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In vitro Competition of *Betula alnoides* Pollens from Two Types of Habitats in a Heterogeneous Landscape in South China

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Abstract

Pollens of *Betula alnoides* were collected in its natural forests at two types of sites with soils originated from limestone (Site L) and granite (Site G) in a heterogeneous landscape at Jingxi County, Guangxi in 2010 to 2012 to investigate whether nutritional differences between the sites influenced its pollen competition. *In vitro* pollen germinations were conducted separately and in pairs for these samples as well as nutrient contents of pollens and soils were measured. There was no significant difference in total nitrogen, phosphorus, potassium and calcium contents of dried pollen and in efficient ones of aqueous extracts of pollens between two types of sites although the soil nutrient contents were quite different between them, and less remarkable correlation was found between these nutrient contents of soil and pollen. The results of pollen competition of *B. alnoides* were much diverse among three years, it seemed that pollen-pollen interaction did not depend on site type, and might vary according to genotype and/or combination of genotypes.

Key words: Betula alnoides, genotype, habitat, heterogeneous landscape, *in vitro*, pollen competition, pollen germination, pollen nutrition, pollen tube growth, soil nutrition.

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Introduction

Pollen competition can take place among pollen grains both from one plant and from several plants in natural

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pollination since pollen grains deposited onto stigma come generally from more than one donors and their number is usually higher than that of ovules. Natural selection would occur before fertilization due to difference of their germination properties, particularly variation of pollen tube growth rates lead to pollen competitions to obtain efficient ovules (PASONEN and KÄPYLÄ, 1998; SNOW and SPIRA, 1996).

The effect of environmental factors such as temperature and site conditions of pollen donors on pollen performance has been widely investigated. In particular, whether soil fertility influences pollen chemical composition and pollen performance is of great concern. For examples, LAU and STEPHENSON (1993) determined the effects of soil nitrogen on pollen performance of *Cucurbita pepo*, they also studied the effects of soil phosphorus on pollen phosphorus content and pollen competition (LAU and STEPHENSON, 1994). OLEKSYN *et al.* (1999) tested pollen nutritional status of *Pinus sylvestris* plantations grown at sites with different soil fertility under contrasting pollution. LANKINEN (2008) studied the effects of soil pH and phosphorus on pollen competitive ability of *Viola tricolor*. TRAVERS (1999) investigated soil fertility and pollen performance of *Clarkia unguiculata* plants in recently burned and unburned oak woodlands, and pollen germination and tube growth were also estimated for chamber-growth plants of this species watered with full strength fertilizer and distilled water (SMITH-HUERTA *et al.,* 2007). These studies were mostly conducted in green-house or for cultivated plants, and up to date, there is less relevant case study in natural community or landscape.

Pollen competition is usually investigated through either *in vivo* or *in vitro* germinations of pollen mixtures. There perhaps exists unknown substance influencing pollen germination and pollen tube growth rate in female gametophyte, we can hardly distinguish this kind of female gametophyte effect and effects of pollen-pollen interaction on pollen performance, and this makes it difficult to study pollen-pollen interactions by pollination of mixed pollens (PARANTAINEN and PASONEN, 2004). While using *in vitro* pollen germination can not only study direct interaction between pollens from different plants, but also is helpful to find out influencing factors of pollen competition, and is thus a simple and efficient way to study pollen competition. It was found out in previous studies that there existed significant positive correlations between *in vitro* and *in vivo* pollen germination (PASONEN *et al.,* 1999; STEINER and GREGORIUS, 1999), this infers that *in vitro* germination can to certain extent reflect status of *in vivo* germination of pollens. *In vitro* pollen germination are still utilized in studies on pollen competition by a number of scholars (PASONEN and KÄPYLÄ, 1998; STEINER and GREGORIUS, 1998; PARANTAINEN and PASONEN, 2004; PULKKINEN *et al.,* 2009; VARIS *et al.,* 2010).

