IN VITRO POLLEN SELECTION FOR HEAT-TOLERANCE IN LILIES

H.S. Chi, Th.P. Straathof, H.J.M. Löffler and J.M. Van Tuyl

DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO), PO Box 16 6700 AA Wageningen, The Netherlands (correspondence to J.M. Van Tuyl)

Abstract

Heat-tolerance is an important character for lily bulb and flower production under subtropical and tropical conditions. Since a gametophytic-sporophytic genetic overlap has been demonstrated in many plant species, pollen selection at high temperatures might be an efficient method to achieve heat-tolerance.

An in vitro pollen germination assay was performed with a range of lily genotypes at various temperatures (20 to 35°C) to determine the differences in pollen germination and pollen tube growth. At 35°C differences in pollen tube length and percentage of pollen germination were found among accessions. Accessions in which heat-tolerance was expected, i.e. wild types of *L. formosanum*, *L. neilgherense* and *L. longiflorum* , gave the longest pollen tubes at 35°C.

An in vitro pollination system was used to select heat-tolerant seedlings within *L. longiflorum* by pollinating at 25, 30 and 35°C. The preliminary results are discussed.

1. Introduction

The lily (*Lilium* L.) is one of the most important crops in the flower bulb industry. Lilies are used worldwide as cut flower, as pot plant or as garden plant. With an area of about 3900 hectares (CBS 1996) The Netherlands is the world leading country in the production of lily bulbs, followed by the United States and Japan. In The Netherlands approximately one billion bulbs are produced annually and 65% of the bulbs is exported (CBS 1996), mainly for cut flower production. The remaining 35% of the bulbs is used for year round flower production in The Netherlands. Most of the flowers produced in The Netherlands are exported, with an economic value of approximately 300 million guilders (CBS 1996).

The genus *Lilium* comprises about 85 species, classified into seven sections (De Jong 1974). More than half of the species originate from Asia. The ancestors of the modern lilies such as the Asiatic and Oriental hybrid lilies originate mostly form China and Japan (Beattie and White 1993). However, since most of the lilies are bred and cultivated in The Netherlands, the modern commercial cultivars are only suitable for cultivation in a moderate climate. Only few commercial cultivars are adapted to tropical or subtropical areas. Therefore, the subtropical lily industry is hampered by poor growth of the bulbs and a low quality of the cut flowers. Widening of the genetic basis to introduce heat-tolerance from wild species into the commerical lily assortment is therefore very important in subtropical lily breeding programmes. Three interesting species originate from tropical or subtropical countries, i.e. *L. neilgherense* from south of India, *L. formosanum* from Taiwan and *L. longiflorum* from south of Japan

and Taiwan (Woodcock and Stearn 1950, Shii 1983). The culture characteristics of these species are vigour, earliness, heat-tolerance and suitability for year-round forcing (Chin et al. 1996).

To breed efficiently for heat-tolerant cultivars, male gametophytic selection (MGS) could be an important tool. The MGS is based on the assumption that the selective trait is controlled by genes expressed both in the gametophytic and the sporophytic phase (Sari-Gorla and Frova 1997). A gametophytic-sporophytic genetic overlap (GSGO) of 60-80% has been demonstrated in many plant species (Ottaviano and Mulcahy 1989). Because of haploidy and the very large size of pollen populations, MGS is expected to be extremely efficient. Three steps are necessary to develop an in vitro pollen selection system for heat-tolerance. First, genetic variation in heat-tolerance must be detected in the species (or genus). Besides a plant test, a bioassay for determining the heat-tolerance of a genotype at pollen level could hereby be useful. Second, a selection system must be developed. Selection pressure can be applied to the pollen before pollination (Frova et al. 1995) or during the pollen tube growth in the style and during fertilization (Petolino et al. 1990). Third, selected and control seedlings must be tested for heat-tolerance and selection response must be found. If the selection system is carried out in vitro, an in vitro assay for heat-tolerance can be efficient.

In this study we investigated the possibilities of a bioassay for determination of heat-tolerance of pollen from different *Lilium* species. Furthermore, an in vitro pollination system (Van Tuyl et al. 1991) was used to select for heat-tolerant seedlings. The possibilities for an in vitro pollen selection system for heat-tolerance in lilies is discussed.

