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ACKNOWLEDGEMENTS. We thank the CSIR, Govt. of India, New Delhi, for generous grant (No 5(136)/88-EMR-II) and associateship to DM. DKM wishes to thank Dr J. P. Mittal and Dr K. V. S Rama Rao, BARC, for constant encouragement.

Received 3 August 1994, revised accepted 21 June 1995

High-temperature tolerance of *Petunia* and *Nicotiana* pollen

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High-temperature tolerance of the pollen of *Petunia hybrida* L. and *Nicotiana sylvestris* L. was investigated by treating dry pollen to temperatures of up to 75°C for 6–48 h and by studying their viability (by fluorochromatic reaction (FCR) test), vigour (by a semi-vivo method), and their ability to set fruits and seeds. In *Petunia*, temperatures of up to 60°C for 48 h did not affect pollen viability, vigour and their fruit- and seed-setting ability. A temperature of 75°C for 24 h reduced pollen viability and vigour, but fruit- and seed-setting ability existed. However, a 75°C exposure for 48 h proved lethal for *Petunia* pollen. In *Nicotiana*, pollen exposed to temperatures of up to 75°C for 6–12 h were able to set seed. With a longer exposure the majority of pollen were FCR-positive, but they were unable to set seed. These results show that pollen grains of *Petunia* and *Nicotiana* can withstand exposures of temperatures as high as 75°C and retain pollen function. This study also indicates that FCR test may not reflect true viability in pollen subjected to extreme stresses.

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IN general, the activity of most metabolizing cells and tissues is best maintained within a moderate temperature range. Extreme low or high temperatures are detrimental to the activity or survival of most cells. Many plant cells respond to heat stress by the synthesis of heat shock proteins[1]. However, desiccated systems such as seeds can withstand a high degree of temperature range[2]. Pollen grains are also desiccated systems and show many physiological similarities to seeds[3]. The tolerance of pollen grains to low temperatures is well documented in studies on their storage under low and ultralow temperatures[4,5]. In contrast, much less is known of the high-temperature tolerance of pollen grains.

Earlier studies on high-temperature stress of pollen were restricted to examining the viability or *in vitro* germinability of treated pollen[6,7]. However, the effects of high temperatures on pollen function, i.e. pollen vigour and the ability of stressed pollen to induce fruit and seed set were not examined. Elsewhere, we have shown that *Brassica juncea* pollen exposed to 75°C for 24 h failed to germinate on the stigma and were unable to induce seed set[8].

In this paper we report on the viability, vigour and fruit- and seed-setting ability of *Petunia hybrida* and *Nicotiana sylvestris* pollen subjected to high temperatures of 45–75°C.

Materials and methods

Plants of *P. hybrida* L. grown under field conditions and of *N. sylvestris* L. grown under glasshouse conditions were used for investigations. Pollen grains from freshly dehisced anthers were uniformly spread on microslides, kept in dry Petri plates, and exposed to different temperatures: laboratory (22 ± 2°C), 45 ± 2°C, 60 ± 2C° and 75 ± 2°C, for various durations. After the treatment, pollen samples were tested for viability, vigour and their ability to set fruits and seeds.

Pollen viability was assessed by the fluorochromatic reaction (FCR) test as described by Heslop-Harrison and Heslop-Harrison[9]. Pollen grains were either used directly after the treatment or after prehydration of 30 min under high humidity by placing the samples in Petri plates lined with moist filter paper. Each treatment was repeated at least two times with two replicates each time. For each replicate at least 300 pollen grains were scored for the FCR test. Test of significance was performed by the group comparison test[10].

Pollen vigour was tested by the semi-vivo method[11,12]. Flower buds were emasculated before anthesis and pollinated on the day of anthesis with pollen exposed to different temperatures. Three hours after pollination, 1.0 cm of the style along with the stigma was cut with a sharp blade, and the cut end implanted in the pollen germination medium (sucrose 10% + boric acid 100 mg/l + calcium nitrate 300 mg/l + 0.8% agar) set in Petri plates. The cut ends of styles were observed under a stereomicroscope at regular intervals to record the time of pollen tube emergence and the number of tubes emerged. In one experiment, pollen tubes emerging from cut ends of styles were stained with DAPI (4',6-diamidino-2-phenylindole), a DNA fluorochrome[13], to examine the division of the generative cell.