Betula alnoides Buch. Ham. ex D. Don is an anemophilous, moneocious and self-incompatible tree species distributed in tropics and warm subtropics. It is a valuable timber species as well as an ecologically orientated tree species (ZENG *et al.,* 2006). In the previous studies, we found an ectone of two types of soils originated from limestone (site L) and granite (site G) in Jingxi County, Guangxi. *B. alnoides* is normally not suitable to grow at limestone hills, however, there exist local sites suitable for growth of this species in this kind of mountains due to slope sedimentation of soil and vegetation acidification. Therefore mosaic landscape including several big or small patches of *B. alnoides* is formed (CHENG *et al.,* 2010).

From 2010 to 2012, pollens of trees were collected separately from each site, and pollen competitions were investigated by *in vitro* pollen germination. Meanwhile, nutrient contents of soil and pollen were measured. The aims of the present study is to answer the following questions: (1) Are there any nutrient differences of soil and pollen between two types of sites? (2) Whether does significant correlation exist between soil and pollen nutrition? and (3) Is pollen competition different between *B. alnoides* trees at two types of sites? The

Table 1. – Nutrient status of soils under canopy of 19 sampled *Betula alnoides* trees at two types of sites (9 from site G and 10 from site L) in the heterogeneous landscape in Jingxi County, Guangxi.

Soil Layer	Site	pH	Total N(g/kg)	Total P(g/kg)	Total K(g/kg)	Eff. N(mg/kg)	Eff. P(mg/kg)	Eff. K(mg/kg)	Eff. Ca(mg/kg)
0-20cm	G	4.45 (0.09) **	1.094 (0.083)	0.544 (0.071) **	13.748 (1.028) **	63.53 (6.68)	0.97 (0.12)**	52.73 (3.84) **	64.35 (21.89) *
	L	5.24 (0.14) **	0.955 (0.130)	0.284 (0.045) **	1.469 (0.133) **	57.00 (10.56)	1.85 (0.10)**	26.91 (3.04) **	394.40 (121.90) *
20-40cm	G	4.66 (0.19) **	0.854 (0.069) **	0.580 (0.102) **	14.466 (1.147) **	40.15 (4.00) *	0.67 (0.14)	32.92 (3.55) **	42.23 (10.20) *
	L	5.52 (0.15) **	0.421 (0.062) **	0.220 (0.031) **	1.650 (0.123) **	24.26 (4.46) *	0.88 (0.17)	14.54 (2.76) **	347.43 (96.59) *
40-60cm	G	4.77 (0.09) **	0.728 (0.067) **	0.585 (0.098) **	14.551 (1.107) **	27.42 (2.83)	0.79 (0.21)	29.00 (3.50) **	45.77 (15.62) *
	L	5.82 (0.16) **	0.365 (0.050) **	0.196 (0.028) **	1.772 (0.100) **	19.73 (4.36)	0.79 (0.17)	13.00 (1.82) **	445.06 (119.40) *

Notes: G and L refer to soils originated from granite and limestone, respectively; Figures in parentheses are standard errors; "Eff." is the abbreviation of "efficient"; *: $P < 0.05$, **: $P < 0.01$.

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study will not only establish foundations for breeding without breeding of *B. alnoides* on basis of molecular markers (WANG *et al.,* 2010) so as to facilitate its hybrid breeding, but also provide scientific evidences for seed orchard establishment of this species.

Materials and Methods

The pollens were collected from trees in an ectone of two types of soils originated from limestone (site L) and granite (site G) in Jingxi County, Guangxi (latitude 22°58'N, longitude 106°20'E). This area belongs to subtropical monsoon climate with annual mean air temperature of 19.1°C and annual mean rainfall above 1500 mm. The altitude of these sites ranged from 800 m to 900 m.

Six to ten individuals of *B. alnoides* were selected for pollen collection from each type of sites in late October or early November from 2010 to 2012 with distances between sampled trees above 50 meters. Before the expected beginning of flowering, branches with male inflorescences of each tree were cut and incubated in water immediately. Once the yellow anthers opened and pollens could be seen, branches were shook on sulfuric acid papers, and pollens were collected and sieved by 200 mesh, then stored in medical vials under vacuum and cold condition (3 to 5°C) for 1–6 weeks before the germination tests (CHENG *et al.,* 2007a).

