Male gametophytic generation and a possible approach for selective pollination in carnation (*Dianthus*) breeding program

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ABSTRACT

Present study focuses on making best possible use of male gametophytic generation in carnation breeding program. Exploration of pollen population revealed the existence of variability in terms of pollen morphology and histochemical content among as well as within varieties and species of *Dianthus caryophyllus* and *D. chinensis* sufficient to make selection. Pollen grain size and histochemical content were found to be associated with germination capacity and pollen tube growth rate. In addition, pollen germination capacity and elongation of pollen tube in response to presence of culture filtrate from *F. oxysporum*. f.sp. *dianthi* causal organism of fusarium wilt in carnation was found to be governed by pollen grain size and histochemical content of pollen grains. Entire result suggests the possibility of selecting the desired pollen grains from a pollen population and possibility of attempting selective pollination in carnation breeding program.

Keywords: carnation; pollen; *Fusarium oxysporum*; *Dianthus chinensis*

Carnation (*D. caryophyllus*) is a commercially cultivated cut flower crop with a great demand in international floriculture trade. *D. chinensis* a relative species of carnation is being used in carnation breeding program for its unique characteristics such as disease resistance, faster growth rate, wider adaptability, better yield distribution, high yield etc.

Plant breeding program in general concentrates on sporophytic generation. Variation and selection, the two basic concepts of breeding is never explored or attempted in gametophytic generation. Any form of selection is attempted only in the resulting progeny i.e., in sporophytic stage and not prior to that. However, it is interesting to realize that each plant produces millions of pollen grains. For instance in maize, a single plant produces 14×10^6 to 50×10^6 pollen grains (Miller 1982). Besides this enormous number, gametophytic generation is in haploid state and thus makes pollen grains an interesting phase where in recessive alleles remain uncovered making it possible to pick up the right pollen grains with desired allelic combinations. Further, overlapping of gene expression in both gametophytic and sporophytic generation (Tanskley 1980, Willing and Mascarenhas 1984a, b, Thi et al. 1992) leads one to the possibility of selective pollination to develop the desired progeny. However, the basic criterion for any selection is variability. Thus, it is important to find out the existence of any form of variability in pollen population. Hence, the present work is attempted with first objective being estimation of variability existing in terms of morphological and histochemical content in pollen population of *Dianthus*. The second objective of this study is to understand the association of the morphological features and histochemical content of pollen grains with that of pollen performance during germination. Thirdly, resistance breeding for fusarium wilt caused by *Fusarium oxysporum* f.sp. *dianthi* being major objective in carnation improvement; it was tried to evaluate the association between pollen grain features with that of tolerance to disease.

MATERIAL AND METHODS

Five varieties of carnation namely Golden rush, Internet, Monaco, Regina, and Trendy imported from Hilverda company of Holland were used for the study. A plant population of *D. chinensis* was grown in the field and three genotypes G2, G3, and G7 were used for detailed study of pollen grains.

Pollen grain morphology

In each variety, pollen grains were collected from developed anthers of different flowers and pooled pollen was used for morphological analysis. Pollen diameter was measured using a calibrated ocular scale.

Pollen germination

Sitting drop method was used for culturing of pollen grains (Shivanna and Johri 1985). Pollen grains for the study were collected from well-grown plants and fully matured anthers. Pollen grains were collected after anther dehiscence and were pre-hydrated before germination by placing them inside Petri plates lined with moist filter paper. Drops (50 µl) of standardized pollen germination medium (calcium nitrate 3.2M, magnesium sulphate 0.8M, potassium nitrate 1.0M, boric acid 2.43M and sucrose

20%) were dispensed into cavities of microscope cavity slides, and pollen grains were cultured in these drops. After two hours, pollen tube elongation was arrested using a drop of one per cent acetocarmine.

Pollen germination in response to culture filtrate

Culture filtrate of *F. oxysporum* f.sp. *dianthi* was produced with a modification in the method of Rowe et al. (1985). Effectiveness of culture filtrate was confirmed before using it in the experiment. The symptoms produced in cuttings incubated in culture filtrate extract were similar to that of disease symptom confirming its efficacy. Culture filtrate was added to pollen germination media in different concentrations (0.5, 1, 5, 10, 20, 30, 40 and 50%). Further, culturing of pollen grains was similar to that explained earlier.

