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Breeding tomato for pollen tolerance to low temperatures by gametophytic selection

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Summary

Haploid selection for traits related to pollen cold tolerance in tomato was performed in segregating populations derived from a *Lycopersicon esculentum* × *L. pennellii* hybrid. BC_1 populations were obtained by combining normal and low temperature treatments on two stages of pollen development: pollen formation, and germination and pollen tube growth. F_1 hybrids were cultivated under low and normal temperatures and their pollen was used to pollinate *L. esculentum* plants at low and normal temperatures. The four BC_1 populations obtained were tested for the quality and quantity of pollen produced at low temperatures. The population obtained by cold treatment at both stages had a significantly improved pollen germination ability at low temperatures. The two other cold-selected BC_1 populations showed no differences compared with the unselected BC_1 population. A second cycle of pollen selection, corresponding to BC_2, was applied in order to test its persistence in the subsequent generations and the possibility to further improve the character. This second cycle showed no improvement although some plants retained the high pollen germination ability at low temperatures that was observed in the first cycle. Hence, gametophytic selection of some characters related with tomato pollen performance may be feasible, at least for the first cycle of selection.

Introduction

Temperatures below 10 °C reduce vegetative growth of tomato (*Lycopersicon esculentum* Mill.), and greatly decrease natural fruit set (Picken, 1984). This means that, in temperate climates, winter tomato production is impeded unless costly artificial methods such as greenhouse heating or hormonal treatment of flowers are employed. Poor winter fruit set is mainly due to a reduction in both pollen quality and quantity since low temperatures (≈6 °C) do not negatively affect ovule viability and the early embryo development, or alter the appropriate flower morphology for the optimum pollen transfer (Fernández-Muñoz & Cuartero, 1991). Cultural practices that improve pollination of unheated greenhouse tomatoes such as the use of bumblebees or vibration of flowers require plants producing sufficient amounts of viable pollen to be effective (Pressman et al., 1999). Breeding tomato cultivars able to produce sufficient amounts of high-fertility pollen that ensure natural winter fruit set in temperate climates cannot be limited to *L. esculentum* alone as in this species, at most a few cultivars with moderate cold tolerance have been found (Fernández-Muñoz et al., 1995a; Maissoneuve & Philouze, 1982). Fortunately, donors of pollen cold tolerance can be found among accessions from the green-fruited, wild tomato species *L. peruvianum* (L.) Mill., *L. hirsutum* Humb. & Bonpl., and *L. pennellii* (Corr.) D'Arcy (Fernández-Muñoz et al., 1995a). Use of *L. peruvianum* is complicated by its poor cross-compatibility with *L. esculentum*. In a more recent study, we observed that *L. hirsutum* had a higher pollen cold tolerance than *L. pennellii* (Domínguez et al., submitted). For *L. pennellii* and

L. hirsutum, crosses and subsequent segregating generations with *L. esculentum* showed reduced pollen fertility which was related to interspecific hybrid sterility (Domínguez et al., submitted; Fernández-Muñoz et al., 1995b). Partial hybrid sterility of *L. esculentum* × *L. pennellii* and *L. esculentum* × *L. hirsutum* has been traditionally attributed to chromosome pairing problems during meiosis (Khush & Rick, 1963; Popov, 1972). More recently, hybrid pollen sterility has been strongly related to interlocus epistasis, i.e., the action of specific genes from the wild species in the background of *L. esculentum* (Moyle & Graham, 2005). In practice, the study of tomato pollen cold tolerance in segregating populations derived from interspecific crosses cannot easily distinguish between low pollen fertility due to interspecific hybrid sterility and to reduced pollen fertility under low temperatures. Since hybrid pollen sterility in *L. esculentum* × *L. pennellii* F_1 hybrids has been observed to be less severe than in *L. esculentum* × *L. hirsutum* F_1 hybrids (Domínguez et al., submitted; Fernández-Muñoz et al., 1995b), *L. pennellii* would be more suitable for genetic studies of this character without interferences of factors other than the effect of cold.

A breeding program for tomato cold tolerance using *L. pennellii* would thereby face major practical obstacles. First, the genetic problems derived from the interspecific cross with *L. esculentum* disfavour its utility by reducing the number of potentially selectable plants. Also, tomato pollen cold tolerance is polygenic in nature (Domínguez et al., submitted; Fernández-Muñoz et al., 1995b), which makes the positive genotypes to be selected in the segregating generations relatively rare. Therefore, breeding for cold tolerance would require screening of large populations. Nevertheless, effective selection can only be ensured in greenhouses with controlled temperature conditions, which, in practice, limits the number of plants that can be tested. Therefore, it would be necessary to find a way to enrich the populations with cold tolerant genotypes. A potentially successful way to overcome these limiting factors could be gametophytic selection.

