Protoplasma 109, 249–255 (1981)

PROTOPLASMA

Environmental Control and Evidence for Predetermination of Pollen Embryogenesis in *Nicotiana tabacum* Pollen

E. Heberle-Bors * and J. Reinert

Institut für Pflanzenphysiologie und Zellbiologie, Freie Universität Berlin

Received May 21, 1981
Accepted June 26, 1981

Summary

The effect of daylenght and temperature for the donor plants (*Nicotiana tabacum* var. Badischer Burley) on the formation of pollen competent for embryogenesis (P-pollen) by the three possible routes (during normal flower development *in situ* (pollen dimorphism), during cold-treatment of excised flower buds, in cultured anthers) was studied. In all three routes, P-pollen frequency (premitotic pollen, before 1. sporophytic division, PPF) was affected in essentially the same way. At 24 °C and long days, PPF was low and short days had only a slightly increasing effect. At 18 °C and long days, PPF was higher and short days further increased it. Correlated with PPF under the different growth regimes was the percentage of units with more than one vegetative-type nucleus (normal embryos + abortive embryos + multinucleate pollen) in 3 weeks old anther cultures. Under greenhouse conditions, PPF was generally higher than at 24° in growth rooms and showed a maximum in the winter months. Plant age did not affect PPF. These results give further evidence that pollen embryogenesis is predetermined before excision and culture of the pollen or anthers.

Keywords: *Nicotiana tabacum;* Pollen embryogenesis; Predetermination.

1. Introduction

In tobacco, the pollen competent for embryogenesis, called E grains (Sunderland and Wicks 1971), S grains (Horner and Street 1978) and P-pollen (Heberle-Bors and Reinert 1979) are formed in cultured anthers (Sunderland and Wicks 1971), in nearly mature flowers *in situ* (Horner and Street 1978) and in cold-treated excised flower buds (Sunderland 1977).

Final proof that P-pollen found *in situ* and *in vitro* are identical structures were results showing that P-pollen formed *in situ* can be cultured and are able to produce embryos after separation from the normal pollen (Heberle-Bors and Reinert 1980). In these experiments it turned out that P-pollen

* Correspondence and Reprints: Institut für Pflanzenphysiologie und Zellbiologie, Königin-Luise-Strasse 12–16 a, D-1000 Berlin 33.

0033-183X/81/0109/0249/$ 01.40

were formed in low frequency (1%) when the pollen were taken from long-day plants at 24 °C, but rather high (10%) when the pollen were taken from short-day plants at 18 °C. Practically identical P-pollen frequencies could be found in isolated pollen cultures with preculture of anthers leading to corresponding differences in plantlet yield (HEBERLE-BORS and REINERT 1979). These results clearly indicated environmental control of pollen embryogenesis prior to culture. Predetermination of pollen embryogenesis has been suggested by DALE (1975) for barley and HORNER and STREET (1978) for tobacco, however, P-pollen formation in cold-treated flower buds was not considered and the inductive factors were not identified. Experiments on the control of pollen embryogenesis are important since its understanding in the model plant tobacco might lead to a more rational search for plant formation from pollen in recalcitrant and low-yielding species such as many cereals.

2. Material and Methods

Tobacco plants (*Nicotiana tabacum*, var. Badischer Burley) were started in growth rooms under long days (16 hours) at 24 °C with additional nutrient supply (HEBERLE-BORS and REINERT 1979). After 40 days, young plants (minimum four per treatment) were transferred to other growth rooms with different daylengths and temperatures including a greenhouse. Light intensity in the growth rooms ranged from 14–20 kLux.