All varieties were replicated thrice in each one of the culture filtrate concentrations. Observations were recorded in ten randomly selected microscopic fields for number of germinated and ungerminated pollen grains in each replication. Pollen grains having a minimum tube-length of half of its diameter were considered as germinated. In each replication, randomly selected 30 germinated and 30 ungerminated pollen grains were considered for measuring diameter. Pollen tube length was measured for all the 30 randomly selected pollen grains considered as germinated. All measurements under microscope were made using calibrated ocular micrometer.

Histochemical stainability of pollen grains

Pollen grains were collected and mixed from randomly selected flowers of each variety. Pollen grains were uniformly spread in a drop of gelatin placed on slide and were air-dried. These were fixed using Carnoy's B solution (6:3:1 of alcohol, chloroform and acetic acid respectively) for 15 minutes. Soon after fixing, they were dehydrated serially with 70, 80, 90, and 100 per cent alcohol keeping for ten minutes in each dilution.

These slides were subjected to histochemical staining for the localization of different cellular chemical compounds viz., total insoluble polysaccharides, proteins, and nucleic acids. Periodic Acid Schiff's test (Jensen 1962), Mercuric Bromophenol Blue test (Mazia et al. 1953) and Toluidine Blue test (Feder and Obreen 1968) were respectively used for testing the presence of total insoluble polysaccharides, protein, and nucleic acid respectively. Further Methyl Green Pyronin (MGP) test was used for differential staining of pollen grains for DNA and RNA (Jensen 1962).

Pollen grains were counted for differential intensity of staining. Pollen grains were mainly classified into stained and unstained. Stained pollen grains were classified further into condensed and diffusely stained. In each slide, number of stained, unstained, diffuse as well as condense stained pollen grains were counted in ten randomly selected microscopic fields. Under each microscopic field, counting was done for all the three stained types of pollen grains. Each slide was considered as single replication. Three such replications were used for each one of the histochemical stains for all the selected varieties in both the species.

Statistical analysis

Means of various morphological features of pollen grains obtained in different culture filtrate concentration and varieties were analyzed according to completely randomized design for two factors. Frequency of pollen grains in different size groups was worked out. Observed frequency distribution was compared with expected frequency distribution under normal distribution using Kolmogorov-Smirnov one sample test (Siegel and Castellan 1988). Similarly, frequency distribution of germinated and ungerminated pollen grains in different size groups within various genotypes of both species under normal germination media, as well as in presence of culture filtrate in the germination media were compared using Kolmogorov-Smirnov two sample test (Siegel and Castellan 1988).

Available variability for differentially stained pollen grains for various histochemicals were evaluated using one way ANOVA. To understand the control of histochemical content on performance of pollen grains, Spearman's rank correlation (MSTATC package) was worked out between number of stained pollen grains with germination percentage and tube-length.

Disease resistance ability of a pollen grain is considered in terms of its germination capacity and tube-length in presence of culture filtrate. The influence of culture filtrate concentration in germination media on germination percentage was found to be logarithmic and on tube length, it was found to be exponential. Hence, slope of logarithmic relationship and exponential values of exponential relationship were used to work out Spearman's rank correlation between response of pollen grains to culture filtrate and histochemical stainability.

RESULTS

Morphological variability

Wide range for pollen grain size was noticed in all the varieties of both *D. caryophyllus* and *D. chinensis* (Table 1) suggesting the existence of wide variability within both the species. Each variety was found to be composed of more than one population of pollen grains based on size. Existence of multiple groups within species and few varieties in each species is represented (Figure 1) as an example of existing situation.