Fresh pollens from sampled trees were used to make three kinds of mixtures (1:1): a. pollens from site L mixed with pollens from site L (L × L); b. pollens from site L mixed with pollens from site G (G × L); and c. pollens from site G mixed with pollens from site G (G × G). The germinabilities of pollen mixtures and of the individuals were tested separately. The basal aqueous incubation medium contained 15% sucrose and 0.02% boric acid (CHENG *et al.,* 2007b). A drop of aqueous incubation medium was placed on the groove of glass microscope slides, then pollens were scattered on the medium using a Q-tip, and a cap of film canister was covered on the medium drop with vaseline around the brim so as to avoid medium drop becoming dry. The groove glass slides were placed in moist 12-cm Petri dishes and cultured in the incubator (30°C) for 3 h (CHENG *et al.,* 2007b). Each test was replicated four times.

After incubation, four views were randomly selected and photographed in each microslide using the camera lens of Olympus microscope (BX51, Japan). And in each view, there should be more than 300 pollen grains. Pollen grains with pollen tubes longer than grain diameter were considered as germinated ones. The amount of pollen grain and the number of germinated pollen in each view were counted using Adobe Photoshop CS software. And 10 germinating pollens were selected randomly in each view, and their pollen tube lengths were measured by CAD software. Pollen germination percentages and pollen tube growth rates of pollen mixtures were compared with those of paired individual pollens.

In 2012, pollens from nine trees at site G and ten trees from site L were used for nutrient analysis. Two samples of 0.75 gram for each tree were placed under 45°C for three days. One was used to directly measure total contents of nitrogen, phosphorus, potassium and calcium in pollens, which were digested in a block digester using a H_2SO_4 and K_2SO_4-$CuSO_4$ mixture catalyst for total N by diffusion method, while HNO_3-$HClO_4$ mixture solution for P, K and Ca by inductively coupled plasma optical emission spectrometry (ICP); another was used to make aqueous extracts of pollens with 20 mL double distilled water in Endorf tube, after centrifugation, supernatant solutions were obtained and their contents of soluble N, P, K and Ca were measured directly with

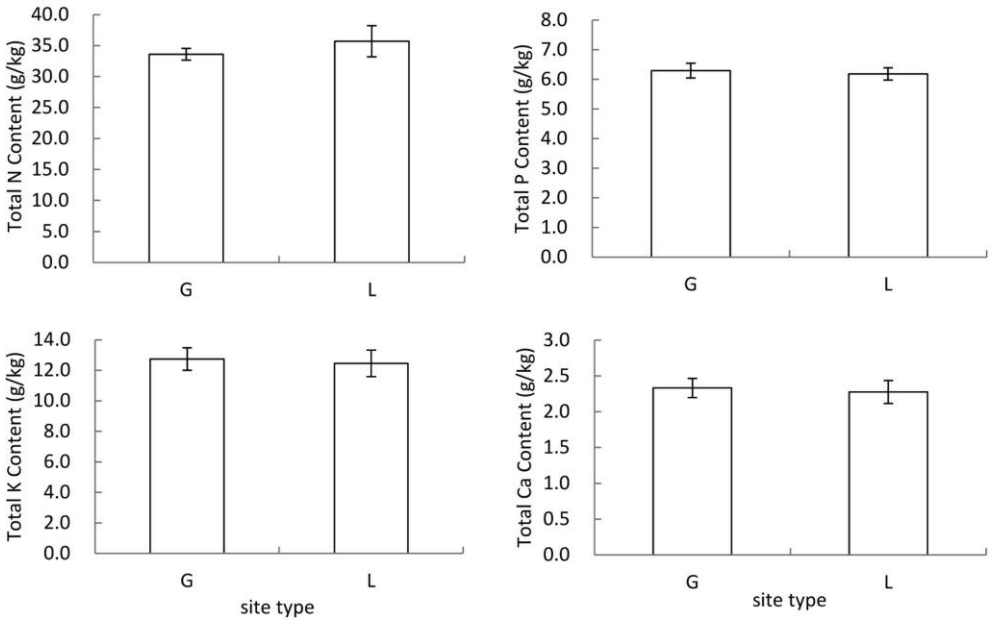

Figure 1. – Differences of nutrient content in dried pollens from two types of sites. G and L refer to soils originated from granite and limestone, respectively. Error bars represent standard errors.

