Measurement and marker-trait association analysis of pollen viability in the K5 tetraploid rose population

By

Xiao Tingting

(Regd.No. 840712979080)

Supervisors:

Dr F.A. Krens

Dr C.A. Maliepaard

Ir C.F.S. Koning-Boucoiran

MSc V.M. Gitonga

Examiners:

Dr F.A. Krens

Dr C.A. Maliepaard

Department of Plant Breeding

Wageningen University

November, 2009

Acknowledgement

Here I would like to thank my supervisor Dr. Frans Krens for giving me this opportunity to study in his group. At the beginning of my experiment, I encountered some problems, you tried to encourage me and gave me valuable advices. Furthermore, you also helped me with lab work. It would have been hard for me to overcome all the difficulties without your guidance. I also appreciate your critical reading and comments on my report. I learnt a lot from you. Sir, thank you.

I would also like to express my appreciation to Dr. Chris Maliepaard. You helped me to understand how TetraploidMaps are generated. You are so kind. I learn a lot statistical knowledge from you. Every time after our meeting, you either help me to solve problems or help me to get new ideas. I never came back with nothing. Although you become my supervisor in the last two weeks, the help you have given to me was precious. Thank you so much.

I would also like to thank my daily supervisor Ir Carole Boucoiran and Msc Virginia Gitonga. Dear Carole, thank you very much. During the period of my thesis, you gave me lots of help. Although you were very busy and bothered by your physical condition, you always tried your best to give suggestions when I asked for help. That was really touching. Thank you. And dear Virginia, you give me so much encouragement during those time, especially when I felt hopeless. You really like my older sister. You taught me how to face problems and how to find the way to solve problems not only in research but also in life. Thanks a lot.

I also want to thank to my parents and my sister here. I could study abroad all because of their help, support and encouragement. I love my family. This is what I want to say at the moment.

Also there are so many thanks to my teachers and my friends here, especially Songlin and Ningwen. From teachers, I learnt knowledge about sciences and how to do research. From friends, I got different ideas and knowledge also. Without your support, I could not finish this. Thank you to everyone.

Abstract

In this research the K5 tetraploid rose population was being used to study pollen viability. Two methods, acetocarmine staining and pollen germination, were used to check pollen viability. By comparing the results, we observed acetocarmine staining to be unreliable as indicator to detect pollen germination. However, to a certain extent, the percentage of middle size (35-45μm) pollen is positively correlated to pollen germination. In the K5 rose population, pollen from flower development stage 1 (semi-open) has the highest pollen germination. These pollen germination data were used in marker-trait association analysis. E32M48-087 and M3-06 markers were detected as significantly correlated to pollen germination. Additionally, TetraploidMap program was tested in this research to generate a genetic linkage map for this population.

Keywords: acetocarmine staining, pollen germination, TetraploidMap, marker-trait association

Contents

1 Introduction

1.1 Pollen viability

Many plants release viable pollen after anther maturation. However, this is not always the case, many plants producing sterile pollen grains or do not produce any pollen at all. This phenomenon has multiple reasons, such as adverse growth conditions, diseases or mutations. Inability to produce or release functional gametes in plants is called male sterility (Alonso 2002). Pollen viability is important for plant sexual reproduction via pollination and subsequent fertilization, leading to fruits and berries for example. Pollen inviability can be caused in many ways: genetic or environmental introduced pollen inviability. One genetic reason, reported by Pagliarini (2000), described that disturbed meiosis can influence pollen viability. Another reason is gene controlled pollen inviability, such as, genes from male gametophyte. Environmental causes include: sensitivity of pollen to temperature, humidity or length of store time (Issarakraisila and Considine, 1994, Zlesak *et al.,* 2007). In tetraploid plants, Zhang et al. (2007) reported abnormal segregation in each stage of meiosis of autotetraploid broccoli pollen mother cell is 31.5%, which means that abnormal meiosis is the main reason of fertility reduction of broccoli. Similar results were also published by Buyukkartal and Colgecen (2007) on tetraploid *Trifolium pratense* L., where abnormal cell division caused sterile pollen.

Although pollen inviability as a kind of male sterility is important for plant hybrid breeding, this could also become an undesirable character for breeders, because they need a large number of new combinations of parental genes. In rose breeding, low seed production in crosses, between rose breeding lines or cultivars, is a widely occurring phenomenon and presents a major problem. Besides insufficient pollination (De Vries and Dubois, 1983), low temperature during pollination (Visser *et al*., 1977), low fertility of the crossing parents is another major reason for seed production. Low pollen viability is a big problem for interspecific rose hybrids, when parents are often of different ploidy levels. The main reason is disturbed meiosis (Kroon and Zeilinga, 1974; Werlemark *et al*., 1999). In interspecific tetraploid rose which is generated from same ploidy levels abnormal meiotic behavior also occurs (Yan *et al.,* 2000). But the frequency of abnormal meiosis in interspecific tetraploid roses has not yet been reported.

1.2 Genetic studies of rose

Wild rose species are usually diploid ($2n = 2x = 14$), while almost all cultivated roses are tetraploid ($2n = 4x = 28$). Most modern cultivated roses are complex hybrids and are considered to be derived from about ten wild species (Zhang 2003). Despite the commercial importance of rose, little is known about the inheritance of interesting agricultural characteristics in rose. Roses are difficult in sexual reproduction. Low seed production and poor seed germination caused by inbreeding depression lead to difficulties in genetic population development. Another limitation for genetic studies is the high level of heterozygosity and varying ploidy levels between species (Dugo *et al.* 2005).

There are several genetic maps of diploid populations published (De Vries and Dubois 1984). Since most of the modern roses are tetraploid, genetic maps of tetraploid roses are required. The heterozygosity and ploidy level have proven to be barriers for mapping of tetraploid roses. Firstly, “pseudo-testcross” strategy (Grattapaglia and Sederoff 1994) was used successfully in rose (Debener and Mattiesch 1999; Rajapakse *et al.* 2001; Crespel *et al.* 2002) for overcoming the heterozygosity. Moreover, ploidy level has become a barrier on mapping due to several reasons. Firstly, the relationship between linked markers, which refer to linkage phase (coupling: markers from same homologous chromosomes, repulsion: markers from different homologous chromosome), is more complex than diploid. Furthermore, if markers are not single dose, the phase problem is more obvious. Hackett *et al.* (1998) showed that a reliable phase can only be obtained from markers between simplex–simplex (coupling and repulsion), duplex–simplex (coupling and repulsion) and duplex–duplex (coupling) in tetraploid. All of these factors could influence marker configurations. Another problem is distorted segregation of markers. One reasons for distorted segregation of markers is double reduction. In the meiosis process of a tetraploid plant, the sister chromatids can end up in a same gamete and introduce more homozygous gametes. In tetraploid rose, double reduction can influence markers segregation (Yan *et al.* 2005a). Another reason for distorted segregation of markers is lack of complete homology resulting in non-random pairing of chromosomes. In meiosis, when four homologs are pairing, highly heterologous chromosomes results in the same chromosome pairs always being formed and disomic inheritance.

Bi-parental simplex markers, duplex markers as well as triplex markers were employed in tetraploid sugarcane (Da Silva *et al.* 1993), alfalfa (Yu and Pauls 1993) and potato (Meyer *et al.* 1998) to identify and merge homologous linkage groups. Uni-parental simplex AFLP and SSRs markers were used in rose by Rajapakse *et al.* (2001), due to lack of common markers they were unable to link the two maps together to form an integrated map and ended up with two separate genetic maps for the two parents. Zhang *et al.* (2006) increasing the number of SSR markers and integrated four out of seven rose chromosomes of the two parental tetraploid maps. The second tetraploid population (K5) was generated by Yan *et al.* (2005b). They followed the same tetraploid mapping method used by Rajapaksa *et al.* along with AFLP and SSR markers. QTLs for powdery mildew resistance were also detected and mapped to this linkage map.

1.3 Background of this study

The underlying mechanism of male sterility in rose has not been studied to date in heterozygous tetraploid rose. Whether the main cause of male sterility has a genetic basis or comes from abnormal chromosome behaviour during meiosis or other reasons, has not been identified. The K5 population is suitable to study pollen viability in tetraploids. This population was obtained by crossing two tetraploid parents ($2n=4x=28$) and used as a mapping population in our group. To study the segregation of pollen viability in this population, a reliable protocol to measure pollen viability should be established.

In this study, two methods were used to investigate pollen viability of the K5 population, acetocarmine staining and pollen germination. Staining is a fast method to estimate pollen viability, and acetocarmine gives more stable results than other staining methods such as 3,5-triphenyl tetrazolium chloride, methyl green/phloxin (Vieitez, 1953 and Owczarzak, 1952). Acetocarmine staining is a method for staining the nucleolus in plant and animal cells (Rattenbury, 1952). Viable pollen grains will show a dark orange colour, while non-viable pollen grains will give a pale red colour or be colourless (Jones, 1955). The pollen germination method, in which pollen grains are placed on an artificial medium and induced to germinate, is widely used to test pollen viability. Several studies reported that sucrose and boric acid at different concentrations in the medium could stimulate or inhibit the germination percentage. Voyiatzi (1995) showed that calcium nitrate reduced the germination percentage in five rose tea hybrid cultivars, whereas Brewbaker and Kwack (1964) showed that calcium improves pollen germination due to its action on pectin of the pollen tube wall. In this study we used the optimal sucrose and boric acid concentration suggested by Voyiatzi (1995) and Richer *et al.* (2007). We also included calcium in order to test its effect on germination in rose.

Flower development is a commonly used indicator for pollen collecting (Devries and Dubois 1983; Marchant *et al.,* 1993, and Richer *et al.,* 2007). The development stage of the flower could seriously influence the germination. For example, in the research of Richer *et al.* on three rose cultivars, low germination was shown in fully opened flowers. The optimal pollen collection stage was before the flowers fully opened. On the other hand, Visser *et al.* (1997) suggested that collecting pollen at a fresh opening stage on hybrid rose was the optimal. Pearson and Haney (1984) reported collecting pollen when buds are fully coloured and one or two petals are open on *R. hybrida.* In this study, the optimal developing stage for pollen collection of the K5 tetraploid rose population also needed to be determined and we used the classification procedure of flower stages from Richer *et al.* (2007). In addition, anther development was also observed, because anther development correlated to pollen development more tightly than flower development.

Pollen germination was used in a marker-trait association analysis, in order to get a general idea of genetic factors involved in pollen germination. Our marker data combines all AFLP and SSR marker data generated by Yan (2005b) and the SSR markers from Verlinden (2007). Furthermore, we included all NBS markers from Koning-Boucoiran *et al*. (2007).

TetraploidMap is a program designed especially for tetraploid plants. In this study, I tried to use TetraploidMap on the K5 tetraploid rose population for the generation of genetic maps using the molecular markers mentioned above.

The objectives of this study are 1) to see the reliability of acetocarmine staining as a method to assess pollen viability by determining the relationship with pollen germination 2) to assess differences between flower development stage and anther development stages with regard to pollen germination, in order to identify the optimal development stage for pollen collecting 3) generate a genetic linkage map using TetraploidMap using the markers segregating in the K5 population and 4) find markers correlated to pollen germination by doing a marker-trait

association analysis in the TertraploidMap program.

2 Materials and methods

2.1 Plant material

The tetraploid cultivar P540 was used as the female parent in a cross with another tetraploid cultivar, P867; the F1 progeny consisted of 175 genotypes (K5 population) and was grown in the greenhouse for experimental use.

Pollen collection

The flowering stages were separated into four groups (0, 1, 2, 3) according to the flower developmental stages as described by Richer *et al.* (2005) (Table 1, Figure 1a).

Table 1: Classification of flowering stages

Flower Stage	Flower appearance
0	Closed bud
1	Closed petals but opened sepals
2	Bud with petals three-quarters open
3	Fully open

Flowers in different stages were collected in the greenhouse and taken to the lab, petals of each flower were removed and anthers were collected into 5-cm^2 Petri dishes by using a pair of tweezers.

Anther Observation

Anthers were checked under a microscope after being collected, in order to define their development stage. The anther development was also classified into four stages (A, B, C, D) according to morphological traits (table 2 Figure 1b-d).

Table 2: Classification of anther development stages

Anther Stage	Anther appearance
A	Closed anthers: anthers are smaller than mature ones
B	Anthers start opening: less than 30% or around 30% are opened
C	Opening: around 40% to 70% are opened
D	Opened: around 70% or more than 70% are opened

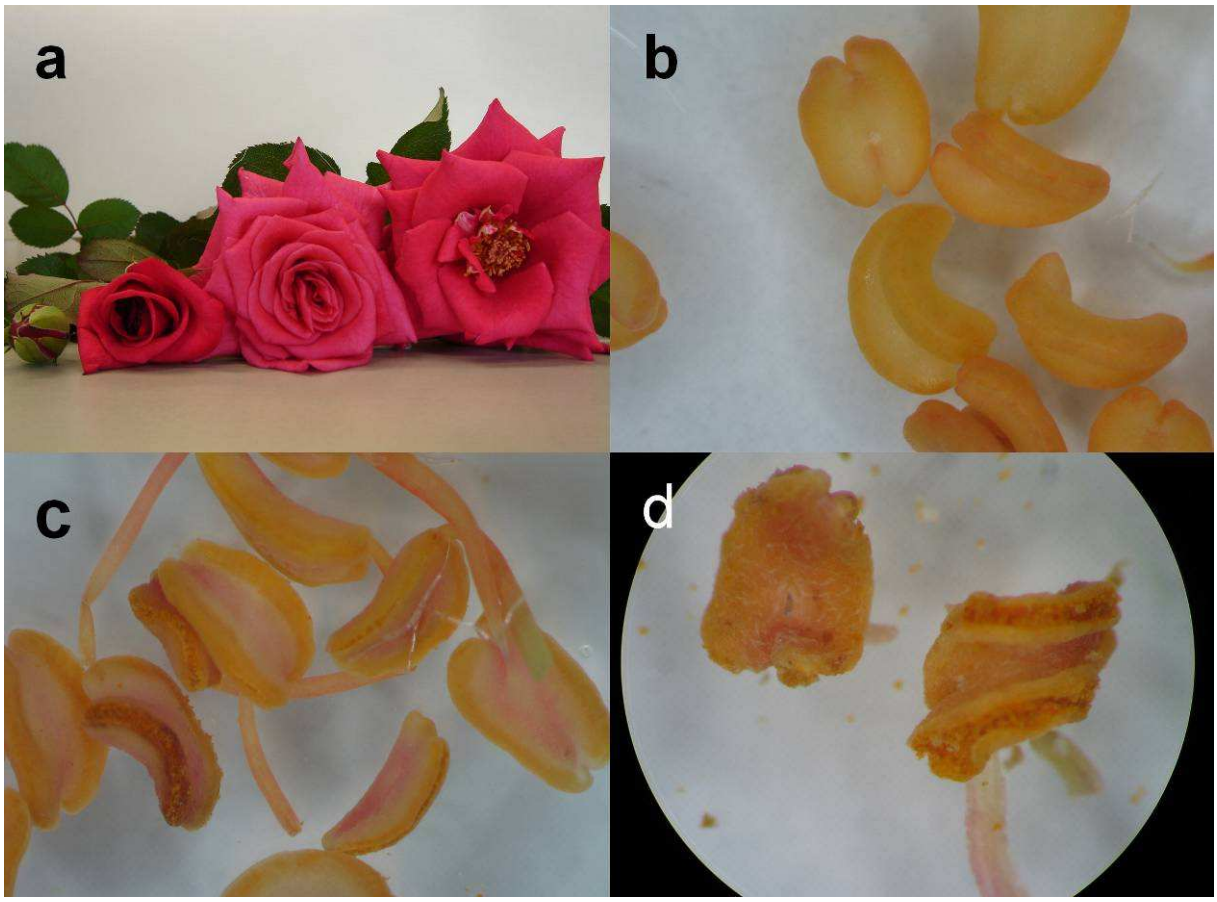

Figure: 1 Morphological traits of different flower and anther stages (a) shows flower stages, from left to right stage: 0, 1, 2 and 3; (b), (c) and (d) show anther stages A, C and D respectively.

