J. Japan. Soc. Hort. Sci. 79 (1): 34–39. 2010.
Available online at www.jstage.jst.go.jp/browse/jjshs1

Simulating Hybrid-seed Contamination Risk with Selfed Seeds from Residual Fertility in a Male-sterile T-4 Mutant Tomato, *Solanum lycopersicum* L.

Masaharu Masuda[1]*, Christopher Ochieng Ojiewo[1], Mayumi Nagai[1], Kenji Murakami[1], Peter Wafula Masinde[2] and Wang Yu[3]

[1]*Graduate School of Natural Science and Technology, Okayama University, Okayama 700-8530, Japan*
[2]*Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000, Nairobi, Kenya*
[3]*Guangxi University, Nanning, Guangxi 530004, P. R. China*

Environmental dependence of male sterility may sometimes result in residual fertility under 'sterility conditions', causing hybrid-seed contamination risk. An experiment was conducted to assess the risk factor and methods to increase hybrid-seed purity in a thermosensitive male-sterile tomato mutant, T-4, whose fertility is partially restored in autumn, but largely remains sterile in spring, with some residual fertility. Examination of pollen germination and the subsequent pollen-tube growth *in vitro* and on stigma revealed that a small proportion of the T-4 pollen was viable, with 10–20% germination, while normal pollen from 'Tiny Tim' had 60–85% germination 3–6 h after pollination. A stable male-sterile mutant T-3, whose pollen development collapses at the microspore stage, was pollinated with T-4 pollen followed by normal 'Tiny Tim' pollen with time lags of 2, 4, and 8 h. Concurrently, the T-4 mutant was self-pollinated by hand followed by normal pollen from an inbred line (M) with time lags of 2, 4, 8, 24, and 48 h. The progeny was scored for contamination based on differences in leaf characteristics. The percentage of T-4 seedlings (narrow leaved) in the F_1 progeny was lowest at 2 h (0.2–6.3%), highest at 8 h (16.9–17.7%) and declined at 24 h–48 h (13.5–10.3%) time lag. The contamination rate was extremely low when pollination was done with normal pollen at (0.4%) and 24 h after (1.4%) anthesis without prior hand pollination with T-4 pollen. It was concluded that with pollination timing soon after anthesis, the T-4 mutant could be effectively applied in a two-line hybrid-seed production system with lower roguing cost of undesirable seedlings as opposed to the conventional three-line system.

Key Words: hybrid seed, male-sterility, pollen competition, residual fertility, two-line system.

Introduction

Development of hybrids has proved to be an effective way of improving crop productivity in many food crops (Fasoula and Fasoula, 2002). The advantages of heterosis in improved yields and fruit uniformity in tomato (*Solanum lycopersicum* L.) were appreciated by the early 1930s and reviewed in 1955 (Haskell and Brown, 1955). To produce F_1 hybrid-seeds, a pollination control system is required to prevent unwanted self-pollination. In crop species with hermaphrodite flowers like the tomato, this can be a major challenge. Conventionally, pollination control is achieved by emasculation (removal of anthers) of the seed parent, but this is time, labour and cost intensive (Sawhney, 2004). The risk of hybrid-seed impurity resulting from contamination of emasculation equipment is high. Hybrid-seed impurities may also result from human error, such as wrong tagging or harvesting of untagged fruits. Manual emasculation is even impractical in crop plants with very small bisexual flowers such as rice or onion. On a commercial scale, production of hybrids is only feasible if a reliable and cost-effective pollination control system is available.

Barrons and Lucas (1942) first suggested that male-sterile plants can be used as female parents, thereby reducing the time, labour and cost associated with emasculation. Commercial hybrid-seed production requires a large number of male-sterile plants as female (seed) parent. This introduces a challenge of developing strategies to multiply the male-sterile plants. The most common method for hybrid-seed production using male-sterile lines is the three-line breeding system that requires, in addition to the seed and pollen parent, a

Received; July 16, 2008. Accepted; June 5, 2009.
* Corresponding author (E-mail: mmasuda@cc.okayama-u.ac.jp).

third line, the maintainer line. Although effective, the system is expensive and cumbersome. Maintenance of (genic) male-sterile lines requires backcrossing with a heterozygous line, but the progeny produced are 50% fertile and 50% male-sterile, and 50% of fertile plants must be discarded at the seedling stage (Perez-Prat and Campagne, 2002).