2. Materials and Methods

2.1 Development of a bioassay for heat-tolerance of pollen

Bulbs of one accession of *L. formosanum*, one accession of *L. neilgherrense* and 14 accessions of *L. longiflorum* (Table 1) were planted in pots in a greenhouse of CPRO-DLO in April and flowered in June and July. Fresh pollen was collected from the plants and directly used. Pollen was germinated on a modified BK-medium (Brewbaker and Kwack 1963). As a modification, the component H_3BO_3 was 3-times as high as in the BK-medium and supplemented with 10% of sucrose and solidified with 0.5% phytagel. To stimulate germination, a thin linear row of pollen was brought on the pollen germination media in the middle of a 9 cm petri-dish. Petri-dishes were placed in climate rooms at four different temperatures, i.e. 20, 25, 30 and 35°C. The experiment was carried out in 5 repeats.

After 24 hours, the length of pollen tubes was measured under the stereomicroscope using millimeter-paper under the petri-dish. The length of the pollen tubes was measured from the edge of the pollen row. Furthermore, the germination percentage was estimated in classes of 10 percent. All data were subjected to analysis of variance.

2.2 In vitro pollination at different temperatures

Bulbs of the *L. longiflorum* cultivars White American, Indian Summer and Gelria were planted in pots in a greenhouse of CPRO-DLO in June and flowered in September. One day before anthesis, the flower buds of the *L. longiflorum* cultivars White American and Gelria were collected from the greenhouse and transferred to the laboratory. The flower buds were placed on water in a laminar flow cabinet at a temperature of 22-25°C till bloom. The aseptic pollen was collected and used for in vitro pollination. The viability of aseptic pollen was determined by an in vitro germination assay as described in paragraph 2.1.

Flower buds with pedicle of the *L. longiflorum* cultivar Indian Summer were cut from the plants in the greenhouse two to four days before anthesis. The complete flowers were sterilized in 70% ethanol for 3 min, and commercial bleach containing 2% NaOCl for 30 min, and subsequently rinsed three times, i.e. 5, 10 and 20 min, in sterile distilled water. After rinsing, petals and anthers were dissected with a scalpel and the pistil, style, ovary and pedicle were placed vertically in a 20 cm test tube partly filled with medium. Tubes were closed with cotton plugs covered by a double layer of alluminium foil. Tubes were placed in a climate room with a light intensity of 12 Wm^2, a photoperiod of 16hrs and a temperature of 25°C. A MS-medium (Murashige and Skoog 1962) supplemented with 8% of sucrose, solidified with 0.4% phytagel and a pH of 6.0 was used to culture the flower bud.

When exudate production indicated stigmatic receptivity, the pistils were pollinated by aseptic pollen in the flow cabinet. After in vitro pollination, the test tubes were placed in climate rooms with three different temperatures, i.e. 25, 30 and 35°C. Five days after pollination, all test tubes were placed at 25°C. Pollen tube growth in the style was observed using aniline blue fluorescence (Kho and Baer 1968). Ovule culture was applied to the seed pods which were ripe between 70-90 days after pollination. The ovules were incubated on a MS-medium supplemented with 5% sucrose and 0.1 mg/l NAA, solified with 0.4% phytagel and a pH of 5.8. The ovules were incubated in the dark. Emerged plantlets were transferred to a medium containing half strength of MS salts, 5% sucrose and 0.4% phytagel and a pH of 5.8 (Van Tuyl et al. 1991).

3. Results

3.1 Development of a bioassay for heat-tolerance of pollen

The analysis of variance of the pollen tube length measurements (Table 2) and the pollen germination measurements (Table 3) show a highly significant temperature and genotype effect. Also a smaller but significant interaction between cultivar and temperature is present, which means that not all the accessions react the same at different temperatures.

In Table 4 the optimum temperature for pollen tube growth, the length of the pollen tubes at 35°C, the optimum temperature for pollen germination and the germination percentage at 35°C is presented for all 16 accessions. The optimum temperature for pollen tube growth is for most accessions at 30°C. Only the *L. longiflorum* cultivar White American has a much lower optimum of 20°C. It was observed that 80 to 90% of the pollen tubes of a certain accession at a certain temperature had the same length. A large variation in pollen tube growth at 35°C was found between the accessions ranging from 1.0 of *L. longiflorum* cultivar Blancivetta to 6.1 mm of *L. neilgherrense*. The wild accesion of *L. formosanum*, *L. neilgherrense* and *L. longiflorum* which originate from warm climate zones (Table 1) showed long pollen tubes at 35°C. At a temperature of 40°C the pollen tube length of all the accesions was almost zero (data not shown). The optimum temperature for pollen germination is for most accesions between 25 and 30°C. The *L. longiflorum* cultivar White American had again the lowest optimum (20°C). Also variation in pollen germination at 35°C was found between the accesions ranging from 10% of *L. longiflorum* Nellie White to 96% of *L. longiflorum* Lorina. No relation between pollen germination at high temperature and the origin related to climate zone (Table 1) could be detected.

