, Hefei, China, under conditions of regular cultivation and management. In this study, we used different dosages of alpha-particles (5 to 100 Gy) and gamma-rays (5 to 50 Gy) to irradiate mature upland cotton pollen grains. By comparing the germination rates of control and irradiated pollen grains, we found that alpha-particle radiation of less than 1 Gy and γ-ray radiation of less than 20 Gy had no effects on the pollen grains and that 100 Gy of alpha-particles and 40 Gy of γ-rays are lethal doses to upland pollen grains. Therefore, we choose 1, 10, 30, 40 and 100 Gy of alpha-particles and 20, 30 and 40 Gy of γ-rays to study the different biological effects of these two types of radiation.

2.2 Alpha-particle irradiation

The alpha-particle irradiation machine was devised by ASIPP (Institute of Plasma Physics, Chinese Academy of Sciences). The penetration depth of alpha particles into cotton pollen grains was simulated by Stopping and range of ions in matter (SRIM) software (2003). The result showed that the penetration depth was about 30 μm.

Before alpha-particle irradiation, some buds were harvested and some were emasculated the day before flowering. The pollen grains were collected from the harvested buds in the morning. Alpha-particle irradiation of the pollen grains was made with a planar ^{241}Am source (alpha-particle energy of 3.5 MeV). Just before alpha-particle irradiation, pollen grains were sprinkled into separate glass culture plates (4 cm diameter). To avoid superposition, the amount of pollen grains to be irradiated was carefully limited. About 100 pollen grains were prepared for alpha-particle irradiation each time. A portion of the pollen grains was set aside as a control. The prepared pollen grains were exposed to alpha particles with doses of 1, 10, 30, 40 and 100 Gy from the side of the Mylar-film base. The alpha-particles had an average energy of 3.5 MeV at the cell-Mylar-film interface with a dose rate of 1.37 cGy/min. The irradiation process was performed three times.

The next step was to pollinate pistils that were emasculated the day before with the irradiated pollen grains, with each pistil receiving as equal an amount of pollen grains as possible.

2.3 γ- ray irradiation

The γ-ray irradiation equipment was supplied by the Institute of Atomic Application in Agriculture, Anhui Academy of Agricultural Sciences in China.

The irradiation was performed on the cotton pollen grains during flowering and fruiting in August 2006, August 2007 and August 2008. The collection of mature pollen grains for γ-ray irradiation was performed in the same manner as the collection of pollen grains for alpha-particle irradiation. The pollen grains were put in the irradiation chamber, and were then irradiated with 20, 30 and 40 Gy of γ-rays. The other parameters for irradiation were unchanged for each irradiation. The flux-density was 0.48 Gy/min. The irradiation process was performed three times.

The next step was to pollinate pistils that were emasculated the day before with the irradiated pollen grains. Each pistil received as equal an amount of pollen grains as possible.

2.4 Scanning electron microscopy (SEM)

Some of the pollen grains irradiated with alpha-particles and γ-rays were affixed onto slide platforms separately and were sprinkled with gold to be observed by SEM (RilinS-300N). For each irradiation, 200 pollen grains were observed to count the number of damaged pollen grains.

2.5 Transmission electron microscopy (TEM)

A portion of the treated pollen grains was fixed first in 4% glutaraldehyde solution and then in a mixture of glutaraldehyde and osmic acid. Later, the pollen grains were dehydrated with an alcohol and acetone gradient and embedded in epoxide resin. The thickness of the ultrathin sections was 70 nm. The ultrastructural changes inside the pollen grains were observed using TEM (Rili H-7650).

2.6 Staining pollen tubes with decolorized aniline blue

Pollen tubes in styles were examined according to the procedure described by Yu et al (2008). After pollination for 11 h, the styles were harvested, fixed in an ethanol-acetic acid mixture (3:1, v/v) for 24 h and rehydrated with an alcohol gradient from 70% to 10%. Then the styles were rinsed with ddH2O, softened in 2 M NaOH and stained with 0.01% (w/v) decolorized aniline blue in 0.1 M K_3PO_4 (pH 9.0) for12 h. After staining, the styles were mounted on glass slides and spread by pressing by hand so that the pollen tubes were exposed and ready for observation by a fluorescence microscope (Olympus BH-2). The whole staining process was repeated for at least five styles per irradiation.

2.7 Pollen grain cultivation and fluorescence labeling of F-actin in pollen tubes

Five milligrams of pollen grains were spread in a film on the surface of 1.0 ml of MW-20% sucrose culture medium containing 0.01% (w/v) H_3BO_3, 0.03% (w/v) $Ca(NO_3)2$, 0.02% (w/v) $MgSO_4$ and 15%(w/v) Macrogol-8000 in a plastic Petri plate and incubated at 28 ℃ and 80% humidity. The pH of the medium was brought to 6.2 before adding sucrose. After 2-3 h of pollen grain germination, pollen tubes that elongated greater than 200 nm were prepared for staining. Pollen grains whose germinated pollen tube length was longer than the pollen grain diameter were recorded as germinated pollen grains. The germination rate of pollen grains was calculated by observing the number of germinated pollen grains in 200 cultured grains.

F-actin within the pollen tube was detected with TRITC-phalloidin (Sigma). The pollen tubes were fixed with 4% paraformaldehyde in fixation buffer (50 mM PIPES, 10 mM EGTA, 5 mM $MgSO_4$, pH 6.9) for 2 h at room temperature. After three washes with PIPES buffer (fixation buffer without paraformaldehyde), the pollen tubes were incubated with staining buffer (fixation buffer + 0.1% Triton X-100 + 1.2 μg/ml TRITC-phalloidin) for 2 to 3 h in the dark at 25 ℃. The pollen tubes were washed with staining buffer (without TRITC-phalloidin) and observed under a Zeiss LSM Leica TCS-SP2 laser-scanning confocal microscope (CLSM) with an excitation wavelength of 541 nm and an emission wavelength of 571 nm. The parameters of optical path were set as follows: scan mode: xyz, scan format: 1024×1024, scan speed: 400 Hz, Beam Exp.: 6, Pinhole: 1, scan direction=Uni. Optical sections (n=20) were acquired at 1-μm intervals on the Z-axis. Three dimensional (3D) reconstructions were assembled using Quantify software. The whole staining process was repeated at least three times.

3. Results and Analysis

3.1 Effects of alpha-particles on the exine wall of pollen grains

Control pollen grains looked approximately like spheres by SEM, dense, orderly, spinescent protuberances could be observed evenly distributed on the surface. Germination pores were symmetrically distributed on the pollen exine wall. There was a furrow in the germination pore of mature pollen grains (Fig. 1-A). Compared with control grains, the surface structure of pollen grains irradiated with alpha-particles (1, 10, 30, 40 and 100 Gy) was disrupted in a dose-dependent manner. Of the pollen grains irradiated with 1 Gy alpha-particles, 2.46% abnormal pollen grains were found with holes of different sizes in the exine wall. 10.46% in the abnormal pollen grains were found with a few long cracks in the exine wall of some pollen grains (Fig. 1-B). Of the pollen grains irradiated with 10 Gy alpha-particles, 22.02% abnormal pollen grains were found with a few more holes of different sizes and long crannies in the exine wall (Fig.1-C). Of the pollen grains irradiated with 30 Gy alpha-particles, 40.15% abnormal pollen grains were found with the exine wall was broke without complete separation of parts (Fig.1-D). Of the pollen grains irradiated with 40 Gy alpha-particles, 53.12% abnormal pollen grains were found with large numbers of holes of different sizes and long crannies in the exine wall. 12.37% in the abnormal pollen grains were found with the spinescent protuberance were ruptured, part of the exine wall was scrapped (Fig.1-E). Of the pollen grains irradiated with 100 Gy alpha-particles, 92.14% abnormal pollen grains were found with the most holes in the exine wall than in the other groups. Most fissures appeared and most spinescent protuberances were ruptured when compared with the other irradiated groups. The cytoplasm of 20.82% abnormal pollen grains flew outward owing to perforation of the exine wall by the alpha-particle irradiation. Of the pollen grains irradiated with 100 Gy alpha-particles, 92.14% abnormal pollen grains were found with the most holes in the exine wall. Most crannies appeared and most spinescent protuberances were ruptured compared with the group treated with 1 Gy, 10 Gy, 30 Gy, 40 Gy alpha-particles. And the inclusion of 23.78% in the abnormal pollen grains was flow outward, owing to the perforation induced by the alpha-particles irradiation of exine wall of the pollen grain (Fig.1-F).