2.2 Pollen staining

One hundred and seventy five individuals of the K5 population as well as the parents were screened. Before staining, anthers from different flower developmental stages, stage I or stage 2 were dried by placing them separately in dark at room temperature for 24 hours (Richer *et al.*, 2005).

Staining procedure

1. Place 8 to 10 dried anthers onto a clean microscope slide with one or two drops of acetocarmine staining solution (appendix 1).
2. Release the pollen grains by pressing the anthers with a pair of tweezers.
3. Incubate for 10 min after placing the cover slip on the slide before observation.

Observation

The pollen grains were observed using a light microscope (Carl ZEISS B.V. 003-08967) with a magnification of 20x10. Size measurements were performed using an ocular equipped with a size bar. According to Crespel *et al.* (2006) pollen of tetraploid rose cultivars from 30 to 54µm with an average size 41.7±3.6µm. In order to identify the relationship between pollen size and

germination (the next step of this study), pollen were separated into two groups depending on shape: normal shape; abnormal shape. Normal shape pollen was separated into three groups: Large (>45μm), medium (35μm-45μm), small (<35μm) and abnormal. Five views per sample were randomly chosen, and the number of each type of pollen was counted.

2.3 Medium selection

Based on the medium of Voyiatzi (1995) and Richer *et al.* (2007) which was developed for rose, four different media were designed and tested (table 3). Forty-one individuals from the K5 population were randomly selected and tested on the four types of media (Table 3). There was no replication for genotypes. Subsequently, the best medium will be selected and used in further germination tests.

Table 3: Medium recipes for germination test

Medium No.	Sucrose (g/L)	Boric acid (mg/L)	Calcium nitrate (mg/L)	Micro agar (g/L)	PH	Germination temperature (℃)	Germination time (hour)
1	250	10	0	10	6.0	28	24
2	250	50	0	10	6.0	28	24
3	250	50	1	10	6.0	28	24
4	250	50	1.5	10	6.0	28	24

2.4 Germination test

Germination procedure

Dried pollen was collected from Petri dishes by gently dabbing them with a cotton swab. Then, the cotton swab was rubbed gently on the surface of the medium to allow the pollen to spread evenly. Caution should be taken to spread pollens evenly for proper observation. Before observation, Petri dishes were wrapped with aluminum foil, and incubated upside down at 28℃ for 24 hours.

Germinated pollen observation

Germination ratio was calculated by visual estimation and counting. Counting was done by drawing circles on the back side of the Petri dishes and counted the pollens inside these circles (more than 200 pollen were counted); the ratio was calculated as the number of germinated pollen divided by the total number of counted pollen × 100.

In order to exclude no-germinated pollen that was caused by indisposed of the medium ingredients, only genotypes showed more than 10% acetocarmine stainability (n=50) were selected for germination analysis. A few progeny that showed lower than 10% stainability (n=21) were included as well. The medium that was found to give the best germination was used in further germination tests.

2.5 Map construction

Linkage analysis was carried out using the software program TetraploidMap (Hackett 2005). In total, marker patterns of 345 molecular markers on the 171 individuals were used for the map construction.

Segregation assessment and preliminary clustering of marker data

The segregation ratio for each marker in the offspring was assessed using TetraploidMap and tested with Chi-square tests for different possible segregation ratios: 1:1, 3:1, 5:1, 11:1 and 35:1. The possibility of occurrence of double reduction was detected by calculating the difference between the observed number of offspring phenotypes and expected number of phenotype based on the parental genotype (goodness of fit test). Markers with evidence of double reduction were excluded. For the single dose markers showing a 1:1 segregation, firstly Chi-squared test was used to check their independence, and then a preliminary cluster analysis was performed to identify which markers were linked in coupling phase. These markers were then given a code to stand for their coupling groups.

Selection of markers and grouping into linkage groups

Markers that did not satisfy any of the expected segregation ratios (1:1, 3:1, 5:1, 11:1, 35:1) or showed evidence of double reduction ($p<0.05$) were excluded.

Single linkage clustering and average linkage clustering analysis were both performed for determining the linkage groups, depending on the distances (distance=$1-10^{-2*\text{significance}}$, significance: p value of Chi-square test on independence of markers). By observing the dendrograms resulting from these two clustering methods and the coupling code, the preliminary groups were determined. In each group, the coupling code should be less than four types for its simplex markers, due to the maximum number of homologs per chromosome. Finally 19 and 16 groups were generated for P540 and P867 respectively.

Marker order and identification of the linkage phases

Ordering of the markers was done for each group using the two point analysis available in TetraploidMap. Due to only pairs of simplex–simplex markers (coupling and repulsion), duplex–simplex (coupling and repulsion) and duplex–duplex (coupling) were accurate for linkage estimations in tetraploid mapping. The recombination and LOD score were calculated for each pair of markers in each possible linkage phase and the most likely phase among these with a recombination frequency smaller than 0.5 was inferred. Markers were checked in each group and excluded if they showed a significant relationship with less than 2 markers (LOD score smaller than 3) in that linkage group. After that, an overall map can be generated by TetraploidMap. If separate maps (markers shown on individual chromosomes) are needed, phase has to be added manually.

2.6 Marker-trait association analysis

The pollen germination data were recorded from genotypes which have more than 10% stainable pollen. Genotypes with lower percentage of stainable pollen were excluded and finally 40 genotypes were used in this analysis.

Xie and Xu (2000) showed that uni- and bi- parental simplex markers are more efficient for mapping QTLs on tetraploids. Benefit from simplex marker followed the Mendelian segregation, mapping QTLs can use the existing procedures in diploid organisms. Another reason is the most reliable linkage estimation is form simplex-simplex coupling pairs of markers (Hackett *et al.,* 1998). Therefore, simplex markers were used in this marker-trait association analysis. There were two steps in this analysis.

First, most genotypes in the K5 population have 0% of pollen germination, this led to a non-normal distribution of the pollen germination data, a non-parametric Kruskal-Wallis test was performed to pre-select markers significantly ($p<0.05$) correlated to pollen germination (Siegel, 1956).

The next step was to use these markers, which were selected in the previously Kruskal-Wallis test, in a multiple regression analysis. The model for this analysis is:

$$y_i = \mu + \sum \beta_k m_{ik} + \varepsilon_i$$

y_i : germination of individual i
μ: expected mean germination in the population
β_k: regression coefficient for marker k
m_{ik}: genotype class for individual i for marker k (0, absent and 1, present)
ε_i : error

The purpose of this step was to evaluate the contribution to germination for each marker that was included in the model and choose a final set of markers that shows a relationship with germination ($p<0.05$).

One assumption of multiple regression analysis is a normal distribution of the pollen germination. The data set we used here did not meet the assumption by checking the Q-Q plot of residuals. Therefore, the accuracy of results on regression needs to be further confirmed.

3 Results

3.1 Acetocarmine staining

One hundred and sixty three individuals including parents of the K5 population were used for

pollen staining. Fifty individuals showed stainability above 10%. Among them, genotype K101 showed the highest stainability of 43.58%, followed by K059 at 41.81%. For the parents (P540 and P867), the percentages were 28.93% and 17.30% respectively. The remaining progenies had lower percentages (<10%) of stainable pollen (Appendix 2). Within these progeny, 19 progeny produced abnormal pollen with abnormal shapes and could not be stained orange. Furthermore, K191 produced anthers but no pollen was formed; and K004 and K007 did not produce any anthers. Complete staining results can be found in Appendix 2. Figure 2 shows pollen staining results for both parents, progeny K088 (high stainability) and K142 (no stainable pollen).

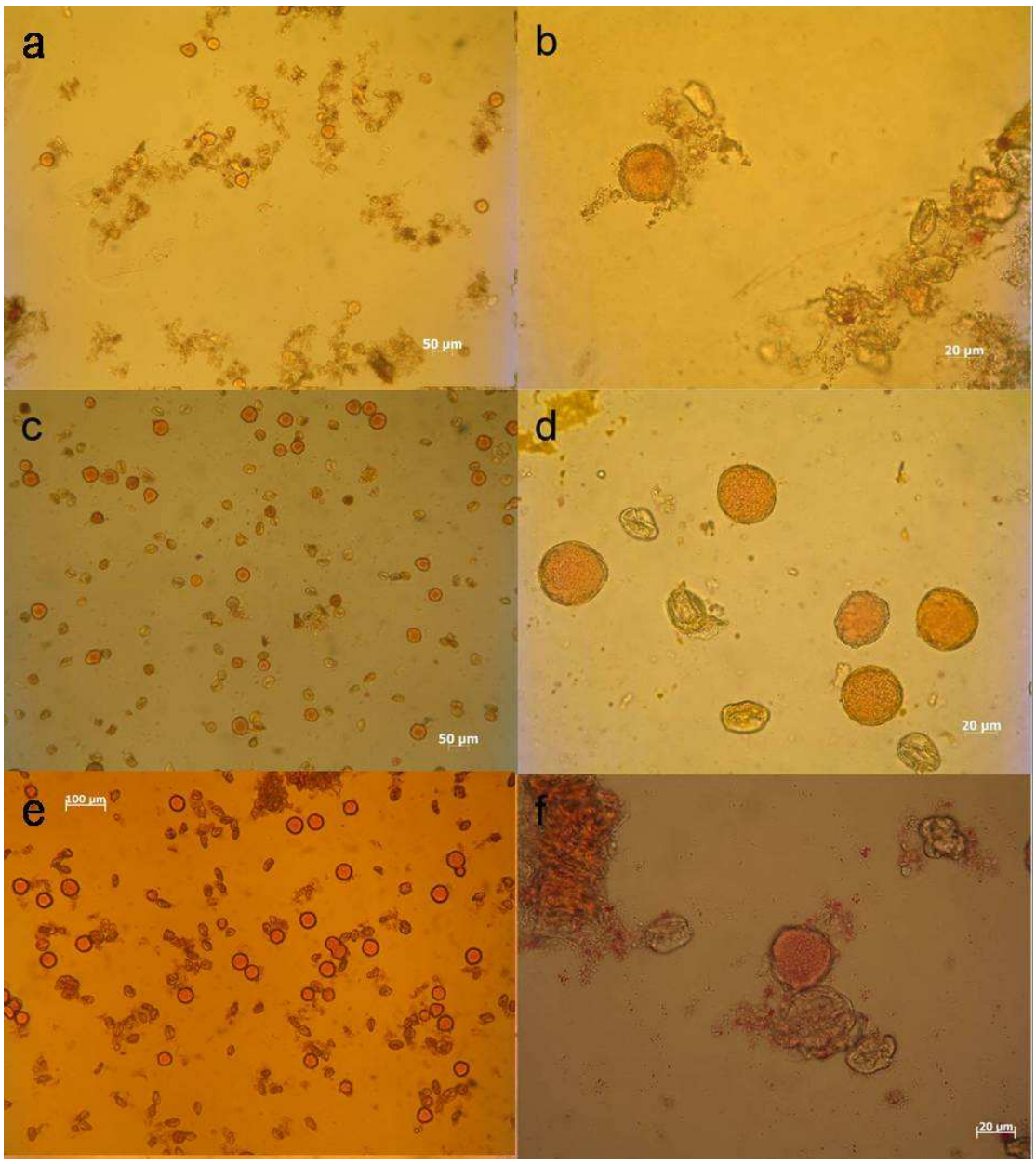

Figure 2: Stained pollen; (a) and (b) from parent P540; (c) and (d) from P867; (e) from K088; (f) from K142. All of these pollen samples are from flower stage 1.

3.2 Germination medium

Forty-one randomly selected genotypes were tested. Pollen mixtures were taken from different flower stages and anther stages of each genotype (Appendix 3). The pollen germination of all 41 genotypes of pollen samples (mixed flower stages) were tested in four types of medium, respectively. An ANOVA analysis was applied to these 41 samples to test for differences between the 4 kinds of medium. The mean germination percentage of medium 2 was 1.91% which was the highest among the 4 tested media, whereas the lowest germination rate of 1.47% occurred for medium 4 (Table 4). No significant differences between the medium types ($p=0.866$) was found. In subsequent pollen germination tests we used medium 2 as it had the highest mean pollen germination value (but not significantly different). Figure 3 gives examples

of germination for the parents and two progeny individuals on medium 2.

Table 4: Mean and standard deviation for germination in germination test

Medium No.	Number of replicate progenies	Mean germination percentage (%)	Standard deviation
1	41	1.81[a]	2.90
2	41	1.91[a]	2.90
3	41	1.56[a]	2.59
4	41	1.47[a]	2.25

[a] indicates significant mean differences from other characters.

