Protoplasma 99, 11–17 (1979)

PROTOPLASMA

High Frequency Production of Embryos in *Datura innoxia* From Isolated Pollen Grains by Combined Cold Treatment and Serial Culture of Anthers in Liquid Medium

AKHILESH K. TYAGI *, A. RASHID, and S. C. MAHESHWARI

Department of Botany, University of Delhi

Received November 1, 1978
Accepted in revised form December 6, 1978

Summary

This study concerns the development of pollen embryos as affected by various physical conditions of culture in media devoid of hormones. Freshly isolated pollen, from anthers of *Datura*, failed to form embryos regardless of whether they were cultured on liquid or solid medium. In contrast, pollen isolated from anthers precultured on solid medium did form embryos and the response could be increased by prior cold treatment of anthers at 4 °C for 4 days. However, the best results were obtained when anthers were cultured from the very beginning in liquid medium and transferred serially to fresh medium. Under such conditions, the anthers dehisced, allowing spontaneous shedding of pollen grains. It was thus possible to have several fractions of shed pollen continuing their development into embryos. When serial culture was started with anthers from cold-treated buds not only were embryos formed in all the fractions of shed pollen but the frequency was also considerably higher than in any mode of culturing.

Keywords: Cold treatment; *Datura innoxia*; Haploids; Pollen embryos.

1. Introduction

After the discovery of the anther culture technique for production of haploid plants in this laboratory (GUHA and MAHESHWARI 1964, 1966), many workers have been trying to refine it. In particular, much effort has been directed in the past two or three years to obtain embryoids in isolated pollen cultures on account of the several potential advantages this technique offers. For example, in anther culture it is neither possible to selectively eliminate regeneration of embryoids from anther wall, nor can one easily quantitate production of embryoids. On the other hand, culture of isolated pollen grains may eliminate the problem of "mix-up" with embryos of other origin and offer all advantages of microbial cultures.

* Correspondence and Reprints: Department of Botany, University of Delhi, Delhi 110007, India.

0033-183X/79/0099/0011/$ 01.40

Induction of haploids in isolated pollen was first achieved by NITSCH and NORREEL (1973). In this procedure (we refer it as Technique 1) pollen squeezed out of the anther by physical disruption of the anther wall are cultured. Unfortunately, however, in the hands of most workers yields have been low despite subjecting excised anthers to cold treatment which is known to be beneficial for embryoid development (NITSCH and NORREEL 1973, NITSCH 1974 a, and SANGWAN-NORREEL 1977). NITSCH (1974 b), REINERT, BAJAJ, and HEBERLE (1975) and WERNICKE and KOHLENBACH (1977) found in *Nicotiana tabacum* that results are much better if pollen grains are isolated from anthers after preculturing them (Technique 2) for four or five days. On the other hand, SUNDERLAND and ROBERTS (1977) tried another technique (Technique 3) in which anthers taken from cold-treated buds were cultured in liquid medium and where they dehisced spontaneously. By transferring the anthers to fresh medium, at intervals, they could obtain fractions of shed pollen, which continued their development into embryos.

In this investigation, we have cultured pollen grains of *Datura innoxia* according to all the three procedures mentioned above, *with* and *without* cold treatment. For a comparison, whole anthers were also cultured on both solid and liquid media. We show here that pollen cultures raised with anthers from cold-treated buds according to the original procedure of NITSCH (1974 a), showed only a marginal increase in the number of haploid embryos over controls. However, when anthers from cold-treated buds were used according to procedure 3 (*i.e.*, serial culture), the best results were obtained.

2. Material and Methods

The detailed procedures adopted for each technique are given below. For the major part of work pollen grains at premitotic stage were used, and in every experiment cultures were started from two sets of buds. One did not receive cold treatment while the other set was kept at 4 °C for 4 days, found by us to be optimal. For cold treatment the buds were placed in a beaker with their lower ends dipped in water. The beaker was covered with polythene bag and wrapped in aluminium foil.