3.2 In vitro pollination at different temperatures

Pollen tube growth in the style was studied. The style of *L. longiflorum* Indian Summer is in general 10 cm long. Most of the pollen tubes of *L. longiflorum* Gelria had a length of 4 cm four days after in vitro pollination and incubation at 35°C. At the same time and temperature, an average of 30 to 50 pollen tubes of White American reached the ovary. Furthermore in this combination, about 10-15 pollen tubes were found in one-third to half of the ovary one week after in vitro pollination.

The results of in vitro pollination of two cross combinations at 25, 30 and 35°C is presented in Table 5. In total, 83 ovaries were pollinated in vitro. At 35°C, all the embryos were rescued by ovule culture. All the embryos rescued at 35°C were found in the upper half of the ovary. The number of seeds per ovary in the combination of the *L. longiflorum* cultivars Indian Summer x White American were higher than in the combination of Indian Summer x Gelria at all three temperatures. Particular at 35°C, the combination of Indian Summer x White American resulted in 9 seeds where the combination of Indian Summer x Gelria did not result in any seeds. The nine seeds from the 35°C treatment germinated 2-4 weeks after in vitro culture of the mature seeds.

4. Discussion

For pollen germination as well as for the pollen tube length, highly significant temperature and genotype effects were found in the bioassay. Pollen germination and pollen tube growth was less at 35°C than at 25 to 30°C for all accessions. But at 35°C still large differences between the genotypes were detected. Three accesions native from warm climates, i.e. *L. formosanum*, *L. neilgherrense* and *L. longiflorum*, and the *L. longiflorum* cultivar Avita had the longest pollen tubes at 35°C. A correlation between the origin related to the climate zone and the pollen tube length at 35°C of the accessions could, however, not be confirmed. A plant test to establish the heat-tolerance of the accessions studied at pollen level will probably provide more information.

Not only differences between genotypes are a condition for a succesful pollen selection system, also segregation of pollen within an accession is required. However, at a certain temperature 80 to 90% of the pollen within an accession had almost the same length.

Two cross combinations were made in which the pollen tube growth was exposed to three temperatures. In both cross combinations the mother plant was *L. longiflorum* Indian Summer. The origin of this cultivar is related to a cold climate. Five days after pollination the ovaries incubated at 30 and 35°C were placed at 25°C to prevent premature yellowing of the seed pot. Still embryos from the 35°C treatment had to be rescued by ovule culture to prevent abortion.

Also both fathers, i.e. *L. longiflorum* White American and Gelria originate from cold climates. White American has a low optimum temperature for pollen tube growth compared to Gelria. This resulted at 35°C in a three times longer pollen tube of Gelria compared to White American. At 35°C the pollen germination percentage of both cultivars is the same. On the basis of these results it was expected that more seeds should be obtained from Gelria than of White American at the 35°C treatment. However, microscopic studies of the pollen tube growth in the style gave opposite results. The pollen tube growth of Gelria was retarded at 35°C, while the pollen tubes of White American reached the ovary. Furthermore, only embryos were obtained at 35°C with White American as father.

To establish if MGS can be useful to select for heat-tolerance in lilies the seedlings obtained at 35°C will be compared in a plant test with seedlings obtained at 25°C. Furthermore, new crosses combined with a high temperature treatment during pollen tube growth will be made with heat-tolerant accessions as father.

5. References

Beattie DJ, White JW 1993. *Lilium* - hybrid and species. In: The physiology of flower bulbs (De Hertogh A, Le Nard M, eds.) Elsevier Amsterdam, pp. 423-342.

Brewbaker JL, Kwack BH 1963. The essential role of calcium ion in pollen germination and pollen tube growth. American Journal of Botany 50(9): 859-865.

CBS 1996. Tuinbouwcijfers. Centraal Bureau voor de Statistiek, pp. 29-74.

Chin SW, Kuo CE, Liou JJ, Shii CT 1996. Dominant expression of vigor and heat tolerance of *Lilium longiflorum* germplasm in distant hybridization with Asiatic and Oriental lilies. Acta Horticulturae 430 (II): 495-502.

De Jong PC 1974. Some notes on the evolution of lilies. North American Lily Yearbook 27: 23-28.