Gametophytic selection has been widely considered a potentially useful tool for plant breeders (Hormaza & Herrero, 1992, 1996). Because of the large population sizes and haploid nature of male gametophytes, the possibility of applying a stress to pollen and hence modifying the subsequent diploid sporophytic generation, is tempting. In order for the pollen selection to be effective it would be necessary that, on one hand, the pollen grains were able to express their own genome and, on the other hand, that an overlap exists between genetic expression in both, the gametophyte and sporophyte. The use of pollen grains as a screening system is based on several reports indicating an overlap of genes that are expressed in the gametophytic and sporophytic generations. In tomato (Tanksley et al., 1981), approximately 60% of the genes expressed in the pollen grain were estimated to be also expressed in the sporophyte, 72% in maize (Sari-Gorla et al., 1986a,1986b), 81% in *Prunus* (Weeden, 1986), 74–80% in *Populus* (Rajora & Zsuffa, 1986), and 60% in barley (Pedersen et al., 1987).

There are few clear results that indicate the usefulness of pollen selection in increasing the tolerance of a given generation to a specific stress, or persistence of this tolerance over generations. For instance, Chikkodi & Ravikumar (2000) improved *Alternaria* resistance in sunflower, Frascaroli & Songstad (2001) introduced chlorsulfuron resistance in maize and proved its persistence during several subsequent generations. More recently, Ravikumar et al. (2003) reported increased drought tolerance in sorghum populations. In tomato, Zamir et al. (1982), Zamir & Vallejos (1983), and Zamir & Gadish (1987) showed deviations in the expected frequencies of gametes with a specific allele from a tomato pollen population subjected to a low temperature treatment. Maisonneuve et al. (1986) applied pollen selection to the improvement of cold tolerance in cultivated tomato with no positive results. This was probably due to the fact that the degree of cold tolerance within the cultivated tomato is very low (Fernández-Muñoz et al., 1995b; Maissoneuve & Philouze, 1982) and better sources of tolerance are needed to provide the selectable genetic variability.

Many reports of gametophytic selection attempt to improve sporophytic traits through pollen selection but, in the case of tomato fruit set at low temperatures, a gametophytic character such as the ability of pollen to grow and germinate is the most limiting factor (Picken, 1984). In the present paper, we aimed to test if gametophytic selection could be a suitable tool to enrich small segregating populations with positive genotypes of a microgametophyte-related trait such as cold tolerance. This was attempted by producing and testing different BC_1 populations after applying cold stress to *L. esculentum* × *L. pennellii* F_1 pollen. Most previous reports of pollen selection tested only a single cycle of pollen selection. A few also tested several additional cycles of selection (Frascaroli et al., 1995; Kovács & Barnabás, 1992; Landi et al., 1989; Sari-Gorla et al., 1994). To test if the second cycle of pollen selection was effective

for cold tolerance of tomato pollen, we also produced and tested different BC_2 populations.

Materials and methods

Plant material and experiments

Lycopersicon esculentum cv. 'Moneymaker' was chosen as the cold sensitive parent because of its poor pollen viability under low temperatures. The wild accession *L. pennellii* PE-45 was chosen as the cold tolerant parent based on the results by Fernández-Muñoz et al. (1995a). PE-45 was collected by our research group in the Ancash Department, Perú at 350 m altitude, where the mean max/min summer temperatures are about 28/16 °C and 20/10 °C in winter. BC_1 populations were used to test if the first cycle of selection on pollen from F_1 ('Moneymaker' × PE-45) plants was effective. Four BC_1 ('Moneymaker' × F_1) populations were produced from crosses using pollen of the F_1 plants submitted to different temperature regimes. 'Moneymaker' and the F_1 plants were divided into two groups, one grown in a refrigerated glasshouse with low night temperatures (18/5 °C, day/night) and the other one in a heated glasshouse at 25/16 °C. The night temperatures in the refrigerated glasshouse were low enough to affect pollen development and germination but not to impede flower development. In order to ensure that pollen was affected by specific temperatures, the temperature treatment started at least 3 weeks before pollination. Cold stress was applied either during pollen formation and/or pollen germination and tube growth. Pollen grains with cold tolerance alleles would be more likely to result in fertilization, and hence produce the next sporophytic generation. 'Moneymaker' flowers grown at low and normal temperatures were emasculated and pollinated at anthesis with pollen collected from the F_1 hybrid grown at low and normal temperatures. Thus, four BC_1 populations were obtained, each representing one of four treatments: pollination at normal temperature with pollen produced at normal temperature (NN), pollination at normal temperature with pollen formed at low temperature (NL), pollination at low temperature with pollen produced at normal temperature (LN), and pollination at low temperature with pollen produced at low temperature (LL).