Table 1. Variability for pollen characters in different genotypes of *D. caryophyllus* and *D. chinensis*

Genotype	Diameter of pollen (μm)			
	mean	variance	range	*CV*
		D. caryophyllus		
Goldrush	32.98	3.52	12.36–67.98	5.69
Internet	32.66	2.08	15.45–52.53	4.42
Monaco	33.87	2.75	15.45–67.98	4.90
Regina	31.29	2.34	15.45–46.35	4.89
Trendy	33.42	5.14	15.45–93.6	6.78
		D. chinensis		
G2	33.25	6.74	12.36–95.79	7.81
G7	37.25	6.88	18.54–52.53	7.04

CV – coefficient of variation

Table 2. Test for the similarity of germinated and ungerminated pollen grain size distribution in genotypes of *D. caryophyllus* and *D. chinensis*; the table shows D_{max} values computed from *K.S.* test

Genotype	Sample size		D_{max}
	germinated	ungerminated	
D. caryophyllus	2155	3953	0.171*
Goldrush	374	733	0.261*
Internet	573	808	0.424*
Monaco	409	816	0.191*
Regina	441	783	0.177*
Trendy	358	813	0.241*
D. chinensis	1456	631	0.692*
G2	552	187	0.716*
G7	426	444	0.49*

* significant at $P < 0.05$

Pollen germination

Kolmogrov-Smirnov test comparing the similarity between the pollen groups that germinated and remained ungerminated within a pollen population of various genotypes and species of *Dianthus* suggested differential response of pollen grains based on size (Table 2). Analysis of variance suggested significant variance due to genotypes for size of germinated pollen grain and length of pollen tube (Table 3).

Pollen germination response in presence of culture filtrate

With the presence of culture filtrate in germination media, pollen grains that germinated were different from those germinated under normal condition. Pollen group that germinated in presence of culture filtrate was different from those under normal condition and the total pollen grain population. Frequency distribution of germinated pollen grains in culture filtrate based on size differed from that of total pollen grain population (Table 4). Germinated pollen grain size was found to be negatively associated with pollen tube length (Table 5).

Histochemical variability

Stainability of pollen grains was found to be dependent on their genetic background (Figure 2). Intensity of staining varied among pollen grain population within each variety of both *D. caryophyllus* (Figure 3) and *D. chinensis* (Figure 4). Thus, it is possible to differentiate pollen grains within a pollen population based upon stainability and intensity of staining.

Table 3. Analysis of variance for germinated pollen grain size and pollen tube length in *Dianthus* spp.

Source	*d.f.*	Mean sum of squares	
		diameter of germinated pollen	pollen tube length
		D. caryophyllus	
Genotypes	4	3.17**	376.05**
Error	90	0.19	61.91
		D. chinensis	
Genotypes	2	23.05**	1826.34*
Error	30	2.25	52.86

* significant at $P < 0.05$, ** significant at $P < 0.01$

Table 4. Test for the similarity of germinated and ungerminated pollen grain size distribution in different conditions compared to total pollen grain population among genotypes of *D. caryophyllus* and *D. chinensis*

Genotypes		Total pollen	Germinated pollen	
			culture filtrate	control
D. caryophyllus	sample size	6108	450	1705
	D_{max}	–	0.11*	0.121*
Goldrush	sample size	1107	91	283
	D_{max}		0.172*	0.173*
Internet	sample size	1381	90	483
	D_{max}	–	0.127	0.112*
Monaco	sample size	1225	88	321
	D_{max}	–	0.148	0.122*
Regina	sample size	1224	91	350
	D_{max}	–	0.291*	0.218*
Trendy	sample size	1171	90	268
	D_{max}	–	0.159*	0.17*
D. chinensis	sample size	1609	266	1634
	D_{max}		0.161*	0.151*
G2	sample size	739	89	463
	D_{max}		0.353*	0.148*
G7	sample size	870	87	783
	D_{max}		0.28*	0.031