The surface structure of the control pollen grains was intact. However, the number of abnormal pollen grains increased rapidly with an increasing of dose of alpha-particles. The percentage of abnormal pollen grains in the control, 1 Gy, 10 Gy, 30, 40 and 100 Gy alpha-particle irradiation groups was 1.46%, 2.46%, 22.02%, 40.15%, 53.12% and 92.14%, respectively (Fig. 2). There was a significant difference ($p<0.01$) in the percentage of abnormal pollen grains between the control and alpha-particle irradiatedpollen grains. There were also significant differences ($p<0.01$) between the groups of pollen grains irradiated with the five doses of alpha-particles.

These results indicate that alpha-particle irradiation distinctly etched the pollen grain wall, forming holes and cracks of different sizes and depths. There was a positive relationship between the degree of etching and the dose of alpha-particles, with a higher alpha-particle dose resulting in deeper etching.

3.2 Effects of γ-rays on the exine wall of pollen grains

γ-ray irradiation failed to damage the exine wall of upland cotton pollen grains at any of the dosages tested (Fig. 3-A-D). Similar results were observed in previous experiments in our laboratory (data not shown).

3.3 Effects of alpha-particles on the interior ultrastructure of pollen grains

In control pollen grains, the exine and interior walls were spaced uniformly apart from each other (Fig. 4-A). The cytoplasm of control pollen grains abounded with endoplasmic reticulum, plastids, liposomes and starch grains. A few small vacuoles (Fig. 4-B) and many round vesicles (Fig. 4-C) that contained lipid bodies and groups of coated vesicles were also present within the cytoplasm.

Alpha-particle irradiation of 1 Gy had no effect on the interior wall of the treated pollen grains (Fig. 4-D). In the pollen grains irradiated by 10 and 30 Gy alpha-particles, the interior wall became concave and turned thicken (Fig. 4-E, F). In the pollen grains irradiated by 40 Gy alpha-particles, the interior wall also became concave and turned thicken (Fig. 4-G). The incrassation of the interior wall increased as the radiation dose increase. At 100 Gy of alpha-particle irradiation, the concavity of the interior wall became most severe than that of pollen grains treated with 1, 10, 30 and 40 Gy alpha-particles. The interior wall turned thinner than that of the control pollen grains (Fig. 4-H). Furthermore, the amount of endoplasmic reticulum and vacuole increased rapidly in the 1 Gy alpha-particle irradiated pollen grains. The electronic density in the coated vesicles changed from hoariness to black (Fig.4-I, J). Alpha-particle irradiation at 10, 30, 40 and 100 Gy caused some similar phenotypes: the amount of endoplasmic reticulum and vacuole decreased rapidly, the distribution of cytoplasm was not compact, the cytoplasm was in a degradation state. The electronic density in the coated vesicles changed from greyto black. There were fewer, but larger, coated vesicles (Fig.4-K-R). However, the size of the coated vesicles turned small in pollen grains treated with 10 Gy of alpha-particle irradiation (Fig.4-K, L). In pollen grains treated with 100 Gy of alpha-particle irradiation, the round vesicles and bulk organelles (such as starch grains and liposomes) were disintegrated. The inclusion of the vesicles and organelles flew outward. The size of the coated vesicles was reduced (Fig. 4-Q, R).

3.4 Effects of γ- rays on the interior ultrastructure of pollen grains

The damage caused to the interior structure of pollen grains irradiated with γ-rays was more severe than the damage caused by 30, 40 or 100 Gy of alpha-particles. In pollen grains irradiated with γ-rays, the interior wall was concave and thinner, the damage became stronger as the dose increased (Fig. 5-A-C). When compared with control grains, the distribution of cytoplasm turned much more pyknotic in the pollen grains treated with 20 Gy of γ-rays. And there were also fewer but larger coated vesicles (Fig. 5-D, E). When the γ-ray dose was 30 and 40 Gy, the plastids in the round vesicles became small, its electronic density changed from hoariness to black, and many organelles that became larger also became concave (Fig. 5-F-I). The damage induced by 40 Gy γ-rays of radiation was stronger than that induced by 30 Gy.

These results indicated that the irradiation of pollen grains with alpha-particles and γ-rays had similar effects on the interior structure of pollen grains, with γ-rays causing more severe defects. Alpha-particles, whose penetration distance is very short, caused more severe damage to the exine wall of the grains but also caused mild internal defects. In contrast, γ-rays directly penetrated the pollen grains and severely damaged the organelles. Because the internal structure of the pollen grain cytoplasm was damaged by irradiation, the DNA of the sperm within the pollen grains may have been destroyed or mutated. Therefore, we examined whether the germination of pistils would be altered when they are pollinated with irra diated pollen grains.

3.5 Effects of alpha-particles and γ- rays on the germination rate of pollen grains

The germination rate of control pollen grains was 62.45%. The germination rates of grains irradiated with 1, 10, 30, 40 and 100 Gy of alpha-particles were 85.78%, 43.78%, 35.67%, 30.14% and 18.67%, respectively (Table 1). The germination rate of pollen grains treated with 1 Gy of alpha-particles was initially higher, but at higher alpha-particle doses the germination rate decreased. There was a significant difference ($p<0.01$) between the germination rate of the control pollen grains and that of the pollen grains irradiated by alpha -particles (least significant difference (LSD) multiple comparison test). There were also significant differences ($p<0.01$) between the germination rates of the different treatment groups. The germination rates of grains irradiated with 20, 30 and 40 Gy of γ-rays were 54.24%, 20.08% and 13.77%, respectively (Table 1). The germination rate of the pollen grains decreased rapidly as the dose of γ-ray irradiation increased. There was a significant difference ($p<0.01$) between the germination rate of the control and γ-ray-irradiated pollen grains. There were also significant differences ($p<0.01$) between the three γ-ray-irradiated groups.

The average number of pollen tubes in the styles after pollination with control pollen grains (Fig. 6-A) was

694.25; the average number of tubes in 1, 10, 30, 40 and 100 Gy alpha-particle irradiated grains (Fig. 6-B-D) was 867.81, 576.23, 412.38, 367.38 and 261.75, respectively (Table 1). The number of pollen tubes in the style was initially higher at 1 Gy of alpha-particle irradiation, but it then decreased as the dose of alpha-particles increased (Table 1). This trend was consistent with the germination rate of pollen grains in liquid culture medium. There was a significant difference ($p<0.01$) in the germination rate between the control and the irradiated groups. Additionally, there were also significant differences ($p<0.01$) between the germination rates of the irradiated groups themselves. The average number of tubes in 20, 30 and 40 Gy γ-rays irradiated grains was 564.27, 208.83 and 89.42, respectively (Table 1). The number of pollen tubes in the styles decreased rapidly as the γ-ray irradiation dose increased (Table 1). This effect was consistent with the germination rate of γ-ray-irradiated pollen grains in liquid culture medium. There was a significant difference ($p<0.01$) between the germination rates of the control and irradiated pollen grain groups. There were also significant differences ($p<0.01$) between the irradiated groups themselves.

The average length of the control pollen tubes was 326.78 μm. The average lengths of the pollen tubes from pollen grains irradiated with 1, 10, 30, 40 and 100 Gy of alpha-particles was 417.66 μm, 332.38 μm, 263.75 μm, 191.62 μm and 82.81 μm, respectively (Table 1). There was a significant difference ($p<0.05$) in the average pollen tube length between the control and alpha-particle irradiated groups. Additionally, there were significant differences ($p<0.05$) between the irradiated groups themselves. The average lengths of pollen tubes irradiated with 20, 30 and 40 Gy of γ-ray was 279.33, 162.58 and 67.14 μm, respectively (Table 1). There was a significant difference ($p<0.05$) in average pollen tube length between the control and irradiated groups. There were also significant differences ($p<0.05$) between the three γ-ray-irradiated groups.