Note: In this table there are no significant different between medium groups, so there is only one character indicated.

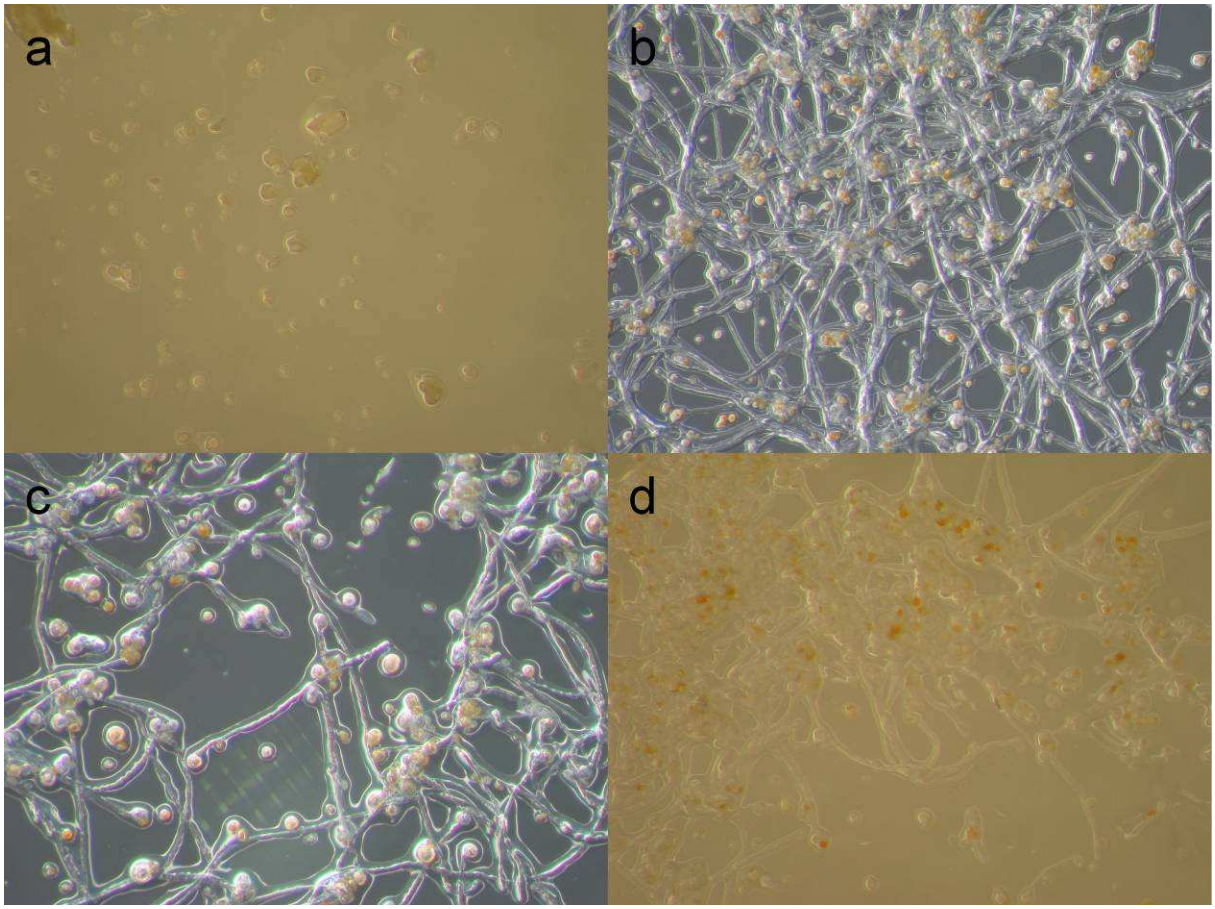

Figure 3: Pollen germination; (a) from P540; (b) from P867; (c) from K088; (d) from K160. All of these pollen samples are from flower stage 1. (The colour difference is due to the background sheet)

3.3 Pollen size and germination

In this experiment we used 42 genotypes of the 50 genotypes (Appendix 6) that showed stainability higher than 10%. Here, less than 50 genotypes were used, because the pollen staining was done before the germination and the 8 genotypes which were not included did not produce flowers in the period in which the pollen germination test was performed. From these 42 genotypes, nine did not germinate. Pollen of the remaining 33 germinated on medium 2. K106 showed a germination rate of 25.5%, which was the highest among the 42 genotypes followed by K088 with a percentage of 21%. For the parents, P540, used as mother, gave 0% and P867, used as father, gave 22.5% germination of pollen.

In order to observe the relationship between pollen size and germination percentage, regression analysis was done in this step. From the pollen staining test, pollen were grouped as abnormal (not stainable) and normal (stainable). This last group was separated further into large, middle and small sized groups of pollen. The percentages of the pollen of these groups were used as predictors and the germination percentage as a depending variable in the regression equations. The nine genotypes which did not germinate were excluded. Three regression model were used depending on the different predictors included (Table 5). The first model uses the percentage of normal (stainable) pollen as independent variable and it was shown normal pollen percentage is correlated to pollen germination positively (p=0.43), with a $R^2_{adjusted}$ value=0.098. In the second model in order to detect which pollen size affected germination most, normal pollen percentage was replaced by 3 subgroups, which were percentages of large, middle and small sized of pollen. Actually this model did not improved compare to the first one. There were only slightly increases in $R^2_{adjusted}$ value (0.118); only middle size pollen significantly associate to pollen germination ($p<0.05$) and large and small size pollen percentages were not detected as significantly correlated to the pollen germination percentage (p=0.453 for large, p=0.933 for small). A third model was obtained by omission of large and small size pollen percentage from model two. This model showed that middle size pollen percentage is significantly (p=0.012) correlated to the percentage of pollen germination and $R^2_{adjusted}$ value=0.159. This means the model only including middle size pollen explains pollen germination the most.

Table 5: Regression equations describing germination percentage contributed by the percentage of normal pollen in different size

Equation No.	Regression equation	$R^2_{adjusted}$
1	GP=4.099+0.269NP	0.098
2	GP=5.102-0.372LP+0.322MP-0.027SP	0.118
3	GP=3.966+0.359MP	0.159

GP: germination percentage

NP: normal pollen percentage

LP: large size pollen percentage

MP: middle size pollen percentage

SP: small size pollen percentage

3.4 Flower stage and anther stage

Anther developmental stages were checked on 388 flowers from 110 genotypes. These flowers covered different stages of these genotypes (Appendix 6). Their distribution shows 76 flowers at stage 0, 97 flowers at stage 1, 110 flowers at stage 2, and 105 flowers at stage 3. Linear regression analysis on flower and anther stage showed a highly significant correlation between flower stage and anther stage ($P<0.05$) with a $R^2=0.626$. Figure 4 shows that flower stages 0, 1 and 3 gave the highest percentage of anther stages A, B and D respectively. In flower stage 2 most of the anthers had developed to stage C and D. Every flower stage contained anthers in all 4 stages, except flower stage 0.

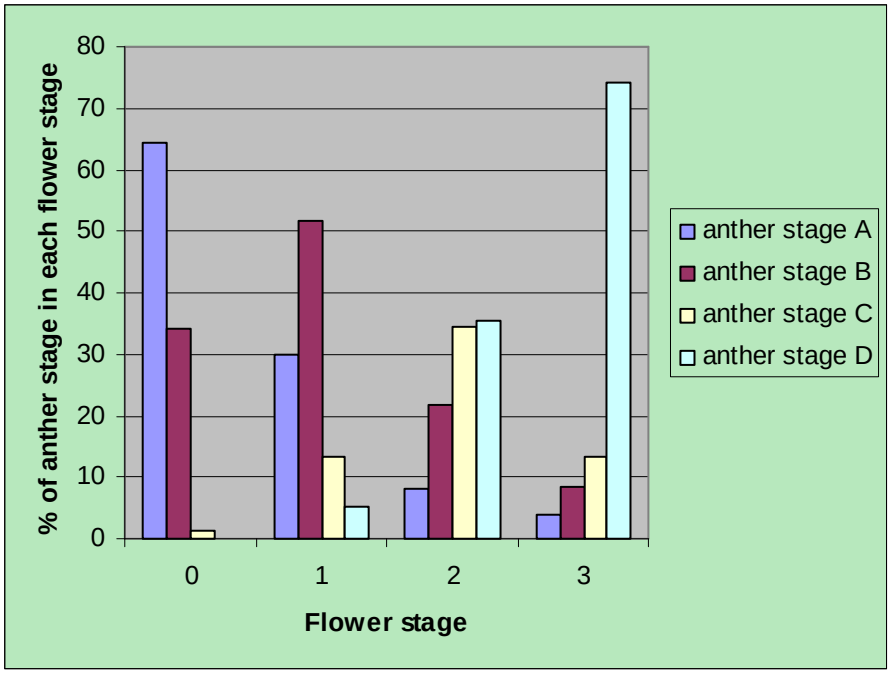

Figure 4: Anther developmental stage corresponding to flower stages

3.5 Flower-anther stage and germination

Flowers used in this part are the same as mentioned in 3.4 (Appendix 6). First a log10 transformation was done on (germination percentage+1) to obtain more equal variance than for untransformed data. In order to find the difference of pollen germination between different development stages ANOVA was performed (Appendix 5) on flower and anther stages separately on pollen germination to identify whether there were significant differences between the different flower stages and anther stages with respect to pollen germination. Furthermore, pairwise comparisons of development stages were carried out after ANOVA testing, to examine differences between different development stages.

First of all, pollen germination was significantly different in different flower development stages

(p<0.05) from the ANOVA test (Table 6). The highest mean germination rate of 3.96% occurred in flower stage 1, followed by stage 2 with 2.81%. Between these two groups, there was no significant difference (p=0.145). A germination rate of 1.63% was observed for flower stage 0. The lowest germination rate was observed for flower stage 3 with only 0.67%, but the difference between stage 0 and 3 was found to be not significant (p=0.537). With respect to germination rates, flower stages 1 and 2 have a higher germination compared to flower stage 0 and stage 3 (p<0.05). This means from the flower development point of view, that flower stages 2 and 3 were optimal to collect pollen for crossing (Figure 5).

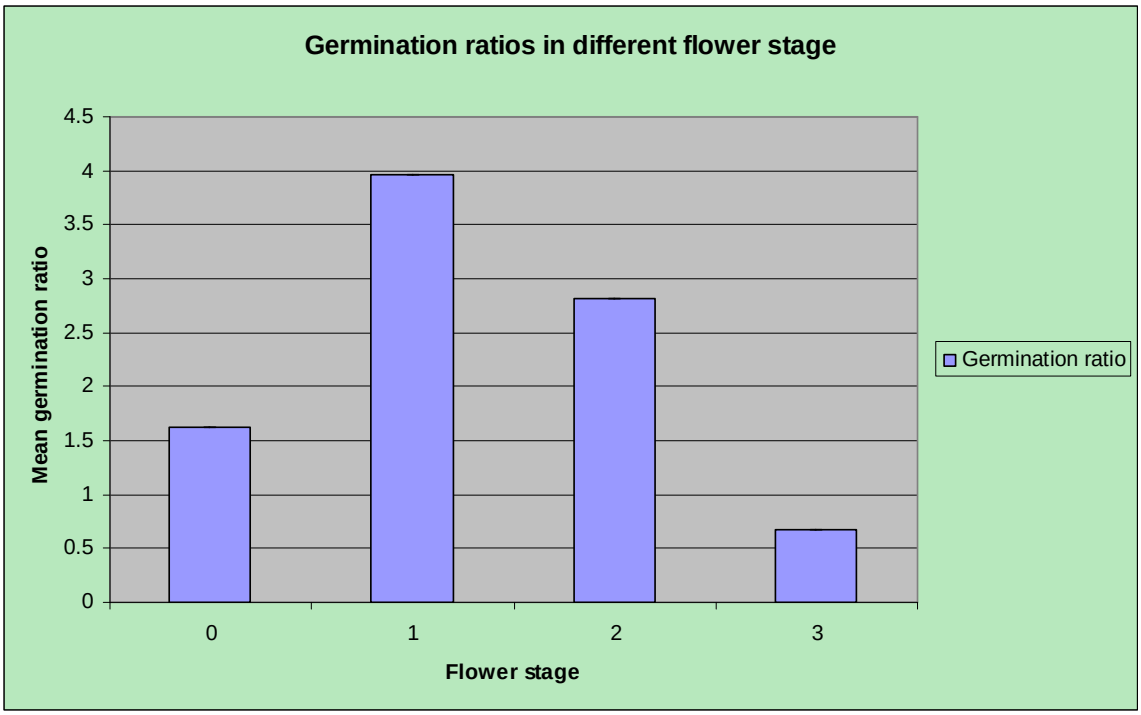

Figure 5: Germination ratios at different flower stages

Table 6: Mean germination percentage for each flower stage

Flower stage	Number of progenies	Mean germination percentage (%)	Standard deviation
0	99	1.63[a]	4.66
1	115	3.96[b]	6.12
2	127	2.81[b]	4.97
3	110	0.67[a]	1.57
Total	451	2.32	4.82

[a] indicate the significant mean differences from [b], and vice versa in [b] Different characters indicate significant differences

Significant differences in pollen germination were also found between different anther development stages. The relationship between anther development and pollen germination is shown in Table 7 and Figure 6. The highest mean germination rate of 4.07% is observed for anther stage C, and then followed by anther stage B with a germination rate of 3.40%. For the

remaining anther stages D and A, a germination rate of 1.58% and 0.86% were observed respectively. Anther stages could be separated into three groups according to ANOVA analysis. Stage C and B with a high germination percentage showed no significant differences ($p=0.154$). On the other hand, stage D and A with a low mean germination percentage were significantly different from stage B and C ($p<0.05$).

In this mean germination analysis process we did not include the genotype effect, which is a very important factor for pollen germination. This was done in order to observe the influence of different flower or anther stages in the whole population instead of in one or two genotypes.