Environmental dependence of male-sterility and partial fertility restoration under certain temperature and daylength conditions have been reported to effectively overcome the problems associated with the three-line hybrid-seed production system and enable the use of a two-line system in rice breeding (He et al., 1999; Wu et al., 2003). We have developed a season-dependent male-sterile tomato mutant by irradiating seeds of 'First' with gamma rays (Masuda et al., 1999). Several studies conducted in the past have established that this novel mutant (referred to as T-4) exhibits a number of desirable traits useful for hybrid tomato development, providing a good opportunity to replace the widely used "three-line" system with a "two-line" system in hybrid tomato production. The mutant bears morphologically normal flowers and acetocarmine-stainable mature pollen. However, while the wild-type pollen stains red with iodine solution, most of the T-4 pollen stains black, probably due to failure to break down starch. It has normal carpels, which are functional and compatible with wild-type pollen.

Exposing T-4 tomato mutant plants to low ambient temperatures in autumn resulted in partial restoration of fertility, with a 15% pollen germination rate on artificial medium and 60% red pollen proportion when stained with iodine. In addition, there was a 50% fruit-set and an average of 26 seeds per fruit in autumn. Hand-pollination with T-4 pollen in the F_3 generation of the first backcross (BC_1F_3) resulted in no fruit-set, 4% pollen germination rate and 55% black pollen in spring (Masuda et al., 2000). In an experiment to determine night temperature effects, fertility restoration was better enhanced when pollination was done in autumn than in spring even when the night temperature during pollination was similar in both seasons. However, there were significant differences in fruit/seed set between night temperatures tested. It was concluded that fertility restoration in the T-4 mutant is night-temperature-sensitive and that, for fertility restoration, the temperature conditions during plant growth were as important as those during pollination (Masuda et al., 2007).

In theory, therefore, the T-4 male-sterile female line can be maintained by growing under fertility-restoring conditions in autumn and used for hybrid-seed production under sterility conditions in spring. However, experiments with BC_2F_3 have shown residual fertility in spring, with a potential risk of hybrid-seed contamination, albeit at low levels (Masuda et al., 2007). Similar problems with residual fertility under "sterility" conditions have been reported in rice thermo-sensitive genic male-sterile lines (He et al., 1999) and ps-2 tomato functional male-sterile lines (Kalloo, 1993). It is important to minimize or eliminate this selfing in T-4 during the hybrid-seed production season to reduce contamination risk.

This study was conducted to assess the contamination risk potential of T-4 pollen in competition with normal pollen during hybrid-seed production and the possible control strategies to minimize this risk and maximize hybrid-seed purity.

Materials and Methods

Competition between T-4 and normal pollen on obligate male-sterile T-3 stigmas

A backcross progeny of a single recessive male-sterile tomato mutant, T-3, with its heterozygous line was used as the pollen recipient in an experiment conducted in 2004. The progeny produced was 50% fertile and 50% male-sterile as expected. The T-3 is an obligate male-sterile mutant induced by irradiation of dry tomato seeds of 'First' with γ-rays. It was reported to develop normal pistil and stamen, but its pollen degraded from tetrad to early microspore stages, leaving a perfect male-sterile trait without the need for emasculation during hybrid-seed production (Masuda et al., 1998).

To examine the competitive ability of T-4 pollen with normal pollen on T-3 stigma, T-4 tomato mutant was used as the source of sterile pollen while 'Tiny Tim' was the source of normal pollen. Seeds of T-3, T-4, and 'Tiny Tim' were sown on 25 March 2004 in vermiculite. Ten seedlings were transferred to 18 cm pots at the 4th leaf stage and fertigated daily with half strength Enshi nutrient solution. At anthesis, the fertile T-3 progeny plants (50%) were rogued off and the remaining sterile plants were hand-pollinated with T-4 pollen followed by pollen from 'Tiny Tim' on the same stigma at time lag periods of 2, 4, and 8 h. The F_1 seed was harvested and screened for contaminants as described below.