* significant at $P < 0.05$

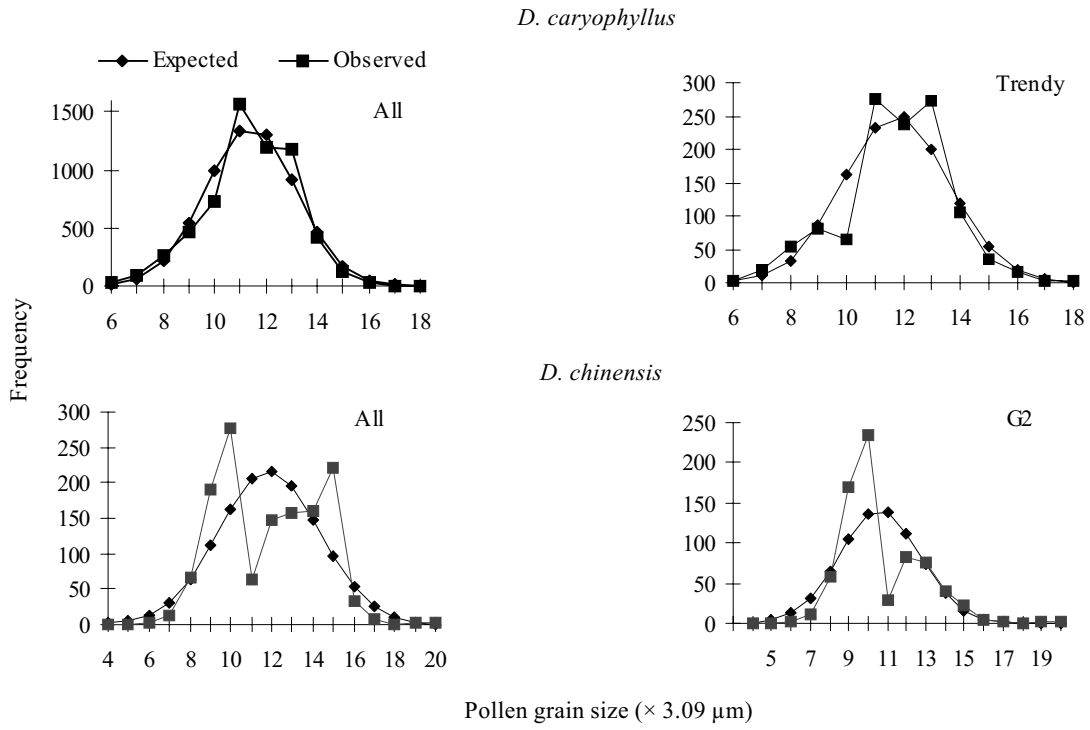

Figure 1. Observed frequency distribution of pollen grains size in comparison with expected normal distribution in different genotypes of *D. caryophyllus* and *D. chinensis*; all refers to the frequency distribution of pollen grains at species level

Histochemical stainability and pollen germination response

Existence of histochemical variability is more meaningful provided an association exists between stainability and pollen performance. Spearmans' rank correlation indicated association of differential stainability of histochemical with that of germination and tube length (Table 6). Stainability for nucleic acid is found to have negative control on germination percentage. However, DNA content in particular was found to have positive impact on germination in presence of culture filtrate. Stainability of pollen grains for starch was found to have positive association with germination percentage in presence of culture filtrate in germination media. Percentage of pollen grains diffusely stained for protein was negatively related with tube length in control.

Table 5. Correlation between pollen diameter and tube length observed in control and in culture filtrate in different genotypes of *D. caryophyllus* and *D. chinensis*

	CV	
	control	culture filtrate
	D. caryophyllus	
Goldrush	–0.1008	–0.1719*
Internet	0.0001	–0.1616*
Monaco	–0.1669	–0.3271*
Regina	0.0468	–0.1548*
Trendy	0.0605	–0.1405*
	D. chinensis	
G2	–0.3546*	–0.2122
G4	–0.4348*	–0.1984*
G7	–0.1560	–0.5683*

* significant at $P < 0.05$

DISCUSSION

Variability in pollen grain size within a variety suggested the possibility of selecting pollen grains based on size. It is possible to separate pollen grains based on size by passing through sieves of different sizes (Walden and Greyson 1985). This sort of variability in pollen grain population based on size is a common feature reported in several species: Johanson et al. (1976) in *Z. mays*, Pallais et al. (1988) in potato, Bottraud et al. (1992) in *Vicia faba*, Harder (1998) in *Pedicularis* species, Knapp et al. (1998) in *Solanum*. Variation in pollen tube length within a variety further suggested the possibility of selecting pregerminated pollen grains with longer tube length for crossing which in turn would result in vigorous progeny. It is possible to separate germinated and ungerminated pollen grains by *in vitro* centrifugation (Bino et al. 1988, Mulchay et al. 1988) as well as by screen and column method (Zhang et al. 1993).

