Euphytica (2015) 206:555–566
DOI 10.1007/s10681-015-1458-4

A preliminary study on the crossability in *Robinia pseudoacacia* L.

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Received: 19 December 2014 / Accepted: 15 April 2015 / Published online: 2 June 2015

Abstract To explore the feasibility of intra specific hybridisation between *Robinia pseudoacacia* (common diploid) and other *Robinia* varieties, tetraploid *R. pseudoacacia*, *R. pseudoacacia var. decaisneana* (Carr.) Voss., *R. pseudoacacia Frisia*, and *R. pseudoacacia* "Idaho" were collected as male parents, and hybridisation trials were conducted over a period of three consecutive years. The average seedling emergence rates of the five hybridised combinations were ~2.3,2.0, 3.3, 1.3, and 0 % per year, respectively. To ensure maximum seedling emergence rates, we found that the best pollinating times were the blooming days. To investigate the causes of low crossability, we also examined pollen-tube growth and fruit setting rates. The results indicated that the causes of low crossability were abnormal pollen-tube growth, failed development of fertilised ovule, and poor seed germination. Low fruit-setting rates caused by emasculation may also lead to low crossability.

Keywords *Robinia* L. · Crossability · Pollen tube · Fruit setting rate

Introduction

Robinia pseudoacacia is a fast-growing, multipurpose tree species native to south-eastern North America. *R. pseudoacacia* has been introduced to Europe, Asia, Australia, South America, and Africa, where it has undergone rapid expansion and become naturalised in

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Electronic supplementary material The online version of this article (doi:10.1007/s10681-015-1458-4) contains supplementary material, which is available to authorized users.

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many countries (Ru et al. 2005). Black locust was first introduced to China in 1877, and is now a popular variety that is extensively cultivated in many parts of the country (Ru et al. 2005; Wang et al. 2009). It is suitable for timber, fuel, land reclamation, bee-keeping, feedstock, raw material for energy plantations, wood fibre and forage (Keeler 1907). Because black locust can tolerate multiple stress conditions, such as salty and alkali soil, cold and drought, etc., it has also been cultured in poor soil conditions for environmental restoration purposes (Keeler 1907).

Robinia pseudoacacia is considered to be a highly outcrossing species(Surles et al. 1990; Yuan et al. 2014; Dini-Papanastasi and Aravanopoulos 2008), due to the physical separation of the stigmatal and antheral surfaces, as well as its protogynous flowering. Since black locust is a highly outcrossing species, we expect artificial hybridisation to provide a potential method for *R. pseudoacacia* breeding, and for study of heredity. However, since its introduction to China, *R. pseudoacacia* breeding strategies have been mainly optimum clonal selection and optimum species introduction (Xun et al. 2009). Few attempts at artificial pollination have been reported(Dini-Papanastasi and Aravanopoulos 2008; Rédei and Intézet 1998; Yuan et al. 2013), and these studies focus mainly on intraspecific hybridisation; to our knowledge, intra specifichybridisation between *R. pseudoacacia* and other *Robinia* L. varietieshas not been reported to date.

We selected *R. pseudoacacia*(diploid) as the female parent, and four *Robinia* L. species: Tetraploid *R. pseudoacacia* L, *R. pseudoacacia var. decaisneana* (Carr.) *Voss*., *R. pseudoacacia* Frisia, and *R. pseudoacacia* "Idaho" as the male parents for evaluation of the hybridisation crossability between *Robinia pseudoacacia L.* and other germplasms.

Tetraploid *R. pseudoacacia* was first introduced to China by Beijing Forestry University from South Korea in 1997. Compared to normal diploid *Robinia pseudoacacia,* tetraploid *R. pseudoacacia* clones have a significantly higher yield, with large leaves and a high leaf protein content; moreover, they are polyanthous, long blossoming, and suitable for fodder and beekeeping (Li and Jiang 2006; Zhang et al. 2009).

Robinia pseudoacacia var. decaisneana (Carr.) Voss. is native to North America. Its tree form and crown are similar to those of *R. pseudoacacia,* but no thorns, or only small thorns, are present on the branches and stocks of the former, and it has an amaranth corolla. *R. pseudoacacia var. decaisneana* (Carr.) Voss can tolerate saline alkali soil, but has relatively low tolerance to cold (Song et al. 2006).

Robinia pseudoacacia Frisia is native to North America, and the colour of its leaves changes seasonally: auratus in spring, yellow-green in summer, and aurantiacus in autumn (Li and Xu 2005).

Robinia pseudoacacia "Idaho" is native to Spain. It has an aubergine corolla, and blooms twice per year, giving it a high ornamental value. It has an abundant root system and is easily propagated (Xu et al. 2013).

The objectives of this study were to investigate the crossability between *Robinia pseudoacacia* and four other germplasms of *Robinia* L. by examining the pollen germination on the stigma and the characteristics of pollen-tube development, and calculating the seed setting and seedling emergence rates. The results provide a foundation for the hybrid breeding of *Robinia* L. and hybrid seeds for future research.

Materials and methods

Plant materials

From 2011 to 2013, two *Robinia pseudoacacia* L. plants (Fig. 1a) were selected as the female parents in a plantation forest at Mijiabu tree farm (40°30′302″N, 116°00′015″E), Yanqing, Beijing, China. Two male parents of *Robinia pseudoacacia* L. were also selected: R. *pseudoacacia var. decaisneana* (Carr.) Voss. (Fig. 1c) and R. *pseudoacacia Frisia* (Fig. 1d) were selected at the Beijing Forestry University campus; R. *pseudoacacia* "Idaho" (Fig. 1e) was selected at the National Nursery in Guan Xian County (Shandong Province, China); and Tetraploid *R. pseudoacacia* L (Fig. 1b) was selected in Beizhangzhuang, Yanqing, Beijing, China (40°58′703″N, 116°87′746″E).