In vivo pollen germination and pollen tube growth was checked in pistils after 3–48 h of pollination. Pistils were fixed in FAA (formalin:glacial acetic acid: 70% ethanol; 5:5:90 v/v) for 24 h, cleared in 4 N NaOH overnight, and mounted in a drop of 0.005% decolourized aniline blue (Gurr) (in 0.05 M Na_2HPO_4 at pH 8.2). Pollen tube growth was examined in a Zeiss/Nikon fluorescent microscope.

Moisture loss in pollen grains was determined in 100 mg pollen samples. Pollen grains were placed in preweighed aluminium cups, and the samples maintained at different temperature conditions. After 24 and 48 h, pollen grains were weighed and the loss in weight was recorded as the loss of moisture content.

Pollen grains from all treatments were used for pollination of emasculated and bagged flowers to determine their ability to set fruit and seed. The flowers were rebagged after pollination. The mature fruits were harvested and the fruit size (diameter), weight of seeds per fruit, and the weight of 100 seeds of each treatment were recorded.

Results

Petunia hybrida

Moisture loss

There was no detectable loss of water in pollen samples maintained under laboratory conditions for 24 or 48 h, in comparison to fresh pollen (control). However, pollen exposed to 45 or 60°C temperature for 24 or 48 h resulted in approximately 5% water loss. An exposure of 75°C for 24 or 48 h caused an 8% water loss in pollen, and there was no further loss in pollen exposed for 48 h.

Pollen viability

Pollen viability was expressed as percentage FCR response. Exposures of 60°C for 24 h (Figure 1 *a*) and 45°C for 48 h (Figure 1 *b*) reduced the FCR values in comparison to fresh pollen (control) or lab conditions. However, 30 min prehydration of pollen restored the FCR score to the control level. Temperatures of 75°C for 24 h (Figure 1 *a*) and 60°C for 48 h (Figure 1 *b*) reduced significantly the percentage FCR response, and prehydration at these temperatures was not effective in restoring the FCR score. Pollen grains exposed to 75°C

