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Influence of Storage Time on Pollen Traits in *Actinidia eriantha*

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Abstract

We examined the influence of storage time on germinability and tube growth of freeze stored pollens collected from 25 wild male plants in *Actinidia eriantha* variety. Pollens were stored in freezer at −20˚C for six months and one year periods to determine changing at germinability in time. *In vitro* germination was conducted in certain cultural medium defined for *Actinidia* genus. The results showed that the germination percentages and tube lengths of genotypes decreased at the end of storage period. MH22, MH45, MH47, MH56, MH67, MH70, MH71, MH72, MH74, MH55 and MH61 genotypes were evaluated as vigor genotypes, because they maintained their viability and germination capability displaying statistically insignificant decreasing although their tube lengths significantly decreased except MH67. This investigation provided to determine some robust wild male germplasm resources in *A. eriantha* in point of durability of pollens against long term conservation for using at future pollination and breeding programs.

Keywords

***Actinidia eriantha*, Genotype, Freeze Storage, Storage Time, Pollen Germination, Pollen Tube Growth**

1. Introduction

Kiwifruit (genus *Actinidia*) is well known for its great medicinal importance and has been considered for various treatments and onsets [1]. The variety *Actinidia eriantha Benth* is a valuable breeding material, as the vitamin C content of the fruit is 1013.98 mg/100g fresh weight (f.wt.), which is much higher than that of *A. deliciosa* (200 - 370 mg/100g f.wt.) and *A. chinensis* (250 - 300 mg/100g f.wt.) [2]. Its fruit size is the third largest for variety in

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this genus [3]. Therefore, cultivated and wild germplasm resources of *A. eriantha* variety carry considerable importance to utilize in breeding and propagation studies for getting productive, high quality and new varieties. High quality and yielding at fruit production absolutely depend on good fertilization following successful pollination. In this regard, the pollens of *A. eriantha* variety as genetic source were also used at researches made on pollen morphological and physiological traits.

Recently the studies on pollen physiological traits especially germination and viability have received substantial attention for their application in plant breeding, conservation and so on [4]. Pollen has remarkable potential to achieve genetic transformation [5]. Long-term storage of pollen grains is a useful method for conservation of genetic factors to use in the breeding programs; on the other hand, storage of pollen is necessary for controlled pollination, getting desired characters by breeding program and to overcome complications emerging at cultural implementations [6]. Studies on pollen traits especially germination percentage and tube growth in stored pollens should be carried out for their viability and longevity in different researches and horticultural exercises [7].

Effects of storage conditions on pollen viability have been reported by many researchers. For instance, according to an investigation about germination capacity of stored pollen of *Solanum melongena* L., maintaining the germination capacity of stored pollen can be useful in time saving in hybridization programs and also in crops improvement, the most important factors for successful pollen conservation are storage temperature and moisture content of material; lowering both tends to increase the period of viability so pollen stored in freezer (−30˚C, −20˚C) showed better germination percentage as compared to pollen stored at +4˚C and in organic solvents [4]. Pollens kept their viability high after 18 months of storage at −20˚C and also controlled pollination with pollens stored for 12 months resulted well in seed set which was comparable to that obtained using fresh pollen in *Salix* [8]. Another research on pear indicated that pollens from early blooming varieties can be stored for very long period without any appreciable loss of viability and germination so that pollen stored at low temperature (−120˚C and −20˚C) showed better viability and germination percentage as compared to pollen stored at room temperature and 4˚C [6]. Best results were obtained after 24 months at −20˚C in date palm pollens [9]. Pollen stored at low temperature (−30˚C, −20˚C) showed better germination as compared to pollen stored at 4˚C in the fresh *Abelmoschus esculentus* L. [5].

Although low temperature storage is an effective way to maintain viability for lots of species, long time preservation can cause decreasing in viability and germinability for some species. Such as pollen stored in freezer at −20˚C and −30˚C showed good germination but progressive storing time caused the gradually decreasing at germination percentage in *Pisum sativum* L. [10]. The viability showed a decreasing trend with increase in storage period and thus an inverse relation between viability and duration of storage was observed in three pear cultivars Pathernakh, Punjab Beauty and Shinseiki [6]. Similar observations were also obtained from cherimoya [11] and potato pollens [12].

Decreasing in pollen germination along long storage period can be derived from physiological or genetic causes. According to study related with *Anigozanthos manglesii*, year-long storage will probably require isolation of pollen because successful freezing requires rapid energy transfer between tissue and coolant, probably sufficiently to allow ice crystal formation and damage to the pollen [13]. Sometimes, cultivars produce high quantity of pollens but not with high quality such as low pollen germination percentage or low tube growth also, some of the pollens may be sterile or not viable [7].