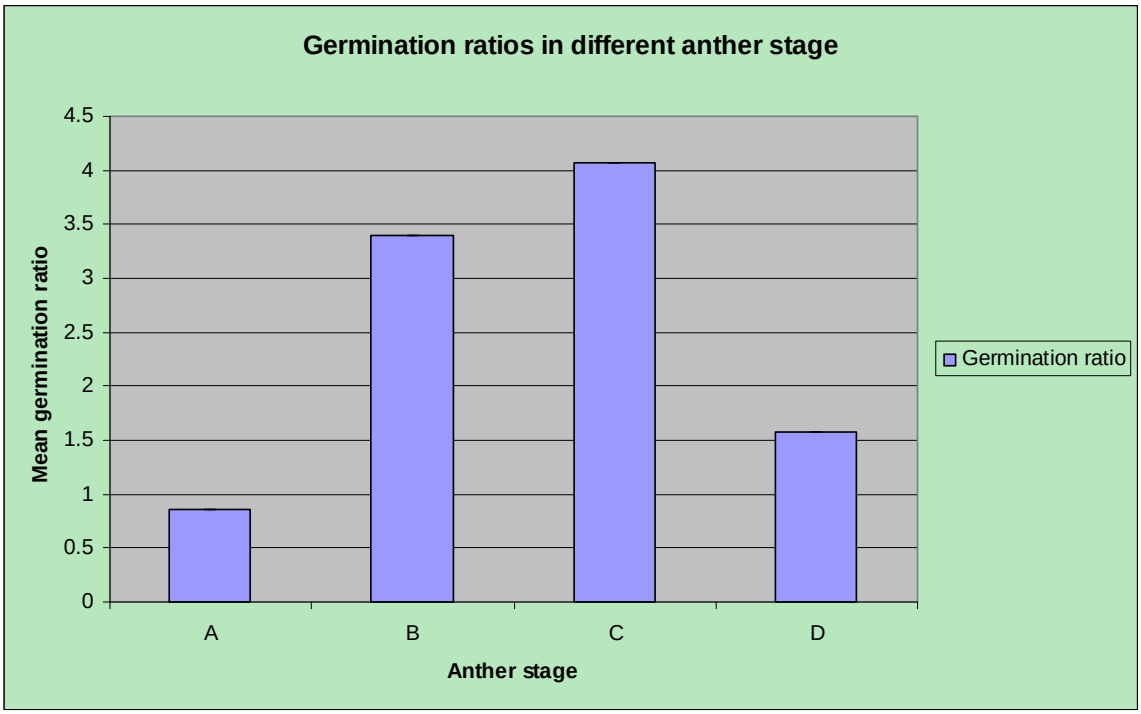

Figure 6: Germination rates at different anther stages

Table 7: Mean germination percentage for each anther stage

Anther stage	Number of progenies	Mean germination percentage (%)	Stand deviation
A	84	0.86[a]	2.71
B	117	3.40[b]	6.21
C	85	4.07[b]	6.23
D	142	1.58[c]	3.09
Total	428	2.43	4.92

[a] indicate the significant mean differences from [b] and [c], and vice versa in [b] and [c]

3.6 Other factors influencing germination

Seventy genotypes were used in this analysis and data is described in 3.4. In order to

investigate the effect of genotype and co-effect of genotype and flower development on germination, multiple linear regression analysis was performed. Both flower stage and genotype as main factors together with the interaction between those two main factors were considered in this analysis. Anther stages were not used, because the experimental design for anther stages was highly unbalanced. A log10 transformation on (germination percentage+1) was used to account for expected unequal variance in the percentage data.

Genotype and flower stages (same result as 3.5) both significantly (p=0.000, p=0.000) influenced pollen germination. Interaction between these two main factors was also significant (p=0.000). The effects of genotype and flower stages on pollen germination were quite visible. For example, P867 had a high germination percentage and P540 does not have germinated pollen and in different flower stages germination percentage was greatly changed. The interaction here means genotype influenced the effect of flower stages on pollen germination. In those 70 genotype, the effects of flower development stages on pollen germination depended on genotype.

3.7 Genetic linkage map construction by TetraploidMap

One hundred and seventy one individuals were used in the mapping construction. Among the available 345 markers there were 321 markers that could be used in further analysis after identifying their segregation ratio by goodness of fit ($p<0.05$). This means that 189 and 210 markers for P540 and P867 respectively, were used to construct a linkage map per parent. Due to the fact that markers with double reduction can not be used in TetraploidMap, 18 markers were excluded from P540, and 19 markers were excluded from P867. In the analysis of each linkage group, those markers that showed little relationship (LOD<3.0) to any other markers in the same group were also excluded.

For parent P540 (Figure 7.1), 165 markers were mapped including 85 bi-parental markers. In this map we obtained 19 LGs with a total length of 1368cM. In the other map, for P867, (Figure 7.2), 16 LGs were obtained with a total length of 1279cM, including 191 markers of which 88 bi-parental markers. Many SSR markers were not mapped on this map.

Although TetraploidMap can in principle also be used to perform QTL analysis once the map is calculated and linkage phase of all the markers is available, this was not done due to quality problems with the resulting map.

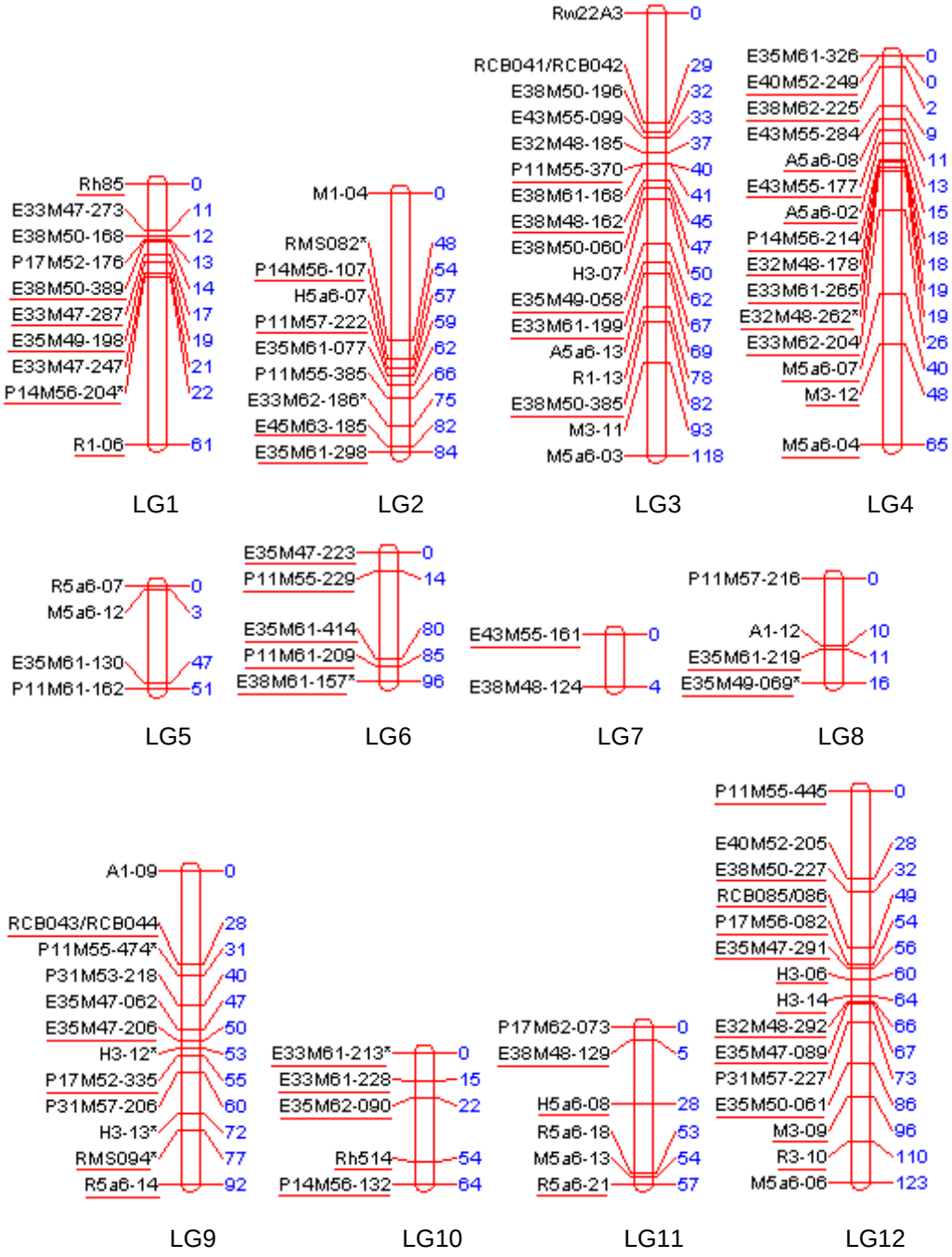

Figure 7.1: Linkage groups for P540. Bi-parental markers are underlined.

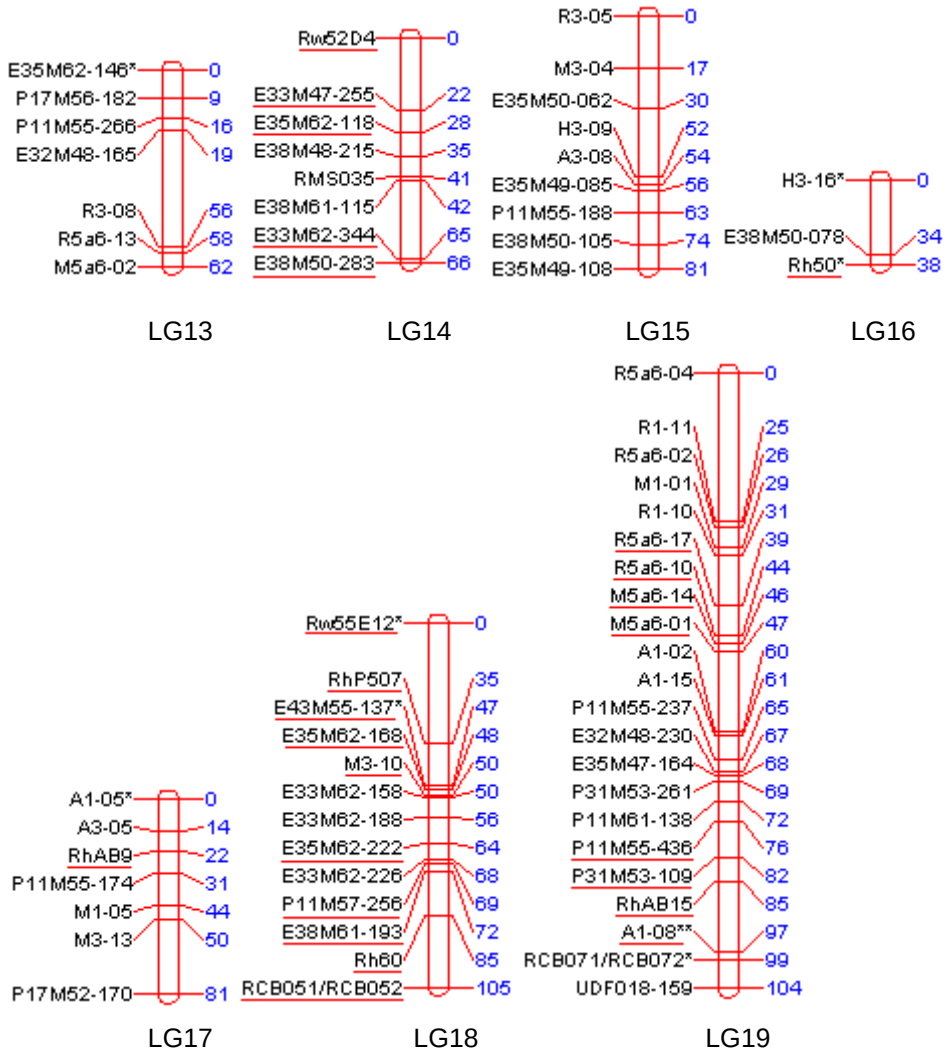

Figure 7.1: Linkage groups for P540. Bi-parental markers are underlined.

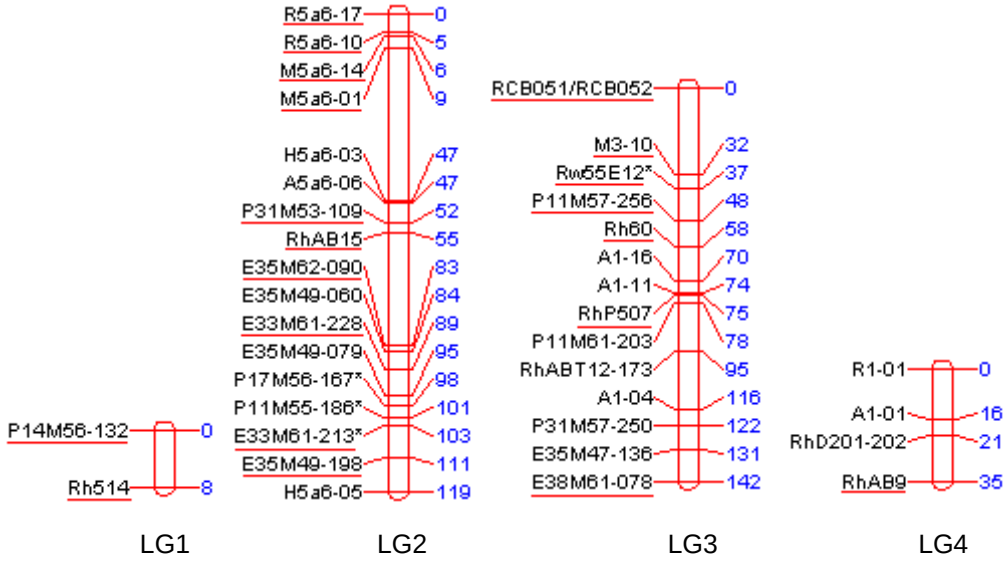

Figure 7.2: Linkage groups for P867. Bi-parental markers are underlined.