Determination of stigmatic receptivity and pollen vitality

To investigate the optimum hybridisation period, flowers from the female parents were collected every 4 h from 8:00 to 16:00 on the day before blooming, the day of blooming, the day after blooming, and 2 days after blooming. To improve the accuracy of the results, 10 flowers were collected at each time point. The stigmatic receptivity was detected by dropping a drop

Fig. 1 *Robinia L* germplasms used in this study: **A** *R. pseudoacacia* L.; **B** Tetraploid *R. pseudoacacia* L. **C** *R. pseudoacacia var. decaisneana* (Carr.) Voss. **D** *R. pseudoacacia* Frisia. **E** *R. pseudoacacia* "Idaho"

of benzidine–H_2O_2 (1 % benzidine: 3 % hydrogen peroxide: water = 4:11:22) onto the stigma and observing under a microscope. Those stigma with high receptivity normally produce a large number of bubbles (Dafni and Maués 1998).

The pollen viability was tested using a chloride-3-phenyl tetrazolium (TTC) staining method on the hybridisation day (Supplemental Fig. 2), according to Huang (2004). Pollen was stained with TTC (1.0 % by weight in 50 % sucrose), and was dusted onto a microscope slide with a brush to which four or five drops of stain had been added. A coverslip was immediately placed on the slide, and its edges sealed with nail varnish. After a 15-min incubation at 40 °C, the pollen was observed under a microscope, and approximately 300 pollen grains from each replicate from the four different areas were counted (three replicates for each staining treatment) to determine pollen viability.

Emasculation, pollination, changes in fruit setting rates, and crossability

To avoid self-pollination and unwanted crosses with nearby plants, the flowers of the seed parent were emasculated and covered with 400 mesh nets (0.037 mm) 1 day before opening in early to middle May (Supplemental Fig. 1). Pollen was applied to the female parent stigma 1 day after emasculation. Table 1 shows the total number of pollinated flowers and all combinations. All nets were removed 3 days after pollination. All pollen from the male parents was collected on their blooming day and stored at 4 °C with silica gel, no more than 5 days prior to our pollination procedure.

The fruit setting rates of all treatments were calculated at 7, 30, 60, and 90 days after pollination, and were calculated as the percentage of pollinated flowers of the total number of remaining flowers.

The seeds were collected from August to September and placed in plastic bags containing silica gel. Each seed (ovule) was classified according to its condition as a mature, aborted or insect-attacked seed. The seeds were placed on moist filter paper in Petri dishes and soaked with 70 °C water for 24 h. Then, all seeds were placed on new moist filter paper in Petri dishes for a further 24 h before planting in seedling bags (diameter = 12.5 cm, height = 12.5 cm) containing potting soil ("turf soil": roseite: sand: perlite = 3:2:2:2). Plants were grown in a phytotron under the following conditions: 12-h light/dark; 25 °C light/18 °C dark; 6.7 flux lumen output; and 70 % humidity. The seedling emergence rate was measured after 4 weeks (The emergence rate did notincrease with longer time). The cross compatibility was calculated as the percentage of pollinated flowers that yielded seedlings.

The differences in fructification percentages, seedling emergence rates and other statistical values among the treatments were assessed by one-way ANOVA using the Statistical Package for the Social Sciences (SPSS) software, version 19.0.

Pollen-tube growth test

Pistils from each treatment were collected 2, 6, 12, 24, 48, and 72 h after pollination, and fixed using FAA (formalin: aceto: alcohol = 18:1:1). The fixed samples were removed from the FAA and treated with 70 % ethanol, 50 % ethanol, 30 % ethanol, and distilled water, successively, for 1 h each. After hydration, all samples were soaked in 5 mol/L sodium hydroxide solution (NaOH) and incubated at

Table 1 Hybridised combinations of *Robinia* L.

Stage	Hybridised combinations (♀ × ♂)	Emasculation	Disposal after pollination	Number of pollinated flowers
Hybridisation	*Robinia pseudoacacia* L. 1 × *Robinia pseudoacacia* L. 2	E	With net	100
	Robinia pseudoacacia L. 2 × *Robinia pseudoacacia* L. 1	E	With net	100
	Robinia pseudoacacia L. 1, 2 × tetraploid *R. pseudoacacia* L.1, 2	E	With net	200
	Robinia pseudoacacia L. 1, 2 × *R. pseudoacacia var. decaisneana* (Carr.) Voss.1, 2	E	With net	200
	Robinia pseudoacacia L. 1, 2 × *R. pseudoacacia* Frisia 1, 2	E	With net	200
	Robinia pseudoacacia L. 1, 2 × *R. pseudoacacia* "Idaho" 1, 2	E	With net	200
Control	Artificial self-pollination	E	With net	200
	Natural self-pollination	NE	With net	200
	Natural seeding	NE	Without net	200
	With net after emasculation	E	With net	200
	Without net after emasculation	E	Without net	200

E emasculation, *NE* no emasculation

56 °C for 50 min. Then, all samples were rinsed three times using distilled water, (5 min for the first and second rinsings, and 2 h for the third rinsing), and soaked in 0.1 % Aniline blue fluorescent staining solution for 2 d. The pollen tubes in the pistils were then observed under a fluorescence microscope (Martin 1959).

DNA extraction and sequence: related amplified polymorphism (SRAP) analysis

Genomic DNA was extracted from the leaves of all male parents and putative hybrid seedlings, using a Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China). SRAP loci primers were selected from Yuan et al. (2011). and are listed in Supplemental Table 1.

