Effects of Streptomycin Sulfate on Seedlessness in Oval Kumquat (*Fortunella margarita* Swingle)

Nimmannoradee Promtong [1)] Ching-Lung Lee [2)] Yau-Shiang Yang [3)]

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Summary

Ten years old potted kumquat plants were sprayed with 120, 240 and 480 ppm streptomycin sulfate 7 days before flowering. The viability, number and germination rate of pollen, growth of pollen tube, seed number and fruit quality were investigated to clarify the effect of streptomycin sulfate on seedlessness of fruits. Results were shown that both viable pollen percentage and normal pollen number were lower in streptomycin sulfate treatments than control. However, no significant difference was found among streptomycin sulfate treatments of different concentrations. Streptomycin sulfate treatments had more abnormal pollen, consequently pollen germination rate was low and pollen tube was short. Pollen tube of streptomycin sulfate treatments appeared abnormal in wavy wall and swollen tips may due to callose deposition. The normal seed number per fruit was 0.4~0.6 in streptomycin sulfate treatments comparing to 2.7 of control. Abnormal seed numbers were higher as 1.2 ~ 1.3 in streptomycin sulfate treatments compared to 0.5 of control contrarily, and there was no significant difference among streptomycin sulfate treatments. High percentage of 2-3 seeds was found in control whereas lots of seedless and 1 seed fruits in streptomycin sulfate treatments. The seedless fruit percentage in streptomycin sulfate treatments were 70.3-76.6% comparing to 16.8% in control. There was no significant difference on the percentage of seedless fruit among different concentrations of the streptomycin sulfate treatments. In addition, streptomycin sulfate treatments resulted in small fruit size, but no effect on fruit quality.

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1) Graduate student in Ph.D. Program, Department of Horticulture, National Chung Hsing University.

2) Chair professor, National Chung Hsing University.

3) Professor, Department of Horticulture, National Chung Hsing University. Corresponding author.

Introduction

Kumquat (*Fortunella* spp.) is an evergreen shrub or small tree, originated in Southeast China. It was cultivated very popular in tropical Malaysia and Thailand, also in subtropical regions of China, Japan and other countries. Kumquat is closely related to *Citrus* and *Poncirus* in subfamily Aurantiodideae (Citroideae) of the family Rutaceae (Swingle and Reece, 1967). Normally kumquat fruits have relatively thick, fleshy, sweet, edible peel, and can be processed to make wine, jam, marmalade and candy. They also can be preserved in syrups or put into alcoholic beverages such as gin, rum and brandy to make cocktail. Some varieties can be used as ornamentals also. Seedless and small seeds are applicable by consumers for fresh consumption, *e.g*. citrus, grape, wax apple, litchi, watermelon and tomato. Each kumquat fruit has 2 ~ 5 seeds usually, which cause the inconvenience of taste or utilization, and it will be easier to eat or process if fruits have few seeds only or all seedless.

Seedlessness can be induced by many methods, includes plant hormones or biochemical treatments and genetic factors. Breeding is the best way to create a seedless cultivar. The applications of plant growth regulators or off-season fruit production are good methods to produce seedless fruits. Seedlessness induction in grapeberry by application of gibberellic acid (GA_3) before full bloom was reported in many previous research. However, the effect was incomplete and the berries were not absolutely seedless (Kimura et al., 1996; Pommer *et al*., 1996). In some cases, GAs can improve the quality of grapeberry also, such as the Sharad seedless (Ramtake *et al*., 2011). Streptomycin sulfate, a sort of antibiotic, was successfully used to induce seedlessness of ‘TosaBuntan’ pummelo (Kitajima *et al*, 2004) and several grape cultivars such as ‘Kyoho’ (Fukunaga and Kurooka, 1988; Ishikawa *et al*. 1996-1998), ‘Muscat Bailey A’ (Ogasawara,1986), ‘Fijiminori’ and ‘Italia IP65’ (Pommer *et al*, 1996). However, the mechanism of seedlessness in kumquat was not sufficiently studied yet, and cultivar variation, climate factors and environment effects might also occur. The objectives of this experiment were to clarify the effects of streptomycin sulfate on seedlessness in kumquat, figure out the optimum concentration and establish seedless fruits production technique in Taiwan.