These four populations were evaluated for pollen ability to develop and grow at low temperatures. Two experiments were carried out, one with low night temperatures in the refrigerated glasshouse with the temperature regime set to 24/4 °C and another in a heated glasshouse set to 28/16 °C. In the low night temperature experiment, 30 plants of each population and five plants of the parents and the F_1 hybrid were cultivated. The four backcross populations were distributed in five randomised blocks of six plants each and the parents in five blocks of one plant per genotype. The experiment at the normal temperature regime was the control for the effect of low temperature on pollen of the parents, F_1, and the unselected NN population. In this experiment, 30 plants of the NN backcross population and five plants of the parents and F_1 were cultivated.

Samples of pollen produced by all the cultivated plants were collected on three dates. The average ± standard deviations of daily maximum and minimum temperatures (°C) registered on the six thermosensitive days of pollen formation (14th, 13th, 12th, 6th, 5th, and 4th days before anthesis following Mutton et al., 1987) for each sampling date for both experiments were: $23.5 \pm 1.4/4.3 \pm 0.5$ (31 March), $25.5 \pm 3.0/4.5 \pm 0.5$ (7 April), and $26.3 \pm 1.7/4.3 \pm 0.8$ (14 April) in the refrigerated greenhouse; and $29.8 \pm 5.3/16.5 \pm 1.4$ (31 March), $28.0 \pm 3.8/17.5 \pm 0.5$ (7 April), and $28.0 \pm 3.0/16.8 \pm 0.7$ (14 April) in the heated greenhouse.

By the end of the first cycle experiment, plants of the LL and NN BC_1 populations produced new populations to test the effectiveness of the second cycle of pollen selection. Four BC_2 populations were obtained, with a similar procedure as that in the first cycle. A mixture of pollen was collected from all 29 plants of the LL population at low temperatures. The LLL and LLN BC_2 populations were obtained by using that mixture of pollen to pollinate flowers of 'Moneymaker' grown at low and normal temperatures, respectively. Pollen of the three most cold tolerant plants from the LL population was mixed and used to pollinate 'Moneymaker' flowers at low temperatures to obtain the LLS population. Finally, pollen mixture from the 28 plants of the NN population grown in the heated glasshouse was used to pollinate 'Moneymaker' flowers at normal temperature to obtain the population NNN.

The same experimental design as in the first cycle experiment was applied to these four new populations to test them for pollen performance at low temperatures. To increase the differences in responses of BC_2 populations, the day temperature was lowered compared to that of the BC_1 evaluation. So, the temperature regime was 19/3 °C in the refrigerated glasshouse and 27/16 °C in the heated glasshouse. The average ± standard deviations of daily maximum and minimum

Table 1. Summarized explanation of pollen sources and temperature treatments for obtaining and testing of the BC_1 and BC_2 populations

		Temperature regime at		
	Pollen source	Pollination stage	Pollen formation stage	Testing
BC_1 population				
LL_L	F_1	low	low	low
LN_L	F_1	low	normal	low
NL_L	F_1	normal	low	low
NN_L	F_1	normal	normal	low
NN_C	F_1	normal	normal	normal
BC_2 population				
LLS_L	Three best LL_L plants	low	low	low
LLL_L	LL_L	low	low	low
NLL_L	LL_L	normal	low	low
NNN_L	NN_C	normal	normal	low
NNN_C	NN_C	normal	normal	normal

temperatures (°C) registered on the six thermosensitive days of pollen formation (Mutton et al., 1987) for each sampling date for both experiments were: $19.3 \pm 1.4/3.4 \pm 0.7$ (29 December), $20.7 \pm 0.8/3.6 \pm 0.4$ (8 January), and $16.5 \pm 3.5/3.3 \pm 0.8$ (13 January) in the refrigerated greenhouse; and $26.8 \pm 0.4/16.3 \pm 1.0$ (29 December), $26.5 \pm 0.8/15.8 \pm 0.4$ (8 January), and $26.1 \pm 1.3/16.5 \pm 1.2$ (13 January) in the heated greenhouse.

A summarized explanation of how the backcross populations were obtained and tested is given in Table 1. Letters $_L$ and $_C$ after the name of a genotype or population refer to the low and control temperature regimes, respectively, at which they were tested.

Assessment of pollen germination, viability, and quantity

Pollen viability was estimated by the acetocarmine staining method, which measures the proportion of pollen grains taking up the stain. This method provides an accurate estimate of viability of tomato pollen formed at low temperatures (Fernández-Muñoz et al., 1994). The ability of pollen to germinate at low temperatures was estimated by *in vitro* germination at 10 °C. Each sample was a mixture of pollen from five flowers at anthesis of the same plant. Pollen was collected by vigorously shaking the anthers with a needle over a microtube. Following this, 1 mL of germination medium (15% sucrose, 1.27 mM $Ca(NO_3)_2$, 1.62 mM H_3BO_3, 1 mM KNO_3, and 0.1 mM KH_2PO_4) was pipetted into the microtube. Immediately after homogenisation, 0.5 mL were pipetted into a 4 mL assay tube, the tube was capped, and then incubated in a shaker at 10 °C overnight. The contents were stained and fixed by adding 200 μL of a 1% acetocarmine solution. Each sample was assessed by inspecting more than 200 grains in at least five microscope fields to calculate the percentage of pollen grains germinated *in vitro*. Prior to pollen germination, 200 μL of the 1% acetocarmine solution were added to the remaining 0.5 mL in the microtube to calculate pollen viability by counting the percentage of grains stained. Pollen quantity was estimated by counting pollen grains on the grids of two Fuchs-Rosenthal clinical haemacytometers (E. Hartnack, Berlin, Germany) and averaging them.