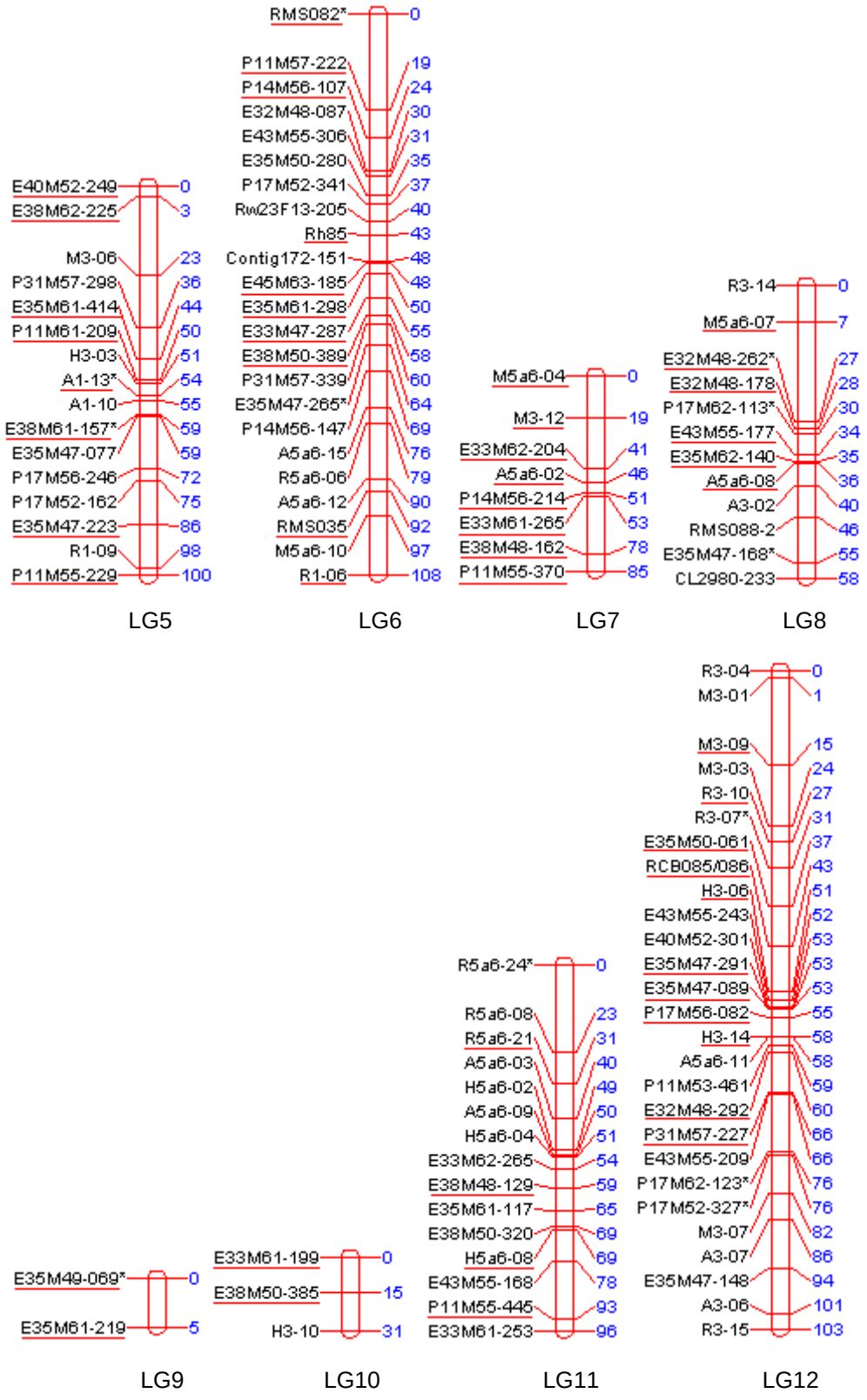

Figure 7.2: Linkage groups for P867. Bi-parental markers are underlined.

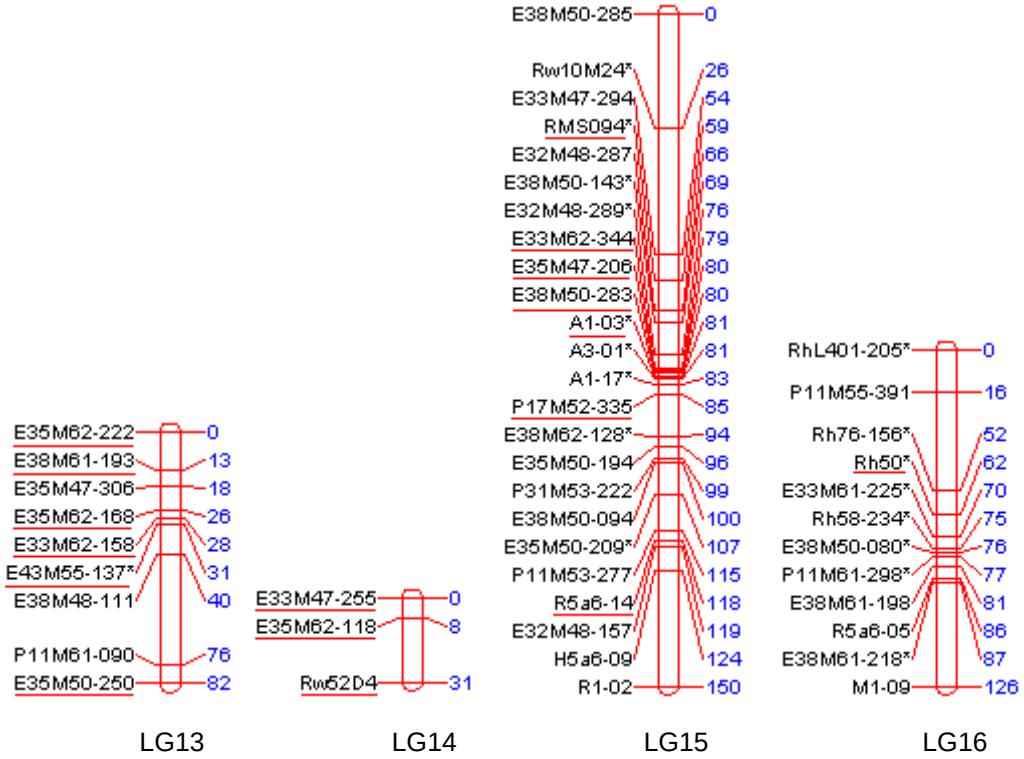

Figure 7.2: Linkage groups for P867. Bi-parental markers are underlined.

3.8 Marker-trait association analysis

Pollen germination data (Appendix 6) was used in genetic mapping analysis.

Firstly, in order to determine which markers were correlated to germination, a non-parametric Kruskal-Wallis test was done for each marker. Fourteen markers were obtained which were significantly associated with germination under a 0.05 confidence level (Table 8).

Table 8: Target markers significantly associated to pollen germination

Marker code	Marker	Kruskal-Wallis Test P-value	Multiple Regression Test P-value
M_1	A1-13*	0.00796	0.208
M_2	E38M48-226	0.02498	0.969
M_3	E32M48-087	0.0014	0.041
M_4	E38M50-196	0.02439	0.116
M_5	H3-03	0.00486	0.689
M_6	M3-06	0.03455	0.049
M_7	P11M55-385	0.04722	0.623
M_8	P31M53-218	0.02675	0.456
M_9	P31M57-248	0.02838	0.678
M_{10}	P31M57-298	0.04011	0.206

M_{11}	P11M57-222	0.00906	0.624
M_{12}	P14M56-107	0.00299	0.510
M_{13}	R3-15	0.03874	0.841
M_{14}	RhL401-205*	0.01312	0.752

After that, multiple regression analysis was performed, 14 markers from the Kruskal-Wallis test were used as predictors of pollen germination as the dependent variable. Resulting p-values are shown (Table 8) and the $R^2_{adjusted}$ was 0.42. Subsequent regression analysis of pollen germination on all 14 target markers showed that only E32M48-087 and M3-06 out of the 14 could be considered as significantly associated with pollen germination at a confidence level of 0.05 (Table 8).

The final regression model contained E32M48-087 and M3-06 as predictors of pollen germination. $R^2_{adjusted}$ was 0.389 in this equation (Table 9), which means 38.9% of the total variation of pollen germination can be explained by the present or absent of markers E32M48-087 and M3-06. The β value shows the effect of each marker on pollen germination. Therefore, from this equation we concluded that marker E32M48-087 has a negative affect on germination and M3-06 has a positive effect on germination.

Table 9: Selection of marker loci putatively correlated to pollen germination.

Marker code	Marker Name	β value	t probability
M_3	E32M48-087	-0.472	0.002
M_6	M3-06	0.311	0.034
$Y=10.196-0.472M_3+0.311M_6$ ($R^2_{adjusted}=0.389$)			

4 Discussion

4.1 Does acetocarmine staining reflect pollen viability?

There were 163 individuals present in the acetocarmine staining experiment and all of them had a stainable pollen percentage lower than 50%. Only K101 and K059 had greater than 40% stainable pollen. This result reveals that pollen quality in the K5 tetraploid rose population is quite low. Both parents had pollen size ranges between 35µm-45µm, which was relatively uniform (Figure 3 a and c). Although the size of the pollen is quite diverse for the whole K5 population, pollen size of most genotypes was in a range of 30 to 54µm with an average size of 41.7 ± 3.6µm. A similar result was reported by Crespel *et al.* (2006).

In order to identify whether acetocarmine staining can be used to measure pollen viability in the K5 population, results of pollen staining and pollen germination were compared. Results showed that 9 genotypes did not germinate, whereas they showed a stainable percentage

higher than 10%. The ratio of germinated genotypes versus non germinated genotypes is almost 3:1 for genotypes having stainable pollen at levels higher than 10%. This result suggests that acetocarmine staining can not be used as a reliable measurement for pollen viability, though it is faster and easier than pollen germination. In order to determine the actual amount of viable pollen, pollen germination is necessary. Parfitt and Ganeshan (1989) also reported that in Prunus, acetocarmine was not reliable for checking pollen viability in agreement with our result. Pearson and Harney (1984) showed that in rose, acetocarmine staining overestimated percentages of viable pollen compared to germination and Mahmoud *et al*. (1998) found similar results in pomegranate. Therefore, it can be concluded that acetocarmine is not a reliable indicator of pollen viability in roses.

Although acetocarmine staining might not be a suitable measurement for pollen viability, excluding the 9 non-germinated genotypes, the relationship between stainable pollen size and pollen germination was studied by regression analysis. Based on the research of Kelly J.K. *et al.* (2002) on the relationship between pollen viability and pollen size in *Mimulus guttatus*, inviable pollen grains have a smaller or larger diameter than viable pollen grains. From the first regression model we could see normal pollen percentage was significantly correlated to pollen germination. In the second model, normal pollen was classified as large, middle and small pollen to perform the regression in order to investigate the effect of different pollen size on pollen germination. In this research, middle size pollen had a significantly positive relationship with pollen germination (at 95% confidence level), but large and small sizes did not. This result, similar to the funding of Kelly J.K. *et al.* (2002), Crespel *et al.* (2006), also reported viable pollen grains with an average 41.7±3.6µm on tetraploid roses. From this point of view, pollen staining can be a reflection of germination rate to a certain extent when taking out non-germinated genotypes. The middle size pollen which has similar size with the parents can explain more of the pollen germination than the other sizes by comparing model 3 to model 2. Furthermore, a high positive correlation between size and ploidy level of pollen grain was reported by Jacob and Pierret (2000) and pollen with a size superior or equal to 42µm were classified as 2n Crespel *et al.,* (2006). This may suggest that 2n pollen contributes to most of the germination in the K5 tetraploid rose population and those small and large sized pollen could be the result of abnormal meioses.

In conclusion, as a faster and easier method to check the pollen viability, acetocarmine staining is not reliable because it gives an overestimation of the percentage of viable pollen compared to germination. In some genotypes it also gives stainable pollen when the pollen does not germinate. Therefore, compared to acetocarmine staining, germination is a more reliable method to identify pollen viability.

4.2 Germination medium

Instead of obtaining optimal medium for a specific genotype, we were more interested in determining one high-performing medium type that can be used in the whole K5 population. Therefore, we chose 41 genotypes for testing randomly and ignored genotype factors in the analysis. Four types of media with different concentrations of boric acid and calcium were

tested. Medium 2, containing 50 mg/l of boric acid, showed higher germination ratios than medium 1 containing 10 mg/l, although not at significant levels. For media 2, 3 and 4, the averages of the pollen germination percentage decreased when calcium concentration increased, as shown in Table 3 and Table 4, though the differences were again not significant. The medium test for germination of the K5 population showed the same results as in Voyiatzi (1995). They reported that the optimal boric acid concentration in germination medium was between 50 and 100 mg/l and that addition of calcium nitrate to the medium reduced the germination rate in five tea hybrid roses. However, Kwack (1967) and Kim (1967) found that presence of calcium affected pollen tube growth positively by controlling the permeability of the cell walls. Similarly Končalová *et al.* (1976) reported that presence of calcium resulted in increased pollen germination and development of longer pollen tubes. Although Voyiatzi (1995) did not clearly explain why his results were different, this difference could be due to differences in sucrose concentrations. To establish the best germination medium; we followed the protocol of Voyiatzi (1995), which contained 20% to 25% (w/v) sucrose and 50 mg/l of boric acid, whereas the medium used by Končalová *et al.* (1976) contained a low sucrose concentration starting from 5%. In addition, the increased effect of calcium on pollen germination negatively correlated to sucrose concentration (Končalová *et al.,* 1976). Another reason for the different influence of calcium on pollen germination could be the different genotypes used.

Our germination results did not show any significant differences between the different media. These results could be explained by the fact that among the 41 tested genotypes many of them did not germinate at all. Therefore, these genotypes did not contribute to differences in the measurements.

4.3 Flower and anther development

For the K5 population, although flower development and anther development are highly correlated to each other, in the regression analysis, anthers are not exactly at the same development stage at a certain flower stage between genotypes. In this study anther development is a factor, which greatly influences pollen germination. Flower development is widely used in roses as an indicator for efficient pollen collection (Devries and Dubois 1983; Marchant *et al.,* 1993, and Richer *et al.,* 2007). However, flower development as an indicator is highly influenced by genotype. For example, in flower stage 2, anthers of K056 are still closed, but for the whole population of K5, flower stage 1 has the highest pollen germination. This will impede pollen collection at that moment for that particular genotype. When anthers have been already open for a number of days, pollen viability might possibly be affected by age and environmental factors. In order to get higher pollen viability, anther stage can be used as a more reliable indicator for pollen viability and for determination of the proper moment for pollen collection.