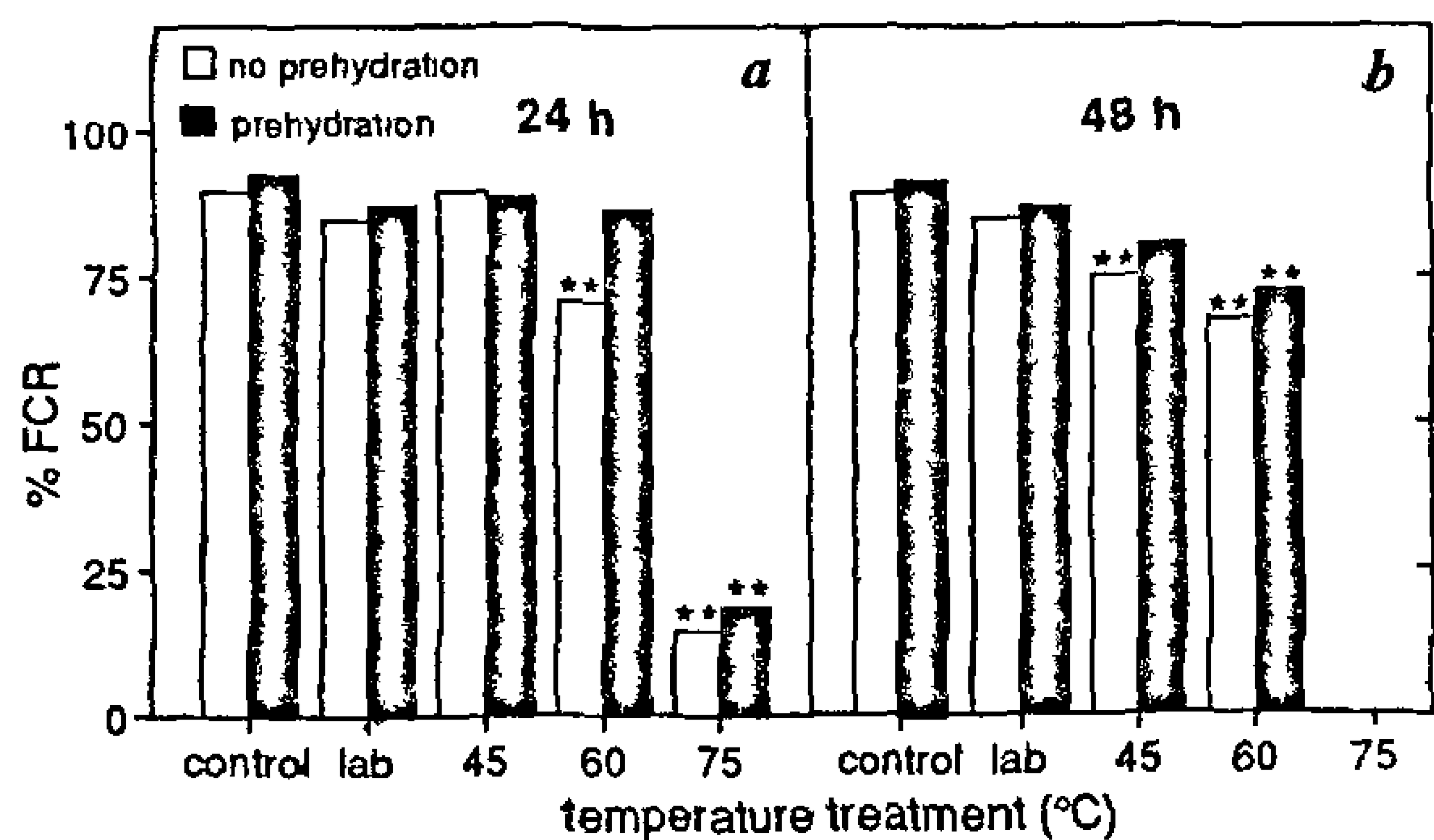

Figure 1 FCR response of *P. hybrida* pollen treated for 24 h (*a*) and 48 h (*b*) at different temperatures tested directly and after 30 min of prehydration The values indicated by (**) are significantly different from the control (fresh pollen) at $P < 0.01$ level