Data analysis

Viability, germination, and number of pollen grains were calculated for each plant with its mean value of the three sampling dates. Prior to analyses, data of % pollen viability and % germination were transformed by the arcsin of the square root of $p/100$, and data of number of pollen grains per flower by the $\log_{10}(x+1)$. However, for convenience, untransformed data are presented in tables and figures. Comparisons between means of genotypes and populations were made by one-way ANOVA in which homogeneity of variances was tested, followed by either Tukey-b (for the case of homogeneous variances) or Games-Howell (for non-homogeneous variances) procedures (SPSS, 2001).

Results

Responses of the parents and the F_1 controls were very similar and consistent in the experiments of the two cycles of selection (Table 2). The viability of pollen stained with acetocarmine was similar among the three genotypes in the normal temperature regime. In the low temperature treatment, only 'Moneymaker' produced a significantly lower percentage of stainable grains. Pollen germination at 10 °C in the normal temperature regime showed that PE-45 pollen was able to germinate at low temperatures better than 'Moneymaker' pollen; the F_1 was similar to 'Moneymaker'. In the cold temperature regime experiment, PE-45 again showed the highest percentage of pollen germination, although it

Table 2. Means and their standard errors (S.E.), number of plants (*N*), and range of % pollen acetocarmine staining, *% in vitro* germination at 10 °C, and number of pollen grains produced per flower for *L. esculentum* 'Moneymaker' (MM), *L. pennellii* PE-45, and the F_1 cultivated at low and control temperatures in the two consecutive experiments of pollen selection

	% Pollen staining			% Pollen germination 10 °C			No. pollen grains per flower (10^3)		
Genotype	Mean ± S.E.	*N*	Range	Mean ± S.E.	*N*	Range	Mean ± S.E.	*N*	Range
First cycle of selection									
MM_L	75.1 ± 2.3 b[a]	5	66.4–79.2	26.2 ± 3.0 c	5	19.8–35.4	36.7 ± 5.0 d	5	19.7–50.8
F_1 (MM × PE-45)$_L$	81.0 ± 2.3 a	5	72.8–86.2	34.4 ± 1.5 bc	5	31.3–39.3	183.2 ± 33.7 bc	5	117.6–312.0
PE-45_L	84.2 ± 0.4 a	4	83.0–85.0	54.1 ± 2.6 a	4	49.0–60.9	285.2 ± 90.6 b	4	261.2–322.1
MM_C	93.3 ± 1.0 a	5	90.7–96.5	37.9 ± 2.4 b	5	32.1–43.1	107.9 ± 17.7 cd	5	57.7–210.7
F_1 (MM × PE-45)$_C$	90.3 ± 4.3 a	5	79.1–96.0	37.7 ± 2.8 b	5	26.2–40.3	279.8 ± 43.8 b	5	220.3–395.6
PE-45_C	96.2 ± 1.0 a	4	93.7–98.7	61.9 ± 2.6 a	4	51.0–70.0	464.9 ± 39.6 a	4	310.4–562.3
Second cycle of selection									
MM_L	69.9 ± 1.7 b	5	65.4–73.2	28.7 ± 2.5 c	5	23.0–35.5	35.8 ± 8.0 c	5	16.8–54.8
F_1 (MM × PE-45)$_L$	76.0 ± 2.2 b	4	72.3–81.0	35.4 ± 1.9 bc	4	31.1–38.7	181.4 ± 33.0 b	4	120.0–247.9
PE-45_L	91.4 ± 2.2 a	4	85.1–95.9	56.6 ± 3.0 a	4	49.0–63.9	266.4 ± 70.8 ab	4	149.1–451.1
MM_C	93.5 ± 1.8 a	4	88.1–96.0	41.5 ± 2.2 b	4	36.6–47.0	146.7 ± 45.8 b	4	76.7–277.6
F_1 (MM × PE-45)$_C$	91.0 ± 3.7 a	4	80.1–96.3	34.4 ± 3.7 bc	4	24.5–42.7	295.4 ± 45.0 ab	4	166.4–371.4
PE-45_C	97.0 ± 0.7 a	5	95.2–98.5	62.6 ± 2.9 a	5	57.1–70.9	461.4 ± 72.6 a	5	274.7–644.0

[a]Means with the same letter in each experiment are not significantly different (Tukey *b* test, $\alpha = 0.05$).

was lower than the percentage observed in the control experiment. The cold sensitive parent, 'Moneymaker', had a significantly lower germination percentage at low temperatures than at normal temperatures, and the F_1 was intermediate, with a percentage of germinated pollen very similar to that observed in the control experiment. The number of pollen grains produced per flower was affected by the temperature regime in all the genotypes, with a decrease at the cold night temperature regime. PE-45 produced a higher number of pollen grains compared with the other genotypes, and the F_1 hybrid had an intermediate number.