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the methods as mentioned for the total contents. For each sampled tree in 2012, three layer of soils (0–20 cm, 20–40 cm and 40–60 cm) were sampled from three points randomly arranged under canopies, and their pH and contents of total N, P and K, and soluble N, P, K and Ca were measured, respectively.

Pollen performance such as germination percentage and pollen tube growth rate of each paired pollen mixture was compared to the expected performance by Student's *t*-test. The expected performance was calculated as the mean values of the two individuals in each paired pollen mixture. The comparisons of soil and pollen nutrition between two types of sites were done with one-way ANOVA. Relationship between soil and pollen nutrition were analyzed with Pearson's coefficient. All the statistical analyses were carried out in the SPSS 13.0 for Windows.

Results and Discussion

Soil and pollen nutrient

As revealed by one-way analysis of variance (*Table 1*), there existed significant differences ($P<0.05$) in pH and contents of total phosphorus, total and efficient potassium, and exchangeable calcium in all three layers of soils between site G and L. While no significant difference ($P>0.05$) was seen in contents of total nitrogen in the soil at 0–20 cm depth, and contents of efficient nitrogen in the soils at both 0–20 cm and 40–60 cm depth, and content of efficient phosphorus in soils of lower two layers.

From *Figure 1* and *Figure 2*, no significant difference was seen between the two types of sites in not only contents of total nitrogen, phosphorus, potassium and calcium in dried pollen, but also efficient nitrogen, phosphorus, potassium and exchangeable calcium in aqueous extracts of pollens although there existed much significant difference of mineral nutrition between the soils. To assess relationships between mineral nutrient contents of soil and pollen, correlation analysis was further conducted in which mineral nutrient contents of soils at 0–20 cm depth were used (*Table 2*). It revealed that mineral nutrient contents of pollens were mostly not remarkably correlated with the mineral nutrient contents of soils, except that efficient phosphorus content in aqueous extract of pollen was in significantly negative correlation with exchangeable calcium content of soil. These were indicated that soil nutrition had no effect on nitrogen, phosphorus, potassium and calcium contents of *B. alnoides* pollens. This was in accordance with Oleksyn *et al.*'s (1999) study on *Pinus sylvestris* in which phosphorus, potassium and calcium concentrations in pollens were also similar between two sites with contrasting pollution although these nutrient concentrations in soils of both sites were quite different. But this did not cohere with Lau & Stephenson's study (1994) on *Cucurbita pepo*, they revealed that soil phosphorus had positively significant effects on pollen phosphorus, and an 8% increase in phosphate concentration per pollen grain for plants growing on phosphorus rich soil.

Pollen competition

The results of pollen competition of *B. alnoides* were summarized as ratios of positively or negatively significant differences between the expected and observed pollen germination percentage and pollen tube growth rate for three mixture types (G×G, G×L, L×L) in three years (*Table 3*). 28 combinations of pollen mixtures were tested in 2010, the percentages of positive effects were higher than those of negative effects on both germination rate and pollen tube growth for all