3.6 Effects of alpha-particles on the F-actin of pollen tubes

F-actin bundles of the control pollen tubes extended continuously along the longitudinal axis of the pollen tubes (Fig .7-A) but were absent from the last 10 to 20 μm of the pollen tube tip (Fig. 7-B). In the pollen tubes from grains irradiated by 1 Gy of alpha-particles, about 90% of the F-actin bundles were interlaced and they extended to the last 10 to 20 μm of the pollen tube (Fig. 7-C). In the pollen tubes from grains irradiated by 10 Gy of alpha-particles, there were no F-actin bundles from the middle of the tube to the tip of the tube (about 148.72 μm) (Fig. 7-D). In the pollen tubes from grains irradiated by 30 Gy of alpha-particles, the F-actin was depolymerized (Fig.7-E). In the pollen tubes from grains irradiated by 40 Gy of alpha-particles, the F-actin was disappeared from the middle region to the tip of the pollen tubes (Fig.7-F). In the pollen tubes from grains irradiated with 100 Gy of alpha-particles, the F-actin extended dispersedly away from the middle of the pollen tube (Fig. 7-E).

3.7 Effects of γ-rays on the F-actin of pollen tubes

The F-actin bundles of pollen grains irradiated with 20 Gy of γ-rays did not extend continually and uniformly; instead they were unevenly distributed throughout the tube. Notably, F-actin was present in the 10- to 20-μm tip region of the irradiated pollen tubes (Fig. 7-F). The F-actin bundles of pollen grains irradiated with 30 Gy of γ-rays were more severely affected then those irradiated with 20 Gy. The distribution of the bundles was more irregular and the bundles were more entangled (Fig. 7-G). In the pollen grains irradiated with 40 Gy of γ-rays, there was no F-actin in the entire pollen tube (Fig. 7-H). The F-actin within the pollen tubes of γ-ray irradiated grains was more severely affected than the F-actin from the group irradiated with alpha-particles.

4. Discussion

4.1 Different biological effects caused by alpha-particles and γ-ray radiation of pollen grains

Little is known about the cytological effects of alpha-particle radiation on plant cells. We found that alpha-particle and γ-ray radiation had different biological effects on the exine wall of upland cotton pollen grains. One possible reason is that the γ-ray is a high energy ray with a short wavelength (0.001-0.0001 nm) and strong penetrating power. This power is in stark contrast to the short penetrating distance of alpha-particles. We suggest that the cytological effects of alpha-particles on pollen grains may be caused by a similar mechanism to that of nitrogen ions. Nitrogen ions also cause etching of the exine wall, and this etching causes a disruption of the ion flux within the pollen grain; the degree of etching and the ion fluence are positively correlated (Yu, *et al*., 2008). Therefore, a possible mechanism for the effects of alpha-particles could be the disruption of the ion flux within the pollen grain.

γ-ray radiation induced stronger damaging effects on the interior ultrastructure of the pollen grains than that induced by alpha-particle irradiation. Due to the dysmorphic appearance of the interior cytoplasm, it is possible that the DNA of sperm in some irradiated pollen grains might be destroyed or mutated; these mutations might be transmitted to the next generation after pollination and fertilization. Thus, alpha-particles and γ-rays with the proper energy and dose have the potential to induce gene mutations in cells. The effects of alpha-particle

irradiation on plant mutation have not been previously reported. In this study, we showed that alpha-particles enter the pollen grains by etching and cause a range of biological effects. It is possible that alpha-particles might induce mutations by a mechanism similar to that of low-energy ions (Yu, *et al*., 2008). We found that the vigor of pollen grains irradiated by alpha-particles was initially higher at a low level of irradiation (1 Gy) and then decreased as the dose increased. This phenomenon was similar to that observed by Calabrese and Linda in animal cells (2003). Their results showed that low doses of alpha-particle radiation have beneficial effects. Our results were also similar to those of Ren et al., who studied the effects of alpha-particles on the germination rate of the *Arabidopsis thaliana* embryo (2006). They showed that 1 Gy of alpha-particle irradiation increased the germination rate of *Arabidopsis thaliana* embryos, but the germination rate decreased rapidly if the irradiation dose exceeded 10 Gy (Ren, *et al*., 2006).

4.2 Relationship between the structure and function of pollen grains and pollen tubes

The interior ultrastructure of pollen grains is very complex. It contains sperm, cytoplasm and many organelles such as ribosomes, endoplasmic reticulum, mitochondria, plastids, liposomes and starch grains. To date, there had been no report on the relationship between the ultrastructure of pollen grains and the function and structure of pollen tubes. In our study, increasing doses of alpha -particle and γ-ray radiation caused increasing amounts of damage to the interior structure of the pollen grain. This damage affected the germination rate of pollen grains in liquid medium, the number of pollen tubes in styles, the F-actin organization within pollen tubes and the average length of pollen tubes after germination. The function and structure of pollen tubes in pollination and fertilization might be affected not only by the gene expression within pollen tubes but also by the interior ultrastructure of pollen grains. Demchik and Day (1996) and Van et al. (1997) observed that high levels of UV-B radiation, which might decrease the effectiveness of pollination and fertilization and consequently change the quantity and quality of seeds, reduce the germination rate and inhibit pollen tube growth. Our results are similar to those of Demchik and Day (1996) and Van et al. (1997).

The irradiation of pollen grains with alpha-particles and γ-rays induced damaging effects on the F-actin in pollen tubes. These effects might be due to the abnormal expression of genes related to the elongation of pollen tubes.

4.3 Possible mechanism of alpha-particle mediated gene transformation

Ion-beam induced gene transformation has been widely used in plants and microorganisms (Bian and Yu, 2005; Yu, *et al*., 1993; Wu, *et al*., 2001). Yu et al. (2008) suggested that the small ionization induced holes created in upland cotton pollen grains irradiated with low ion fluxes might allow exogenous genes to enter pollen grains. Alpha-particles enter pollen grains by etching the exine wall as nitrogen ion implantation induced. Thus, alpha-particle irradiation of a suitable dose might be used to break the pollen wall to induce a transfer of exogenous DNA into the pollen grains without serious damage to the cytoplasm and nuclei of the pollen grain. Thus, our study may provide a new method for genetic transformation.

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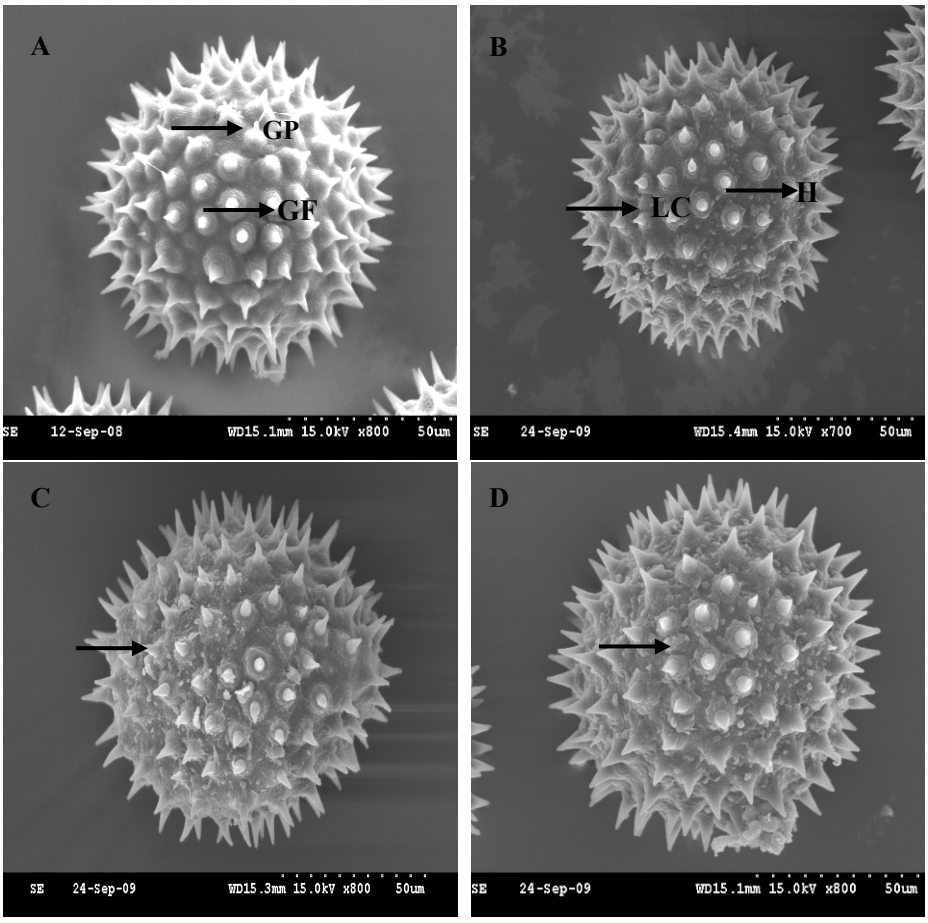
Table 1. Germination rate and pollen tube growth behavior of pollen grains treated with alpha-particles and γ-rays