Frequency distributions and mean values for pollen quality and quantity of the BC_1 populations in the first cycle of pollen selection are given in Figure 1. If the pollen selection worked, two outcomes would be expected: an overall increase of pollen performance at low temperatures in any of the selected populations and/or some plants with better pollen performance. There were no significant differences among the means of the five populations for pollen viability, although the NN_C population showed some plants in higher categories. Several plants within the BC_1 populations produced pollen less viable than that of 'Moneymaker'$_L$.

For pollen germination at 10 °C, the mean of the double-selected population LL_L was significantly higher than those of the rest. The other two populations that were generated by pollen selection under cold stress, LN_L and NL_L, showed similar mean percentages and distributions. The nonselected population grown under low temperatures, NN_L, showed the lowest percentage of pollen germination although it was not significantly different from the means of NL_L and LN_L. Only in LL_L there was a considerable number of plants showing pollen germination between the mean values of 'Moneymaker'$_L$ and PE-45_L (about one-half of plants in that population). Moreover, seven plants of LL_L surpassed the maximum germination value observed for 'Moneymaker'$_L$ (35.5%, Table 2) and one plant had a germination percentage similar to PE-45_L. No plants from the remaining populations were within the 40–50% and 50–60% pollen germination categories.

The five tested populations showed wide frequency distributions for the number of pollen grains per flower, overlapping those of the parents, (Figure 1). There were no significant differences among their means, which were intermediate between those obtained for 'Moneymaker' and the F_1 hybrid (Table 2).

The results from the second cycle of pollen selection are shown in Figure 2. The frequencies of plants with pollen quality inferior to that of the sensitive parent were higher following the second cycle of selection, affecting at least one half of the BC_2 plants. Many plants