Competition between T-4 and normal pollen on facultative male-sterile T-4 stigmas

Seeds of T-4 and line 'M', a medium-fruited pure line established by selfing five generations from fruits sold in an open market of Sweden by the author, were sown in vermiculite on 30 March 2005 and raised in small pots until about 1 week before anthesis when 6 plants were selected and transplanted onto beds in a greenhouse on May 15, 2005. At anthesis of the first inflorescence truss, the T-4 flowers were hand-pollinated with T-4 pollen (selfed) followed by pollen from line 'M' at time lag periods of 2, 4, 8, 24, and 48 h. A schematic representation of these pollination activities was outlined in an earlier report (Masuda and Ojiewo, 2008). The F_1 seed was harvested and screened for contaminants as described below.

Competition between T-4 and normal pollen on T-4 stigmas without prior selfing

Seeds of T-4 and medium-fruited line 'M' were sown in cell trays filled with vermiculite in a heated greenhouse in mid-February 2004. The seedlings were pricked to 4.5 L pots in mid-April. One day (24 h) after anthesis, T-4 stigmas were pollinated with 'M' pollen without prior hand-pollination. Mature fruits were harvested from which F_1 seed was extracted and sown for screening. The experiment was repeated in 2007 with a slight modification. Seeds of T-4 and line 'M' were sown, in a mixture of vermiculite and soil (1 : 1) on March 16, 2007. The seedlings were pricked into 4.5 L pots with the same medium on May 1, 2007. Pollination of the T-4 flowers with 'M' pollen was done at anthesis. The rest of the operations were similar.

Observation of in vitro pollen-tube growth on artificial medium

For *in-vitro* pollen germination, pollen grains from T-4 and 'Tiny Tim' were cultured in drops of a modified Brewbaker and Kwack (1963) medium consisting of 10% sucrose and 50 ppm H_3BO_3 in deionized distilled H_2O supplemented with 100 ppm $CaCl_2$. The pH of the medium was adjusted to 6.0. Hanging drop cultures were prepared by placing 30 mL of growth medium on 30 mm Petri-dishes. The anthers were held with forceps and gently tapped onto each drop to release small amounts of pollen. The Petri dishes were then gently overturned onto Petri dish covers with moist filter papers. Pollen was left to germinate in an incubator set at 25°C and observed under a light microscope after 2 and 4 h.

Observation of in vivo pollen-tube growth on T-3 stigmas

Obligate male-sterile stigmas of T-3 flowers were used in this experiment to eliminate the possibility of pollen mixture. The stigmas were pollinated independently with T-4 and 'Tiny Tim' pollen between late-May and mid-June 2004. Floral styles were collected 3 and 6 h after pollination and fixed in FAA {an 18 : 1 : 1 (v/v/v) mixture of ethanol, formaldehyde, and acetic acid}. Samples were softened in 1 N NaOH for 2 h, washed with water and stained with 0.2% (w/v) aniline blue in 0.1 N K_3PO_4 for 2 h. Dissected styles were squashed and observed under fluorescent microscope (Olympus, Tokyo, Japan). Pollen was considered to have germinated when the pollen tube was equal to or more than the pollen grain diameter. The pollen germination rate was calculated by averaging the percentage germination from 6 slides, with a range of 30–60 pollen grains per film of view.

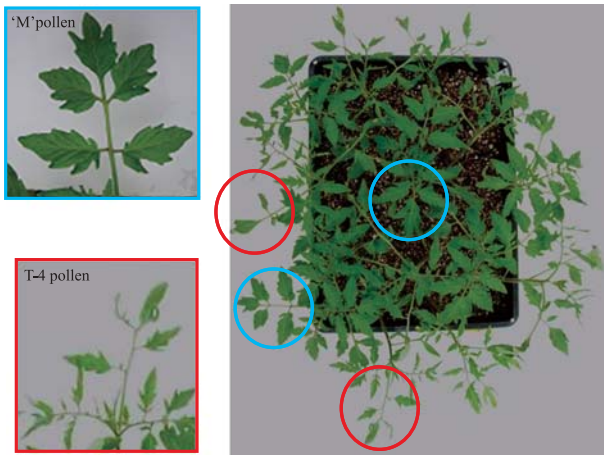

Fig. 1. Leaf shape as a marker of seedlings derived from pollination with T-4 or 'M'. Red circles: Narrow-leaved seedlings from T-4 pollen; Blue circles: Broad-leaved seedlings from 'M' pollen.