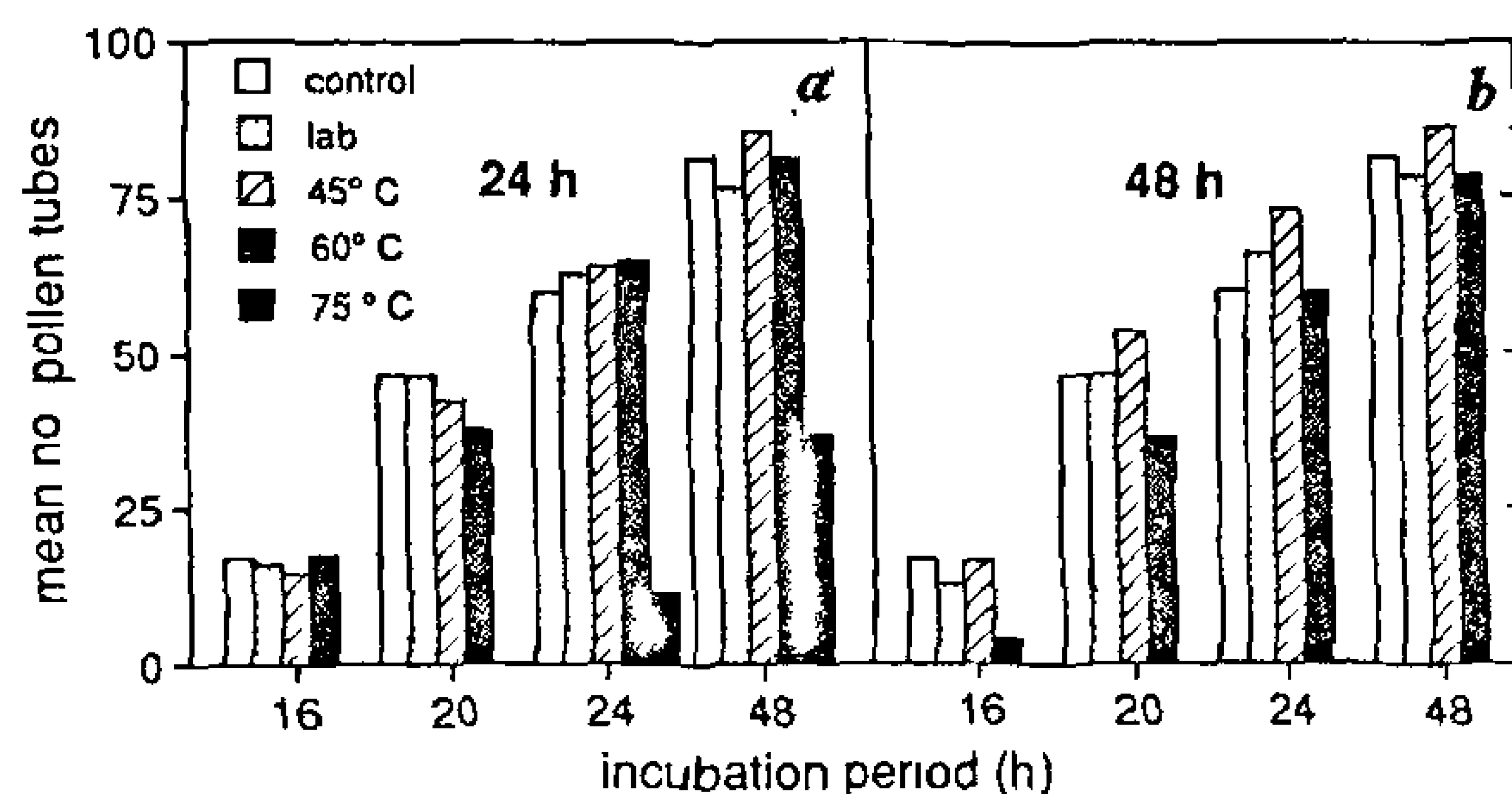

Figure 2. Mean number of pollen tubes of *P. hybrida* emerging from semi-vivo implanted pistils pollinated with pollen exposed to different temperatures for 24 h (*a*) and 48 h (*b*), and incubated for 16–48 h. Pollen tube number was counted up to 100; it was not possible to count accurately beyond 100.

for 48 h failed to show the typical FCR fluorescence (Figure 1 *b*); they mostly showed dull fluorescence, although strong background fluorescence was observed in the medium.

Pollen vigour

The vigour of pollen grains, i.e. the time taken by pollen tube to travel through the style, was analysed using the semi-vivo method. The mean number of pollen tubes that emerged from the cut end of pistil varied depending on the temperature and duration of the treatment. In pollen exposed to 75°C for 24 h there was a marked delay in pollen tube emergence; no pollen tubes emerged for 20 h, there was some emergence by 24 h, and by 48 h the number of tubes emerged was less than one-half of that in the other treatments (Figure 2 *a*). In pollen grains exposed to 60°C for 48 h, there was reduction in the number of tubes emerging during the first 20 h; however, by 24 h the number was comparable to the control (Figure 2 *b*). There was no pollen tube emergence in pollen grains exposed to 75°C for 48 h (Figure 2 *b*).

Pollen tubes that emerged from styles were stained with DAPI to check the presence of sperm cells. In the control as well as in the treatments where pollen tubes emerged, two sperm cells were observed.