Although anther stage is a more reliable indicator for collecting pollen for crossing, it is not easy in practice considering the anther is tiny and inside the petals. Therefore we also recommend flower stages as the indicator for collecting pollen. However, breeders should be aware of the influence of genotype differences on anther development.

In this study, anther stage C gave the highest pollen germination percentage 4.07 and the highest pollen germination flower stage is in flower stage 1, followed by flower stage 2, which are 3.96% and 2.81%, respectively. Similarly, Marchant et al. (1993) observed the highest pollen germination rate on semi-open flower stage, and Richer et al. (2006) observed it at flower stages 1 and 2, although there is a slight difference for each genotype. In this study, we used the flower stages definition of Richer et al., (2006), and found similar results. Although for some genotypes pollen germination achieved 25.5%, the highest mean pollen germination at flowering stage 1 was only 3.96%. This was because most genotypes in the K5 population did not have germinated pollen.

4.4 Other factors influencing pollen germination

From multiple regression analysis, besides flower development (3.5 and 4.3), genotype also significantly ($p<0.05$) influenced pollen germination. In the K5 population, depending on difference in genotype, differences in pollen germination were very large. In addition, there was significant interaction between genotype and the optimal flower stage for germination. In genotypes K019, K088, and K140, pollen viability started decreasing from flower stage 0, while most other genotypes showed their highest pollen germination in flower stage 1 and 2. This indicates that the effects of flower stage on germination were influenced by genotype.

4.5 Tetraploid maps

In genetic maps of P540 and P867, most linkage groups have many dense marker regions, however, there are also many sparsely marker regions. Large gaps between markers from those sparsely marker regions highly influence the quality of the genetic maps. For example in LG6, this may have been caused by the small amount of markers (345) and progeny (171) included in the mapping process. The marker number influences marker density of the map and the preferable population size is 250 (Hackett *et al.* 1998). Another possible explanation is from erroneous grouping of some markers during cluster analysis. If one marker was mistakenly clustered in a group, this marker could introduce a large gap in the linkage group where it supposed to belong. Additionally, recombination was calculated for each pair of markers in each possible linkage phase and the most likely phase was chosen, still phase can not be 100% correct, especially for duplex-duplex pairs. The large gaps could also be due to the misidentification of the marker phase. This can also be excluded by sub-grouping the linkage group. Another solution could be excluding that particular marker from the linkage group.

There are 85 and 88 bi-parents markers mapped on P540 and P867. This difference is due to the fact that some bi-parental markers were excluded from one parent due to low LOD values with other markers, during marker selection after the linkage group was formed. It is possible that some bi-parent markers were only mapped in one parent. This indicates that some bi-parents markers actually are not able to be integrated on the parent maps. In these two

maps many SSR markers were not mapped. Those maps include less SSR markers compared to maps from Koning-Boucoiran (personal communication). This is another disadvantage of those two maps. Many linkage groups from homologous chromosomes can not be linked because of the missing SSR markers. Those missing markers can be added again by omitting the individuals with any missing allele data.

Finally, although the recombination and LOD calculations were deduced from the most likely phase, the way, the phase information was calculated on the program was not given. If maps for individual chromosomes are needed, inserting the phase for the parents can be a problem, especially for duplex markers. In some linkage groups, LOD values between duplex markers and simplex markers are very low. This suggests that for the linkage group, no accurate phase for the duplex markers can be set.

The maps are not very informative. The main purpose of this exercise was to test the TetraploidMap program. This lead to the following advices before constructing maps: first, use subgrouping to reduce gap distances. In the grouping analysis, if a group of markers shows difference with the others (LOD scores), it is better to separate them in a subgroup than force them into one big group. It is possible that in the group those markers are from different chromosomes, which lack homology. Secondly, you can use another mapping program (JoinMap) to do grouping for all markers, in order to avoid the complex demdrograms. Then use TetraploidMap to do further analysis, due to its advantages for tetraploid species (double reduction checked and recombination calculated based on tetraploid). Another point is to use simplex–simplex (coupling and repulsion), duplex–simplex (coupling and repulsion) and duplex–duplex (coupling) marker pairs when entering phase information for parents. If there still is a problem of small LOD values between simplex and duplex markers, maybe sub-grouping can help, although more small linkage groups will result.

4.6 Marker-trait association analysis

Marker-trait association analysis was employed to detect marker loci correlated to pollen germination. In the Kruskal-Wallis test analysis step a set of 14 markers was obtained, which was found to be significantly correlated ($p<0.05$) to germination. In the second step, E32M48-087 and M3-06 were selected in regression analysis as significantly correlated to pollen germination.

Different amounts of markers could be explained by multiple reasons. First, the calculation process can show differences in the set of significantly correlated markers. Kruskal-Wallis is based on mean squares from the ranks of the pollen germination, T.S. $H=(K-1)MSB(rank)/MST(rank)$(k-1: degree of freedom, MSB: mean square between group, MST: mean square of total). But in regression analysis pollen germination percentage itself was used as the response variable. Furthermore, only 40 progeny from the K5 tetraploid rose population were used in this analysis. The low number of progeny could also decrease power to detect the relationship of marker-trait association. In addition, Kruskal-Wallis was done for each marker, but in regression analysis the markers could influence each other. For the

Kruskal-Wallis test, the mean value of genotypes containing the allele contrasted with those of individuals without the allele. However, in the regression model markers were added in the model one by one. If two markers are linked together and they were both correlated to germination, one of them could absorb the influence of the other one when both were included in the regression model at the same time. Those two linked markers could end up with only one marker correlated to germination.

In the regression equation for marker E32M48-087 and M3-06 (Table 9), $R^2_{adjusted}$=0.389 can be interpreted as the 38.9% of the total variation of pollen germination is explained by those two markers. Based on our results we cannot conclude that major genes influence/affect pollen germination. A possible explanation could be the limited amount of studied markers which do not cover the regions of genes related to pollen germination. Also, only a few progenies from the K 5 population germinated, and could be used for the marker-trait association analysis, resulting to a low power analysis to detect a major gene. Another explanation is that the main factor influencing the variation of pollen germination is not a major gene. Beside genes and QTLs, there are many reasons that cause pollen non- viability, such as abnormal meiotic behavior (Yan *et al.,* 2000; Zhang *et al.*, 2007; Buyukkartal and Colgecen 2007) and environmental influence as mentioned before. Furthermore, the values of the regression coefficients β, showed that marker E32M48-087 is negatively correlated to pollen germination, while marker M3-06 is positively correlated to pollen germination. Male sterility genes or QTLs could be the most possible reasons to explain the negative effect of a marker on pollen germination. It is possible that male sterility phenomenon in the K5 population (progenies producing no pollen mentioned in 3.1) is caused by the accumulation of genes or QTLs that are negatively related to pollen viability, although only one marker was found negatively correlated to pollen germination for these 40 progenies. The small amount of markers explaining variation of pollen germination could be the result of the limited amount of the studied progenies or markers as mentioned. The physical mechanism of lack of pollen germination could be similar to male sterility, according to the observation of the genotypes showing no pollen germination, such as no male organ or abnormal anthers with no pollen inside. Although we did not include those progenies showing no pollen germination (progenies producing no pollen mentioned in 3.1) in the marker- trait association analysis, they still share large amount of similar genes as they come from the same population. Therefore, there are potential male sterility genes or QTLs which could have been missed in the marker- trait association analysis since they were not included. The marker having a positive effect on pollen germination is very interesting since the only report in literature concerns fertility restoration QTLs on cytoplasmic male sterile (CMS) on pepper (Wang *et al.,* 2004) and wheat (Ahmed *et al.,* 2001), which are positively correlated to pollen viability. But CMS has a different kind of working mechanism compared to purely nuclear controlled male sterility. Therefore, further research is necessary, in order to explain the positive effect of the genes or QTLs nearby the region of the marker M3-06.

Due to the low quality of the maps obtained with TetraploidMap, we did not did not identify QTLs with TetraploidMap program. In the future, pollen germination can be checked again and analyzed using other maps.

5 Conclusion

The rose K5 population, resulting from the crossing between two tetraploid cultivars, consisting of 184 individuals, was screened for pollen viability. Both pollen staining with acetocarmine and pollen germination methods were investigated and compared. The pollen staining method was proved to be not reliable as an indicator to evaluate pollen germination. However, acetocarmine staining reflects the germination rates based on linear regression analysis of pollen germination and different size of pollen. In addition, the suitable medium for pollen germination of the K5 tetraploid rose population was composed of 25% (w/v) sucrose, 50 mg/l boric acid and 1% (w/v) agar. Results of the pollen germination tests showed that anther development stage was a better indicator than flower development stages for pollen germination, however, it is hard to implement. Therefore, in order to obtain the highest germination rate, pollen should be collected from flower development stage 1 (semi-open), corresponding to anther development stage C. In factor analysis, not only genotype and flower development significantly influenced pollen germination, there was also an interaction between germination, flower stage and genotype. In the genetic analysis process, two linkage maps were generated by using TetraploidMap, AFLP, SSRs and NBS markers were included. Although the maps are not very informative, the same marker data was used in marker-trait association analysis. By performing Kruskal-Wallis and multiple regression tests on pollen germination data and marker data, markers E32M48-087 and M3-06 were detected as significantly correlated to pollen germination.

6 Future work

Pollen germination is highly influenced by the medium and different genotypes have different optimal medium recipes. We cannot conclude that genotypes from the K5 population all share the same optimal medium recipe. In order to obtain the optimal germination medium, more combinations of ingredients with different concentrations or new medium recipes should be tested for the individuals of the K5 tetraploid rose population.

Repeats of pollen germination experiments are strongly recommended in future research to confirm current results. In the present study, due to time limitations, we could not include replications for each genotype. This fact influenced the statistical analyses and had a serious impact on interpreting our result.

The weather conditions need to be considered while performing pollen germination tests. Pollen viability can be influenced by temperature and humidity. During our research, pollen germination was very low when the temperature and humidity were very high.

TetraploidMap can be tested again, but one should be aware the difficulties of the grouping.

References

Ahmed, TA, Tsujimoto, H and Sasakuma, T 2001, 'QTL analysis of fertility-restoration against cytoplasmic male sterility in wheat' *Genes Genet Syst* vol76, pp33–38

Alonso, JM and Socias i Company, R 2002, 'Study of Male-sterility Transmission in 'ROF' Almond', *Acta Hort,* vol59, pp265-268

Brewbaker, JL and Kwack, BH 1964, 'The calcium ion and substance influencing pollen growth'. Ed: Linskens, HF. *Pollen Physiology and Fertilization*. pp. 143-151, North-Holland Publishing Co., Amsterdam, The Netherlands

Buyukkartal, N and Colgecen, HT 2007, 'The reasons of sterility during pollen grain formation in the natural tetraploid *Trifolium pratense* L.' *International Journal of Botany*, vol3, pp188-195

Crespel, L, Chirollet, M, Durel, C, Zhang, D, Meynet, J and Gudin, S 2002, 'Mapping of qualitative and quantitative phenotypic traits in Rosa using AFLP markers', *Theor Appl Genet,* vol105, pp1207-1214

Crespel, L, Ricci, SC,and Gudin, S 2006, 'The production of 2*n* pollen in rose', *Euphytica*, vol151, pp155–164

Da Silva, J, Sorrells, ME, Burnquist, WL and Tanksley, SD 1993, 'RFLP linkage map and genome analysis of *Saccharum spontaneum* L.', *Genome,* vol36, pp782-791

Debener, T and Mattiesch, L 1999, 'Construction of a genetic linkage map for roses using RAPD and AFLP markers', *Theor Appl Genet,* vol99, pp891-899

De Vries, DP and Dubois, LAM 1983, 'Pollen and pollination experiments. X. The effect of repeated pollination on fruit- and seed set in crosses between the Hybrid Tea-Rose cvs. Sonia and Ilona', *Euphytica*, vol32, pp685-689

De Vries, DP and Dubois, LAM 1984, 'Inheritance of the recurrent flowering and moss characters in F1 and F2 Hybrid Tea x *Rosa centifolia muscosa* (Aiton) Seringe populations', *Gartenbauwissenschaften,* vol49, pp97-100

Dugo, ML, Satovic, Z, Millán, T, Cubero, JI, Rubiales D, Cabrera, A and Torres, AM 2005, 'Genetic mapping of QTLs controlling horticultural traits in diploid roses', *Theor Appl Genet,* vol111, pp511–520

Grattapaglia, D and Sederoff, R 1994, 'Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudotestcross mapping strategy and RAPD markers', *Genet,*

vol137, pp1121-1137

Hackett, CA, Bradshaw, JE, Meyer, RC, Mcnicol, JW, Milbourne, D and Waugh, R 1998, 'Linkage analysis in tetraploid species: a simulation study' *Genetics Research*, Vol71, pp143-153

Hackett, CA, Milne, I, Bradshaw, JE, and Luo, ZW 2005, 'TetraploidMap for Windows: Software suite for calculating linkage maps for autotetraploid populations', Available at: *www.bioss.ac.uk/knowledge/tetraploidmap/* (Accessed on 06-04-2009)

Issarakraisila, M and Considine, JA 1994, 'Effects of temperature on pollen viability in mango cv. 'Kensington'**',** *Annals of Botany*, vol73, pp231-240

Jacob,Y and Pierret, V 2000, ' Pollen size and ploidy level in the genus Rosa.', *Acta Hort*, vol508, pp289–292

Jones, JK 1955, 'Cytogenetic studies in the genera Fragaria and Potentilla', *Ph.D Thesis*, University of Manchester

Kelly, J, Rasch, A and Kalisz, S 2002, 'A method to estimate pollen viability from pollen size variation', *American Journal of Botany,* vol89, pp1021–1023

Kim, JJ 1967, 'Airborn pollens in Seoul.' *Kor.J. Pediatr.,* vol16, pp8-11

Koning-Boucoiran, CFS, Dolstra, O, van der Linden, CG, van der Schoot, J, Gitonga, VW, Verlinden, K, and Krens, FA 2007, 'NBS-profiling as a targeted help to study the genetics of resistance to diseases in tetraploid roses', First International Rose Genomics 2007, Angers, France, 11-13 December 2007

Končalová, MN, Jičínská, D and Sýkorová, O 1976, 'Effect of calcium and sucrose concentration on pollen gremination in vitro of six rosa species', *Biol Plantarum Praha*, vol18, pp26–30