Material and Methods

Plant materials

This experiment was conducted at the Grape Center of Horticulture Experiment Station, National Chung Hsing University, located at Wufeng, Taichung, Taiwan. Ten-year-old healthy kumquat plants grafted on sour orange collected from Lan Yan Branch Station of Hualian District

Agriculture Experiment and Extension Station were planted in plastic containers (36 cm in diameter) with artificial media of 3:1 mixture of soil and peat moss. Moderate vigor plants (90 cm in height) were selected for experiments. These plants were pruned to nature type on January 15, 2010, irrigated three times a week and applied composite fertilizer (15N-15P_2O_5-15K_2O) twice at 1 week after pruning and 1 month after fruit set, respectively. After new shoots growing up and matured, healthy and uniform shoots were selected for streptomycin sulfate treatments.

Methods of experiment

1. Streptomycin sulfate treatment

The completely randomized design (CRD) was used in this experiment with 4 treatments and 5 replications (potted plants). Three shoots were selected for each treatment and each plant composed of 4 treatments. Totally 60 shoots with 10 uniform flower buds on each were used in this research. Streptomycin sulfate (USB Corporation Company) of 3 concentrations consisted of 120 ppm, 240 ppm and 480 ppm were applied to flower buds 7 days before flowering (April 1, 2010). The flower buds sprayed with distilled water only were used as the control. All applications of streptomycin sulfate were mixed with 0.1% Tween-20 as surfactant.

2. Pollen viability test

Flowers on streptomycin sulfate treated and control plants were harvest at full bloom stage. Three flowers were investigated for each treatment and 12 flowers were picked on each plant totally. Pollens were collected from flowers at 7:30-9:00 am when flower were just opened. All pollens were strained with 1, 2, 3- triphenyltetrazolium chloride (TTC) for viability test (Mulugeta *et al*., 1994). Pollens were scattered onto a microscope slide and a drop of TTC was added first, then the slide was covered with a cover slide and the edges was sealed with nail varnish. Viability observation started 15 minutes after pollens were strained. The percentage of viable pollen was counted on 5 point per slide and each treatment consisted of 4 replications (slides). The strained pollens were considered as viable, and the diameter of both viable and non-viable pollen was measured.

3. Pollen germination and pollen tube growth

Pollens for germination and pollen tube growth experiments *in vitro* were collected the same way as viability test. Put pollens into beaker with 10 ml of distilled water and mixed appropriately. Took 1 ml of the pollen solution with pipette, placed on a glass slide containing B&K medium [100 ppm boric acid (99%, H_3BO_3), 200 ppm magnesium sulfate (99%, $MgSO_4 \cdot 7H_2O$), 300 ppm calcium nitrate (98%, $Ca(NO_3)_2 \cdot 4H_2O$), 100 ppm potassium nitrate (99%, KNO_3) (Brewbaker and Kwack, 1963)] supplemented with 1% agar and 15% sucrose, and incubated at 25°C for 4 hrs. Germinated pollens were counted and germination rate was calculated. Subsequently, pollen tube

length were measured and recorded on those pollens incubated continuously for 24 hrs (Buyukkartal, 2003).

4. Fruits quality analysis

Fruits were harvested 150 days after 50% flower blooming. Ten fruits were picked randomly from each treatment for quality analysis.

1) Fresh weight

Electronic weighing scales was used to weigh the fruits and expressed in g unit.

2) Fruit size

Fruit length and width were measured by using Mitutoyo Tesa Mahr Digital Vernier Caliper and expressed in mm unit.

3) Total soluble solids (TSS)

Total soluble solids was determined by handheld tortuous account (Hand refactometer, ATAGO) and exposed in ^{o}Brix.