Conditions		Germination rate of pollen grain [a] (%)	Number of pollen tubes [a]	Length of pollen tubes [a] (μm)
Alpha-particles	control	62.45±1.73**	694.25±38.58**	326.78±5.76*
	1 Gy	85.78±2.38**	867.81±25.80**	417.66±5.63*
	10 Gy	43.78±1.93**	576.23±45.24**	332.38±4.64*
	30 Gy	35.67±1.21**	412.38±3.26**	263.75±2.51*
	40Gy	30.14±2.13**	367.38±4.32**	191.62±3.29*
	100Gy	18.67±2.52**	261.75±20.24**	82.81±4.77*
γ-rays	control	62.45±1.73**	694.25±38.58**	326.78±5.76*
	20Gy	54.24±1.58**	564.27±31.80**	279.33±4.18*
	30Gy	20.08±1.95**	208.83±29.24**	162.58±5.99*
	40Gy	13.77±2.75**	89.42±19.24**	67.14±4.27*

[a] Values are means ± s. d. An analysis of significant variance was performed between the control groups and radiation groups according to the least significant difference (LSD) multiple comparison test. Significance levels are indicated by * and **. * represents significant at P<0.05; ** represents significant at P<0.01.



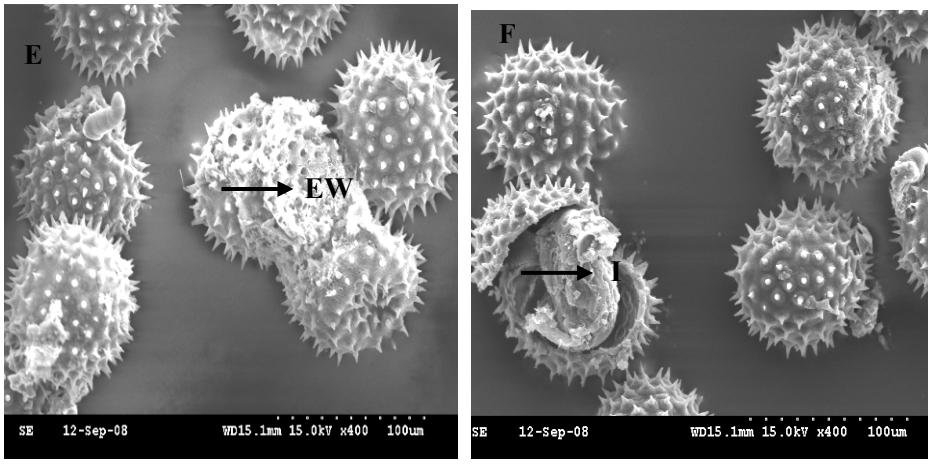


Figure 1. Morphology of pollen grains treated with alpha-particles as observed by SEM

A: Control pollen grains (800×). B: Pollen grains treated with 1 Gy alpha-particles (400×). C: Pollen grains treated with 10 Gy alpha-particles (400×). D: Pollen grains treated with 30 Gy alpha-particles (400×). E: Pollen grains treated with 40 Gy alpha-particles (400×). F: Pollen grains treated with 100 Gy alpha-particles (400×). GF, germinal furrow; GP, germination pore; H, hole; LC, long crack; EW, exine wall; I, inclusion.

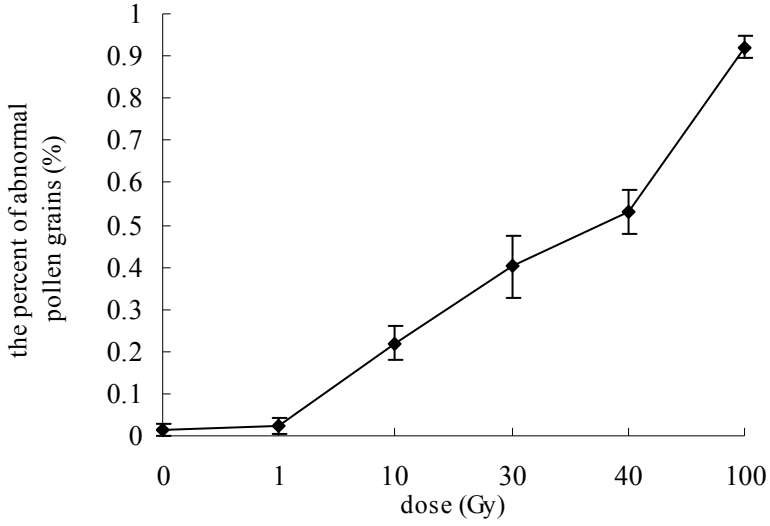
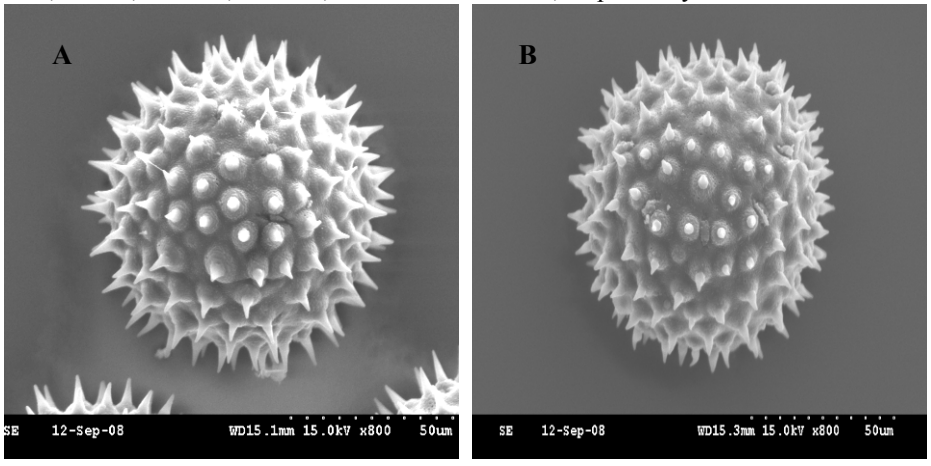


Figure 2. Percentage of abnormal pollen grains treated with alpha-particles as observed by SEM

The percentage of abnormal pollen grains in the control, 1, 10, 30, 40 and 100 Gy alpha-particle irradiated pollen grains was 1.46%, 2.46%, 22.02%, 40.15%, 53.12% and 92.14%, respectively.