Frova C, Portaluppi P, Villa M, Sari Gorla M 1995. Sporophytic and gametophytic components of thermotolerance affected by pollen selection. Journal of Heredity 86: 50-54.

Murashige T, Skoog F 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Ottaviano E, Mulcahy DL 1989. Genetics of angiosperm pollen. Advances in Genetics 26: 1-64.

Petolino JF, Cowen NM, Thompson A, Mitchell JC 1990. Gamete selection for heat-stress tolerance in maize. Journal of Plant Physiology 136: 219-224.

Sari-Gorla M, Frova C 1997. Pollen tube growth and pollen selection. Chapter 16 Biotechnology and Crop Improvement (eds. Sawhney & Shivanna) pp 323-351.

Shii CT 1983. The distribution and variation of *Lilium formosanum* and *L. longiflorum* in Taiwan. Lily Yearbook North Am. Lily Soc. 36: 48-51.

Van Tuyl JM, Van Dien MP, Van Creij MGM, Van Kleinwee TCM, Franken J and Bino RJ 1991. Application of in vitro pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Science 74: 115-126.

Woodcock HBD, Stearn WT 1950. Lilies of the world. Country Life Ltd., London.

Table 1. *Lilium* accessions used in the heat-tolerance experiments.

Code	CPRO-number	origin / cultivar	species	origin related to climate zone*
1	940272	native in Taiwan	L. *formosanum*	3
2	86105	native in India	L. *neilgherrense*	3
3	940317	Avita	L. *longiflorum*	2-3
4	771017	Indian Summer	L. *longiflorum*	1
5	960100	native in Japan and Taiwan	L. *longiflorum*	3
6	970002	Snow Queen	L. *longiflorum*	2
7	970001	Gelria	L. *longiflorum*	1
8	970008	White American	L. *longiflorum*	1
9	83139	Hinemoto	L. *longiflorum*	2-3
10	83293	White Diamond	L. *longiflorum*	2-3
11	78903	Nellie White	L. *longiflorum*	2
12	88257	Romero	L. *longiflorum*	1
13	81284.2	Blancivetta	L. *longiflorum*	2
14	85234	Osnat	L. *longiflorum*	2
15	960155	White Fox	L. *longiflorum*	3
16	960158	Lorina	L. *longiflorum*	1-2

*1 = grown in cold climates; 2 = grown in moderate climates; 3 = grown in warm climates (Van Tuyl, personal communication).

Table 2. Analysis of variance of the pollen tube length.

source	d.f.	s.s.	m.s.	v.r.	F pr.
temperature	3	314.0	104.7	326.8	<0.001
genotype	15	527.5	35.2	109.8	<0.001
temp * geno	45	255.3	5.7	17.7	<0.001
residual	256	82.0	0.3		
total	319	1178.8			

Table 3. Analysis of variance of the pollen germination experiment.

source	d.f.	s.s.	m.s.	v.r.	F pr.
temperature	3	77574.7	5171.6	222.5	<0.001
genotype	15	38873.4	12957.8	557.5	<0.001
temp * geno	45	44481.6	988.5	42.5	<0.001
residual	256	5950.0	23.2		
total	319	166879.7			

Table 4. Results of the pollen tube growth and pollen germination experiment.

code	pollen tube growth		pollen germination	
	optimum temperature	length (mm) at 35°C	optimum temperature	% of germination at 35°C
1	30 °C	4.0	25-30 °C	80
2	30 °C	6.1	25-30 °C	88
3	30 °C	5.0	25-30 °C	86
4	25 °C	3.0	25-30 °C	98
5	30 °C	4.4	25-30 °C	88
6	30 °C	2.3	30 °C	56
7	30 °C	3.0	20-25 °C	68
8	20 °C	1.1	20 °C	68
9	30 °C	2.0	25 °C	40
10	30 °C	2.0	30 °C	98
11	30 °C	1.5	20-30 °C	10
12	30 °C	2.0	30 °C	70
13	30 °C	1.0	30 °C	12
14	30 °C	1.5	30 °C	28
15	25 °C	2.0	30 °C	88
16	25 °C	2.0	30 °C	96
average		2.7		67.1

Table 5. Results of the in vitro pollination experiment at different temperatures.

cross	IS (4) x WA (8)				IS (4) x Ge (7)			
temperature	# ovary	# seeds	seeds/ovary	relative	# ovary	# seeds	seeds/ovary	relative
25°C	13	1615	124	100	12	1140	95	100
30°C	15	1306	87	70	14	364	26	27
35°C	11	9	0.8	0.6	18	0	-	-