4) Titratable acid (TA)

ATAGO FS-2 was used for the measurement of juice acidity. Fruit juice was diluted by adding 9 ml distilled water into 1ml sampled juice, and 0.1% phenolphthalein was dropped into the solution as the indicator. Juice was titrated by using 0.1N NaOH until the solution changed to pink, then calculated as tartaric acid content and expressed in the unit of mg/100ml.

5) Seed distribution

Seeds were counted from 40 fruits per treatment and calculate the percentage of seed distribution inside each fruit.

5. Statistical analysis

Data were subjected to an analysis of variance (ANOVA) to determine whether differences existed among treatments. Duncan's Multiple Range Test (DMRT) was used (Statistical Analysis System; SAS 9.2) and a value of $P < 0.05$ was considered statistically significant.

Results

Results showed that the effect of streptomycin sulfate on pollen viability and number were different on various treatments. Table 1 showed that the highest percentage of viable pollen was found in control (53.5%), while all streptomycin sulfate treatments (39.1-43.3%) were significantly lower than the control. No significant difference was found among streptomycin treatments of different concentrations. The number of normal pollen was 63.9/400 μm^2 in control, significantly higher than streptomycin sulfate treatments. On contrary, abnormal pollen in all

streptomycin sulfate treatments were 35.8-43.3/400 μm^2 which were higher than 23.4/400 μm^2 of the control. However, no significant difference was found on total pollen number among all treatments.

Table 1. Effects of streptomycin sulfate on viable pollen percentage and pollen number in oval kumquat.

Treatments [z]	Viable pollen (%)	Number of pollen/ 400μm^2		
		Normal	Abnormal	Total
Control	53.5a[y]	63.9a	23.4b	87.3a
SM 120 ppm	43.3b	41.3b	43.3a	84.6a
SM 240 ppm	41.2b	35.4b	35.8ab	71.2a
SM 480 ppm	39.1b	41.9b	42.1a	84.0a

[z] Streptomycin sulfate were treated on the 7th day before flowering.

[y] Means within a column followed by the same letter are not significantly different by Duncan's MRT at $p \leq 0.05$.

The effect of streptomycin sulfate on pollen diameter, pollen germination rate and pollen tube length were shown in Table 2. Pollen diameters of all treatments were between 20.3 to 21.7 μm, and no significant difference was found among them. In contrast, pollen germination rate and pollen tube length of control were 35.3% and 278.7 μm, respectively, significantly higher than all streptomycin sulfate treatments. Abnormal pollen tube appeared more frequently in streptomycin sulfate treatments. Some wavy wall structure and swollen tip were observed in abnormal pollen tube (Fig. 1).

Total seed number per fruit of the control was 3.2, significantly higher than all streptomycin sulfate treatments which were 1.6-1.9. Similarly, the normal seed number of control (2.7) was significantly higher than streptomycin sulfate treatments (0.4-0.6). On contrary, abnormal seed number of control was 0.5 per fruit only, whereas streptomycin sulfate treatments were much more up to 1.2-1.3 per fruit. All concentrations of streptomycin sulfate induced the formation of abnormal seeds and diminished total seed number. Therefore the percentages of seedless fruit on streptomycin sulfate treatments were more than 70%, highly different from the control which was 16.8%. The effect of streptomycin sulfate on seed number per fruit and seedless fruit percentage were shown in Table 3.

Table 2. Effects of streptomycin sulfate on germination of normal pollen in oval kumquat.

Treatments [z]	Pollen diameter (μm)	Pollen germination rate [y] (%)	Pollen tube length [x] (μm)
Control	21.7a[w]	35.3a	278.7a
SM 120 ppm	20.7a	19.5b	217.5b
SM 240 ppm	20.3a	19.2b	223.7b
SM 480 ppm	21.4a	20.3b	215.0b

[z] Streptomycin sulfate were treated on the 7th day before flowering.