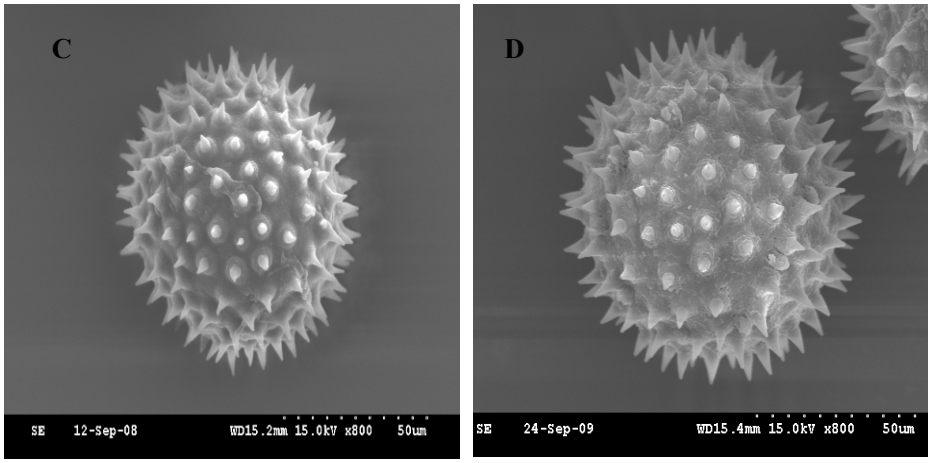
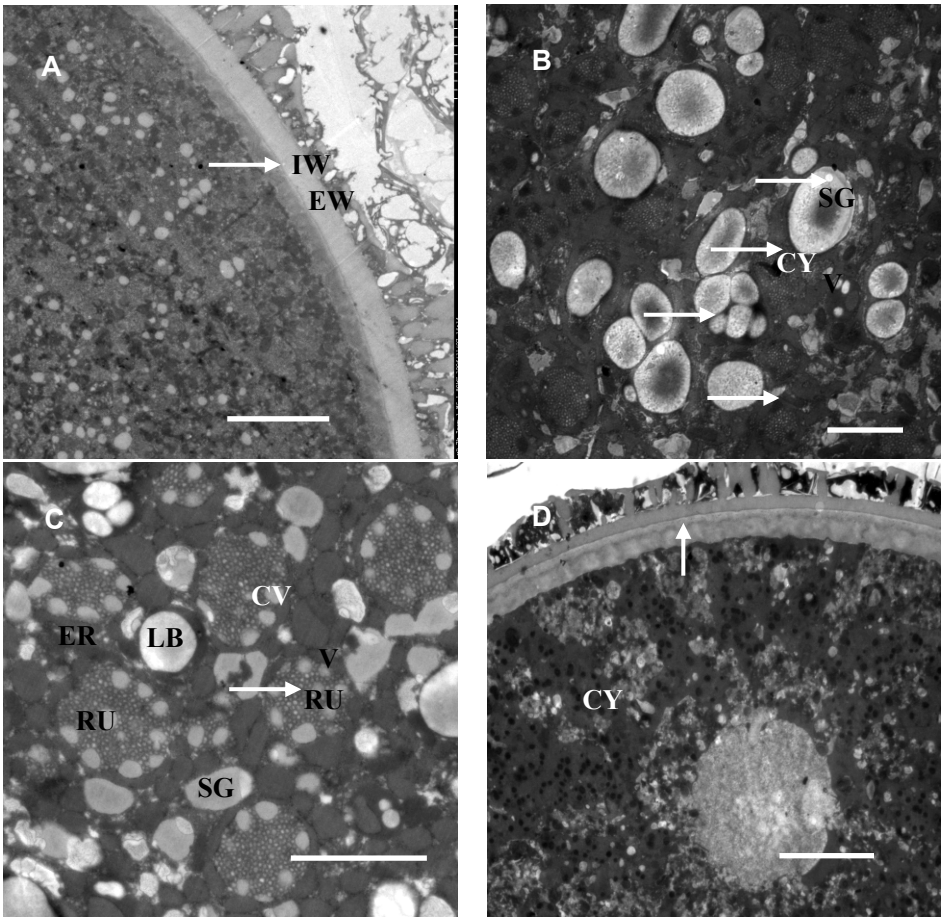
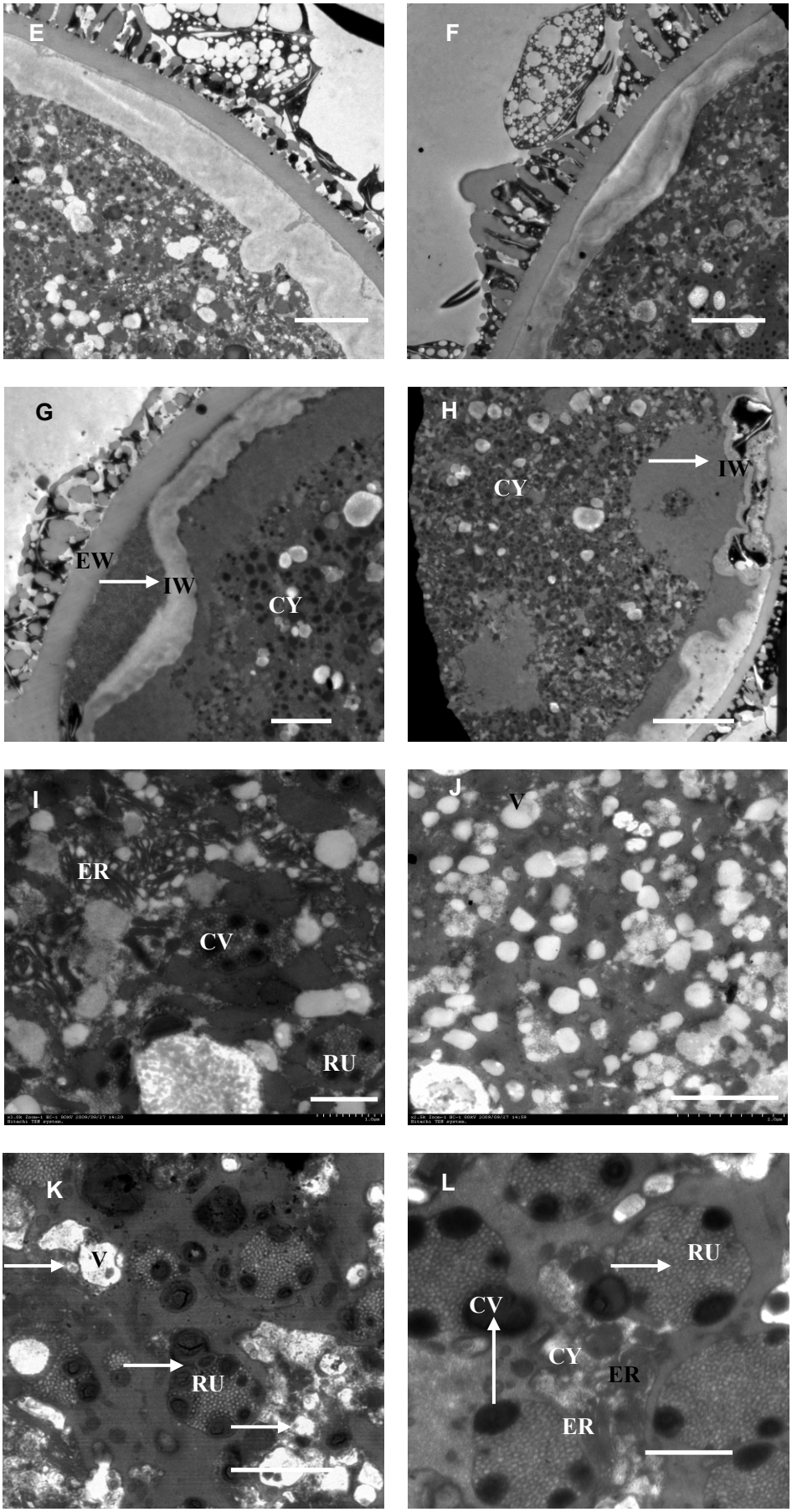


Figure 3. Morphology of pollen grains treated with γ-rays as observed by SEM

A: Control pollen grains (800×). B: Pollen grains treated with 20 Gy γ-rays (800×). C: Pollen grains treated with 30 Gy γ-rays (800×). D: Pollen grains treated with 40 Gy γ-rays (800×).