It is important to record here that; pollen grain size is related with the pollen tube growth rate (Ottaviano et al. 1983). Pollen tube growth rate influences the resulting

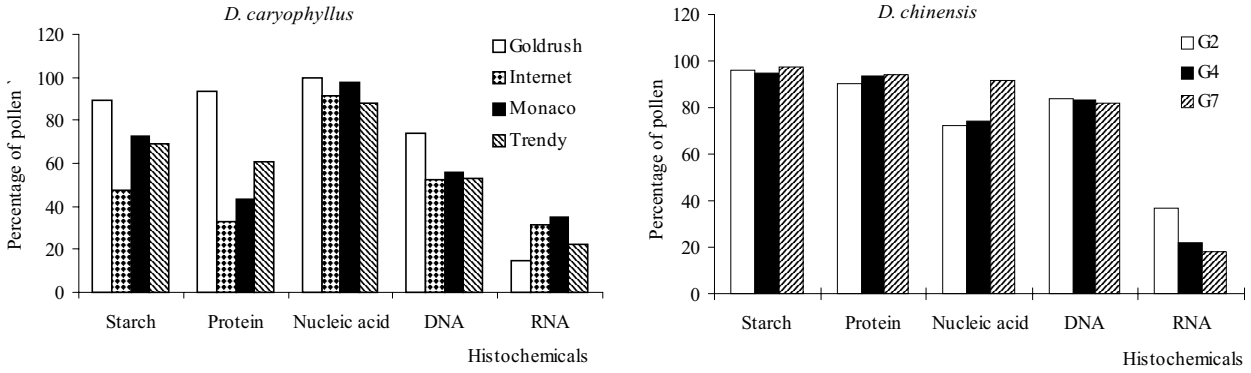

Figure 2. Differences in histochemical stainability of pollen grains in response to genotypic variability in *D. caryophyllus* and *D. chinensis*