[y] 4hrs after culture on medium.

[x] 24hrs after culture on medium.

[w] Means within a column followed by the same letter are not significantly different by Duncan's MRT at $p \leq 0.05$.

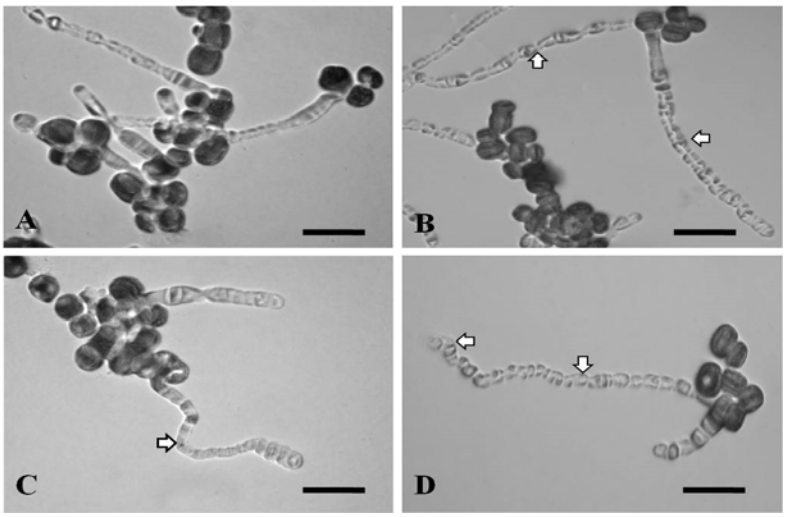

Fig. 1. Pollen tube growth in B&K medium with 1% agar and 15% glucose at 25° C for 24 hrs under microscope.

A: Control, B: Streptomycin sulfate 120 ppm, C: Streptomycin sulfate 240 ppm and D: Streptomycin sulfate 480 ppm. The abnormal pollen tubes were wavy and swollen in the tip (arrows in B, C and D). Bar = 50μm.

Table 3. Effects of streptomycin sulfate on seed number in oval kumquat.

Treatments [z]	Seed number/ fruit			Seedless fruit (%)
	Normal	Abnormal	Total	
Control	2.7a[y]	0.5b	3.2a	16.8b
SM 120 ppm	0.6b	1.3a	1.9b	70.3a
SM 240 ppm	0.4b	1.2a	1.6b	76.6a
SM 480 ppm	0.4b	1.2a	1.6b	73.8a

[z] Streptomycin sulfate were treated on the 7th day before flowering.

[y] Means within a column followed by the same letter are not significantly different by Duncan's MRT at $p \leq 0.05$.

Regarding the distribution of seed number, Figure 2 showed that all streptomycin sulfate treatments of different concentration had very high percentages of seedless and 1 seed fruits, and significantly different from the control. The peak of seedless fruits percentage was found on 240 ppm streptomycin sulfate treatment. The treatment of 480 ppm streptomycin sulfate did not increase seedless fruits percentage any more. In contrast, the control had more 2-3 seeds fruit obviously, and the percentage of 2-3 seeds fruit was significantly higher than all streptomycin sulfate treatments.