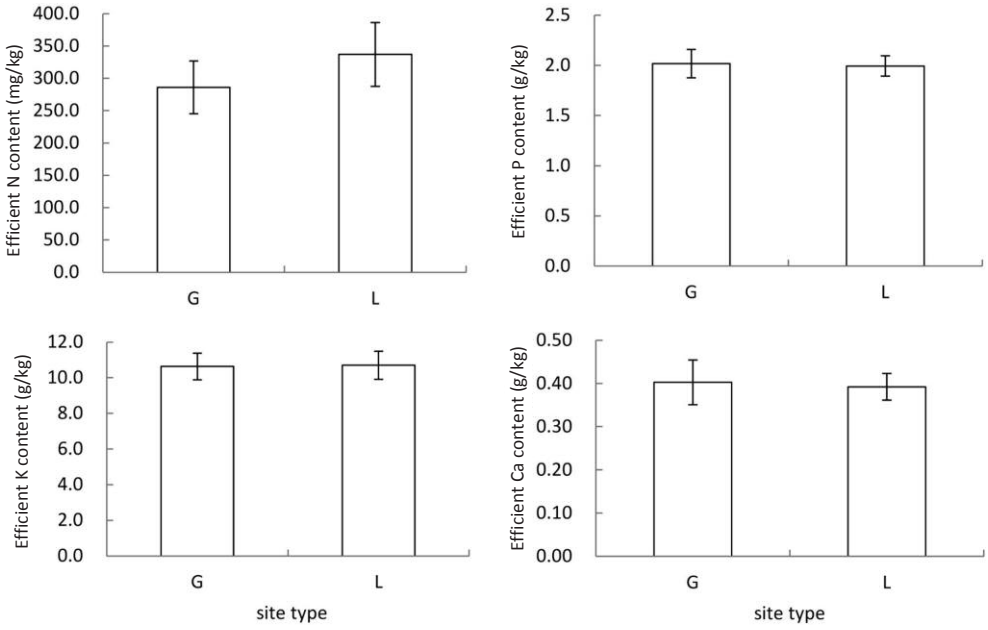

Figure 2. – Differences of nutrient content in aqueous extracts of pollens from two types of sites. G and L refer to soils originated from granite and limestone, respectively. Error bars represent standard errors.

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Table 2. – Relationships between mineral element contents of soil (Layer 0–20cm) and pollen of *Betula alnoides* (N = 19).

Soil chemical traits	Mineral element content of dried pollen (g/kg)				Mineral element content in aqueous extract of pollen (g/kg)			
	Total N	Total P	Total K	Total Ca	Eff. N	Eff. P	Eff. K	Eff. Ca
pH Value	-0.2610	-0.3375	-0.19235	0.1519	-0.0007	-0.4133	-0.1213	-0.0937
Total N (g/kg)	-0.2345	-0.0616	-0.34019	0.0037	0.0449	-0.3829	-0.3471	0.1741
Total P (g/kg)	-0.2378	-0.0142	-0.08119	0.1223	-0.2234	-0.2290	-0.0794	0.1428
Total K (g/kg)	-0.1903	0.1260	0.102182	0.1961	-0.2267	0.0665	0.0390	0.1012
Eff. N (mg/kg)	-0.1930	0.0870	-0.28834	0.3077	0.1700	-0.2276	-0.2615	0.2615
Eff. P (mg/kg)	0.2301	-0.2126	0.07219	-0.0786	0.0273	-0.2738	0.0963	-0.2251
Eff. K (mg/kg)	-0.1335	0.1389	0.007084	0.0357	-0.1388	0.0385	0.0045	0.1102
Eff. Ca (mg/kg)	-0.3117	-0.3781	-0.3917	0.0336	0.1439	-0.4958*	-0.3293	-0.0800

Notes: Data were Pearson's coefficients; "Eff." is the abbreviation of "efficient"; *: $P < 0.05$.

three mixture types, except pollen tube growth for G × G, for which the percentages of positive and negative effects were equal to each other. Among 22 combinations of pollen mixtures in 2011, the percentage of negative effect was higher than that of positive effect on germination rate and equal to that on pollen tube growth for G × G, the percentages of both effects on germination rate and pollen tube growth were equal to each other for G × L, while for L × L, no negative effect was found and 75.0% was positive, much higher than that of no effect. In 2012, a complete combinations of pollen mixtures for eight individuals (four from each site) were tested, the percentages of negative effects were higher than those of positive effects on germination rate and pollen tube growth for all three mixture types. The results of pollen germination and tube growth were quite different among three years, this resulted to certain extent from the fact that less individuals and their combinations were repeated due to climate and other conditions, for example, rainy weather usually occurred during the sea-

Table 3. – *In vitro* competition of fresh pollens from *Betula alnoides* trees at two types of sites. 28 pairs of pollens were tested in 2010, 22 in 2011, and 28 in 2012.