Estimation of contamination rates

To determine the percent of T-4 seedlings, seeds from 20 fruits in each of the pollination treatments were extracted. Fruits were crushed and the extract mixture of seed and pulp fermented at 37°C for 2 days. The fermentation product was thoroughly washed in running tap water, cleaning out the pulp while the seeds were trapped on a sieve. The seeds were then dried at room temperature for 1 week before sowing in vermiculite. On emergence, seedlings were identified using the leaf characteristics; namely, plants with narrow leaves were estimated to be derived from the pollination with T-4, while F_1 plants had broad leaves (Fig. 1). Emerged seedlings were counted and the contamination rate estimated as the proportion of T-4 seedlings (%) per fruit and per total number of germinated seedlings, under each treatment.

Results and Discussion

The frequency of T-4 seedlings (narrow leaved) in the F_1 progeny was lowest at 2 h lag period in 2004 (Table 1). According to the test of independence, there is significant at 1% level by Chi-square between 2 h and 4 h, and also significant at 5% level between 4 h and 8 h. It was lowest at 4 h lag period, increasing to a peak at 8 h and declining thereafter in 2005 (Table 2). There was a significant differences at 1% level between 4 h and 8 h. The apparent peaking of T-4 seedling frequency at 8 h may be due to pistil-mediated pollen-pollen interactions resulting in the promotion of the T-4 pollen by the viable pollen. The viable wild-type pollen seemed to have "paved the way" in the style biochemically and/or mechanically. Studies on pollen-pollen and pollen-

Table 1. Rates of T-4 seedlings in F_1 progeny after T-3 × 'Tiny Tim' cross following prior hand-pollination with T-4 pollen. Male-sterile mutant (T-3), whose pollen development collapses at tetrad stage, was used as a seed parent.

Time lag (h)	No. of tested seedlings (a)	No. of T-4 pollen fertilized seedlings (b)	Contamination rate (b/a × 100)
2	1412	3	0.2
4	770	101	13.1
8	685	121	17.7

pistil interactions have shown that pollination with viable pollen stimulates stigmatic secretions, which facilitate full hydration and germination of pollen grains (Shivanna et al., 1997). Viable pollen pollination also induces accumulation of flavonols in the stigma, which are essential for pollen germination and pollen-tube growth (Vogt et al., 1994). Although T-4 pollen was not sufficiently viable, it is plausible to suggest that the T-4 pollen 'took advantage' of the biochemical activities resulting from pollen-pistil activities after pollination with viable pollen.

Differences in pollen tube growth rates (certation) between foreign and self pollen may strongly influence whether hybrid offspring are produced after mixed pollen loads are delivered to a stigma (Klips, 1999). The promoting effect of wild-type pollen on the T-4 pollen was apparently dependent on the interval between pollinations. The pollen-pistil stimulus facilitating pollen-tube growth seems to be negated by the adverse certation effects where the time lag was too short, as in the 2 and 4 h, where T-4 pollen was out-competed. On the other hand, the presence of a lethal factor (Visser and Mercucci, 1983) could be responsible for T-4 pollen death, failure of pollen-tube growth, or abortion of the selfed embryo where the lag period was too long. The consequence was a reduced proportion of T-4 seedlings in the F_1 progeny, as in the 24 and 48 h.

When pollen germination and the subsequent pollen-tube growth were examined on artificial medium and on T-3 stigmas, a small proportion of the T-4 pollen was shown to be viable. However, the T-4 pollen germination rate was much lower than that of normal viable pollen from 'Tiny Tim'. The germination rate ranged from 13–17% after 2–4 h on artificial medium and 10–20% on T-3 stigmas 3–6 h after pollination. On the other hand, the 'Tiny Tim' pollen germination rate ranged from 35–39% after 2–4 h on artificial medium and 60–85% on T-3 stigmas 3–6 h after pollination (Fig. 2). Masuda et al. (1999) reported that the T-4 pollen germination rate on artificial media was less than 25% as compared to over 50% for pollen from wild-type cultivar 'First'. In later studies, Masuda et al. (2000) reported that the germination rate on artificial media varied seasonally, with 15% germination in autumn and 4% in spring. The results of this study agree with these earlier reports of low pollen viability and vigour and further elucidate the fact that this trend is similar both *in vitro* and *in vivo*. Considering that it takes 24–48 h to complete pollen tube growth in tomato (Picken, 1984), it is plausible to suggest that T-4 pollen germination and pollen-tube growth were still at the initial stages during the 2–4 h lag periods, and that the normal pollen being more vigorous out-competed the sterile pollen leading to fewer T-4 seedlings.