2.1. Technique 1

Pollen grains were isolated from anthers by pressing them with the piston of a 10 ml glass syringe, in liquid medium. The suspension was filtered through a 45 micron sieve to remove coarse debris and the filtrate containing pollen grains (and some fine debris) was centrifuged at 800 rpm for 5 minutes. Centrifugation was repeated twice after suspending the pellet in fresh medium, to wash the pollen grains, which were finally cultured at a density of 10^4 pollen grains per ml of medium as described by NITSCH (1974 a).

2.2. Technique 2

Pollen grains were isolated from anthers after 5 days of preculture on NITSCH's (1969) medium solidified with agar. This was achieved by splitting open the anthers followed by a gentle pressing. The pollen-suspension was cultured at a density of 10^3 grains per ml.

2.3. Technique 3

Anthers were inoculated into 250 ml flasks, containing 10 ml of NITSCH's (1969) liquid medium. Anthers from these flasks were transferred to fresh medium after 5, 10, and 15 days of inoculation. Thus it was possible to have 3 fractions of suspended pollen grains shed during 0–5, 6–10, 11–15 days after culture and an additional fourth fraction comprising the residual pollen shed thereafter 15 days. Pollen counts were made by a haemacytometer, taking an average of ten readings per replicate. The embryos formed in each fraction were counted after 40 days, when the experiment was terminated.

To enable a comparison of the efficiency of this new procedure with the original continuous culture technique, anthers at both premitotic and postmitotic stages of pollen, were inoculated also on liquid as well as agar solidified media, according to the technique of SOPORY and MAHESHWARI (1976).

All cultures were initially incubated in darkness at 24 ± 1 °C for 20 days and then transferred to diffuse light at 26 ± 1 °C. For each observation, at least six replicates were considered. Cultures were transferred to petri dishes for photography. All data in this paper refer to embryos visible to the unaided eyes. Finally, we should make it clear that the entire work, reported here, was done employing basal medium. Addition of hormones would have raised the response to such a level that the distinctive effects of the various cultural procedures would have been obliterated.

3. Results

We first describe the results obtained with the continuous culture of anthers on solid and liquid media without cold treatment. In liquid medium, the percentage of anthers responding and number of embryos produced per anther was distinctly higher than in the solid medium (Tab. 1). In anthers from cold-treated buds, response was further enhanced in solid and liquid media.

Table 1. *Comparison of the Efficiency of Different Culture Techniques With and Without Cold Treatment for Formation of Pollen Embryoids in Datura innoxia.* (For each observation at least six replicates, with 90 anthers, were considered)

Method of culture	Average number of embryoids per anther		Per cent responding anthers		Per cent responding pollen	
	Control	Cold-treated	Control	Cold-treated	Control	Cold-treated
1. Continuous culture						
a) Solid medium	0.21 ± 0.007 [1]	0.97 ± 0.049	12.0 ± 0.70	40.0 ± 3.6	—	—
b) Liquid medium	0.33 ± 0.034	3.92 ± 0.13	17.6 ± 1.28	44.4 ± 2.22	—	—
2. Pollen grains isolated from fresh anthers	0.00	0.07 ± 0.06	—	—	0.00	0.01 ± 0.0015
3. Pollen grains isolated from anthers precultured for five days	3.90 ± 0.16	5.52 ± 0.15	—	—	0.38 ± 0.016	0.55 ± 0.015
4. Serial culture	2.35 ± 0.12	23.85 ± 1.45	30.8 ± 1.35	66.0 ± 3.96	(data not available)	

[1] Standard error.

As far as isolated pollen grains are concerned (Technique 1), our attempts to culture them from freshly excised anthers met with total failure. Even after cold treatment, in several experiments only an occasional culture formed a few embryos. By contrast pollen isolated from 5-day-precultured anthers (Technique 2) on an agar nutrient medium, formed embryos regularly. The response increased further in pollen isolated from cold-treated buds.