Pollen tube growth studies performed *in vivo* were in agreement with those of the semi-vivo method. The pistils pollinated with control pollen and with pollen treated for up to 60°C for 24 h showed high germination in 3 h, and a large number of pollen tubes reached the ovary in 24 h. However, pistils pollinated with pollen exposed to 75°C for 24 h showed very few germinating pollen in 3 h, and by 48 h only a few pollen tubes had reached the ovary. Pollen tubes of 75°C-treated pollen had a thick wall and the tube growth was not uniform compared to other treatments.

Fruit- and seed-setting ability

The fruit- and seed-setting ability of high-temperature-treated pollen showed that pollen grains treated for up to 60°C for 24 or 48 h were as effective (90% fruit set) as fresh pollen. Also, the size (diameter) of fruits was not affected in these treatments (Figure 3 *a*). Pollen exposed to 75°C for 24 h were also effective in inducing fruit set although fruit-set was observed in only 70% of pollinated pistils, and the fruit size was also reduced (Figure 3 *a*). The weight of seeds produced in a fruit was, interestingly, more in 45°C (both 24 and 48 h treatments) and in 60°C for 24 h, in comparison to the control (Figure 3 *b*), but it was reduced in the 75°C treatment (Figure 3 *b*). There was no difference in the weight of 100 seeds produced in most treatments. However, in 75°C (24 h) treatment, there was an increase in the weight of 100 seeds (Figure 3 *c*).

In one experiment 45 and 60°C treatments were extended to 5 days. There was no apparent effect on the fruit- and seed-setting ability of 45°C-treated pollen. However, the 60°C treatment reduced both the fruit size and the seed weight per fruit.

Nicotiana sylvestris

Preliminary experiments with *N. sylvestris* pollen showed that high temperatures of up to 60°C for 48 h had no adverse effect on pollen function. Therefore, studies on *Nicotiana* were largely confined to 75°C treatments for 6–48 h.

The pollen viability tests showed that over 50% of pollen exposed to 75°C for 24 h, were FCR-positive, in comparison to approximately 20% for *Petunia*. Exposures longer than 24 h were, however, lethal to *Nicotiana* pollen.

Pollen vigour tests by the semi-vivo method showed that in control more than 100 pollen tubes emerged in 20 h. In contrast, pollen exposed to 75°C for 12 h took