Končalová, MN, Jičínská, D and Sýkorová, O 1976, 'Studies in rose pollen III. Pollen viability and germinability in eight Czechoslovak *Rosa* species', *Preslia Praha,* vol48, pp347-353

Kwack, BH 1967 'Studies on cellular site of calcium action in promoting pollen growth', *Physiol Plant*, vol20, pp825-833

Kwack, BH, Kim, I 1967, 'Effects of calcium ion and the protective action on survival and growth inhibition of pollen', *Physiol Plant,* vol20, pp73-82

Kroon, GH and Zeilinga, AE 1974, 'Apomixis and heterogamy in rose rootstocks (*Rosa canina* L.)', *Euphytica,* vol23, pp345-352

Mahmoud, AA, Mohamed, AB and Farahat, AF 1998, 'Pollen viability, germination and rates of pollen tube growth in some pomegranate cultivars (Punica granatum, L.)', *Agricultural Sciences,* vol10, pp73-81

Marchant, R, Power, JB, Davey, MR, Chartier-Hollis, JM and Lynch, PT 1992, 'Cryopreservation of pollen from two rose cultivars', *Euphytica*, vol66, pp235-241

Meyer, RC, Milbourne, D, Hackett, CA, Bradshaw, JE, Mcnihol, JW and Waugh, R 1998, 'Linkage analysis in tetraploid potato and association of markers with quantitative resistance to late blight (*Phytophthora infestans*)', *Mol Gen Genet*, vol259, pp150–160

Owczarzak, A 1952, 'A rapid method for mounting pollen grains', *Stain Technology*, vol27, pp249

Pagliarini, MS 2000, 'Meiotic behavior of economically important plant species: the relationship between fertility and male sterility', *Genet Mol Bio*, vol. 23, pp997-1002

Parfitt, DE and Ganeshan, S 1989, 'Comparison of procedures for estimating viability of Prunus pollen', *HortScience,* vol24, pp354–356

Pearson, HM and Harney, PM 1984,'Pollen viability in rosa' *HortScience*, vol19, pp710–711

Rajapakse, S, Byrne, DH, Zhang, L, Anderson, N, Arumuganathan, K and Ballard, RE 2001, 'Two genetic linkage maps of tetraploid roses', *Theor Appl Genet* vol103, pp575-583

Rattenbury, JA 1952, 'Specific staining of nucleolar substance with aceto-carmine', *Stain Technology*, vol27, pp113-122

Richer, C, Poulin, M and Rioux, JA 2007, 'Factors influencing pollen germination in three Explorer™ roses', *Can J Plant Sci,* vol87, pp115–119

Siegel, S 1956, 'Nonparametric statistic for the behavioural science' McGraw-Hill, New York

Verlinden, K 2007, 'Genetic mapping of quantitative traits in a tetraploid rose population using microsatellite and AFLP markers', Master Thesis, Wageningen University, The Netherlands

Vieitez, E 1953, 'The use of 2, 3, 5-triphenyl tetrazolium chloride for determining the viability of pollen' (English summary) *An Edaf Fis Veg Madrid*, vol11, pp297-308

Visser, T, De Vries, DP, Welles, GWH and Scheurink, JAM 1977, 'Hybrid tea-rose pollen. I. Germination and storage', *Euphytica*, vol26, pp721-728

Voyiatzi, CI 1995, 'An assessment of the in vitro germination capacity of pollen of five tea hybrid rose cultivars', *Euphytica,* vol83, pp199–204

Wang, LH, Zhang, BX Lefebvre, V, Huang, SW, Daubèze, AM and Palloix, A 2004, 'TL analysis of fertility restoration in cytoplasmic male sterile pepper' *Theor Appl Genet* vol109, pp 1058–1063

Werlemark, G, Uggla, M and Nybom, H 1999, 'Morphological and RAPD markers show a highly skewed distribution in a pair of reciprocal crosses between hemisexual dogrose species, *Rosa* sect. *Caninae', Theor Appl Genet,* vol98, pp557-563

Xie, CG and Xu, SZ 2000, 'Mapping quantitative trait loci in tetraploid populations', *Genet Res*, *Camb*., vol76, pp105-115

Yan, Z, Denneboom, C, Hattendorf, A, Dolstra, O, Debener, T, Stam, P, and Visser, PB 2005b, 'Construction of an integrated map of rose with AFLP, SSR, PK, RGA, RFLP, SCAR and morphological markers', *Theor Appl Genet,* vol110, pp766-777

Yan, Z, Dolstra, O, Prins, TW, Stam, P and Visser, PB 2005a, 'Assessment of partial resistance to powdery mildew (*Podosphaera pannosa*) in a tetraploid rose population using a spore-suspension inoculation method' *Towards efficient improvement of greenhouse grown roses: genetic analysis of vigour and powdery mildew resistance*, PhD Thesis, Wageningen University, The Netherlands

Yu, KF and Pauls, KP 1993, 'Segregation of random amplified polymorphic DNA markers and strategies for molecular mapping in tetraploid alfalfa', *Genome*, vol36, pp844-85

Zhang, L, 2003, 'Genetic linkage mapping in tetraploid and diploid rose', Dissertation, Clemson University, USA

Zhang, LH, Byrne, DH, Ballard, RE and Rajapakse, S 2006, 'Microsatellite marker development in rose and its application in tetraploid mapping', *J Am Soc Hortic Sci*, vol131, pp380-387

Zhang, SN, Wan, SF, Zhang, W and Hou, XL 2007, ' Meiosis of pollen mother cells in atotetraploid broccoli', *Acta Horticulturae Sinica*, vol34, pp387-390

Zlesak, DC, Zuzek, K and Hokanson SC 2007, 'Rose pollen viability over time at varying storage temperatures' *IVth IS on Rose Research and Cultivation*, Ed. H.B. Pemberton

Appendix

1 Protocol for acetocarmine staining solution (1%)

Ingredients	Quantity
Carmine	1 gram (g)
Glacial acetic acid (45%)	100 millilitre (ml)
Ferric Chloride (FeCl2*6H2O) (10%) Optional	5 millilitre (ml)
Total volume	100 millilitre (ml)

Note: Add carmine powder to boiling 45% glacial acetic acid, cool rapidly, and then filter into a dark glass. Staining can be intensified by adding ferric chloride (FeCl2.6H2O). Caution: the stain stains clothes.

2 Staining results

Genotype	Large (>45μm)	Middle (35μm-45μm)	Small (<35μm)	Abnormal	Total amount	Normal pollen(%)
P540	4	23	8	86	121	28.93
P867	2	25	5	153	185	17.30
K001	0	25	9	410	444	7.66
K002	1	9	5	132	147	10.20
K003						low
K004	3	16	2	147	168	12.50
K007						0
K008	2	4	1	77	84	8.33
K009	0	3	0	118	121	2.48
K010	0	10	6	143	159	10.06
K011	0	27	9	96	132	27.27
K012	0	17	5	222	244	9.02
K013	4	18	0	96	118	18.64
K014						0
K015						0
K016	0	13	0	155	168	7.74
K017	2	1	0	93	96	3.13
K019	0	27	9	96	132	27.27
K021	0	10	5	104	119	12.61
K022						low
K023						low
K024	1	3	4	170	178	4.49
K026		0.7				low
K027	2	10	4	260	276	5.80
K028						low
K029						0
K030	1	6	6	171	184	7.07
K031	5	10	2	181	198	8.59
K032	0	12	6	143	161	11.18
K033						0
K034	3	12	0	151	166	9.04
K035	2	5	2	85	94	9.57
K039	9	32	11	247	299	17.39
K040	1	35	12	158	206	23.30
K041	0	4	2	103	109	5.50
K042	0	6	0	180	186	3.23
K043	4	7	2	115	128	10.16
K044	1	4	3	328	336	2.38
K045	0	3	0	128	131	2.29

Genotype	Large (>45μm)	Middle (35μm-45μm)	Small (<35μm)	Abnormal	Total amount	Normal pollen(%)
K046	1	23	4	183	211	13.27
K049	1	5	3	153	162	5.56
K050						low
K051						low
K052	0	21	5	88	114	22.81
K053						0
K054	0	12	2	121	135	10.37
K055						low
K056	12	4	0	84	100	16.00
K058						low
K059	3	74	20	135	232	41.81
K060						0
K062						low
K063	0	3	1	112	116	3.45
K064						0
K065	1	2	3	95	101	5.94
K066	5	0	1	149	155	3.87
K067	1	7	2	140	150	6.67
K068						0
K069	1	1	0	94	96	2.08
K070	0	3	1	137	141	2.84
K071						
K072	0	3	1	112	116	3.45
K073	0	8	2	156	166	6.02
K074						0
K077	1	1	1	111	114	2.63
K078	0	19	3	204	226	9.73
K080						low
K082						low
K085	0	40	0	189	229	17.47
K086						low
K088	0	31	6	191	228	16.23
K089						low
K090						low
K091						
K093	1	2	1	70	74	5.41
K095						
K096						
K097	2	57	9	163	231	29.44
K098						low
K099	0	17	4	95	116	18.10

Genotype	Large (>45μm)	Middle (35μm-45μm)	Small (<35μm)	Abnormal	Total amount	Normal pollen(%)
K100	0	22	11	142	175	18.86
K101	1	18	18	70	107	34.58
K102	4	17	5	147	173	15.03
K103						low
K104	2	8	0	239	249	4.02
K105						0
K106	0	79	0	165	244	32.38
K107	0	13	0	175	188	6.91
K108	1	3	1	160	165	3.03
K109	0	52	1	158	211	25.12
K111						low
K113	1	15	13	154	183	15.85
K115						
K116						
K118	2	13	2	235	252	6.75
K119	4	2	0	130	136	4.41
K120	1	13	7	169	190	11.05
K121						0
K123	3	5	0	137	145	5.52
K124	0	12	3	97	112	13.39
K126	0	3	0	64	67	4.48
K128	3	30	15	198	246	19.51
K130	6	24	8	106	144	26.39
K131	0	15	3	153	171	10.53
K132						low
K134						low
K135						0
K137	2	13	1	120	136	11.76
K138	3	5	3	139	150	7.33
K140	2	27	5	97	131	25.95
K141						low
K142	0	19	6	206	231	10.82
K144	2	5	0	194	201	3.48
K147	0	5	2	120	127	5.51
K148	2	11	4	105	122	13.93
K151						low
K152						low
K153						0
K154						low
K155	3	14	6	103	126	18.25
K156						low
K157	1	43	6	188	238	21.01

Genotype	Large (>45μm)	Middle (35μm-45μm)	Small (<35μm)	Abnormal	Total amount	Normal pollen(%)
K158						low
K159						0
K160	0	60	3	182	245	25.71
K161	0	8	4	225	237	5.06
K162	3	8	3	198	212	6.60
K163	8	9	3	250	270	7.41
K165	1	2	2	97	102	4.90
K166	0	3	1	103	107	3.74
K167	0	27	14	95	136	30.15
K169						0
K171	4	3	5	260	272	4.41
K172	0	6	0	158	164	3.66
K174	4	7	6	146	163	10.43
K175	3	7	3	160	173	7.51
K177	1	13	9	219	242	9.50
K179						low
K181						
K182						low
K183	3	7	2	325	337	3.56
K184						low
K185	3	9	5	171	188	9.04
K187	6	9	9	114	138	17.39
K189	3	34	1	136	174	21.84
K190						0
K191						0
K192	0	13	2	128	143	10.49
K193	4	25	4	179	212	15.57
K194	3	3	1	140	147	4.76
K195	0	38	1	492	531	7.34
K198						low
K199	0	2	0	79	81	2.47
K200	2	0	0	55	57	3.51
K204	0	27	9	74	110	32.73
K205	1	29	7	296	333	11.11
K206						0
K207	0	18	6	286	310	7.74
K208	1	11	2	133	147	9.52
K210						0
K211						0
K212						low
K215	0	10	0	148	158	6.33
K216						

Genotype	Large (>45μm)	Middle (35μm-45μm)	Small (<35μm)	Abnormal	Total amount	Normal pollen(%)
K217						
K218						
K219	0	6	0	144	150	4.00
K220						low
K221	2	8	8	97	115	15.65
K222	5	12	6	148	171	13.45
K223	1	36	5	135	177	23.73
K224						
K225	8	10	1	163	182	10.44
K228	1	4	0	142	147	3.40
K229						low
K231	2	5	2	120	129	6.98
k244	0	6	0	104	110	5.45

3 Medium test results

Genotype	Germination percentage			
	medium 1	medium 2	medium 3	medium 4
K142	0.5	1.0	1.0	0.7
K160	10.0	10.0	7.0	7.0
K130	1.0	1.5	3.0	3.0
K124	0.5	1.0	0.5	0.1
K035	0.5	0.0	0.5	0.5
K043	0.0	0.0	0.0	0.0
K184	0.0	0.0	0.0	0.0
K200	0.0	0.0	0.0	0.0
K140	0.1	0.1	0.1	0.5
K225	0.5	0.5	1.0	0.4
K140	8.0	7.0	7.0	3.0
K011	10.0	10.0	10.0	7.0
K035	0.1	1.0	1.0	0.5
K169	0.0	0.0	0.0	0.0
K002	8.0	8.0	8.0	8.0
K162	0.1	0.5	0.1	0.1
K154	0.0	0.1	0.1	0.1
K010	0.1	0.5	0.0	0.0
K192	0.1	0.5	0.5	1.0
k050	0.5	0.0	0.0	0.0
K026	0.0	0.1	0.5	0.0
K120	0.5	3.0	1.0	2.0
K019	3.0	0.5	0.5	0.5
K022	0.1	0.0	0.0	0.0
K031	0.5	0.5	0.5	1.0
K097	5.0	5.0	5.0	4.0
K867	7.0	7.0	7.0	8.0
K540	0.0	0.0	0.0	0.0
K119	0.5	0.5	0.5	0.5
K085	2.0	2.0	1.0	1.0
K162	0.0	0.5	0.0	0.5
K082	0.5	0.5	0.5	0.5
K021	0.5	0.5	0.0	0.5
K086	1.0	1.0	0.5	0.5
K003	0.5	0.5	0.0	0.5
K093	3.0	3.0	2.0	3.0
K099	1.5	1.5	0.5	1.0
K100	1.5	1.5	1.0	1.0