Few studies have been published on *in vitro* viability and storage conditions of pollen grains of kiwifruit [14]. It was observed in kiwifruit, after 365 days of storage, the pollen grains had completely lost the ability to germinate in culture medium so strategies for short term conservation of pollen grain may be required, although the viability may be reduced during storage of pollen grains, which could decrease the efficiency of pollination [15].

In this study, we examined the influence of storage time on Pollen Germination (PG) and Pollen Tube Length (PTL) of freeze stored pollens collected from 25 wild male germplasm resources in *Actinidia eriantha* variety.

2. Material and Methods

The well-grown flower clusters were pruned off from twenty five wild male genotypes (MH10, MH22, MH26, MH30, MH31, MH34, MH41, MH43, MH45, MH46, MH47, MH48, MH55, MH56, MH57, MH58, MH60, MH61, MH66, MH67, MH69, MH70, MH71, MH72 and MH74) of *A. eriantha* variety growing at Magu Mountain region in Nancheng county, Fuzhou city, Jiangxi province, southeast of China (27˚25'N - 27˚32'N & 116˚27'E - 116˚32'E) at the beginning of flowering season in early May.

Flower clusters as experimental materials, which were immediately kept into cold preservation box after picked up in field, were transported to the laboratory of Agronomy Department, Jiangxi Agricultural University in Nanchang city. In the laboratory, Sepals and petals were separated and anthers were placed in the sterile labeled petri dishes. Anthers were kept in the laboratory at room temperature until dry in order to dehiscence and then pollens were collected into paper bags by vibrating anthers with traditional hand method. Collected pollens were stored in freezer at −20˚C for six months and one year periods to determine changing at pollen germinability in time.

In vitro germination assays determine the actual germination ability of pollen under suitable conditions [16]. Pollen germination in cultural medium is a useful viability test. Results of many researches showed that calcium, boric acid, sucrose, temperature and pH had important roles in germination tests for different species and varieties. Such as, for almond pollens it was suggested that addition of B to the culture media significantly increased pollen germination and pollen tube growth [17]. The effect of either sucrose or boric acid individually showed good results, but sucrose in combination with boric acid promoted pollen germination as well as tube development, because boron makes a complex with sugar and this sugar-borate complex is known to be capable of better translocation than non-borate, non-ionized sugar molecules [18]. Calcium concentration also was important for pollen germination, but more critical for normal pollen tube growth and to consolidate the view that temperature was the critical factor determining reproducibility of *in vitro* pollen germination [19]. The role of calcium and boric acid were observed at stored almond and peach pollen [20]. The importance of boric acid, calcium, temperature and sucrose in culture medium was also declared for almond pollen [21]. The role of pH was investigated in banana [22].

The most suitable culture medium for kiwifruit pollen germination and tube growth was 10% sucrose + 100 $mg \cdot L^{-1}$ boric acid + 10 $mg \cdot L^{-1}$ $Ca(NO_3)_2$. The suitable culture time was 5 hours, the best temperature for pollen germination and tube growth was 30˚C and the suitable pH value of culture medium was 6 [23]. 8% agar was added into liquid culture medium including distilled water to solidify. Culture medium was heated in the microwave oven. A few drops hot culture medium were added on the microscope slides. Two slides were used for per genotypes. The pollens were gently dusted on the solid culture medium placed on slides using hair brash. The labeled microscope slides were put into the sterile petri dishes with moistened filter paper and incubated in dark. Pollen germination was observed under a light microscope (40 × 10) with Image-Pro Plus 6.0 software. Pollen was considered to have germinated when the length of the pollen tube was equal to or longer than the diameter of the pollen. Germination data were obtained on a sample of 100 pollen grains with 4 replications (four view points in two slides) by Analyzer software program. Mean pollen tube length of each genotype was recorded by averaging of 40 pollen grains using Tsview software program (10 mm = 100.000 photo pixel).

Statistical analysis were carried out using Microsoft Excel (2010), SPSS version 20 software with one way ANOVA and MANOVA test methods. Means were compared using Tukey test ($p \leq 0.05$).

3. Results and Discussion

Pollen grains, being the sexual reproductive unit and the carrier of male genetic material in higher plants, play a vital role in breeding programme and assist successful fruit-set. High crop yield generally depends on viable pollen grains. Pollen fertility and viability have a paramount importance in hybridization programme. Pollen performance in terms of germination ability may have the relative importance with fruit-set [24]. In this research, main pollen traits including germination, tube growth and longevity of 25 wild male plants in *A. eriantha* were examined to determine germinability at two different periods of time intervals as six months and one year in freeze storage (−20˚C). It was said in the research of *in vitro* germination and pollen conservation of some *Musa* species that wild plants are often considered as genes bank and useful to solve problems in cultivated species [25]. Some other works were carried out by several researchers such as in *Arabidopsis thaliana* [26] and *Morus alba* [27].