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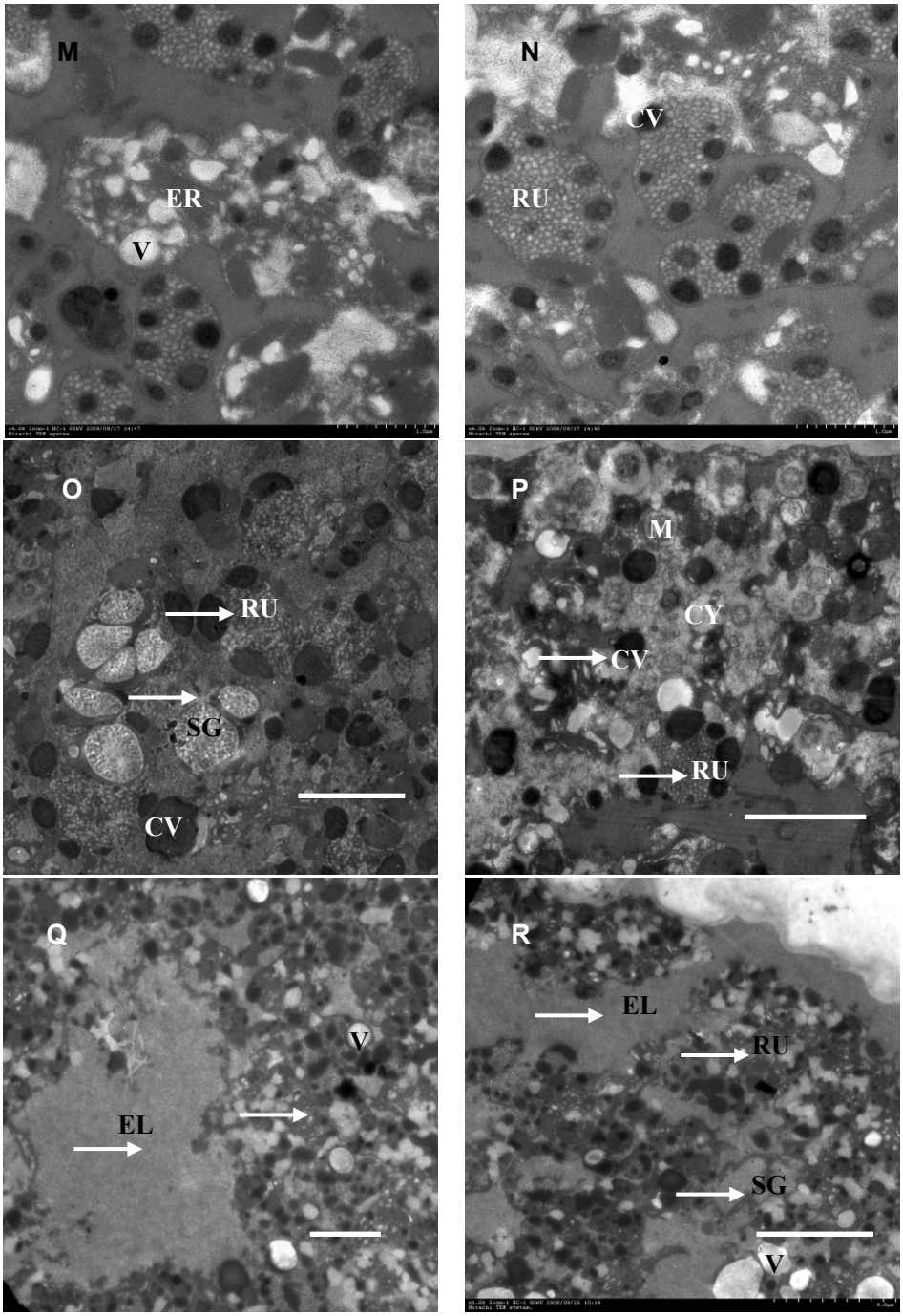
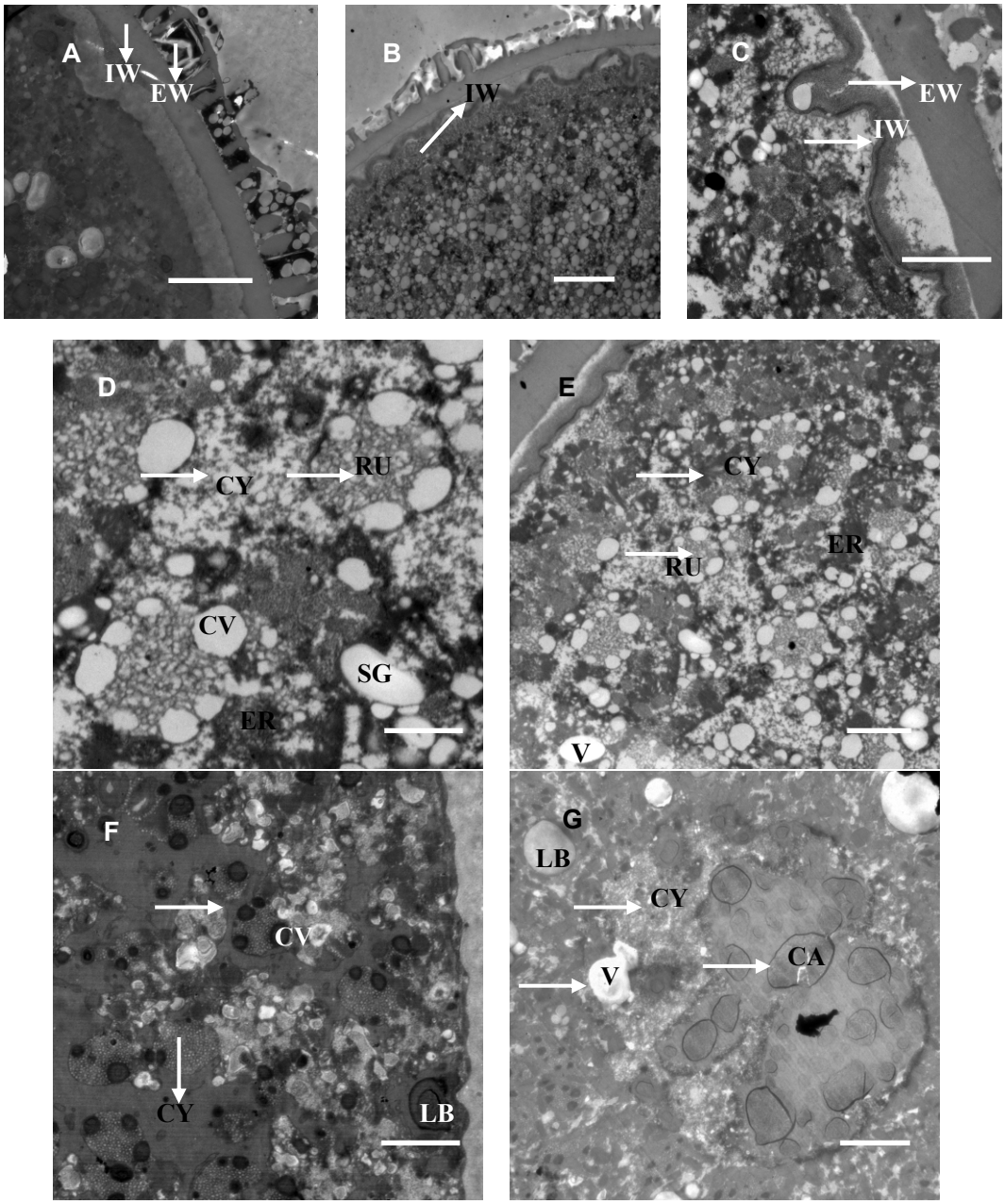


Figure 4. Changes in the interior structure of pollen grains treated with alpha-particles as observed by TEM (section thickness=70 nm)

A, B, C: Control pollen grains. The arrow in A shows that the exine and inner walls of control grains are arranged regularly, 700×, bar=50 μm. The arrows in B show the densely distributed cytoplasm containing ribosomes, endoplasmic reticulum, mitochondria, plastids, liposomes, starch grains and small vacuoles, 1500×, bar=20 μm. The arrow in C shows a round vesicles, 2500×, bar=20 μm. D: 1 Gy alpha-particle-treated pollen grains. The arrow in D shows the exine and inner walls arranged regularly, 500×, bar=100 μm. E: 10 Gy alpha-particle-treated pollen grains. The arrow in E shows damage to the interior wall, 700×, bar=50 μm. F: 30 Gy alpha-particles-treated pollen grains (The arrow in F shows that the interior wall was concaved entad, 700×, Bar=50μm.). G: 40 Gy alpha-particles-treated pollen grains (The arrow in G shows that the interior wall was concaved entad, 700×, Bar=50μm.). H: 100 Gy alpha-particle-treated pollen grains. The arrow in H shows that the concave interior wall became more serious than that of pollen grains treated with 1, 10, 30 and 40 Gy alpha-particles, and the interior wall turned thinner than that of the control, 500×, bar=100 μm. I and J: 1 Gy

alpha-particle-treated pollen grains (3000× bar=10 μm for I, 2500× and bar=20 μm for J). K and L: 10 Gy alpha-particle-treated pollen grains (2500× and bar=20 μm for K, 4000× and bar=10 μm for L). M and N: 30 Gy alpha-particle-treated pollen grains (4000× and bar=10 μm for M, 4000× and bar=10 μm for N). O and P: 40 Gy alpha-particle-treated pollen grains (2000× and bar=20 μm for O, 2500× and bar=20 μm for P). Q and R: 100 Gy alpha-particle-treated pollen grains (1200× and bar=20 μm for Q, 4000× and bar=50 μm for R). The arrows in I-R show that the amount of cytoplasm was significantly reduced and its distribution was less dense. The electronic density in the coated vesicles changed from grey to black, and there were fewer coated vesicles, but these were larger. The round vesicles and many organelles were disintegrated, their inclusion flowed outward, and the size of the coated vesicles was reduced.