For determination of P-pollen frequency *in situ*, flowers formed during a period of one week (minimum 30 flowers from each of five sets of plants) were collected from plants in full bloom at a corolla length of 4–4.5 cm. The anthers were fixed, the pollen isolated and stained in acetocarmine (HEBERLE-BORS and REINERT 1979). For cold-treatment, flower buds with pollen at the early binucleate stage were collected and kept at a temperature of 9–10 °C for 10 days (RASHID and REINERT 1980). Five experiments were conducted each with 15–20 buds per treatment. Anthers were cultured without cold-treatment also at the early binucleate stage on a liquid NITSCH's medium (1969) without vitamins for 7 and 21 days at 24 °C. The cultured anthers were removed and the medium centrifuged at low speed to collect pollen from dehisced anthers. Five experiments were conducted, each with 80–100 anthers per treatment.

In all experiments, the number of P-pollen (premitotic pollen, before 1. sporophytic division, no or only little cytoplasmic staining, generative and vegetative nucleus clearly visible), and of dead pollen (no cytoplasmic staining, no or only collapsed nuclei) was expressed as percentage of the total population. From two drops of stained pollen suspension, 500 pollen from the upper, middle, and lower part of the cover slip were counted. After 21 days of anther culture, all units with more than one vegetative-type nucleus (normal embryos + abortive embryos + multinucleate pollen = induction rate, HEBERLE-BORS) were counted. The data are mean values of the parallel experiments with the threefold standard error. In order to obtain further statistical confirmation for the differences, the mean values were compared by t-test.

3. Results

P-pollen can be formed *in situ* in nearly mature flowers (pollen dimorphism, PD), in cold-treated, young flower buds and in *in vitro* cultured anthers. The effect of the four possible combinations of long (16 hours) days and short

(8 hours) days at 24 and 18 °C for the donor plants (LD 24-, SD 24-, LD 18-, and SD 18-plants) on these three developmental routes of P-pollen formation is shown in Fig. 1. In addition, the percentage of dead pollen is also recorded.

In all three routes, P-pollen frequency (PPF) was very low when flowers were taken from long-day plants grown at 24 °C (0.34% *in situ* to 1.6% *in vitro*). Short days at this temperature had only a slightly increasing effect

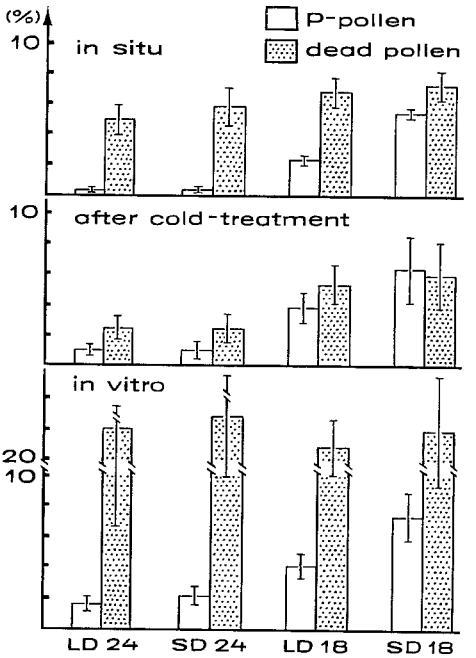

Fig. 1. P-pollen frequency (PPF) and dead pollen frequency (DPF) in nearly mature flowers *in situ*, in cold-treated flower buds and in cultured anthers from LD 24-, SD 24-, LD 18-, and SD 18-plants. The differences in PPF and DPF *in situ* are significant on the P = 0.01 level except the difference in PPF of LD 24- and SD 24-plants. The differences after cold-treatment are significant on the P = 0.05 level except the difference in PPF and DPF of LD 24- and SD 24-plants. The differences in PPF in culture are significant on the P = 0.05 level. The differences in DPF in culture are not significant