In the first experiment, mean pollen germination percentages (%/˚C) and tube lengths (10 mm = 100.000 photo pixel/˚C) of 25 wild male genotypes in *A. eriantha* were compared after six months storage by variance analysis (**Table 1**). The genotypes were separated into nine different statistical groups (a, b, c, d, e, f, g, h, i) in the pollen germination percentages according to variance analysis. Pollen germination was ranged between 25% and 91.50%. As result, MH48 (25%) and MH67 (26.25%) were in the lowest group (a). MH74, MH57 and MH30 were in the second group (ab) with low values. MH66 alone had highest germination percentage in the last group (i). Pollen germination percentages of MH58, MH10, MH43, MH41, MH69, MH70, and MH71 were

Table 1. Comparison of mean pollen germination percentages (%/˚C) and tube lengths (10 mm = 100.000 photo pixel/˚C) of 25 wild male genotypes in *A. eriantha* after six months storage.

Germination percentage			Pollen tube length		
Genotype	N_1	Result	Genotype	N_2	Result
MH48	4	25.00a	MH74	40	26.22a
MH67	4	26.25a	MH57	40	26.53a
MH74	4	37.75ab	MH34	40	32.05ab
MH57	4	39.00abc	MH48	40	32.17ab
MH30	4	44.75abcd	MH72	40	32.66ab
MH26	4	51.00bcde	MH47	40	32.77ab
MH47	4	51.50bcde	MH45	40	35.23abc
MH56	4	52.50bcde	MH67	40	35.46abc
MH31	4	55.00bcde	MH26	40	38.48abcd
MH60	4	56.25bcdef	MH56	40	40.00abcd
MH34	4	56.75bcdef	MH41	40	40.09abcd
MH72	4	56.75bcdef	MH10	40	43.54bcd
MH22	4	57.00bcdef	MH61	40	44.34bcd
MH46	4	57.75bcdef	MH55	40	45.51bcd
MH61	4	62.75cdefg	MH46	40	46.36bcd
MH55	4	65.75defg	MH22	40	48.07cd
MH45	4	68.00defgh	MH60	40	48.44cd
MH71	4	69.25defghi	MH71	40	51.09de
MH70	4	69.75defghi	MH69	40	53.04def
MH69	4	73.00efghi	MH70	40	62.52efg
MH41	4	74.50efghi	MH31	40	65.77fg
MH43	4	79.50fghi	MH30	40	68.08g
MH10	4	82.75ghi	MH43	40	81.30h
MH58	4	89.50hi	MH66	40	86.52h
MH66	4	91.50i	MH58	40	88.95h

"N1" expresses total replication counts; "N2" expresses total pollen grain counts.

ranged between 89.5% and 69.25% following MH66. On the other hand, the genotypes were separated into eight statistical groups in pollen tube length. The tube length was ranged between 26.22 mm (MH74) and 88.95 mm (MH58). MH57, MH34, MH48, MH72 and MH47 showed low values in two groups "a" and "b" following MH74. MH43 (86.52 mm) and MH66 (81.30 mm) displayed highest values in same group with MH58.

In the second experiment, mean pollen germination percentages and tube lengths of genotypes were compared by variance analysis after one year storage (**Table 2**). MH10, MH30, MH31, MH34, MH43, MH57, MH58 and MH66 completely lost their viability at the end of one year storage period. Pollen germination percentage was ranged between 7.5% (MH26) and 61.25% (MH45). Pollen tube length was ranged between 7.09 mm (MH46) and 35.74 mm (MH55).

In the third experiment, two storage terms (six months and one year) were compared in mean pollen germination percentages and mean pollen tube lengths to determine statistical differences for each genotype by variance analysis (**Table 3**). Also proportional percentage changing in germination percentages and tube lengths from sixth month to one year was calculated for each genotype (**Table 4** and **Table 5**).

Table 2. Comparison of mean pollen germination percentages (%/˚C) and tube lengths (10 mm = 100.000 photo pixel/˚C) of 25 wild male genotypes in *A. eriantha* after one year storage.