Variable	Year	G×G				G×L				L×L			
		No. of pairs	No effect	Positive effect	Negative effect	No. of pairs	No effect	Positive effect	Negative effect	No. of pairs	No effect	Positive effect	Negative effect
Germination rate (%)	2010	7	42.86	42.86	14.28	10	70.00	30.00	0	11	54.55	36.36	9.09
	2011	12	50.00	8.33	41.67	6	33.34	33.33	33.33	4	25.00	75.00	0
	2012	6	33.33	16.67	50.00	16	31.25	25.00	43.75	6	0	33.33	66.67
Pollen tube length (um)	2010	7	71.43	14.29	14.28	10	60.00	40.00	0	11	72.73	27.27	0
	2011	12	33.34	33.33	33.33	6	33.34	33.33	33.33	4	25.00	75.00	0
	2012	6	50.00	0	50.00	16	37.50	18.75	43.75	6	50.00	0	50.00

Notes: G and L refer to soils originated from granite and limestone, respectively.

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Table 4. – Difference between observed (Obs.) and expected (Exp.) values for germination of paired fresh pollen mixture of *Betula alnoides* (Results in Year 2010 and 2011).

Pollen mixture	Pollen germination (%)				Pollen tube length (um)			
	Samples	Exp.	Obs.	Effect	Samples	Exp.	Obs.	Effect
				Year 2010				
G×G	G4+G5	9.36	16.11	*	G11+G14	18.69	13.20	#
	G4+G14	7.44	12.74	*	G12+G13	9.78	11.90	*
	G7+G14	6.12	8.94	*				
	G11+G14	16.51	10.56	#				
G×L	L1+G5	13.60	28.55	*	L3+G4	19.25	21.88	*
	L4+G4	9.33	21.06	*	L4+G4	14.68	20.76	*
	L10+G12	11.97	18.16	*	L4+G12	16.39	19.54	*
					L10+G12	14.72	20.71	*
L×L	L1+L4	32.50	23.81	#	L3+L8	17.87	23.03	*
	L3+L4	38.64	50.58	*	L3+L10	18.65	26.96	*
	L3+L8	25.12	44.26	*	L7+L10	14.74	22.50	*
	L3+L10	25.50	33.87	*				
	L7+L10	10.14	15.37	*				
				Year 2011				
G×G	G20+G21	20.82	12.98	#	G20+G21	29.8	23.84	#
	G20+G22	26.50	23.76	#	G20+G23	25.14	22.95	#
	G20+G23	15.10	6.42	#	G21+G5	25.13	19.06	#
	G21+G5	12.10	5.64	#	G21+G12	20.23	18.07	#
	G21+G24	3.24	7.01	*	G21+G24	21.28	25.47	*
	G22+G23	12.89	8.02	#	G22+G23	25.16	32.7	*
					G22+G24	18.97	25.06	*
					G22+G25	21.49	34.02	*
G×L	G20+L4	26.98	32.51	*	G20+L4	32.97	34.68	*
	G20+L20	23.00	14.02	#	G21+L4	29.12	33.48	*
	G21+L20	15.23	8.23	#	G21+L20	25.91	23.2	#
	G22+L20	20.88	23.87	*	G22+L20	28.90	24.64	#
	L4+L20	20.96	26.93	*	L4+L20	29.16	32.94	*
L×L	L20+L27	24.13	26.36	*	L20+L27	25.40	31.1	*
	L20+L28	10.85	21.91	*	L20+L28	21.39	25.77	*

Notes: G and L refer to soils originated from granite and limestone, respectively; The expected value is the mean pollen germination percentage or the mean pollen tube length of the two individuals in each paired mixture; *: positive, $P < 0.05$; #: negative, $P < 0.05$.

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Table 5. – Difference between observed (Obs.) and expected (Exp.) values for germination of completely paired fresh pollen mixture for eight individuals of *Betula alnoides* (Results in Year 2012).