(0.4% *in situ* to 2.2% *in vitro*). Lowering the temperature for the donor plants to 18 °C uniformly increased PPF. In flowers from LD 18-plants 2.4% (*in situ*) to 4.2% (*in vitro*) could be obtained, short days lead to a further increase of 5.4% (*in situ*) to 7.4% (*in vitro*). Instead of PPF to measure induction, induction rate was used, *i.e.*, the percentage of units in 21 days old anther cultures with more than one vegetative-type nucleus (Heberle-Bors 1980). It turned out that induction rate was predetermined by daylength and temperature for the donor plants in the same way as PPF,

i.e., low induction at LD 24-conditions (2.2%), slight but significant increase by short days (2.6%), stronger increase by lowering the temperature (3.6%) and further increase by short days at 18 °C (5.4%, Fig. 2).
Parallel to plants in growth rooms, PD was determined in greenhouse plants over a period of 15 months. During the winter months the greenhouse plants were supplied with supplementary illumination for 16 hours daily. Tem-

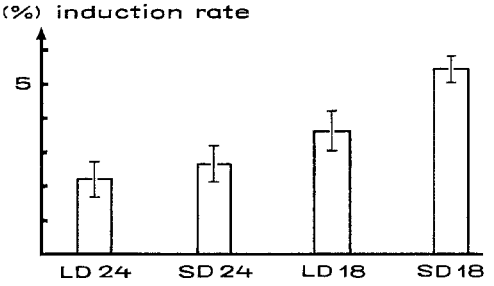

Fig. 2. Induction rate (normal embryos + abortive embryos + multinucleate pollen) in 3 weeks old anther cultures from LD 24-, SD 24-, LD 18-, and SD 18-plants. The differences are significant on the P = 0.01 level

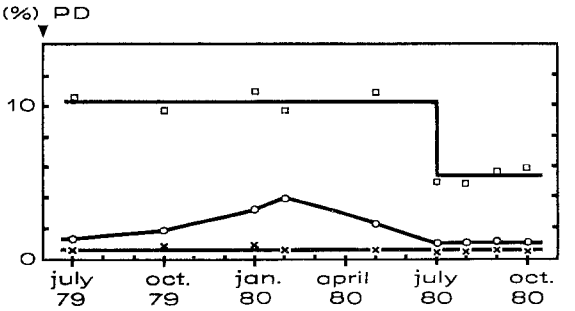

Fig. 3. Pollendimorphism (PD) in LD 24- (×-×-×), SD 18- (□-□-□) and greenhouse (o-o-o) plants during a period of 15 months

perature was set to 24 °C, but it varied considerably depending upon the out-door conditions. In growth room plants, under both LD 24- and SD 18-conditions, PD remained constant throughout the year. In July 1980, however, when temperature regulation was readjusted, it turned out that the time before the temperature in the SD 18-room was some degrees too low and consequently PD fell to a level of 5–6% after readjustment. This decline of PD corrects previously published PD values of 10% (HEBERLE-BORS and REINERT 1980) which resulted from experiments performed in 1979. In greenhouse plants, PD was significantly higher throughout the year than in LD 24-plants from growth rooms and there was a clear maximum of PD in the winter months (Fig. 3).

An endogenous factor on plant development which might affect pollen plant induction is plant age. In two sets of SD 18-plants PD was determined from the start of flowering for eight weeks by collecting the flowers on two successive days in irregular intervalls. In both sets, PD was on the same level and remained constant for the whole eight weeks (Table 1).

Table 1. *Effect of Plant Age on Pollen Dimorphism in Two Sets of SD 18-Plants (A + B). Flowers Were Collected on Two Successive Days from July 25, 1980 (Opening of First Flower) to October 9, 1980*

	July 25 + 26 1980	August 17 + 18 1980	September 3 + 4 1980	September 18 + 19 1980	Oktober 8 + 9 1980
A	5.7%	5.1%	5.2%	5.6%	—
B	—	5.2%	5.9%	5.7%	5.4%