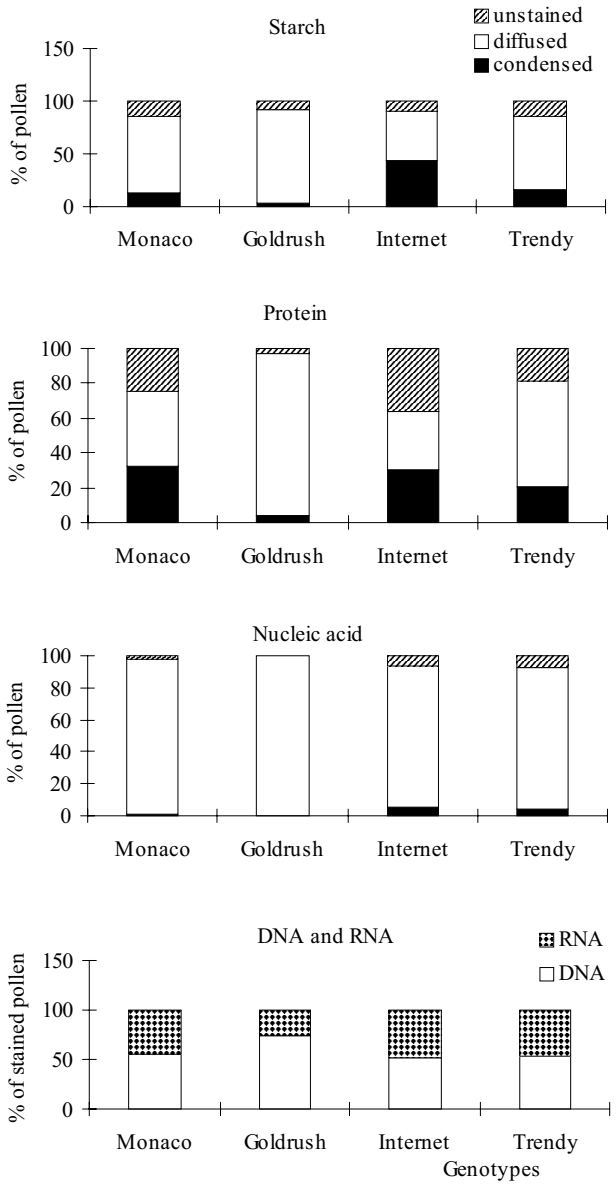

Figure 3. Pollen grain percentage based on intensity of histochemical staining in different genotypes of *D. caryophyllus*; last graph refers to percentage of pollen grains stained for DNA and RNA out of total for nucleic acid

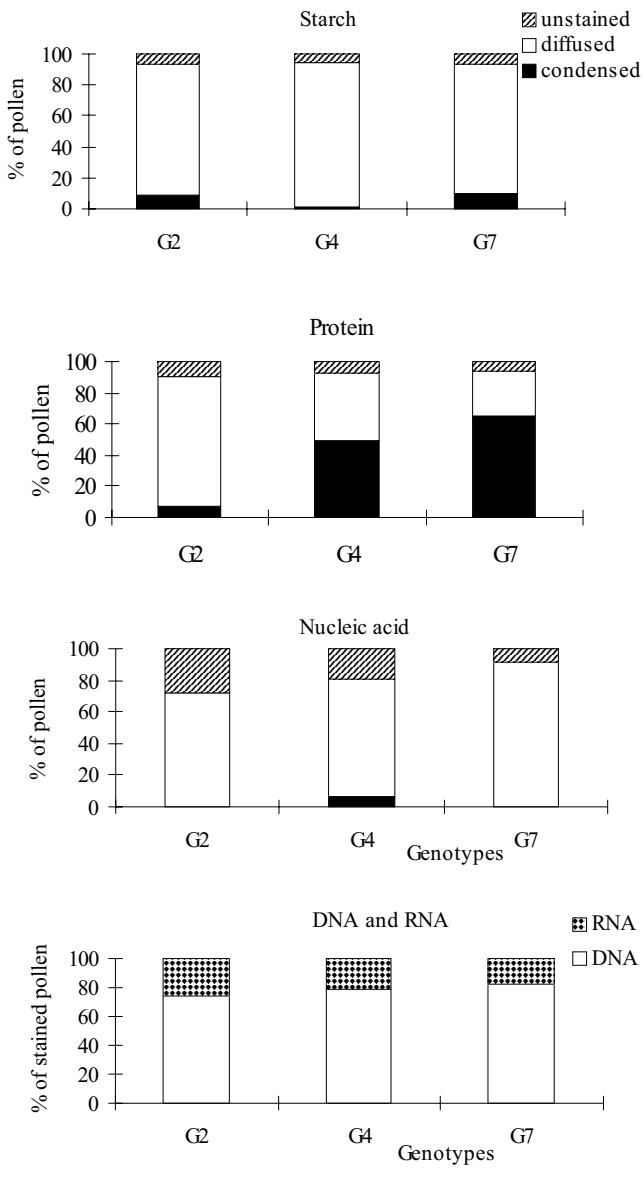

Figure 4. Pollen grain percentage based on intensity of histochemical staining in different genotypes of *D. chinensis*; last graph refers to percentage of pollen grains stained for DNA and RNA out of total for nucleic acid

Table 6. Rank correlation between histochemical stainability and pollen germination characters observed in control and in presence of culture filtrate