IW, interior wall; EW, exine wall; RU, round vesicle; SG, starch grain; CY, cytoplasm; CV, coated vesicle; V, vacuole; LB, lipid body; ER, endoplasmic reticulum; EL, inclusion; CA, cavity.



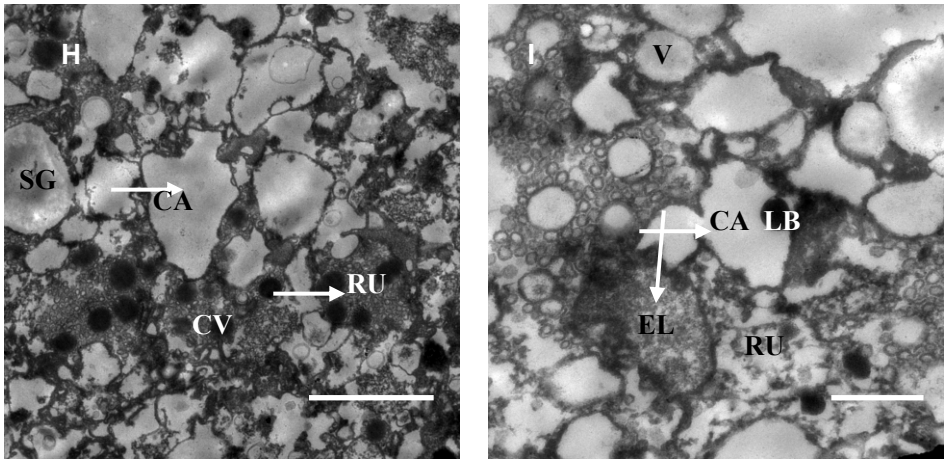
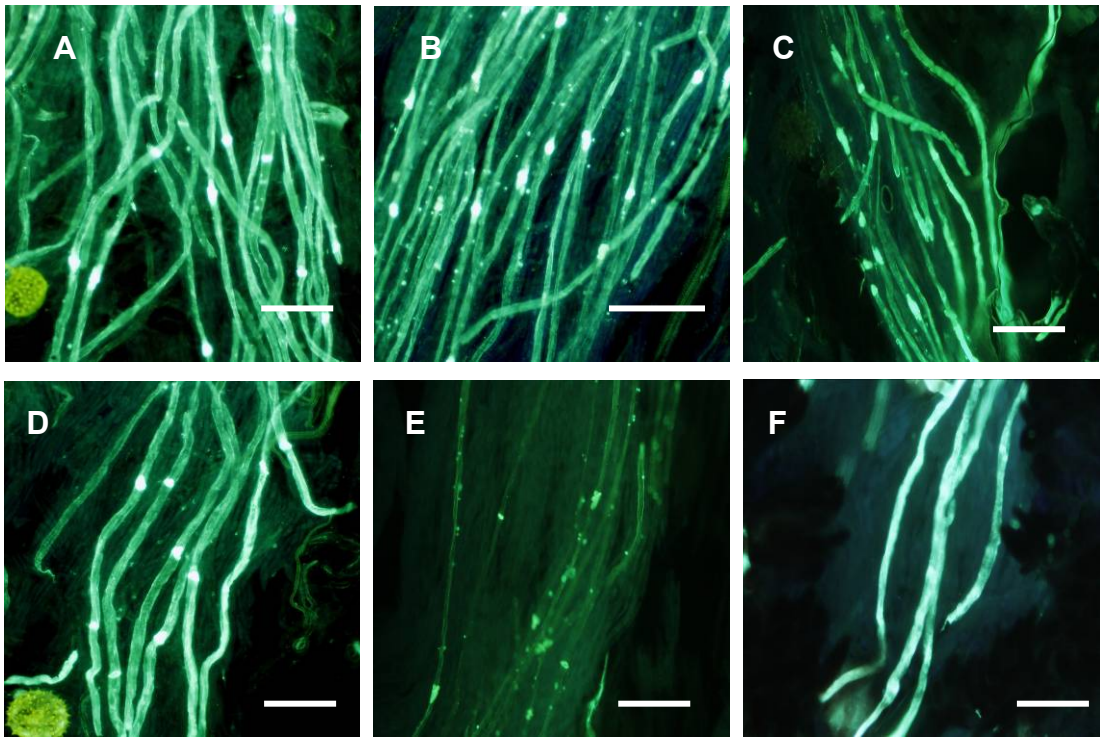


Figure 5. Changes in the interior structure of pollen grains treated with γ-rays as observed by TEM (section thickness=70 nm)

A: 20 Gy γ-ray-treated pollen grains (1000×, bar=50 μm). B: 30 Gy γ-ray-treated pollen grains (700×, bar=50 μm). C: 40 Gy γ-ray-treated pollen grains (2500×, bar=20 μm). The arrows in A-C show that the interior wall became concave and thin, and the damage became more severe as the dose increased. D and E: 20 Gy γ-ray-treated pollen grains (1000× and bar=10 μm for D, 1200× and bar=20 μm for E). F and G: 30 Gy γ-ray-treated pollen grains (1500× and bar=20 μm for F, 1200× and bar=20 μm for G). H and I: 40 Gy γ-ray-treated pollen grains (2500× and bar=20 μm for H, 6000× and bar=5 μm for I). The arrows in D-I show that the amount of cytoplasm was greater and its distribution turned more pyknotic compared to the control. There were fewer coated vesicles but they were larger in size. The plastids in the round vesicles were smaller and its electronic density changed from grey to black.



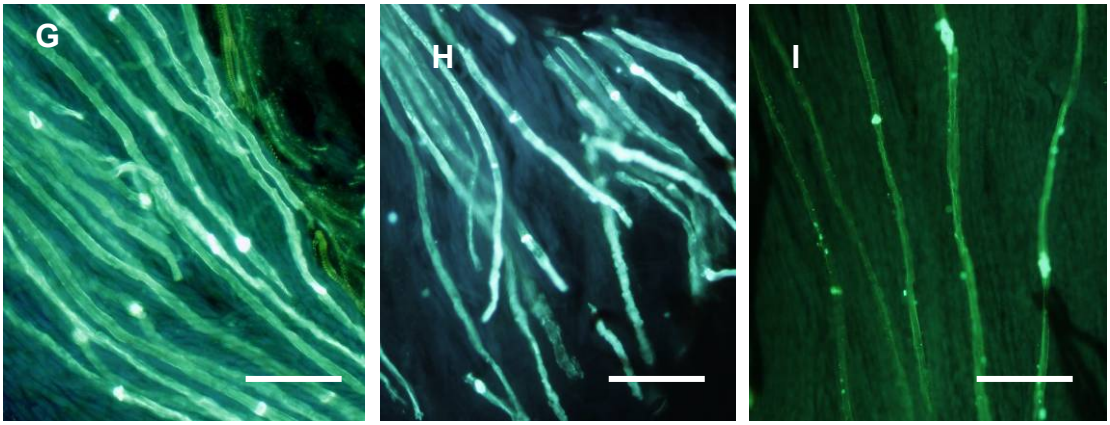
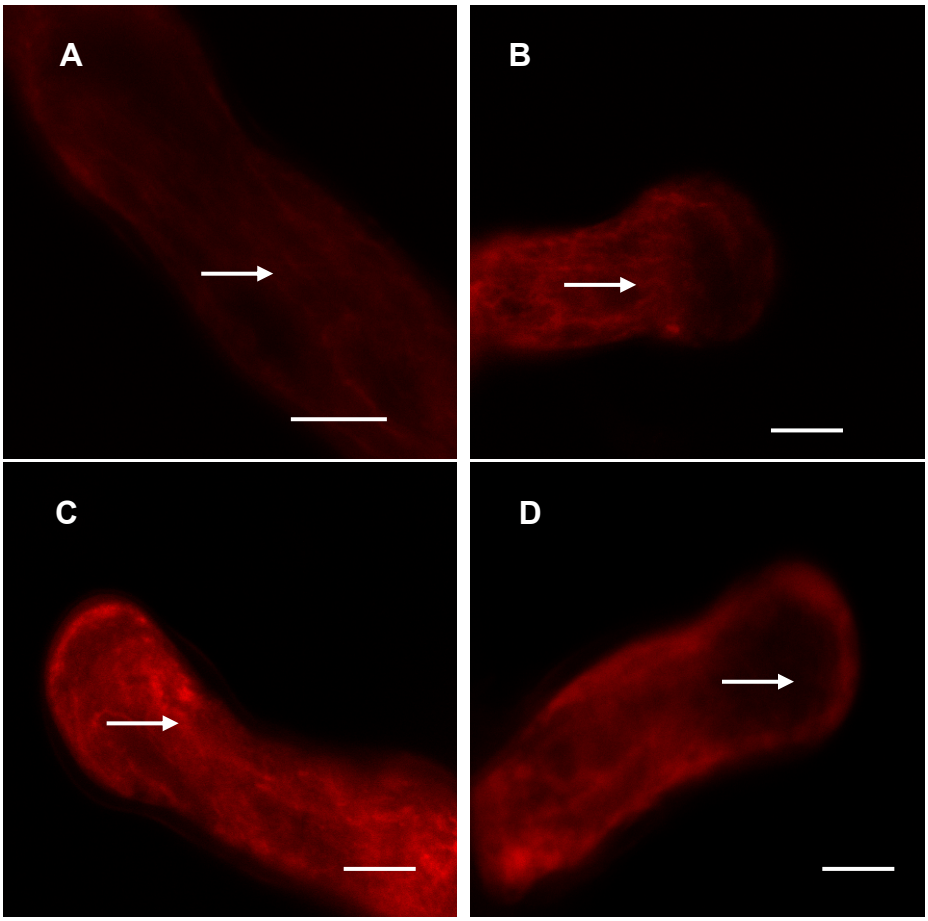