4. Discussion

Previous investigations have shown that in tobacco P-pollen formation is increased *in vitro* as well as *in situ* by growing the donor plants under SD 18-conditions instead of LD 24-conditions (HEBERLE-BORS and REINERT 1979, 1980). This identity of reaction to daylength and temperature could be confirmed in the present investigation and extended to other daylength/temperature combinations. Furthermore, it could be shown that also in excised cold-treated flower buds P-pollen formation was depending in the same way upon daylength and temperature given to the donor plants prior to excision.
Closely related to PPF by these three routes of P-pollen formation were the actual frequencies of pollen regenerants. *In vitro,* the effect of daylength and temperature on PPF after 7 days of anther culture correlated with the induction rate after 21 days of anther culture and, as has been reported earlier, to plantlet yield in isolated pollen cultures from LD 24- and SD 18-plants (HEBERLE-BORS and REINERT 1979). In cold-treated flower buds, PPF from LD 24- and SD 18-plants correlated with plantlet yield in *ab initio* pollen cultures from such flowers (RASHID and REINERT 1981). *Ab initio* pollen cultures from nearly mature flowers *in situ* (HEBERLE-BORS and REINERT 1980) gave rise to plantlets and young embryos only from SD 18-plants but not from LD 24-plants. The failure with LD 24-plants is seen to be caused by the overall low number of pollen regenerants. This strong correlation between PPF and pollen regenerant frequency and the identity of PPF under three rather different routes can only be explained when it is recognized that pollen embryogenesis is determined prior to excision of the flowers from the plant, *i.e.*, before the early binucleate stage. As a consequence of these results, it cannot be maintained that the switch in development takes place during culture (BHOJWANI *et al.* 1973), or during cold-treatment (SUNDERLAND 1977,

1980), or that the act of severing the contact between anther and donor plant provides the trigger for induction (DUNWELL 1981).

The experiments revealed that both temperature and daylength were involved in pollen plant induction. This is in addition to results of HEBERLE-BORS and REINERT (1979) and RASHID and REINERT (1981) who found in pollen cultures from precultured anthers, respectively cold-treated flower buds of SD 18-plants, an increase in embryo yields as compared to LD 24-plants. The beneficial effect of low temperature for the plants and for the excised flower buds have to be clearly distinguished. Raising the plants under low temperature (about 15–18 °C) affects the frequency of embryogenic pollen whereas low temperature (5–10 °C) after excision of the flower buds merely preserves viability of all pollen leading to better survival of the already induced pollen (DUNCAN and HEBERLE 1976).

In connection with the differences in the reaction of the three routes of P-pollen formation to the growth conditions of the donor plants, the first observation to be considered was the overall low PPF *in situ* as compared to *in vitro* and cold-treatment. This suggests that during *in vivo* flower development many of the P-pollen die. It is further indicated by the positive correlation between the number of P-pollen and dead pollen under the different growth regimes.

The fact that PPF in cold-treated flower buds and in cultured anthers was very variable, may result from the difficulty to distinguish P-pollen from other pollen types. Cytoplasmic degradation which according to BHOJWANI *et al.* (1973) preceeds P-pollen formation, seems to take place in nearly all pollen, since the number of degrading pollen (the lightly staining ones) is much greater than that of the P-pollen and is not depending upon the growth conditions of the donor plants (HEBERLE-BORS and REINERT 1979). If, by suboptimal treatment, some of the P-pollen have not completed degradation, they cannot be distinguished from degrading (dying) normal pollen. Such suboptimal treatments are the stage effect (SUNDERLAND and ROBERTS 1979) and perhaps the treatment time of 10 and 7 days, respectively, might also be suboptimal. An additional factor is the formation of brown anthers, which contain dead pollen in large numbers (as judged by examination of single anthers), among them presumably P-pollen. In cold-treated flower buds, brown anthers were not very frequent and consequently the number of dead pollen was very low and in fact the lowest of all three pathways. This confirms earlier results (DUNCAN and HEBERLE 1976) that cold-treatment exerts its effect by preserving the viability of the pollen.