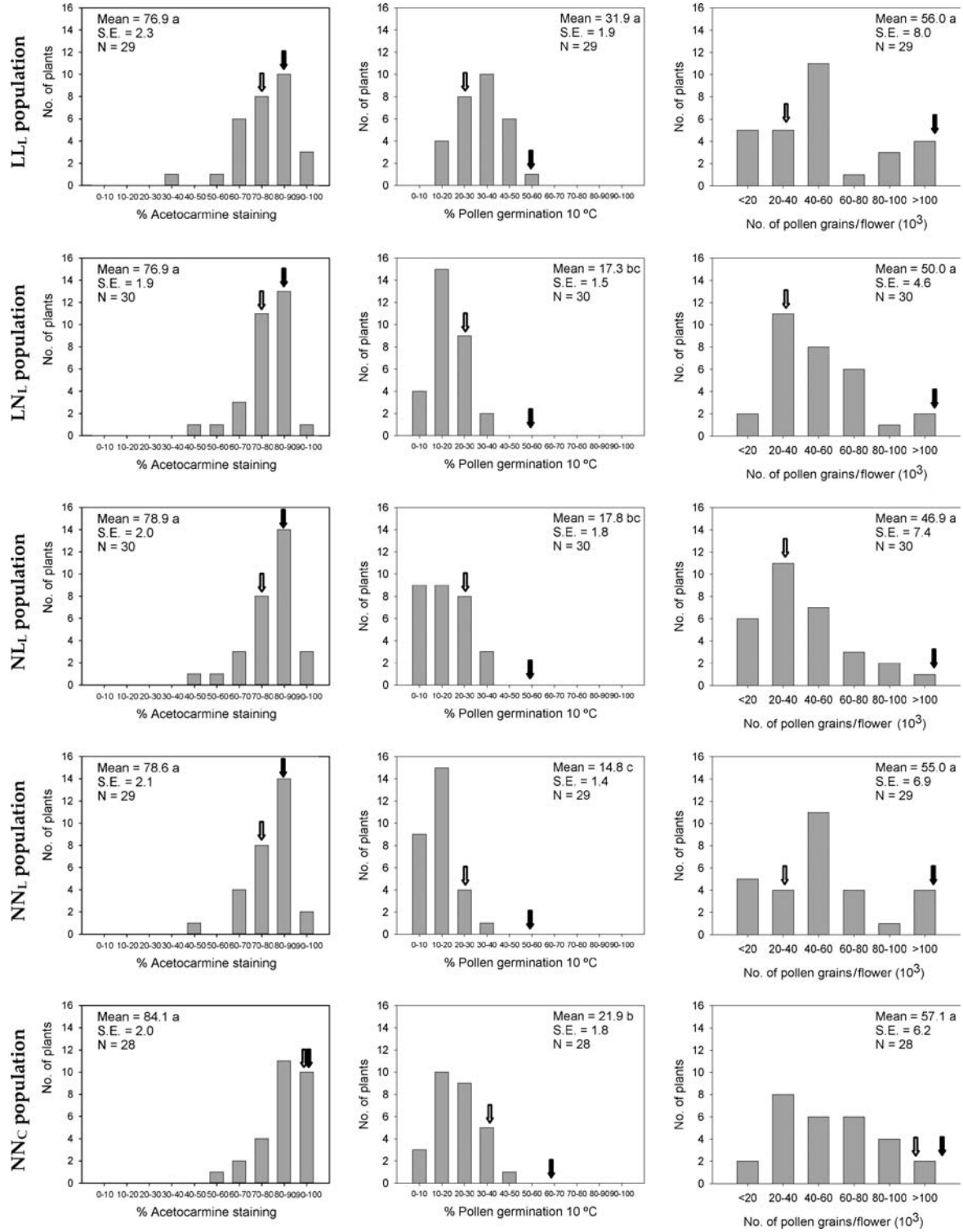

Figure 1. Frequency distributions and basic statistics of % acetocarmine pollen staining, *in vitro* pollen germination at 10 °C, and number of pollen grains per flower of the BC_1 populations corresponding to a first cycle of pollen selection. Means with the same letter for each character are not significantly different (Tukey-*b* test, $\alpha = 0.05$). Arrows indicate the parental means (open for 'Moneymaker', solid for PE-45).