Polymerase Chain Reaction(PCR)was performed in a reaction volume of 25 μL containing 30-ng DNA, Mg^{2+} 2.5 mmo1/, dNTPs 0.2 mmol/L, Taq DNA polymerase 1.5 U, primer 0.3 μmol/L. The PCR profile consisted of denaturation at 94 °C for 5 min, followed by 5 cycles at 94 °C for 1 min, 35 °C for 1 min, and 72 °C for 1.5 min; then 35 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.5 min, with a final extension at 72 °C for 10 min. The PCR products were electrophoresed in an 8 % polyacrylamide gel.

Results

Stigmatic receptivity and pollen vitality

The flower structure of *R. pseudoacacia* is typical papilionaceous, enforcing cleistogamy. In the benzidine–H_2O_2 tests, the stigmas from the blossom day (Fig. 2c), and the second day (Fig. 2d) turned blue surrounded by many bubbles, which indicates high activity on the blooming day, and remained active on the second day, and then became inactive on the afternoon of the thrid day (Fig. 2e).

From the benzidine–H_2O_2 tests of the stigmas performed at different times on three consecutive days, we conclude that the stigma receptivity of *R. pseudoacacia L.* is maintained for up to 3 days, and that the blooming day has the highest stigma receptivity, which decreases gradually over the following 2 days. The stigmas displayed zero receptivity in the afternoon of the third day.

The TTC test (Supplemental Table 2, Supplemental Fig. 2) showed that the pollen vitalities of all five pollen types were >50 %, which is sufficient for hybridisation.

Pollen-tube growth behaviour

Pollen germination took place in all of the hybrid treatments, but the level of germination varied

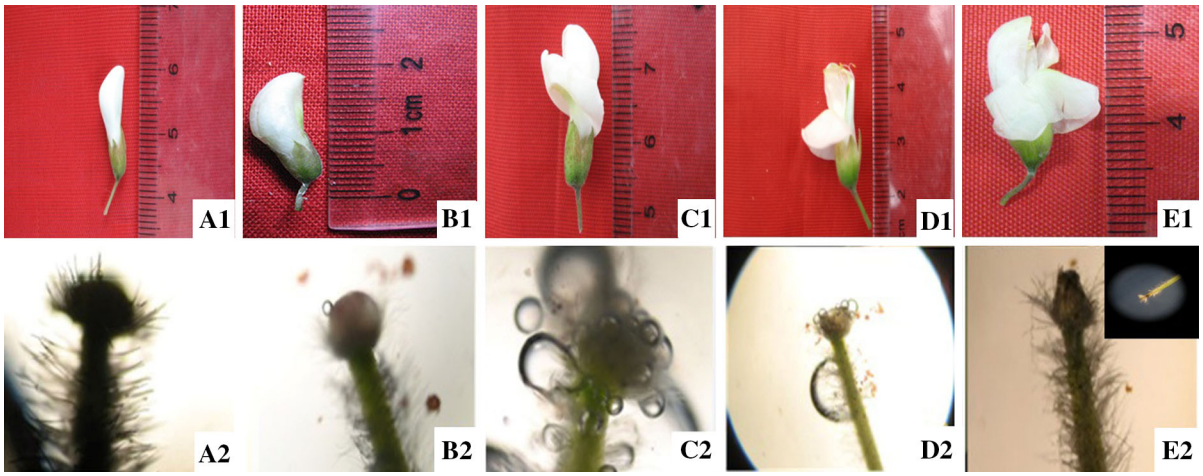


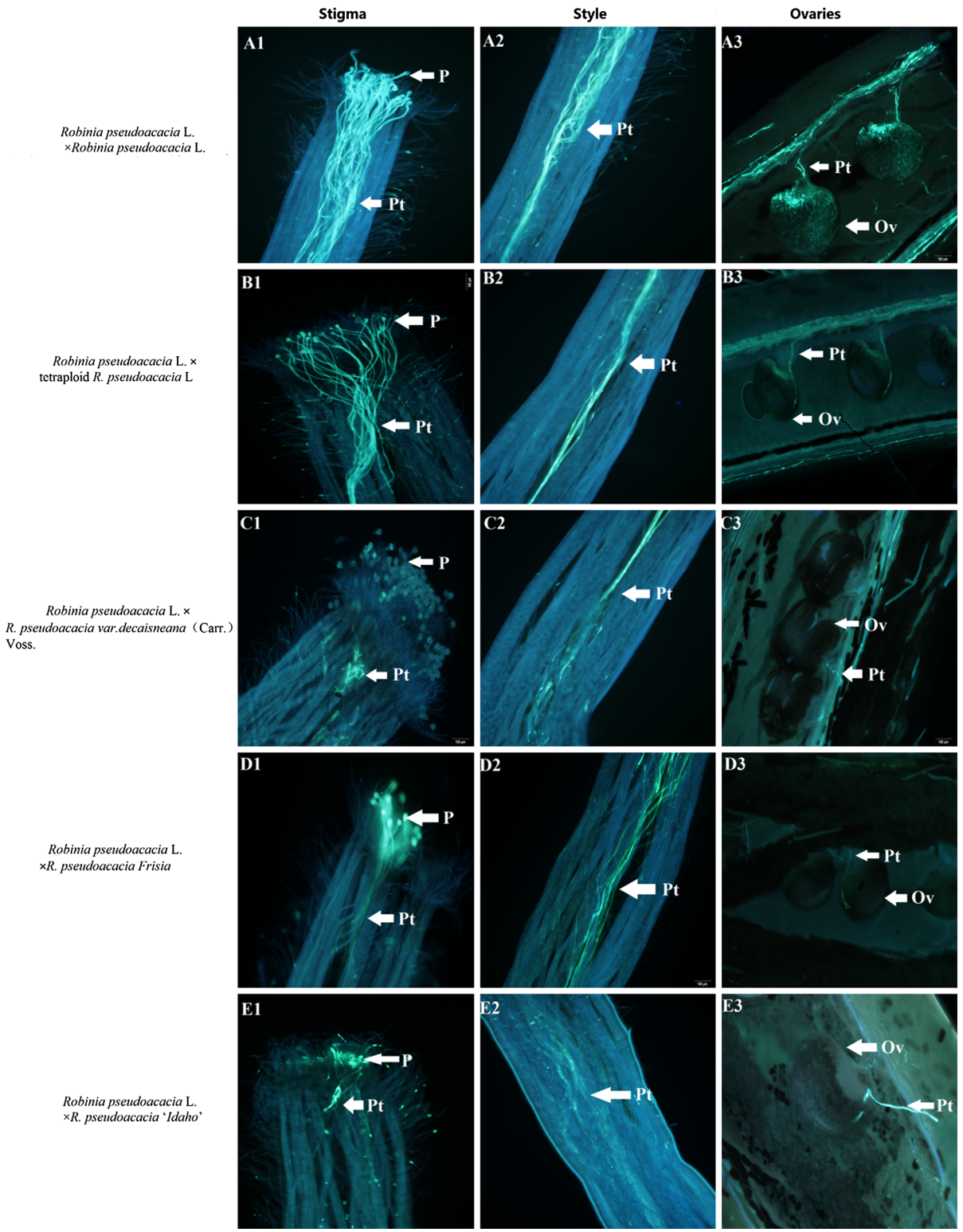
Fig. 2 Floral development and benzidine–H_2O_2 test of stigma at the indicated time points. *Top row* floral development at different periods **A1** 1 day before bloom; **B1** Just before bloom; **C1** First day of blooming; **D1** Second day of blooming; **E1** Third day of blooming. Second row: benzidine–H_2O_2 test of stigma at the indicated time points. **A2** Stigmas had no receptivity 1 day before blossoming; **B2** Stigmas had weak receptivity, when flora were about to open; **C2** Stigmas had their highest receptivity on blooming day. **D2** Stigma receptivity was still existing on the second day after blooming **E2** Stigmas had zero receptivity on the third day after blooming, and the stigma became *black*