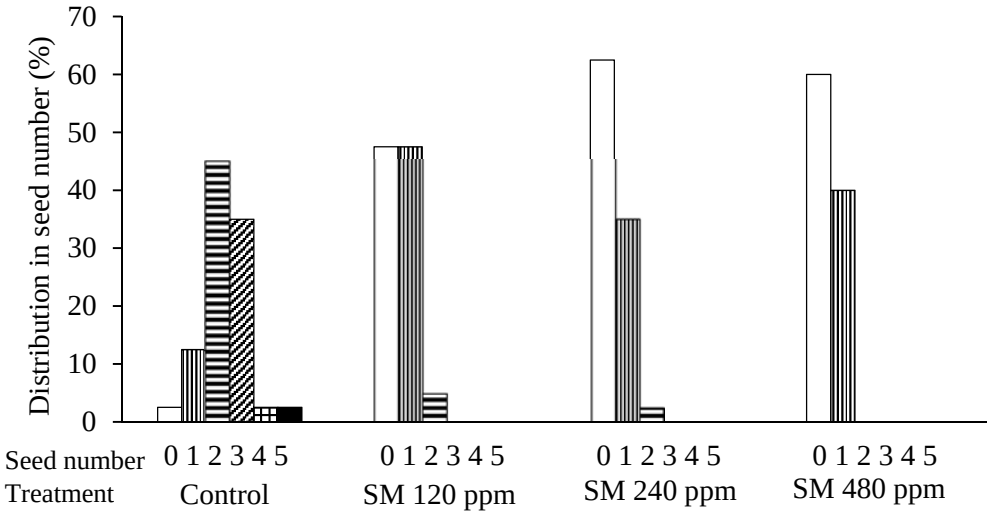

Fig. 2. Effects of streptomycin sulfate on distribution of seed number in oval kumquat fruits.

For the fruit quality (Table 4), fruit weights of streptomycin sulfate treatments were significantly lower than the control. The fruit weight of 3 streptomycin sulfate treatments were 6.8 g, 6.6 g and 6.4 g, respectively, markedly declined with the increase of streptomycin sulfate concentration. Fruit sizes in all streptomycin sulfate treatments were smaller than the control definitely. Fruit length and width of control were 30.4 mm and 22 mm, respectively, and streptomycin sulfate treatments were approximately 28 mm and 21 mm. No significant difference was found on fruit shape of 120 ppm, 240 ppm streptomycin sulfate treatments and control, while the fruits of 480 ppm streptomycin sulfate treatment showed a lower fruit shape index, i.e. the fruit shape tended to be round instead of oval. In terms of fruit quality, the total soluble solids and acidity were not significantly different among all treatments. Total soluble solids and acidity of all treatments were 7.4-7.9 °Brix and 1.7-1.9 mg/ 100 ml, respectively.

Table 4. Effects of streptomycin sulfate on fruit quality in oval kumquat.

Treatments [z]	Fresh weight (g)	Size		Fruit shape index (length/ width)	TSS (^{o}Brix)	Titratable acidity (mg/ 100 ml)
		Length (mm)	Width (mm)			
Control	7.7a[y]	30.4a	22.0a	1.4a	7.9a	1.8a
SM 120 ppm	6.8b	28.7b	20.9b	1.4a	7.8a	1.9a
SM 240 ppm	6.6bc	28.5b	20.8b	1.4a	7.6a	1.7a
SM 480 ppm	6.4c	28.2b	20.9b	1.3b	7.4a	1.8a

[z] Streptomycin sulfate were treated on the 7th day before flowering.

[y] Means within a column followed by the same letter are not significantly different by Duncan's MRT at $p \leq 0.05$.

Discussion

Pollens from streptomycin sulfate treated plants had less normal pollen, lower viability and germination rate, and shorter pollen tube. Streptomycin sulfate can inhibit pollen development and induce seedlessness of oval kumquat obviously. Results of this research are the same as the conclusions of Thanarut *et al*. (2010) on 'Kyoho' and 'Honey Red' grapes. Polito *et al*. (2002)

reported that although streptomycin sulfate inhibited pollen growth of walnut strongly *in vivo*, but no effect on pollen germination and pollen tube growth *in vitro*. The inhibition effect of streptomycin sulfate occurred in the style. Only few pollen tubes of streptomycin sulfate treated plants can grow and reach the ovary, whereas pollen tubes of control can grow through to the base of style at bloom period. The result of the present research showed that the inhibition effect of streptomycin sulfate on kumquat occurred *in vitro*, which was different from the result of Polito *et al*.(2002). Different species of fruit crops may have different response to the same biochemical treatment. Some previous research indicated that callose formation in incompatible pollen was higher than normal growth pollen (Tupy, 1959), and incompatible pollens of tomato had thick wall and callose deposition at the tip of pollen tube (De Nettancourt, 1973). The wavy wall structure and swollen tip found in abnormal pollen tube of this research were probably due to dense callose deposition also. Advanced observation and research need to be carried out to clarify the reason and mechanism.