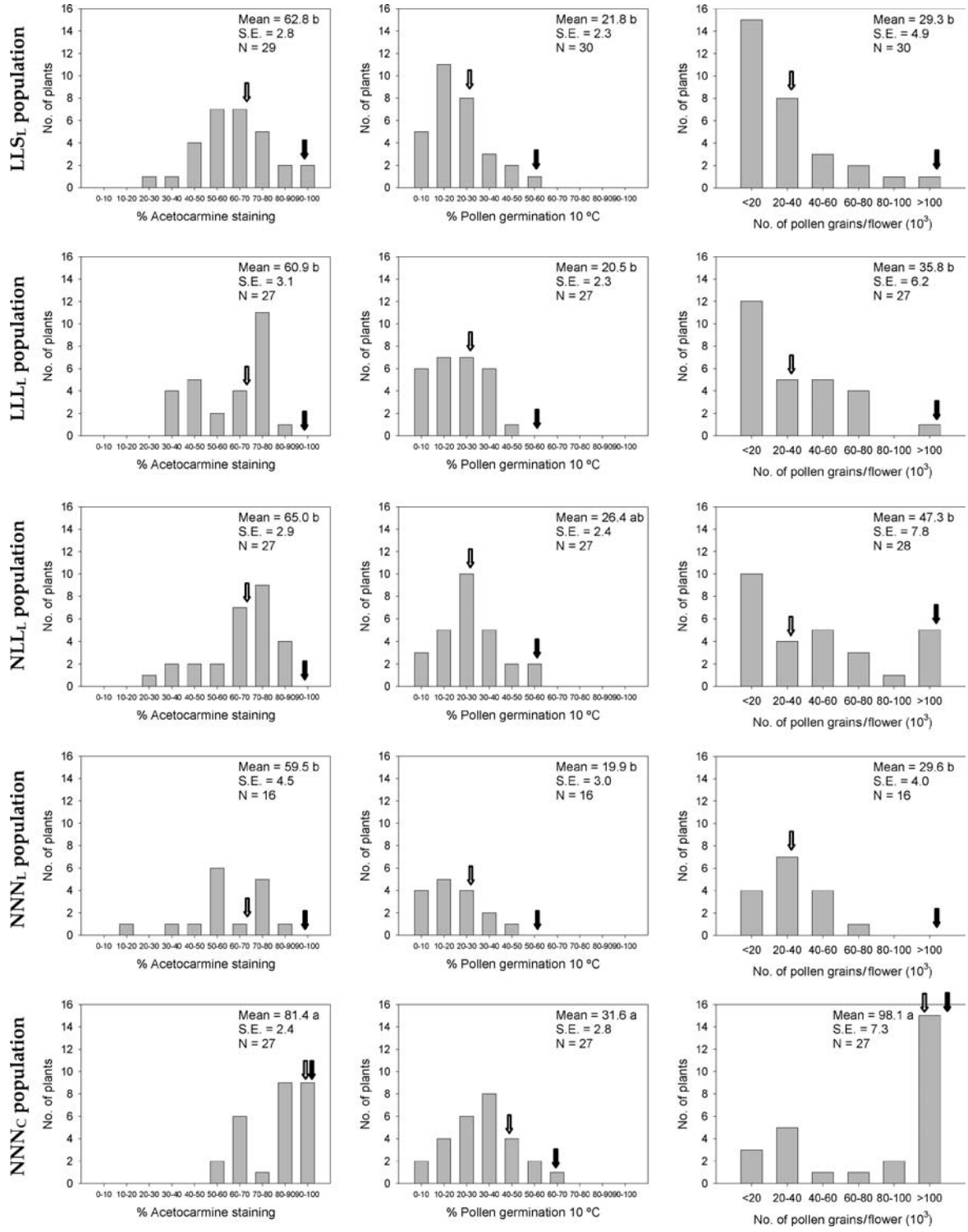

Figure 2. Frequency distributions and basic statistics of % acetocarmine pollen staining, *in vitro* pollen germination at 10 °C, and number of pollen grains per flower of the BC_2 populations corresponding to a second cycle of pollen selection. Means with the same letter for each character are not significantly different (Tukey-*b* test, $\alpha = 0.05$). Arrows indicate the parental means (open for 'Moneymaker', solid for PE-45).

under the cold treatment had higher numbers of aborted pollen grains than of the viable ones. There were no differences in pollen viability among the populations except for NNN_C that showed a higher mean percentage of viability, as expected. There was also an overall decrease in pollen germination at 10 °C in the populations of the 2nd cycle compared with the 1st cycle. LLS_L, LLL_L, and NLL_L showed clearly lower means of percentage of germination at 10 °C than that of the population from which they originated, LL_L. Moreover, from seven plants in LL_L population that clearly surpassed the pollen germination rate of 'Moneymaker'$_L$ in the first cycle of selection, only three remained in the second cycle experiment in LLS_L, one in LLL_L, and four in NLL_L (categories 40–50% and 50–60% in Figure 2). Again, only the mean of the population NNN_C was significantly higher than the rest. Several plants within BC_2 populations produced lower numbers of pollen grains than the 'Moneymaker'$_L$ plants. The NNN_C population showed the highest number of pollen grains produced and, among the populations submitted to cold treatment, the nonselected NNN_L was the only population without any plants in the highest categories. It is noticeable that in the second cycle of selection, there was a clear difference in the number of pollen grains produced between NNN_L and NNN_C. This difference was not observed for the corresponding BC_1 populations NN_L and NN_C in the first cycle experiment. It must also be mentioned that half of the NNN_L population could not be screened simply because the plants died or did not grow to flower due to chilling.

Discussion

In the experiments of both cycles of pollen selection, the responses of the nonsegregating generations (parents and F_1) were very consistent. As expected, low temperatures clearly affected pollen quality, as indicated by viability and germination at 10 °C, and pollen quantity of cv. 'Moneymaker' compared to the tolerant parent *L. pennellii* PE-45. This consistency emphasizes the importance of applying cold stress under controlled conditions as it helps to reduce the differences due to variable environmental factors. The tolerance of PE-45 was evident as demonstrated by its better performance for the three pollen traits in the low temperature experiments compared to 'Moneymaker'. Pollen cold tolerance of PE-45 is intriguing as it was collected at a low altitude where it grows at mild temperatures. This is in contrast to many reported cold tolerant wild accessions of tomato, e.g., from *L. hirsutum* and *L. peruvianum* (Fernández-Muñoz et al., 1995a), which come from high altitudes where temperatures can be very low.

Two main stages of pollen development can be affected by cold temperature: pollen formation in the anther, and the ability of pollen to germinate and grow through the style. The numbers and viability of pollen produced are two characters that are the result of microgametophyte formation. Pollen selection was ineffective in the improvement of pollen viability and the number of pollen grains per flower. This result could be explained if genes controlling cold tolerance for pollen formation were not expressed in the microgametophyte but rather in the sporophyte. The tapetum is the main sporophytic tissue responsible for pollen formation (Goldberg et al., 1993). Winter environmental conditions cause early degeneration of tomato tapetal tissue and subsequent abortion of pollen mother cells (Bonner, 1988). The number of viable pollen grains per flower depends on the number of pollen mother cells able to successfully complete meiosis and pollen development. Two periods of tomato pollen formation are especially sensitive to low temperatures, principally meiosis and to a lesser degree, pollen maturation (Mutton et al., 1987). Since tapetal tissues and pollen mother cells are sporophytic in origin, expression of haploid pollen genotype can only be expected in the less cold-sensitive stage of pollen maturation.