markedly, as did the growth of the pollen tubes in the styles. A large number of the *R. pseudoacacia* pollen grains which germinated on the stigma and pollen tubes passed successfully through the styles, and a high proportion of the pollinations completed their development in the pistil and reached the ovary tissue (Fig. 3a). The majority of the pollen grains of tetraploid *R. pseudoacacia* L. were also able to germinate on the stigma and pollen tubes of *Robinia pseudoacacia*, and successfully reached the ovary tissue within 72 h. In *R. pseudoacacia var. decaisneana* (Carr.) Voss, *R. pseudoacacia* Frisia and *R. pseudoacacia* "Idaho", only a small quantity of pollen germinated on the stigma (Fig. 3c1, d1 and e1), compared with tetraploid *R. pseudoacacia* L. Twisted pollen tubes were observed in the styles during germination trials in *R. pseudoacacia var. decaisneana* (Carr.) Voss and *R. pseudoacacia* "Idaho", however, a few pollen tubes reached the ovary tissue (Fig. 3c3, e3).

Results of artificial hybridisation and crossability

The artificial crosses and their results are presented in Table 2. All five hybridisation treatments ultimately produced seeds; the proportion of seeds was ~17.2 % in *Robinia pseudoacacia* L., 14.9 % in *R. pseudoacacia var. decaisneana* (Carr.) Voss., 15.4 % in *R. pseudoacacia Frisia*, 52.9 % in tetraploid *R. pseudoacacia* L., and 4.2 % in *R. pseudoacacia* "Idaho". Some of the seeds, however, failed to reach maturity or were attacked by insects, especially those from the *Robinia pseudoacacia* L. × *R. pseudoacacia Frisia* treatment. All seeds from the *Robinia pseudoacacia* L. × *R. pseudoacacia* "Idaho" treatment suffered serious insect pest damage, and produced no mature seeds. Only a proportion of the seeds reached maturity, and the mature seed rates were 35.1 % in *Robinia pseudoacacia* L., 45.3 % in *R. pseudoacacia var. decaisneana* (Carr.) Voss., 68.3 % in *R. pseudoacacia Frisia*, and 62.8 % in tetraploid *R. pseudoacacia* L. Additionally, severe insect attack occurred on the seeds and reduced seed numbers.

Robinia pseudoacacia L. × *Robinia pseudoacacia* L. (artificial crosses) suffered greater seed abortion (61.9 %) compared with the other three hybrids (21.3, 27.6 and 37.0 %). Among all five hybridisation treatments, tetraploid *R. pseudoacacia* L. × *R. pseudoacacia* L. produced the greatest number of seeds (105.7 per year); however, the seedling emergence rate was only ~1.3 %; *Robinia pseudoacacia* L. × *pseudoacacia Frisia* displayed the highest seedling emergence rate (3.3 %). All four intra specific hybridisation treatments showed very low seedling emergence rates.