Finally, anthers containing pollen grains were inoculated also on liquid medium (Technique 3), and transferred to new medium at intervals of 5 days. In this technique the pollen grains are liberated spontaneously. Pollen grains from buds not subjected to chilling did not form embryos in the 0–5 day fraction (Fig. 1 *a*) but did so in later fractions (Figs. 1 *b–d*). It may be noted, that this simple expedient of transferring anthers to new medium, two or three times, increased the frequency of anther response as well as number of embryos per anther. With combined cold treatment and serial culture the results were even more striking (Figs. 1 *f–i*). In these cultures embryos were formed also in the 0–5 day fraction (Fig. 1 *f*); cold treatment enhanced also their number per culture (Tab. 1).

The results described above pertain to culture of anthers containing pollen grains at the premitotic stage. This stage proved distinctly superior to the postmitotic stage at which embryos were formed only occasionally; the average number being only four embryos per one hundred anthers as against about 2000 embryos when anthers were cultured at premitotic stage of pollen after cold treatment.

4. Discussion

The present work gives information about four factors influencing induction of pollen embryos: 1. Cold treatment, 2. Stage of pollen at the time of inoculation, 3. Suitability of different culture techniques and 4. Probable role of anther wall.

To divert the pollen towards its new developmental pathway of forming an embryo cold treatment does seem a very important factor as originally discovered by NITSCH and NORREEL (1973). The stage of pollen at the time of inoculation is also important. A higher frequency of haploid formation was observed in *Datura* both in pollen as well as anther culture, when the starting

---

Fig. 1 *A–J*. Formation of pollen embryoids in serial and continuous cultures. Anthers at premitotic stage were inoculated in 10 ml NITSCH's (1969) basal medium and transferred serially to new medium at intervals. The cultures were incubated in dark for 20 days and then transferred to diffuse light. *A–D* Embryoids developing from 0–5, 6–10, 11–15-day shed fractions of pollen and residual pollen from untreated anthers. *E* Embryoids developing in control continuous culture of untreated anthers. *F–I* Embryoids developing from 0–5, 6–10, 11–15-day shed fractions of pollen and residual pollen from cold-treated anthers. *J* Embryoids developing in continuous culture of cold-treated anthers