Figure 6. Pollen tubes in styles pollinated with alpha-particle- and γ-ray-irradiated grains

A: Pollen tubes in a style pollinated with control pollen grains (320×, bar=90 μm). B: Pollen tubes in a style pollinated with 1 Gy alpha-particle-treated pollen grains (320×, bar=100 μm). C: Pollen tubes in a style pollinated with 10 Gy alpha-particle-treated pollen grains (320×, bar=90 μm). D: Pollen tubes in a style pollinated with 30 Gy alpha-particle-treated pollen grains (320×, bar=100 μm). E: Pollen tubes in a style pollinated with 40 Gy alpha-particle-treated pollen grains (320×, bar=90 μm). F: Pollen tubes in a style pollinated with 100 Gy alpha-particle-treated pollen grains (320×, bar=100 μm). G: Pollen tubes in a style pollinated with 20 Gy γ-ray-treated pollen grains (320×, bar=100 μm). H: Pollen tubes in a style pollinated with 30 Gy γ-ray-treated pollen grains (320×, bar=100 μm). I: Pollen tubes in a style pollinated with 40 Gy γ-ray-treated pollen grains (320×, bar=100 μm). The number of pollen tubes in styles pollinated with control pollen grains was the greatest, and the number of pollen tubes in styles pollinated with 40 Gy γ-ray-treated pollen grains was the smallest.



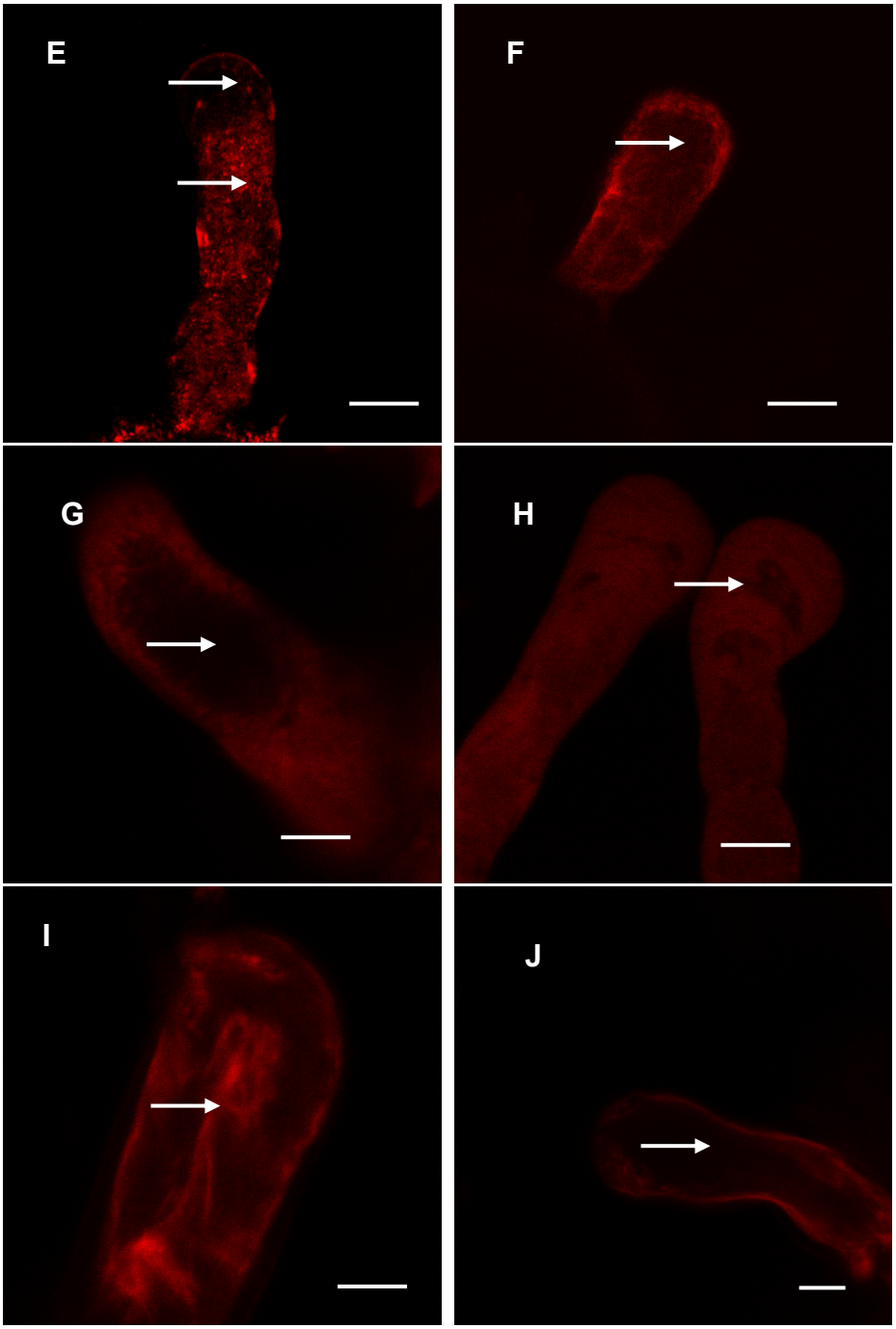


Figure 7. F-actin distribution of pollen tubes germinated from pollen grains irradiated with alpha-particles or γ-rays

A and B: F-actin distribution of pollen tubes developed from control pollen grains. C: F-actin distribution of pollen tubes developed from 1 Gy alpha-particle-treated pollen grains. D: F-actin distribution of pollen tubes developed from 10 Gy alpha-particle-treated pollen grains. E: F-actin distribution of pollen tubes developed from 30 Gy alpha-particle-treated pollen grains. F: F-actin distribution of pollen tubes developed from 40 Gy alpha-particle-treated pollen grains. G: F-actin distribution of pollen tubes developed from 100 Gy alpha-particle-treated pollen grains. H: F-actin distribution of pollen tubes developed from 20 Gy γ-ray-treated pollen grains. I: F-actin distribution of pollen tubes developed from 30 Gy γ-ray-treated pollen grains. J: F-actin distribution of pollen tubes developed from 40 Gy γ-ray-treated pollen grains. The arrows in A-H show the distribution of F-actin in pollen tubes. The bar=10 μm.

 ISSN 1916-9752 E-ISSN 1916-9760