Stigma
Style
Ovaries
A1
A2
A3
P
Pt
Pt
Pt
Ov
Robinia pseudoacacia L. ×Robinia pseudoacacia L.
B1
B2
B3
P
Pt
Pt
Pt
Ov
Robinia pseudoacacia L. × tetraploid R. pseudoacacia L
C1
C2
C3
P
Pt
Pt
Ov
Pt
Robinia pseudoacacia L. × R. pseudoacacia var.decaisneana（Carr.）Voss.
D1
D2
D3
P
Pt
Pt
Pt
Ov
Robinia pseudoacacia L. ×R. pseudoacacia Frisia
E1
E2
E3
P
Pt
Pt
Ov
Pt
Robinia pseudoacacia L. ×R. pseudoacacia 'Idaho'

◀**Fig. 3** Pollen germination and pollen-tube growth in *Robinia pseudoacacia* L., tetraploid *R. pseudoacacia* L., *R. pseudoacacia var. decaisneana* (Carr.) Voss., *R. pseudoacacia* Frisia, and *R. pseudoacacia* "Idaho" on the hybrid *Robinia pseudoacacia* L. stigma, style and ovaries. *P* pollen; *Pt* pollen tube; *Ov* Ovary

Compared to *Robinia pseudoacacia* L. × *Robinia pseudoacacia* L. (artificial crosses), the natural pollination and emasculated (without nets) treatments resulted in markedly higher seed yields, however, their seedling emergence rates were 2.7 % and 4.3 %, respectively, only slightly higher than the artificial crosses. The two self-treatments produced a few seeds, ~16.7 (self-pollination) and 11.7 (artificial self-pollination) seeds per year, significantly lower than the artificial hybrid treatment (34.3 seeds per year). The "emasculated with bags" treatment produced no seeds (Figs. 4, 5).

SRAP primer screening and hybrid identification

From the 35 pairs of primers, we selected 20 that displayed male parent characteristics with polymorphism: em1/me3, em1/me6, em1/me10, em2/me4, em2/me9, em2/me11, em3/me1, em3/me4, em3/me13, em4/me5, em4/me10, em5/me5, em5/me6, em6/me12, em6/me10, em6/me11, em9/me13, em6/me13, em9/me11, and em12/me11. Selected SRAP primer selection results are shown in Fig. 6.

Hybridity was confirmed by the SRAP markers. The results indicated that the five hybrids contained genetic information inherited from their parents (Fig. 7). For the cross-combination *R. pseudoacacia* L. 2 × *R. pseudoacacia* L.1, a total of nine amplicons were produced in the hybrid, among which seven fragments ~120, 200, 230, 380, 450, and 480 bp in length were detected in the hybrid and its parents, and two fragments of ~80 and 220 bp were also observed in the hybrid, which exhibited the same genotypes as the male parent. In the hybrid of *R. pseudoacacia* 2 × Tetraploid *R. pseudoacacia* 1, eight fragments were detected; five of ~50, 120, 150, 450, and 480 bp were detected in both parents; two polymorphic fragments of ~220 and 240 bp were detected in only the female parent; and one polymorphic fragment of ~250 bp was found in only the male parent. In the hybrid *R. pseudoacacia* 2 × *R. pseudoacacia* var. decaisneana (Carr.) Voss. 1 and *R. pseudoacacia* 2 × *R. pseudoacacia Frisia*. 1, we also found significant polymorphic fragments from its own male parents, which indicated that the putative hybrids were true hybrids.

Discussion

An understanding of species' floral development is a prerequisite for studies of sexual compatibility between varieties and for breeding. If the pollen vitality and stigmatic receptivity are not well-known, erroneous conclusions from artificial hybridisation experiments are possible (Douglas and Freyre 2010). Using stigmas and pollen that are at the appropriate developmental stage for maximum fertilisation potential ensures that the production of viable seed reflects true crossability. Using observation under an electron microscope, Sun et al. (2012) noticed a large amount of mucus on the stigma of black locust on the blossom day, which was assumed to represent the best pollination time; the amount of mucus then began to decrease gradually over the next 48 h, and was scarcely visible after 72 h. Our results were similar to Sun et al. (2012); the flowers at blossom day displayed the highest stigma receptivity, and the stigma receptivity was sustained for a maximum of 72 h. The pollen had very high vitality on the blossom day (Supplemental Table 1; Supplemental Fig. 3). Despite the fact that the locations and blossoming time of some male parents were different to those of the female parents, and they were unable to maintain their pollen vitality at the level of the blossom day, when we carried out our pollinations, the pollen vitality remained >50 % with appropriate storage conditions.

The low crossability between *Robinia pseudoacacia* and the four other *Robinia* L. varieties indicated the existence of barriers to breeding. In our study, although the pollen from all four varieties reached the ovary tissue, pollen germination failure and abnormal pollen tubes were observed in the male parents of *R. pseudoacacia var.* decaisneana (Carr.) Voss and *R. pseudoacacia* "Idaho", which are typical post-fertilisation barriers. Similar phenomena were also found in *Populus simonii Carr* × *Populus diversifolia* (Chen et al. 2009). Although all five hybrid combinations ultimately set seeds, *Robinia pseudoacacia* × *R. pseudoacacia* "Idaho" and *Robinia pseudoacacia* × *R. pseudoacacia var.* decaisneana (Carr.) Voss suffered serious insect attack, especially *Robinia pseudoacacia* × *R. pseudoacacia* "Idaho", in which