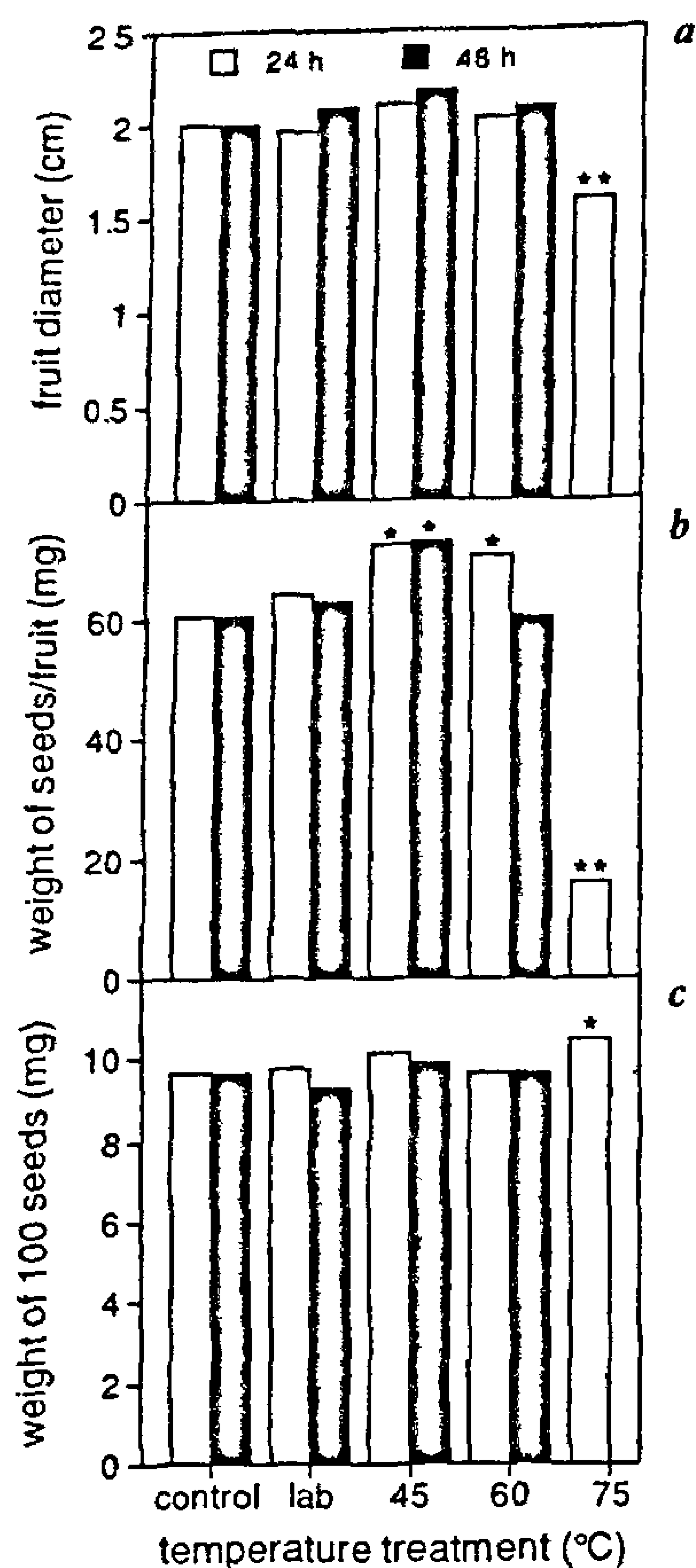

Figure 3. Fruit and seed characteristics obtained after pollination with pollen samples of *P. hybrida* exposed to different temperatures for 24 and 48 h: (*a*) fruit diameter; (*b*) average weight of seeds per fruit; and (*c*) average weight of 100 seeds (*) and (**) are significant at $P < 0.05$ and $P < 0.01$ probability level, respectively, as compared to control (fresh pollen).

48–50 h for tube emergence and in those treated for 24 h there was no tube emergence even by 50 h. Fluorescence studies of pistils 50 h after pollination with pollen treated to 75°C for 24 h revealed germination of only 50–100 pollen grains on the stigma. Of these, only about 20–30 tubes entered the style and 5–10 tubes approached the cut ends of the style.

Fruit and seed sets were obtained with pollen treated at 75°C for 6 h and were comparable to the control (fresh pollen). However, pollen treated for 12 h yielded fruits in only 60% of pollinated flowers and the size of fruits and the number of seeds per fruit were markedly reduced (data not given). Pollen samples exposed to 75°C for 24 and 48 h failed to set fruit and seed.

Discussion

The results presented here have shown that pollen grains of *Petunia hybrida* and *Nicotiana sylvestris* are able to withstand a high temperature stress of up to 75°C, albeit for short periods, and retain not only the viability but also the ability to set fruits and seeds. However, the 75°C treatment reduces pollen vigour; treated pollen take much longer time to germinate and for the pollen tubes to reach the ovary. The reduction in the size of fruits and weight of seeds/fruit by 75°C (24 h) treatment in *Petunia* may be attributed to the fewer number of pollen tubes reaching the ovary before the ovules lose receptivity. However, there was a small increase in the weight of 100 seeds for this treatment. This may be explained on the basis of reduced competition among the fertilized ovules for nutrients.