Pollination with 'M' pollen at anthesis and 24 h after

Table 2. Rates of T-4 seedlings in F_1 progeny after T-4 × 'M' cross following prior manual self-pollination with T-4 pollen.

Time lag (h)	No. of tested seedlings (a)	No. of T-4 selfed seedlings (b)	Contamination rate (b/a × 100)
2	430	27	6.3
4	420	15	3.6
8	272	46	16.9
24	312	42	13.5
48	426	44	10.3

Table 3. Contamination rates of F_1 progeny with T-4 seedlings after pollination with normal 'M' pollen without prior manual T-4 self-pollination.

Pollination time	No. of tested seedlings (a)	No. of T-4 selfed seedlings (b)	Contamination rate (b/a × 100)
At anthesis	4100	15	0.4
24 h after anthesis	3028	43	1.4

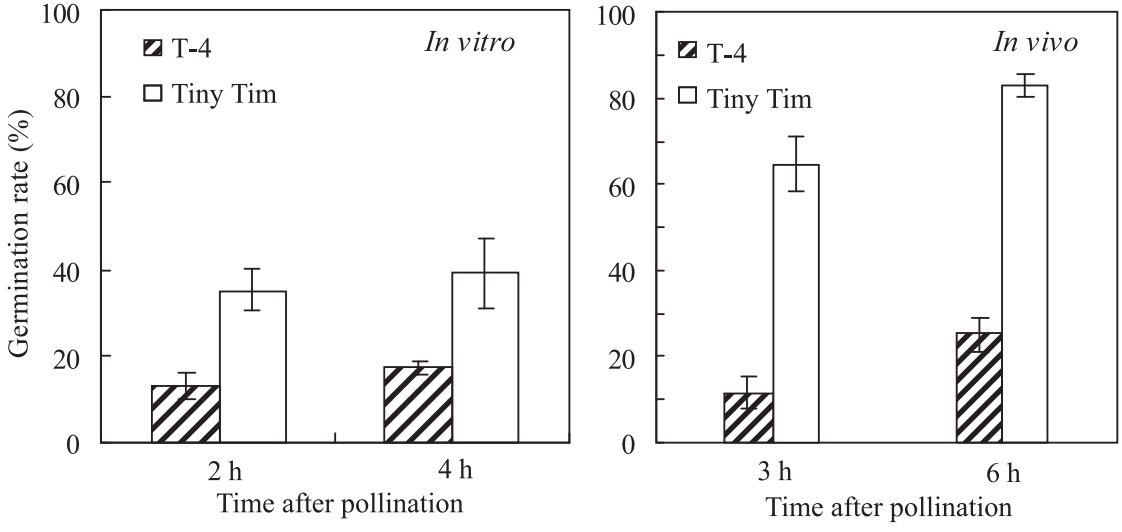

Fig. 2. *In vitro and in vivo* germination rate (%) of T-4 and 'Tiny Tim' pollen.