Plants with a considerably lower pollen viability, germination, and the number of pollen grains than the sensitive parent 'Moneymaker' were observed in all BC_1 and BC_2 populations studied regardless of whether they were evaluated at low or normal temperatures. This could be explained by hybrid sterility reported to occur in interspecific tomato hybrids and in segregating generations (Domínguez et al., submitted; Fernández-Muñoz et al., 1995b; Liu et al., 1995; Moyle & Graham, 2005; Mutschler & Liedl, 1994). The resulting lower means of pollen quality and quantity in the populations hindered the observation of their differences. It should also be mentioned that the response to selection was assessed on different backcross generations with ≈30 plants each. Given the small population sizes as well as the polygenic nature of sterility factors and traits related to pollen cold tolerance, it could be argued that any difference between backcross populations can be attributable to the appearance of favourable or unfavourable genotypes in one of the generations simply by chance.

Pollen selection was only effective for the percentage of pollen germination at 10 °C in the first selection

cycle where the selected populations showed a better response than the unselected NN_L. Moreover, in the double-selected population LL_L the bulk of the population shifted toward higher categories of the percent pollen germination and it was also the only population displaying plants in higher categories to those of the sensitive parent. Germination at 10 °C of a pollen grain depends on two factors: its viability and its ability to initiate tube growth at low temperature. Since there were no differences in means and distributions of % pollen viability among tested BC_1 populations, better performance of LL_L must be a result of selection for genes directly related to germination and pollen tube grow at low temperatures. Evidence of selection of pollen genes during germination and pollen tube growth at low temperatures were given by Zamir et al. (1982) and Zamir & Vallejos (1983). They obtained BC_1 populations from a F_1 *L. esculentum* × *L. hirsutum* hybrid with and without cold stress during the pollination phase and demonstrated significant skewing towards *L. hirsutum* alleles in the cold-pollination BC_1. However, they did not test their populations for the pollen cold tolerance, but only for several vegetative characters, and the results were unclear (Zamir & Gadish, 1987). In this study, LN_L showed little improvement over NN_L in spite of stress application to the germination and pollen tube growth phases. The fact that % pollen germination within NL_L population did not improve that of NN_L was somehow expected since cold stress was not applied to the phase more related to this character. These results suggest that the application of cold stress to only one of the phases of the pollen life cycle, regardless which one, is insufficient to improve pollen response at low temperatures via gametophytic selection.

Given that no significant differences were observed for pollen quality and quantity between the selected and unselected populations grown at low temperatures, the second cycle of pollen selection was ineffective. The loss of one half of the plants of the population NNN_L during the evaluation at low temperatures may indicate that pollen selection at low temperatures applied in our experiments had at least some effect on the vegetative tolerance to cold, consistent with the observation that pollen selection leads to responses at sporophytic level for many traits (Mulcahy et al., 1996). The ineffectiveness of the second cycle of pollen selection could be explained in several ways. One possibility would be that, since the populations are BC_2's, there is a greater portion of genome from the sensitive parent. This explanation is unsatisfactory because in the unselected population NNN, which also correspond to a BC_2, no significant decrease was observed in comparison with the NN population. Another explanation could be that the lower temperatures applied to the second cycle masked the possible additional response to selection. Pollen performance of BC_2 populations could be underestimated by excessive cold stress. Nevertheless this explanation seems to be unsatisfactory because parents and F_1 controls had similar responses in the two cycles of selection. On the other hand, many cold sensitive plants at the vegetative level that were lost in NNN_L population because of chilling most probably were also the most cold sensitive at the reproductive level. Thus, this unintentional bias toward higher vegetative cold tolerance in NNN_L could explain the absence of response in the second cycle of selection not by underestimation of pollen performance at low temperatures of selected populations but by its overestimation in the control population.

Most research on gametophytic selection attempted to correlate pollen selection with an improvement in sporophytic traits, with mixed results (Darakov, 1995; den Nijs et al., 1986; Rodríguez-Garay & Barrow, 1988; Sari-Gorla et al., 1992; Searcy & MacNair, 1993). Other authors relate it with gametophytic traits such as pollen functionality, also with ambiguous results (Frova et al., 1995; Quesada et al., 1996). This study shows that the first cycle of pollen selection was successful for a gametophytic trait such as pollen germination at low temperatures but the second cycle of pollen selection was ineffective for further improvement in any of the pollen cold tolerance traits evaluated. This is in agreement with other studies that showed no significant improvement beyond the first cycle (Kovács & Barnabás, 1992; Sari-Gorla et al., 1994), although in other cases a response to the second cycle was observed (Frascaroli et al., 1995; Landi et al., 1989).

In conclusion, pollen selection may be useful for increasing the number of plants able to germinate and grow pollen at low temperatures in segregating populations, at least for the first cycle of selection. Nevertheless, this conclusion is somehow weakened by the finding of an apparent inefficiency of the second cycle of selection and by the small size of backcross populations tested that was imposed by experimental constraints.

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