Germination percentage			Pollen tube length		
Genotype	N_1	Genotype	Genotype	N_2	Result
MH10	4	0.00a	MH10	40	0.00a
MH30	4	0.00a	MH30	40	0.00a
MH31	4	0.00a	MH31	40	0.00a
MH34	4	0.00a	MH34	40	0.00a
MH43	4	0.00a	MH43	40	0.00a
MH57	4	0.00a	MH57	40	0.00a
MH58	4	0.00a	MH58	40	0.00a
MH66	4	0.00a	MH66	40	0.00a
MH26	4	7.50ab	MH46	40	7.09b
MH60	4	7.75ab	MH60	40	9.49b
MH46	4	10.25ab	MH26	40	11.83bc
MH48	4	12.25ab	MH48	40	16.78cd
MH67	4	18.25abc	MH56	40	18.46d
MH69	4	26.75abcd	MH69	40	18.53d
MH74	4	28.50bcde	MH74	40	19.07de
MH41	4	33.75bcde	MH45	40	19.21de
MH72	4	43.25cdef	MH41	40	21.44def
MH61	4	44.00cdef	MH70	40	23.21def
MH47	4	45.25cdef	MH47	40	25.48efg
MH55	4	46.75def	MH72	40	25.86fg
MH70	4	47.75def	MH22	40	26.41fg
MH56	4	49.75def	MH61	40	27.18fg
MH71	4	51.50def	MH71	40	29.98gh
MH22	4	55.50ef	MH67	40	35.23h
MH45	4	61.25f	MH55	40	35.74h

"N1" expresses total replication numbers; "N2" expresses total pollen grain numbers.

In the result, the differences between mean pollen germination percentages among six months and one year storages were found significant according to variance analysis for other genotypes except MH22, MH45, MH47, MH56, MH67, MH70, MH71, MH72 and MH74 genotypes. The proportional percentage changing of decreasing pollen germination percentage from sixth month to twelfth month in freeze storage for MH22, MH45, MH47, MH56, MH67, MH70, MH71, MH72 and MH74 were 2.63%, 9.93%, 12.14%, 5.24%, 30.48%, 31.54%, 25.63%, 23.79% and 24.50% respectively. In pollen tube length, only MH67 (from 35.46 mm to 35.23 mm) had not statistically significant difference with 0.9% changing from 6^{th} month to 12^{th} month.

Table 3. Paired comparison of two storage terms in mean PG percentage (%) and PTL (10 mm) for each *A. eriantha* genotype.

Genotype	Germination rate differences	SD	Tube length differences	SD
MH10	82.750*	3.637	43.542*	4.318
MH22	1.5	11.63	21.665*	3.588
MH26	43.500*	6.513	26.648*	2.774
MH30	45.750*	2.428	68.082*	3.76
MH31	55.000*	2.273	65.780*	3.804
MH34	56.750*	5.75	32.057*	1.63
MH41	40.750*	6.466	18.654*	2.369
MH43	79.500*	1.323	81.301*	3.453
MH45	6.75	12.592	16.025*	2.057
MH46	47.500*	7.38	39.273*	2.757
MH47	6.25	9.486	7.292*	2.513
MH48	12.750*	3.224	15.394*	2.411
MH55	19.000*	6.052	9.773*	3.183
MH56	2.75	10.423	21.538*	2.249
MH57	39.000*	8.377	26.530*	2.154
MH58	89.500*	1.041	88.958*	6.999
MH60	48.500*	4.56	38.947*	2.645
MH61	18.750*	5.483	17.159*	2.533
MH66	91.500*	1.708	86.529*	5.384
MH67	8	3.932	0.224	4.36
MH69	46.250*	5.202	34.505*	3.638
MH70	22	12.143	39.304*	3.129
MH71	17.75	7.526	21.117*	2.85
MH72	13.5	8.895	6.803*	2.365
MH74	9.25	3.934	7.145*	1.901

In the fourth experiment, differences between mean germination percentages and mean pollen tube lengths of variety were compared at the end of storage period (**Table 6** and **Table 7**). Significant differences ($p = 0.02$) were found for both. Proportional percentage changing for PG and PTL were 60.52% and 69.58%, respectively. Average pollen germination percentage of variety decreased from 59.78% to 23.60% and pollen tube length of variety decreased from 48.78 mm to 14.84 mm from 6th month to 12th month.

Consequently, the germination percentages and tube lengths of genotypes decreased along long storage period. It was found in such investigation that the feijoa pollen partially lost its viability after been stored for 90 days in a freezer and had a great loss after 150 days [28]. In present work, Germination percentages and tube lengths of MH10, MH26, MH30, MH31, MH34, MH41, MH43, MH46, MH48, MH55, MH57, MH58, MH60, MH61, MH66 and MH69 genotypes significantly decreased from sixth month to twelfth month, some of them completely

Table 4. Effect of storage time on pollen tube lengths (10 mm) of 25 wild *A. eriantha* genotypes.