K174	0.5	0.5	0.0	0.5
K052	6.0	8.0	3.0	3.0
K199	0.5	0.5	0.5	0.5

4 Germination rates according to pollen sizes

Genotype	Large pollen(%)	Middle pollen(%)	Small pollen(%)	Normal pollen(%)	Abnormal pollen(%)	Pollen germination(%)
P540	3.31	19.01	6.61	28.93	71.07	0.00
P867	1.08	13.51	2.70	17.30	82.70	22.50
K002	0.68	6.12	3.40	10.20	89.80	7.50
K004	1.79	9.52	1.19	12.50	87.50	0.00
K010	0.00	6.29	3.77	10.06	89.94	0.00
K011	0.00	20.45	6.82	27.27	72.73	10.75
K013	3.39	15.25	0.00	18.64	81.36	5.00
K019	0.00	20.45	6.82	27.27	72.73	8.00
K021	0.00	8.40	4.20	12.61	87.39	8.00
K032	0.00	7.45	3.73	11.18	88.82	7.50
K039	3.01	10.70	3.68	17.39	82.61	0.00
K040	0.49	16.99	5.83	23.30	76.70	12.00
K043	3.13	5.47	1.56	10.16	89.84	
K046	0.47	10.90	1.90	13.27	86.73	11.50
K052	0.00	18.42	4.39	22.81	77.19	5.50
K054	0.00	8.89	1.48	10.37	89.63	
K056	12.00	4.00	0.00	16.00	84.00	1.03
K059	1.29	31.90	8.62	41.81	58.19	21.50
K085	0.00	17.47	0.00	17.47	82.53	12.50
K088	0.00	13.60	2.63	16.23	83.77	21.00
K097	0.87	24.68	3.90	29.44	70.56	7.00
K099	0.00	14.66	3.45	18.10	81.90	
K100	0.00	12.57	6.29	18.86	81.14	7.00
K101	0.93	16.82	16.82	34.58	65.42	10.00
K102	2.31	9.83	2.89	15.03	84.97	
K106	0.00	32.38	0.00	32.38	67.62	25.50
K109	0.00	24.64	0.47	25.12	74.88	3.50
K113	0.55	8.20	7.10	15.85	84.15	
K120	0.53	6.84	3.68	11.05	88.95	0.00
K124	0.00	10.71	2.68	13.39	86.61	
K128	1.22	12.20	6.10	19.51	80.49	0.00
K130	4.17	16.67	5.56	26.39	73.61	12.00
K131	0.00	8.77	1.75	10.53	89.47	5.00
K137	1.47	9.56	0.74	11.76	88.24	16.00
K140	1.53	20.61	3.82	25.95	74.05	12.50
K142	0.00	8.23	2.60	10.82	89.18	10.50
K148	1.64	9.02	3.28	13.93	86.07	1.00
K155	2.38	11.11	4.76	18.25	81.75	0.00

K157	0.42	18.07	2.52	21.01	78.99	2.00
K160	0.00	24.49	1.22	25.71	74.29	8.00
K167	0.00	19.85	10.29	30.15	69.85	0.00
K174	2.45	4.29	3.68	10.43	89.57	0.00
K187	4.35	6.52	6.52	17.39	82.61	
K189	1.72	19.54	0.57	21.84	78.16	10.00
K192	0.00	9.09	1.40	10.49	89.51	
K193	1.89	11.79	1.89	15.57	84.43	
K204	0.00	24.55	8.18	32.73	67.27	7.50
K205	0.30	8.71	2.10	11.11	88.89	2.00
K221	1.74	6.96	6.96	15.65	84.35	
K222	2.92	7.02	3.51	13.45	86.55	9.50
K223	0.56	20.34	2.82	23.73	76.27	12.50
K225	4.40	5.49	0.55	10.44	89.56	0.75

5 Important SPSS outputs

ANOVA test on medium test

ANOVA

germratio

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5,205	3	1,735	,243	,866
Within Groups	1142,585	160	7,141		
Total	1147,790	163			

Multiple Comparisons

Germination percentage

LSD

(I) medium	(J) medium	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.1024	.5902	,862	-1.268	1.063
	3	.2512	.5902	,671	-.914	1.417
	4	.3341	.5902	,572	-.831	1.500
2	1	.1024	.5902	,862	-1.063	1.268
	3	.3537	.5902	,550	-.812	1.519
	4	.4366	.5902	,461	-.729	1.602
3	1	-.2512	.5902	,671	-1.417	.914
	2	-.3537	.5902	,550	-1.519	.812
	4	.0829	.5902	,888	-1.083	1.249
4	1	-.3341	.5902	,572	-1.500	.831
	2	-.4366	.5902	,461	-1.602	.729
	3	-.0829	.5902	,888	-1.249	1.083

ANOVA test on germination and flower stage

ANOVA

germratio

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	286,559	3	95,520	6,239	,000

Within Groups	4271,378	279	15,310		
Total	4557,936	282			

Multiple Comparisons

Log(germination percentage)

LSD

(I) flower stage	(J) flower stage	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	1	-,25232*	,05412	,000	-,3587	-,1460
	2	-,17805*	,05292	,001	-,2821	-,0740
	3	,03381	,05468	,537	-,0737	,1413
1	0	,25232*	,05412	,000	,1460	,3587
	2	,07427	,05081	,145	-,0256	,1741
	3	,28613*	,05264	,000	,1827	,3896
2	0	,17805*	,05292	,001	,0740	,2821
	1	-,07427	,05081	,145	-,1741	,0256
	3	,21186*	,05141	,000	,1108	,3129
3	0	-,03381	,05468	,537	-,1413	,0737
	1	-,28613*	,05264	,000	-,3896	-,1827
	2	-,21186*	,05141	,000	-,3129	-,1108

*. The mean difference is significant at the 0.05 level.

ANOVA test on germination and anther stage

ANOVA

germratio

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	338,536	3	112,845	7,146	,000
Within Groups	4153,359	263	15,792		
Total	4491,895	266			

Multiple Comparisons

Log(germination percentage)

LSD

(I) anther stage	(J) anther stage	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	-,24235*	,05760	,000	-,3556	-,1291
	C	-,32426*	,06197	,000	-,4461	-,2025
	D	-,11918*	,05544	,032	-,2282	-,0102
B	A	,24235*	,05760	,000	,1291	,3556
	C	-,08191	,05740	,154	-,1947	,0309
	D	,12317*	,05029	,015	,0243	,2220
C	A	,32426*	,06197	,000	,2025	,4461
	B	,08191	,05740	,154	-,0309	,1947
	D	,20508*	,05524	,000	,0965	,3137
D	A	,11918*	,05544	,032	,0102	,2282
	B	-,12317*	,05029	,015	-,2220	-,0243
	C	-,20508*	,05524	,000	-,3137	-,0965

*. The mean difference is significant at the 0.05 level.

6 Germination rates according to flower-anther stages

Genotype	Flower stage	Anther stage	Germination percentage(%)	
P540	0	A	0	0
P540	1	A	0	0
P540	2	C	0	0
P540	3	D	0	0
P867	0	B	0	0
P867	1	B	1	3
P867	2	C	20	25
P867	3	D	3	3
K002	0	A	0	0
K002	1	C	1	1
K002	2	D	0	0
K002	3	D	0	0
K004	0	A	0	0
K004	1	B	0	0
K004	2	C	0	0
K004	3	D	0	0
K010	0	A	0	0
K010	1	B	0	0
K010	2	D	0	0
K010	3	D	0	0
K011	0	A	0	0
K011	1	B	10	8
K011	2	C	6	4
K011	3	C	4	2
K013	0	A	0	0
K013	1	D	4	6
K013	2	D		
K013	3	D	0	0
K019	0	A	10	10
K019	1	B	7	9
K019	2	D	5	4
K019	3	D		
K021	0	B	3	7
K021	1	B		
K021	2	D	1	15
K021	3	D	0	0
K029	0	A	0	0
K029	1	A	0	0

Genotype	Flower stage	Anther stage	Germination percentage(%)	
K029	2	B	0	0
K029	3	D	0	0
K032	0	A	0	0
K032	1	B	5	10
K032	2			
K032	3	C	4	6
K039	0	A	0	0
K039	1	B	0	0
K039	2		0	0
K039	3	D	0	0
K040	0			
K040	1			
K040	2	D	13	11
K040	3	D		
K043	0	A	0	0
K043	1	B	2	2
K043	2	C	0	0
K043	3	D	0	0
K046	0			
K046	1			
K046	2	D	4	6
K046	3	D	0	0
K050	0	B		0
K050	1	C		0
K050	2			0
K050	3			
K051	0			
K051	1	B	1	2
K051	2	C	3	2
K051	3			
K054	0	A	0	0
K054	1			
K054	2	A		
K054	3	C	0	0
K056	0	A	0	0
K056	1	A	0	0
K056	2	A	3	0
K056	3	C		
K059	0	B	14	17
K059	1	B	21	22
K059	2	C	8	12
K059	3	D	7	5

Genotype	Flower stage	Anther stage	Germination percentage(%)	
K058	0	A	0	0
K058	1	B	1	0
K058	2	D	0	0
K058	3	D	0	0
K060	0			
K060	1	A	0	0
K060	2	C	0	0
K060	3	D	0	0
K063	0			
K063	1		0	0
K063	2		0	0
K063	3			
K066	0			
K066	1			
K066	2	A	1	1
K066	3	C	0	0
K085	0	B	0	0
K085	1	B	14	11
K085	2	C	6	4
K085	3	D	2	3
K088	0	B	25	23
K088	1	C	20	16
K088	2	C	18	17
K088	3	D	0	0
K093	0	B	0	0
K093	1	B		
K093	2	D	2	1
K093	3	D		
K097	0	A		
K097	1	A	7	7
K097	2	B	1	3
K097	3	D	3	2
K099	0	A	0	0
K099	1	A	5	4
K099	2	C	3	6
K099	3	D	0	0
K102	0	B	0	0
K102	1	C	0	0
K102	2	D	0	0
K102	3	D	0	0
K101	0	A	0	0
K101	1	C	10	10

Genotype	Flower stage	Anther stage	Germination percentage(%)	
K101	2	D	4	6
K101	3	D	0	0
K104	0			
K104	1	C	0	0
K104	2	D	0	0
K104	3			
K106	0	B	0	0
K106	1	B	31	20
K106	2	C	19	17
K106	3	D	6	6
K107	0			
K107	1			
K107	2	D	0	0
K107	3	D	0	0
K109	0		3	4
K109	1			
K109	2	D		
K109	3	D	0	0
K113	0	A	0	0
K113	1	B		
K113	2	C	2	2
K113	3	D	1	1
K118	0	B	0	0
K118	1	B	0	0
K118	2	C	0	0
K118	3	D	0	0
K123	0	B	0	0
K123	1	C	0	0
K123	2	C	2	1
K123	3	D	0	0
K128	0	A	0	0
K128	1	B	0	0
K128	2	D	0	0
K128	3	D	0	0
K130	0	B	4	5
K130	1	C	10	14
K130	2	D	6	7
K130	3	D	0	0
K132	0	B	0	0
K132	1	B	0	0
K132	2	D	0	0
K132	3	D	0	0

Genotype	Flower stage	Anther stage	Germination percentage(%)	
K134	0		0	0
K134	1	B	0	0
K134	2	C	0	0
K134	3	D	0	0
K137	0	B	0	0
K137	1	C	13	19
K137	2	D	10	13
K137	3	D	3	2
K140	0	B	8	17
K140	1	D	3	1
K140	2	D	7	5
K140	3	D	2	6
K141	0	A	0	0
K141	1	A	0	0
K141	2		0	0
K141	3			
K144	0	A		
K144	1	B	2	2
K144	2	C	0	0
K144	3	D	0	0
K148	0	B	0	0
K148	1	B	0	0
K148	2	C	1	1
K148	3	D	0	0
K153	0		0	0
K153	1		0	0
K153	2		0	0
K153	3		0	0
K155	0	A	0	0
K155	1	B	0	0
K155	2	D	0	0
K155	3	D	0	0
K157	0	B	0	0
K157	1	B	0	0
K157	2	B	2	2
K157	3	C	0	0
K160	0	A	0	0
K160	1	B	7	9
K160	2	C	3	2
K160	3	D	0	0
K167	0	B	0	0
K167	1	B	0	0

Genotype	Flower stage	Anther stage	Germination percentage(%)	
K167	2	B	0	0
K167	3	C	0	0
K169	0			
K169	1			
K169	2	B	0	0
K169	3	C	0	0
K174	0	A	0	0
K174	1	B	0	0
K174	2	C	0	0
K174	3	C	0	0
K175	0	A		
K175	1	A		
K175	2	C	0	0
K175	3	D		
K179	0	A		
K179	1	A	0	0
K179	2	D	0	0
K179	3			
K185	0	B		
K185	1	C	3	4
K185	2	C	2	3
K185	3	D	1	1
K189	0	B	3	8
K189	1	C	10	10
K189	2	D	8	4
K189	3	D	0	0
K192	0	B	0	0
K192	1	B	4	4
K192	2	C		
K192	3	D	0	0
K200	0			
K200	1	A	0	0
K200	2	B	0	0
K200	3	B		
K204	0	B		
K204	1	B	7	8
K204	2	D	0	0
K204	3	D	0	0
K205	0			
K205	1	B	4	3
K205	2			
K205	3	D		

Genotype	Flower stage	Anther stage	Germination percentage(%)	
K207	0		0	0
K207	1	B	0	0
K207	2	B	0	0
K207	3	D	0	0
K208	0	A	0	0
K208	1	B	0	0
K208	2	D	10	11
K208	3	D	0	0
K212	0	A	0	0
K212	1	A	0	0
K212	2	A	0	0
K212	3	A	0	0
K219	0			
K219	1	A	0	0
K219	2	D	0	0
K219	3	D		
K222	0			
K222	1	A	15	10
K222	2	C	1	1
K222	3	D	0	0
K223	0	A	0	0
K223	1	B	13	11
K223	2	C	0	0
K223	3	D	0	0
K225	0	A	0	0
K225	1	B		
K225	2	B	1	1
K225	3	D	0	0
K228	0			
K228	1	B	0	0
K228	2	D	0	0
K228	3			