Pollen		G3		G6		G13		G15		L13		L15		L17	
candidates		Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.
Pollen germinations (%)	G6	11.32	14.35**												
	G13	5.39	4.36##	11.17	10.83										
	G15	4.26	3.97	10.04	5.63##	4.12	3.05##								
	L13	3.70	3.51	9.48	10.38	3.55	3.93	2.42	2.26#						
	L15	9.11	9.78	14.89	16.96**	8.97	8.36	7.83	5.72##	7.27	5.19##				
	L17	5.90	9.13**	11.67	19.07**	5.75	6.53*	4.62	1.95##	4.05	3.26#	9.47	11.92*		
	L19	28.54	22.95##	34.31	27.46#	28.39	19.36##	27.26	8.26##	26.69	22.39##	32.10	22.28##	28.89	47.38**
Pollen tube length (uM)	G6	25.00	24.93												
	G13	33.27	30.57#	32.58	26.51##										
	G15	28.33	26.58	27.64	17.06##	35.90	34.63								
	L13	30.99	34.17*	30.29	26.25##	38.56	40.74	33.62	26.99##						
	L15	31.68	33.70*	30.99	32.24	39.26	36.95	34.32	35.41	36.97	38.19				
	L17	38.73	32.95	38.03	25.87##	46.30	41.11#	41.36	40.35	44.02	31.02##	44.71	42.50		
	L19	38.35	36.02##	37.66	49.69**	45.93	34.02#	40.99	31.08##	43.64	35.98##	44.34	33.85##	51.38	60.86

Notes: G and L refer to soils originated from granite and limestone, respectively; The expected value is the mean pollen germination percentage or the mean pollen tube length of the two individuals in each paired mixture; *: positive, $P<0.05$; #: negative, $P<0.05$; **: positive, $P<0.01$; ##: negative, $P<0.01$.

son when pollen became mature, it was difficult to collect pollen according to plan. All the same, it seemed that the direction of pollen competition did not depend on the type of pollen mixture, which inferred that soil nutrition had no effect on pollen germination and tube growth of *B. alnoides*. This was in accordance with Smith-Huerta *et al.*'s (2007) study on *Clarkia unguiculata* that no significant difference were found in *in vivo* pollen germination and tube growth between its chamber-growth plants watered with full strength fertilizer and distilled water. While it was inconsistent with studies on *Cucurbita pepo* in which soil nitrogen or phosphorus had obviously positive influences on the pollen germination and tube growth (Lau and Stephenson, 1994; Lau and Stephenson, 1993). As a whole, there was no significant difference on pollen germination rate and pollen tube growth rate of *B. alnoides* between site G and site L in the heterogeneous landscape, this was also supported by the above results that soil nutrition had no effects on the mineral nutrient contents of pollens. It could be deduced that gene flow could not be site specific through pollen dispersal between the two types of sites.

Pollen competition was assessed further for the cases with significant differences in 2010 and 2011 (*Table 4*) and all cases in 2012 (*Table 5*). It was shown from *Table 4* that positive effects were found on pollen germination and pollen tube growth rate for paired pollen mixtures including G4, G12, L10 and L3 in 2010, and L4 in 2011, respectively. While most paired pollen combinations including G21 or G20 in 2011 had negative effects on pollen germination or pollen tube growth rate except combinations of G20 + L4, G21 + L4 and G21 + G24, which showed positive effects. Most paired pollen combinations including L4 had positive effects on pollen germination or pollen tube growth rate in two years except L1 + L4 in 2010. These demonstrated that, it perhaps depended on genetic property whether pollen competition showed negative, positive or no effects. This was more obviously demonstrated in the tests for complete paired combinations of fresh pollen from eight individuals in 2012. From *Table 5* it was indicated that combinations including L19 or G15 showed negative effects on pollen germination except that L19 + L17 (positive effect) and G15 + G3 (no effect), while in combinations of L17, only two combinations with G15 and L13 had negative effects on pollen germination. As to pollen tube growth rate, similar results could be concluded. As a whole, pollen competition depended on specific individuals and their combinations, which inferred that pollen competition was determined by genetic effect, that's to say, genetic effect was very important for pollen competition. This was in accordance with many previous studies, for examples, Parantainen and Pasonen (2004) found out that direct pollen-pollen interaction can be more pronounced among certain genotypes of *Pinus sylvestris*, this was also confirmed by Varis *et al.*'s (2010) study on *P. sylvestris* pollen, they concluded that pollen-pollen interaction may vary according to genotype and/or genotype combination. In the present study, it seemed that pollen-pollen interactions were determined by genetic effect rather than by soil environment in which pollen donors grew. Thus when hybrid breeding or

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breeding without breeding (WANG *et al.,* 2010) are carried out, pollen competition should be assessed for *B. alnoides* so as to obtain good hybrid combination more efficiently.

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