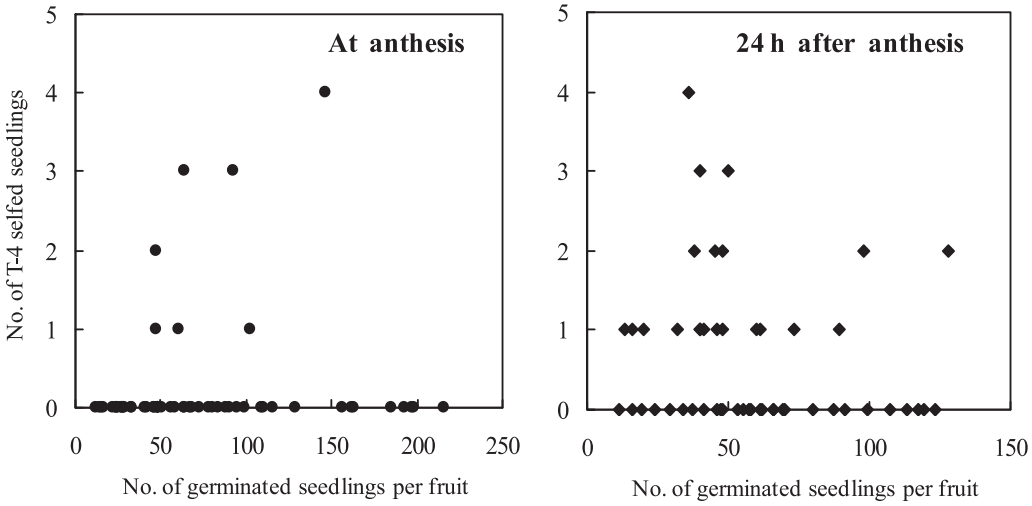

Fig. 3. No. of T-4 selfed seeds per fruit identified at 3rd expanded leaf stage of F_1 seedlings. T-4 male-sterile mutant (seed parent) was pollinated with line 'M' pollen at and 24 h after anthesis.

anthesis without prior hand pollination with self pollen resulted in 0.4% and 1.4% T-4 seedlings in the F_1 progeny, whose values were significantly different at 1% level by Chi-square (= 23.9) (Table 3). The distribution of T-4 seedlings in the F_1 progeny of individual flower pollinations is outlined (Fig. 3). These results indicate that the risk of hybrid-seed contamination resulting from residual fertility in spring could be negligible or reduced greatly with proper timing of pollination. We suggest that pollination of T-4 flowers with the desired pollen be completed by mid-day to ensure that the flowers are at anthesis. Pollinating in the afternoon may increase the contamination risk, as observed in the 8 h time lag, so, it may be less risky to continue with pollination the following morning with reduced contamination risk as observed in the 24 and 48 h time lags and at 24 h without prior selfing.

In practice, hybrid-seed impurity is expected to be minimal, but not ultimately absent in spring. However, purity control in a hybrid-seed production programme using T-4 as the seed parent is simplified by the special narrow leaf characteristic that aids identification and roguing at 2–3 true-leaf stage. The wild-type 'First' from which the T-4 mutant was isolated has uniquely narrow leaves. In the case of hybridization of a similar cultivar with T-4, identification of contaminants may be difficult. To fine-tune this useful breeding strategy, we are introgressing a dwarfing trait into the T-4 that will be used an additional marker (Masuda et al., 2005). The short internode trait is conspicuous at the first internode, making identification even easier.

The conventional hybrid seed production using male-sterile lines involves pollination with a heterozygous (*Msms*) male-fertile line. The F_1 progeny of such a cross would be 50% *Msms* (fertile) and 50% *msms* (sterile). The fertile plants have to be rogued off but their identification is only possible after flowering. This practice has several limitations and demerits. First, it is uneconomical in terms of labour, inputs and space to maintain 50% of the population only to eliminate them after flowering. Secondly, it is difficult to prevent unwanted pollination of the male-sterile plants from this pollen source. Thirdly, it is uneconomical to propagate the maintainer for no other purpose than obtaining seeds from the male-sterile lines. Fourthly, roguing of such a high proportion of undesirable male-fertile off-types is very expensive, labour intensive and time consuming. In this respect, T-4 mutant can be selfed after partially restoring at cooler temperature in autumn, and the desirable crossed seed early identification of T-4 contaminants at the seedling stage ensures minimum wastage of space, time, energy, and money for maintenance. The T-4 mutant therefore offers the hybrid-seed producer a good opportunity to use a two-line hybrid-seed production system, thereby circumventing the cost, labour, time, and space related huddles associated with manual emasculation or the 3-line hybrid-seed production system. The indirect impact of this would be reduced cost of hybrid seed stemming from reduced production costs, an advantage that would be felt greatly by tomato farmers and consumers as well.

Literature Cited

Barrons, K. and H. E. Lucas. 1942. The production of first generation hybrid tomato seed for commercial planting. Proc. Amer. Soc. Hort. Sci. 40: 395–404.

Brewbaker, J. and B. Kwack. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. Amer. J. Bot. 50: 747–758.