Intensity of staining	Correlation with histochemical									
	control					culture filtrate				
	starch	protein	nucleic acid	DNA	RNA	starch	protein	nucleic acid	DNA	RNA
	Correlation with germination per cent									
Stained	0.5714	0	–0.8571**	0.6786		0.8571**	0.6071	–0.7500*	0.7500*	–
Condensed	–0.3571	0.4286	–0.1071	–0.500	–0.50	–0.5000	0.3929	–0.4643	0.0360	0.0357
Diffusely stained	0.2857	–0.2860	–0.6786	0.6071	0.2143	0.5714	–0.286	–0.4643	0.5360	–0.6430
Unstained	–0.6429	0	0.8571**	–0.536	–0.536	–0.7857*	–0.607	0.7500*	–0.1100	–0.1070
	Correlation with pollen tube length									
Stained	–0.0714	–0.2860	0.3214	–0.3214		0.6071	0.1786	–0.2143	0.3570	–
Condensed	0.3929	0.6429	0.3929	0.6429	0.6429	0.6071	–0.0710	–0.1429	0.3210	0.3214
Diffusely stained	–0.2143	–0.821*	0.4286	–0.5714	–0.1430	0.2143	–0.1780	0	0.0360	–0.2500
Unstained	0.2143	0.2857	–0.3214	0.0714	0.0714	–0.1786	–0.1790	0.2143	0.1790	0.1786

* significant at $P < 0.05$ level, ** significant at $P < 0.01$ level

progeny vigor in sporophytic stage (Mulcahy 1974, Ottaviano et al. 1988). This phenomenon of pollen tube vigor influencing the progeny vigor can be exploited indirectly in breeding program by enhancing the pollen load used for pollination (Visser and Verhaugh 1988, Palmer and Zimmerman 1994, Johanson and Stephenson 1997) or by subjecting pollen grains to distance based competition (Ottaviano et al. 1988, Rosellini et al. 1994, Tejaswini 1999).

Association between pollen grain stainability for histochemical content and its germination percentage as well as tube length suggests the possibility of using stainability as marker for pollen grain selection. Pollen grains are known to be packed with different biochemicals like sugar, starch, lipids, phytic acid (Bertin 1988, Wetzel and Jensen 1992, Stephenson et al. 1994) and mRNA (Stephenson 1992). These storage products get metabolized upon germination and elongation of pollen tube, thus play an important role in germination and in initial stages of pollen tube growth (Vasil 1974, Wetzel and Jensen 1992, Stephenson et al. 1994). Presence of these biochemical is dependent on the sporophyte from which they originate and its growing condition (El-Sayed and Kirkwood 1992, Lau and Stephenson 1994) and also on the evolutionary background of the species (Baker and Baker 1983). Thus, one can expect to find association of histochemical stainability with that of pollen performance. Pollen grain stainability indicated its capacity to germinate and length of tube it can produce both in control and in presence of culture filtrate. Thus, pollen grains with desired allelic combination for vigor and resistance can be picked up based on their histochemical stainability.

Thus, in conclusion variability available within pollen population suggests the possibility of selecting pollen grains based on size, tube length and histochemical stainability and which in turn would enhance the probability of developing a progeny of interest by selective pollination.

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ABSTRAKT

Selekce pylových zrn u hvozdíku (*Dianthus*) a její využití ve šlechtění

Studie je zaměřena na co nejlepší využití samčí generace gametofytů ve šlechtitelském programu hvozdíku. Šetřením v pylové populaci jsme zjistili, že mezi odrůdami a druhy *Dianthus caryophyllus* a *D. chinensis* i uvnitř těchto odrůd a druhů existuje dostatečná variabilita pylové morfologie a histochemického obsahu pro provádění selekce. Dále jsme zjistili, že

velikost pylových zrn a histochemický obsah souvisejí s klíčivostí a rychlostí růstu pylových láček. Kromě toho je zřejmé, že se klíčivost pylu a prodlužovací růst pylové láčky jako reakce na filtrát kultury připravený z *F. oxysporum* f. sp. *dianthi*, původce fuzáriového vadnutí hvozdíku, řídí velikostí pylového zrna a histochemickým obsahem pylových zrn. Celkové výsledky naznačují možnost selekce žádoucích pylových zrn z pylové populace i možnost učinit pokus o selektivní opylování ve šlechtitelském programu hvozdíku.

Klíčová slova: hvozdík; pyl; *Fusarium oxysporum*; *Dianthus chinensis*

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