Table 2 Results of artificial pollinations and controls

Female Parent	Stage	Male parent					Self-pollination	Natural pollination	Emasculated without net	Emasculated with net	Artificial self-pollination
		Robinia pseudoacacia L.	*R. pseudoacacia var. decaisneana* (Carr.) Voss.	*R. pseudoacacia* Frisia	tetraploid *R. pseudoacacia* L.	*R. pseudoacacia* "Idaho"					
Robinia pseudoacacia L.	Number of seeds	34.3 ± 4.0^{c}	29.7 ± 4.5^{c}	30.7 ± 3.2^{c}	9.7 ± 1.5^{b}	8.3 ± 0.6^{de}	16.7 ± 3.5^{d}	142.0 ± 9.2^{a}	102.0 ± 11.1^{b}	0 ± 0^{e}	11.7 ± 2.1^{de}
	Fruit setting rate (%)	4.0 ± 0.9^{c}	2.3 ± 0.7^{c}	2.9 ± 1.0^{c}	2.8 ± 1.1^{c}	2.0 ± 1.4^{c}	2.5 ± 1.3^{c}	29.5 ± 14.5^{a}	16.5 ± 8.7^{b}	0 ± 0^{c}	4.00 ± 2.4c
	Seed abortion rate (%)	56.7 ± 6.1^{a}	22.1 ± 5.5^{b}	27.1 ± 3.7^{b}	42.9 ± 19.5^{ab}	–	35.4 ± 4.6^{ab}	52.0 ± 14.3^{a}	40.6 ± 1.4^{ab}	–	39.4 ± 18.4^{ab}
	Mature seed rate (%)	40.6 ± 4.6^{bc}	48.6 ± 3.0^{bc}	65.2 ± 5.3^{a}	49.6 ± 13.1^{bc}	0 ± 0^{e}	51.5 ± 10.8^{abc}	36.5 ± 9.7^{c}	49.4 ± 4.4^{bc}	0 ± 0^{d}	52.2 ± 10.6^{ab}
	Insect attack (%)	3.8 ± 1.3^{c}	29.4 ± 2.7^{b}	7.6 ± 1.7^{c}	7.5 ± 6.6^{c}	100 ± 0^{a}	13.2 ± 6.3^{c}	11.4 ± 4.7^{c}	10.0 ± 3.0^{c}	–	8.4 ± 9.2^{c}
	Seedling emergence number	4.6 ± 0.6^{bc}	4.0 ± 1.0^{bcd}	6.7 ± 2.0^{ab}	2.7 ± 0.6^{cde}	0 ± 0^{e}	4.0 ± 1.7^{bcd}	5.3 ± 1.2^{bc}	8.7 ± 3.2^{a}	0 ± 0^{e}	1.7 ± 1.2de
	Seedling emergence rate (%)	2.3 ± 0.3^{bc}	2.0 ± 0.5^{bcd}	3.3 ± 0.1^{ab}	1.3 ± 0.3^{cde}	0 ± 0^{e}	2.0 ± 0.9^{bcd}	2.7 ± 0.6^{bc}	4.3 ± 1.6^{a}	0 ± 0^{e}	0.8 ± 0.6^{de}
	Mature seed germination rate (%)	34.6 ± 10.3^{abc}	28.6 ± 9.9^{bc}	33.7 ± 11.8^{abc}	56.7 ± 5.8^{a}	–	48.6 ± 22.9^{ab}	10.5 ± 2.0^{c}	18.0 ± 9.2^{c}	–	30.3 ± 25.7^{abc}

The means of the values above with letters are significantly different ($P < 0.05$) according to Duncan's multiple range test

Fig. 4 **A** Mature putative hybrid seeds: *a Robinia pseudoacacia* L. × *Robinia pseudoacacia* L., *b Robinia pseudoacacia* L. × *R. pseudoacacia var. decaisneana* (Carr.) Voss., *c Robinia pseudoacacia* L. × *R. pseudoacacia* Frisia, *d Robinia pseudoacacia* L. × tetraploid *R. pseudoacacia* L.); **B** Normal seeds (*left*) and abortive seeds (*centre* and *right*)