FCR test is generally considered as the best available method to assess pollen viability (ability to effect fertilization)[14,15]. Our earlier studies[11] on pollen subjected to moderate stresses highlighted the limitation of the FCR test in assessing pollen vigour although it did reflect viability. Our studies on heat-treated pollen of *Brassica*[8] indicated that the extent of retention of fluorescence may give a better indication of pollen function than initial fluorescence. Present studies clearly show that the FCR test does not necessarily reflect true viability or vigour in pollen subjected to extreme stresses. Over 50% of *Nicotiana* pollen treated at 75°C for 24 h, were FCR-positive, but failed to effect fertilization. The pistils pollinated with such pollen showed germination of only 50–100 pollen on the stigma and growth of only 5–10 tubes in the style even after 50 h of pollination. Thus, the result of the FCR test on stressed pollen needs to be used with caution.

Previous studies on high-temperature tolerance of pollen were limited to a few taxa and were confined largely to *in vitro* germination tests. Pollen of lily, apple and rose showed[6] 50% germinability when exposed to 70°C for 4–8 h. A small proportion of *Eucalyptus rhodantha* pollen retained[7] *in vitro* germinability after exposure to 70°C for 24 h.

Our earlier studies on *B. juncea*[8] showed that exposure of pollen to 60°C for up to 24 h did not affect pollen viability and their ability to set fruits and seeds. However, pollen exposure to 75°C even for 4 h failed to germinate either *in vitro* or *in vivo*. The pollen of lily, apple and rose[6], *Eucalyptus*[7], and *Petunia* and *Nicotiana* (present studies) are all two-celled pollen systems, and seem to be more tolerant to high-temperature stress than the pollen of *B. juncea*[8], a three-celled system. Although high-temperature tolerance of no other three-celled pollen system, to our knowledge, has been investigated, this difference is in line with a range of other physiological differences between two- and three-celled pollen systems[16–19]

Heat tolerance of *E. rhodantha* pollen has been suggested to be an adaptation to the harsh semi-arid conditions prevailing in the natural habitat of the species (Western Australia), in which pollen grains are likely to remain exposed to prolonged heat and desiccation before pollination[7]. The authors suggested that similar heat tolerance is likely to be present in other species adapted for the same type of harsh environment as of *E. rhodantha*. The present investigation on *Petunia* and *Nicotiana*, and the earlier studies on lily, apple and rose[6], the species which grow under moderate or temperate conditions, have shown that high-temperature tolerance in all these species is comparable to that of *E rhodantha*. Thus, pollen grains in general and two-celled pollen in particular shown high-temperature tolerance, which is likely to be related to low moisture level present in most of the pollen systems. Small differences in the degree of tolerance between species may reflect the differences in the ability of the pollen protoplasts and membranes to withstand extreme desiccation associated with high temperatures.

Studies on pollen grains of some of the cereals such as wheat and maize also show that a low moisture level is an essential requirement for high-temperature tolerance. Pollen of cereals, unlike the pollen of a majority of taxa, are shed under more hydrated conditions[20]; such pollen lose their ability to effect fertilization even under moderately high temperature stress of 40°C for 4 h (ref. 21).

Extensive studies have been carried out on the tolerance of actively metabolizing organisms at supraoptimal temperatures[2,22]. Evidence indicates that the accumulation of metabolites such as proline and betain may provide thermal protection to several enzymes[23]. It is suggested that proline might stabilize protein configuration by maintaining hydration shells around the molecules. Pollen grains in general are rich in proline[4]. In *Petunia* pollen, free proline accounts for 2.6% of dry weight[24]. Zhang and Croes[25] reported that proline protected germination and metabolic function of *Lilium* pollen from unfavourably high and low temperatures. Further studies are, however, needed to understand the biochemical basis of thermotolerance in pollen grains. It has been shown that some heat shock proteins are expressed in developing seeds[26] and pollen grains[27]. These may also play a role in heat tolerance of desiccated pollen grains.

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ACKNOWLEDGEMENTS. We thank Mr Dennis Dyck for assistance in the preparation of figures. G U R. was supported by a Senior Research Fellowship from the Council of Scientific and Industrial Research, New Delhi. This study was supported in part by a Natural Sciences and Engineering Research Council of Canada Operating Grant to V.K S.

Received 14 February 1995, revised accepted 25 May 1995