Genotype	PTL (10 mm)		Mean differences	(%) Differences
	6 months	1 year		
MH10	57.64	0	−57.64	100
MH22	48.08	26.41	−21.67	45
MH26	38.49	11.84	−26.65	69.24
MH30	68.08	0	−68.08	100
MH31	65.78	0	−65.78	100
MH34	32.06	0	−32.06	100
MH41	40.09	21.44	−18.65	46.60
MH43	81.30	0	−81.30	100
MH45	35.24	19.21	−16.03	45.49
MH46	46.37	7.09	−39.28	84.71
MH47	32.78	25.48	−7.30	22.27
MH48	32.18	16.78	−15.40	47.86
MH55	45.52	35.74	−9.78	21.68
MH56	40.00	18.47	−21.53	53.82
MH57	26.53	0	−26.53	100
MH58	88.96	0	−88.96	100
MH60	48.44	9.50	−38.94	80.39
MH61	44.35	27.19	−17.16	38.69
MH66	86.53	0	−86.53	100
MH67	35.56	35.24	−0.32	0.90
MH69	53.04	18.53	−34.51	65.06
MH70	62.52	23.22	−39.30	62.86
MH71	51.10	29.98	−21.12	41.33
MH72	32.67	25.86	−6.81	20.84
MH74	26.22	19.08	−7.14	27.23
Average	48.78	14.84	−33.94	69.58%

lost their viabilities. On the other hand, MH22, MH45, MH47, MH56, MH70, MH71, MH72 and MH74 genotypes had not significant differences in PG, although they had significant differences in PTL. Only MH67 had not significant differences in both pollen germination and pollen tube length, so these genotypes evaluated as vigor genotypes because the changing at their viability didn't have statistically importance among freeze storage.

In this study, the variation in pollen traits of wild genotypes may be due to genetic diversity.

Table 5. Effect of storage time on pollen germination percentages (%) of 25 wild *A. eriantha* genotypes.

Genotype	PG (%)		Mean differences	(%) Differences
	6 months	1 year		
MH10	82.75	0	−82.75	100
MH22	57	55.5	−1.5	2.63
MH26	51	7.5	−43.5	85.29
MH30	45.75	0	−45.75	100
MH31	55	0	−55	100
MH34	56.75	0	−56.75	100
MH41	74.5	33.75	−40.75	54.70
MH43	79.5	0	−79.5	100
MH45	68	61.25	−6.75	9.93
MH46	57.75	10.25	−47.5	82.25
MH47	51.5	45.25	−6.25	12.14
MH48	25	12.25	−12.5	50.00
MH55	65.75	46.75	−19	28.90
MH56	52.5	49.75	−2.75	5.24
MH57	39	0	−39	100
MH58	89.5	0	−89.5	100
MH60	56.25	7.75	−48.5	86.22
MH61	62.75	44	−18.25	29.08
MH66	91.5	0	−91.5	100
MH67	26.25	18.25	−8	30.48
MH69	73	26.75	−46.25	63.36
MH70	69.75	47.75	−22	31.54
MH71	69.25	51.5	−17.75	25.63
MH72	56.75	43.25	−13.5	23.79
MH74	37.75	28.5	−9.25	24.50
Average	59.78	23.6	−36.18	60.52

Table 6. Independent sample test (bootstrap), comparison of mean PTLs (10 mm = 100.000 photo pixel/˚C) of *Actinidia eriantha* variety along storage period.

Pollen tube length		Mean differences	Bootstrap				
			deviation	Standard error	Sig.	95% Confidence	
						Low	Up
PTL	Variance equal	33.93880	−0.29668	4.64815	0.020	24.06459	45.67892
	Variance not equal	33.93880	−0.29668	4.64815	0.020	24.06459	45.67892

Table 7. Independent sample test (bootstrap), comparison of mean PG percentage (%/˚C) of *Actinidia eriantha* variety along storage period.

Pollen germination percentage		Mean differences	Bootstrap				
			deviation	Standard error	Sig.	95% Confidence	
						Low	Up
PG	Variance equal	36.18000	−1.09757	6.31265	0.020	22.33194	49.53085
	Variance not equal	36.18000	−1.09757	6.31265	0.020	22.33194	49.53085

4. Conclusion

This investigation demonstrates that germination ability of each wild genotype from *Actinidia eriantha* variety which was grown in the same origin area was different against long term freeze storage. Our investigation can be used as a basis source for further researches on genetic diversity of wild germplasm resources especially in *A. eriantha* variety as a plentiful source in natural distribution area.

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