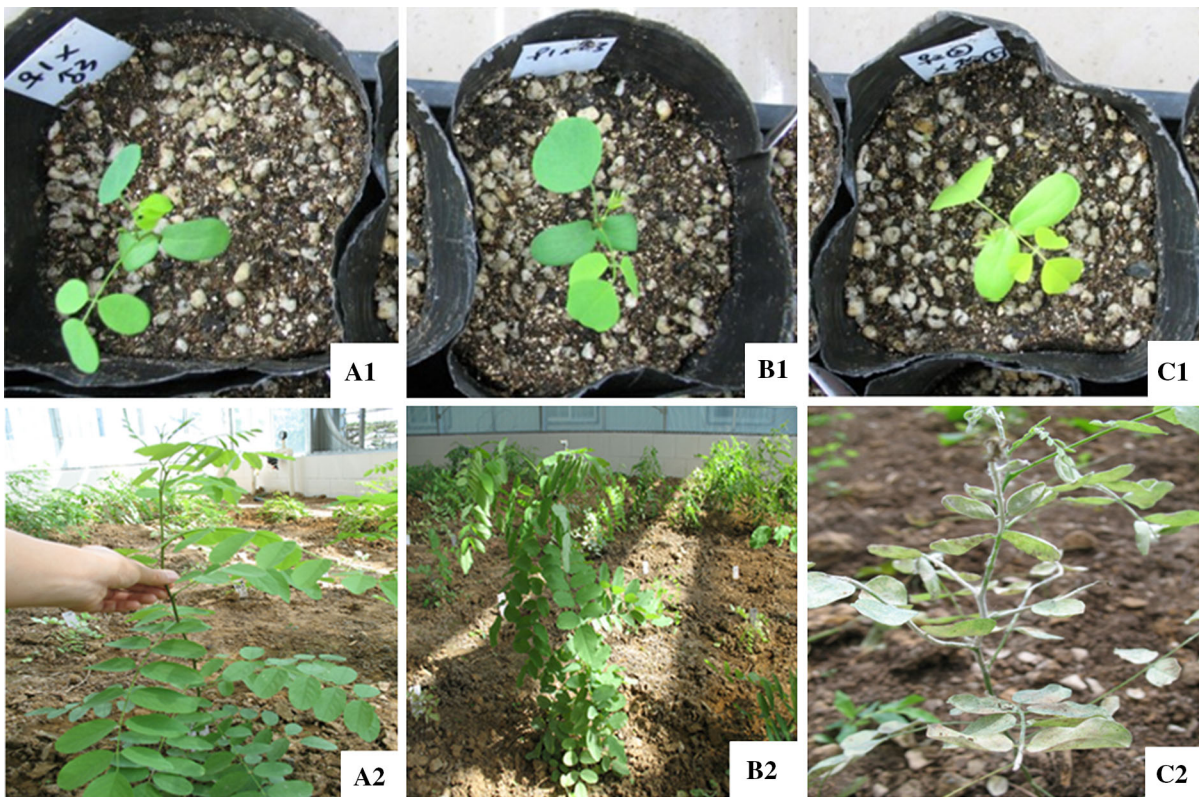


Fig. 5 Putative hybrid seedlings. **A1** and **A2** Putative *Robinia pseudoacacia* L. × *R. pseudoacacia var. decaisneana* (Carr.) Voss. hybrid seedlings; **B1** and **B2**. Putative *Robinia pseudoacacia* L. × tetraploid *R. pseudoacacia* L. hybrid seedlings; **C1** and **C2** *Robinia pseudoacacia.* L × *R. pseudoacacia* Frisia hybrid seedlings

the insect attack rate was 100 %. The seed abortion rates of the remaining four combinations were 56.7 % in *Robinia pseudoacacia* × *Robinia pseudoacacia*, 22.1 % in *Robinia pseudoacacia* × *R. pseudoacacia var. decaisneana* (Carr.), 27.1 % in *Robinia pseudoacacia* L. × *R. pseudoacacia* Frisia, and 42.9 % in *Robinia pseudoacacia* L. × tetraploid R. *pseudoacacia L.*, which suggests that the failure of fertilised ovule development is a key post-fertilisation barrier to take into consideration. Of all the mature seeds, only 34.6 % in *Robinia pseudoacacia* L. × *Robinia pseudoacacia* L, 28.6 % in *Robinia pseudoacacia* × *R.*

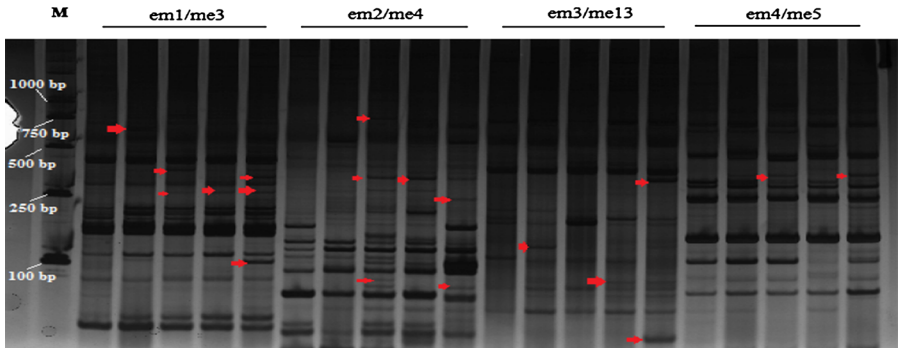


Fig. 6 Selected primer selection results of five male parents. M: D2000 DNA Marker; em1/em3, em2/em4, em3/em13, em4/em5 represent the primer combinations; the experimental material for each primer combination is *Robinia pseudoacacia* L.1, tetraploid *R. pseudoacacia L.* 1, *R. pseudoacacia var. decaisneana* (Carr.) Voss. 1, *R. pseudoacacia* Frisia.1 and *R. pseudoacacia* "Idaho" 1, from left to right, respectively; *arrows* indicate the paternal characteristic bands

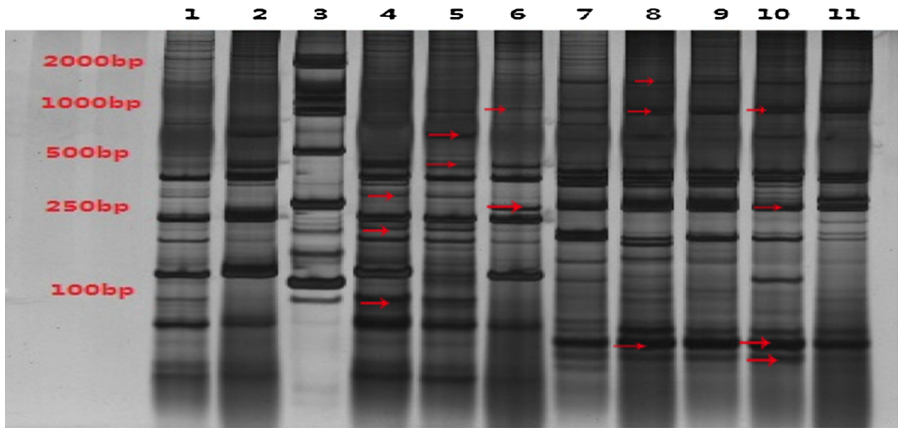


Fig. 7 Profile of PCR products of the hybrids and their parents using em9/em11 primer pairs (*Arrows* indicate the Male specific fragments). *1 R. pseudoacacia* L.1; *2 R. pseudoacacia L.*2; *3* D2000 DNA Marker; *4* Putative hybrids of *R. pseudoacacia* L.2 × *Robinia pseudoacacia* L.1; *5* Putative hybrids of *R. pseudoacacia* L.1 × *R pseudoacacia* L.2; *6* Putative hybrids of *R. pseudoacacia* L.2 × Tetraploid *R. pseudoacacia* L. 1; *7* Tetraploid *R. pseudoacacia* L.1; *8* Putative hybrids of *R. pseudoacacia* L.2 × *R. pseudoacacia var. decaisneana* (Carr.) Voss.1; *9 R. pseudoacacia var. decaisneana* (Carr.) Voss.1; *10* Putative hybrids of *R. pseudoacacia* L.2 × *R. pseudoacacia* Frisia.1; *11 R. pseudoacacia* Frisia.1

pseudoacacia var. decaisneana (Carr.), 33.7 % in *Robinia pseudoacacia* L. × *R. pseudoacacia Frisia*, and 56.7 % in *Robinia pseudoacacia* × tetraploid R. *pseudoacacia* ultimately generated seeds. Poor seed germination was another important post-fertilisation barrier.