Fasoula, V. A. and D. A. Fasoula. 2002. Principles underlying genetic improvement for high and stable crop yield potential. Field Crops Res. 75: 191–209.

Haskell, G. and A. G. Brown. 1955. Hybrid vigour in cultivated tomatoes. Euphytica 4: 147–162.

He, Y. Q., J. Yang, C. G. Xu, Z. G. Zhang and Q. Zhang. 1999. Genetic bases of instability of male-sterility and fertility reversibility in photoperiod-sensitive genic male-sterile rice.

Theo. Appl. Gen. 99: 683–693.
Kalloo, G. 1993. Tomato, *Lycopersicon esculentum* Mill. p. 645–666. In: G. Kalloo and B. O. Bergh (eds.). Genetic improvement of vegetable crops, Pergamon Press, Oxford, New York.
Klips, R. A. 1999. Pollen competition as a reproductive isolating mechanism between two sympatric *Hibiscus* species (Malvaceae). Amer. J. Bot. 86: 269–272.
Masuda, M. and C. Ojiewo. 2008. Induced mutagenesis as a breeding tool for improvement of *Solanaceous* vegetables. Gamma Field Symposia 45: 47–60.
Masuda, M., T. Furuichi, Y. Ma and K. Kato. 1998. Pollen degradation and inheritance of male-sterility in three mutants in tomato, *Lycopsersicon esculentum* Mill. cv. First. J. Japan. Soc. Hort. Sci. 67: 583–588 (In Japanese with English abstract).
Masuda, M., K. Kato, K. Murakami, H. Nakamura, C. O. Ojiewo and P. W. Masinde. 2007. Partial fertility restoration as affected by night temperature in a male-sterile T-4 mutant tomato, *Lycopersicon esculentum* L. J. Japan. Soc. Hort. Sci. 76: 41–46.
Masuda, M., Y. Ma, K. Uchida and K. Kato. 1999. Characterization and genetic analysis of male-sterile mutant induced in tomato cv. First, having mature pollen stainable with acetocarmine. J. Japan. Soc. Hort. Sci. 68: 566–568.
Masuda, M., Y. Ma, K. Uchida, K. Kato and S. G. Agong. 2000. Restoration of male-sterility in seasonally dependent male-sterile mutant tomato, *Lycopersicon esculentum* cv. First. J. Japan. Soc. Hort. Sci. 69: 557–562.
Masuda, M., T. Yuasa and K. Murakami. 2005. Growth characters and genetic inheritance in tomato cv. First mutant with short internodes induced by seed irradiation with ion beam. Sci. Rep. Fac. Agr. Okayama Univ. 94: 25–29 (In Japanese with English abstract).
Perez-Prat, E. and M. M. L. Campagne. 2002. Hybrid-seed production and the challenge of propagating male-sterile plants. Trends in Plant Sci. 7: 199–203.
Picken, A. J. F. 1984. A review of pollination and fruit set in the tomato (*Lycopersicon esculentum* Mill). J. Hort. Sci. 59: 1–13.
Sawhney, V. K. 2004. Photoperiod-sensitive male-sterile mutant in tomato and its potential use in hybrid-seed production. J. Hort. Sci. Biotech. 79: 138–141.
Shivanna, K. R., M. Cresti and F. Ciampolini. 1997. p. 15–39. In: Pollen biotechnology for crop production and improvement, K. R. Shivanna and V. K. Sawhney (eds.). Pollen development and pollen-pistil interaction. Cambridge University Press.
Visser, T. and M. C. Mercucci. 1983. Pollen and Pollination Experiments. IX. The pioneer pollen effect in apple and pear related to the interval between pollinations and the temperature. Euphytica 32: 703–709.
Vogt, T., P. Pollak, N. Tarlyn and L. P. Taylor. 1994. Pollination- or wound-induced kaempferol accumulation in *Petunia* stigmas enhances seed production. Plant Cell 6: 11–23.
Wu, D., S. Shen, H. Cui, Y. Xia and Q. Shu. 2003. A novel thermo/photoperiod-sensitive genic male-sterile (T/PGMS) rice mutant with green-revertible albino leaf colour marker induced by gamma irradiation. Field Crops Res. 81: 141–147.