Kimura *et al*. (1996) pointed out pollen germination and tube growth were not affected by streptomycin sulfate treatment alone. However, when plants were treated with streptomycin sulfate combined with GA_3 at 1-2 weeks before anthesis could inhibit pollen tube growth in pistil and pollen germination in agar medium on 'Muscast Bailey A' grape cultivar. Pollen germination and pollen tube growth were inhibited apparently by streptomycin sulfate treatment alone in this experiment. The effect of streptomycin sulfate on pollen germination and pollen tube growth may different in various fruit crops.

Seedlessness is one of the most important attributes for concern as far as the quality of all fruits for fresh consumption or juice production. For some seedless citrus cultivars, when grown close to sexually compatible varieties, cross pollination may occur and fruits with seed are produced. During pollination phase, the natural mechanism of self-incompatibility occurred *in vitro* and *vivo* on mandarin orange, due to pollen tube growth was inhibited by some substance produced during pollen germination. Mesejo *et al*. (2006) reported that foliar spray of $CuSO_4 \cdot 5H_2O$ at full bloom stage could reduce the number of seeded fruit and number of seeds per fruit under cross pollination conditions. Results of this experiment showed that streptomycin sulfate applied prior to pollination reduced pollen germination and pollen development *in vitro*. Streptomycin sulfate clearly inhibited pollen development as in the presence of abnormal pollen grains.

Pollens treated with streptomycin sulfate had a lower germination rate and wavy wall of pollen tube as well. Lo *et al*. (2007) reported that pollen tube growth and reaching the ovule were usually due to an accumulation of cells. Mesejo *et al*. (2006) indicated that $CuSO_4 \cdot 5H_2O$ prevented pollen tubes from reaching the embryo sac only at the period 24 hours after application,

and no difference was found after pollination *in vivo* comparing to control. More advanced research need to be done to figure out the phenomena and physiological mechanism of seedless fruits formation in kumquat, other citrus fruits or some important tropical and subtropical fruits in Taiwan and Thailand.

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鏈黴素對長實金柑(*Fortunella margarita* Swingle)無子化之影響

石 巧 月[1] 李 金 龍[2] 楊 耀 祥[3]

關鍵字：無子果實、種子數、授粉、果實品質

摘要：為探討鏈黴素(streptomycin sulfate)誘導長實金柑之無子化，本研究以 10 年生盆栽植株為供試材料，在開花前 7 日分別以 120、240 及 480 ppm 之鏈黴素噴施枝梢後，調查花粉活力、花粉管生長、種子數及果實品質。由結果可知，鏈黴素處理會誘使正常花粉數減少、花粉發芽率降低，同時花粉管會變短、波狀且硬化而呈節腫之異狀。該情形在不同濃度鏈黴素處理者之間並未見明顯的差異。每個果實之正常種子數，對照組為 2.7 個種子，鏈黴素處理者相對較少，只有 0.4~0.6 個種子。相反地，鏈黴素處理者之異常種子數為 1.2~1.3，較對照組的 0.5 個高，但是並未發現不同濃度鏈黴素處理後呈現明顯的差異。另外，對照組有高比率的果實有 2~3 個種子，而鏈黴素處理者大部分的果實為無子或只有 1 個種子。至於無子率，對照組為 16.8%，鏈黴素處理者為 70.3~76.6%，不同濃度鏈黴素處理後並未呈顯著差異。除此之外，鏈黴素處理後，發現其果實較小之外，其他品質並未受到影響。

1) 國立中興大學園藝學系博士班學生。
2) 國立中興大學講座教授。
3) 國立中興大學園藝學系教授，通訊作者。