All five hybridisation treatments ultimately produced seeds, but the number of seeds was very small. A key reason for this may be low fruit-setting rates. Approximately 30–70 % of the pollinated flowers fell in the first week (7 days) after pollination, and almost 90 % fell in the first month following pollination (Fig. 8). The fruit setting rates were only ∼ 2–3 % on day 90. The final fruit setting rates of *Robinia pseudoacacia* × *R. pseudoacacia* Frisia, *Robinia pseudoacacia* × tetraploid R. *pseudoacacia*, *Robinia pseudoacacia* × *R. pseudoacacia var. decaisneana* (Carr.) Voss., and *Robinia pseudoacacia* × *Robinia pseudoacacia* "Idaho" were 2.88, 2.75, 2.25, and 2.00 %, respectively (Fig. 8).

The fruit-setting rate of the *Robinia pseudoacacia* artificial crossing was 4 %, significantly lower than that of natural pollination (29.5 %). This result was similar to Sun et al. 2012 and Xie 1994, who reported fruit setting rates for artificial crossing and natural pollination of 3.56 % (artificial crossing) and 40.66 %

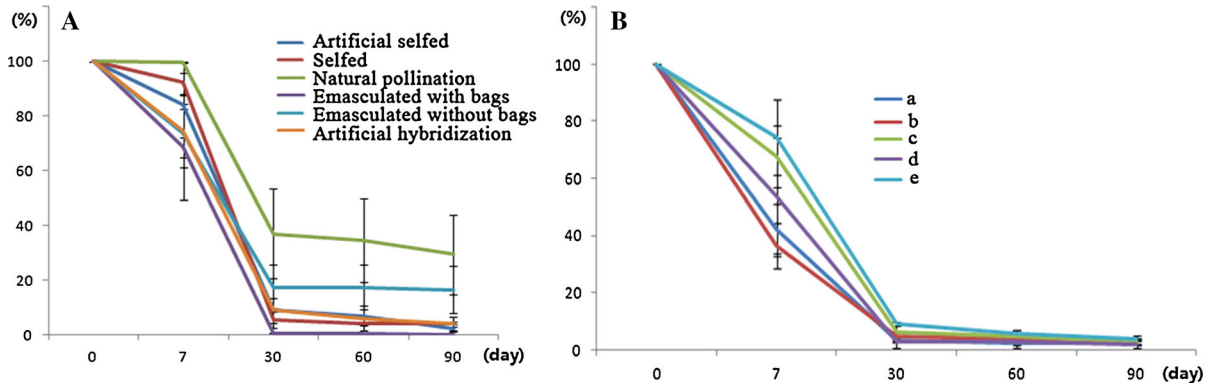


Fig. 8 Changes in fruit-setting rates (from days 1 to 90). **A** Fruit-setting rates of artificial hybridisation and other treatments; **B** fruit-setting rates of five hybridised combinations: *a Robinia pseudoacacia* L. × *R. pseudoacacia* var. *decaisneana* (Carr.) Voss.; *b Robinia pseudoacacia* L. × *R. pseudoacacia* Frisia.; *c Robinia pseudoacacia* L. × tetraploid *R. pseudoacacia*; *d Robinia pseudoacacia* L. × *Robinia pseudoacacia* "Idaho".; *e Robinia pseudoacacia* L. × *Robinia pseudoacacia* L

(natural pollination), and 6.5 % (artificial crossing) and 30 % (natural pollination), respectively, on day 90. A similar phenomenon was also found in the artificial crossing of *Cerasus pseudocerasus* (Hedhly et al. 2009), *Prunus salicina* (Guerra et al. 2010) and *Cerasus vulgaris Mill* (Janick and Moore 1996). The reason that emasculation decreases the fruit setting rate is unclear. Some studies have proposed that emasculation promotes the caducity of flowers. Hedhly et al. (2009) inferred that the increased ethylene content caused by emasculation promotes caducity of the flowers. In our study, all "emasculated" treatments showed significantly lower fruit-setting rates than natural pollination, which indicated that emasculation has negatively affected the fruit setting rates of *Robinia pseudoacacia* artificial hybridisation. Considering that *Robinia pseudoacacia* is a highly outcrossing species, emasculation may not be necessary for its hybridisation. We plan to attempt new artificial pollination methods and crossbreeding identification using molecular markers in future studies to eliminate interference by emasculation.

In this study, we have provided a reference for other researchers to identify an appropriate time for pollination in *Robinia pseudoacacia* hybridisations to ensure maximum fertilisation potential. Although our four hybridised combinations ultimately produced seeds, and three of them produced hybrid seedlings, seed production was limited, and observation under a fluorescence microscope indicated that the low crossability between *Robinia pseudoacacia* and *R. pseudoacacia var. decaisneana* (Carr.) Voss., *Robinia pseudoacacia* and *R. pseudoacacia* "Idaho" was due to abnormal pollen-tube development and failure of pollen generation. Also,in future studies, we plan to attempt new hybrid operations to avoid the detrimental effects of emasculation, and to obtain more hybrid seeds for further study.

Acknowledgments This work was financially supported by the Scientific Research of the Forest Public Welfare Industry (No. 201304116), the National Nature Science Foundation of China (No. 31170629) and the Program for Changjiang Scholars and the Innovative Research Team in University(No. IRT13047).

Conflict of interest The authors declare that they have no conflict of interest.

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