The higher and with season varying PD in greenhouse plants finds a plausible explanation when it is compared with the daylength/temperature studies in the climate rooms. Fluctuations in daylength and temperature as they occur in greenhouses oscillate in a year between long day/high temperature and short day/low temperature and also between high and low temperature during

a day so that LD 24- and SD 18-conditions can be regarded as extremes of the greenhouse conditions resulting in the observed changes in PD.

Plant age is a major factor controlling pollen plant production in tobacco anther cultures (ANAGNOSTAKIS 1974). Only the first flowers formed give high yields whereas after about two weeks of flowering the plantlet yield decreases rapidly. This was also true in our anther cultures, but not so for isolated pollen cultures with anther preculture where pollen plant production remained remarkably unaffected (HEBERLE-BORS and REINERT, unpublished). The stability of PD found in the present study fits to this observation and it appears that the age effect is related to post-induction development.

References

ANAGNOSTAKIS, S. L., 1974: Haploid plants from anthers of tobacco-enhancement with charcoal. Planta **115**, 281—283.

BHOJWANI, S. S., DUNWELL, J. M., SUNDERLAND, N., 1973: Nucleic-acid and protein contents of embryogenic tobacco pollen. J. exp. Bot. **24**, 863—871.

DALE, P. J., 1975: Pollen dimorphism and anther culture in barley. Planta **127**, 213—220.

DUNCAN, E. J., HEBERLE, E., 1976: Effect of temperature shock on nuclear phenomena in microspores of *Nicotiana tabacum* and consequently on plantlet production. Protoplasma **90**, 173—177.

DUNWELL, J. M., 1981: Stimulation of pollen embryo induction in tobacco by pretreatment of excised anthers in a water-saturated atmosphere. Pl. Sci. Letters **21**, 9—13.

HEBERLE-BORS, E., 1980: Interaction of activated charcoal and iron chelates in anther cultures of *Nicotiana* and *Atropa belladonna*. Z. Pflanzenphysiol. **99**, 339—347.

HEBERLE-BORS, E., REINERT, J., 1979: Androgenesis in isolated pollen cultures of *Nicotiana tabacum:* dependence upon pollen development. Protoplasma **99**, 237—245.

— 1980: Isolated pollen cultures and pollen dimorphism. Naturwiss. **67**, 311.

HORNER, M., STREET, H. E., 1978: Pollen dimorphism. Origin and significance in pollen plant formation by anther culture. Ann. Bot. **42**, 763—777.

NITSCH, J. P., 1969: Experimental androgenesis in *Nicotiana*. Phytomorphol. **19**, 389—404.

RASHID, A., REINERT, J., 1980: Selection of embryogenic pollen from cold-treated buds of *Nicotiana tabacum* var. Badischer Burley and their development into embryos in cultures. Protoplasma **105**, 161—167.

— — 1981: Differentiation of embryogenic pollen in cold-treated buds of *Nicotiana tabacum* var. Badischer Burley and nutritional requirements of the isolated pollen to form embryos. Protoplasma **106**, 137—144.

SUNDERLAND, N., 1977: Comparative studies of anther and pollen culture. In: Plant cell and tissue culture: Principles and applications (SHARP, W. R., LARSEN, P. O., PADDOCK, E. F., RAGHAVAN, V., eds.), pp. 203—219. Columbus, Ohio: Ohio State University Press.

— 1980: Anther and pollen culture 1974—1979. In: The plant genome (DAVIES, D. R., HOPWOOD, D. A., eds.), pp. 171—183. Norwich, England: The John Innes Charity.

— WICKS, F. M., 1971: Embryoid formation in pollen grains of *Nicotiana tabacum*. J. exp. Bot. **22**, 213—226.

— ROBERTS, M., 1979: Cold-pretreatment of excised flower buds in float cultures of tobacco anthers. Ann. Bot. **43**, 405—414.