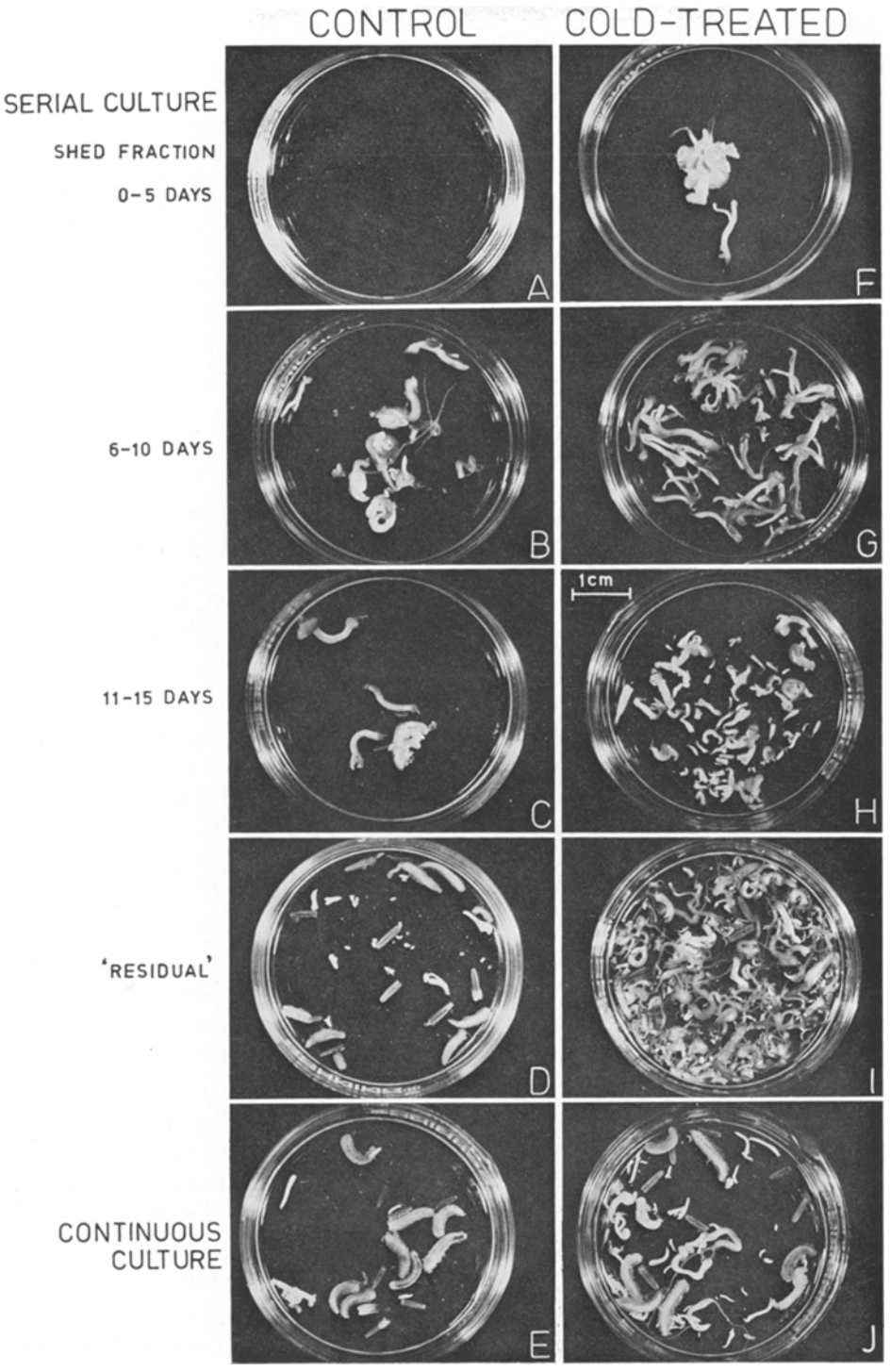
CONTROL
COLD-TREATED
SERIAL CULTURE
SHED FRACTION
0–5 DAYS
6–10 DAYS
11–15 DAYS
'RESIDUAL'
CONTINUOUS CULTURE
1cm
A
B
C
D
E
F
G
H
I
J

Fig. 1 *A–J*

material was at the premitotic stage (NITSCH and NORREEL 1973; present investigation). At postmitotic stage, the response was occasional. However, in pollen cultures of *Nicotiana tabacum* var. Badischer Burley (HEBERLE and REINERT 1977) the binucleate stage of pollen has been found more suitable for embryo formation.

Contrary to the results of NITSCH (1974 a), we failed to induce embryo formation at will from pollen grains isolated from freshly excised anthers even though the nutrient medium as well as the pollen density were the same. Pollen grains isolated from 5-day-precultured anthers proved to be better material for obtaining androgenic embryos. Following this technique it was possible to obtain embryos in *Datura* at low pollen density, 10^3 per ml. However, it remains to be seen whether preculture can be replaced by hormones.

To avoid the tedium of preculturing and crushing of anthers, we finally experimented with the serial culture technique described by SUNDERLAND and ROBERTS (1977) for *N. tabacum* and adopted it for our work. Pollen grains were shed spontaneously from anthers and transfer of anthers to fresh medium at frequent intervals improved the response. While this technique cannot be called pollen culture in the strict sense of the term, it offers distinct advantages over other conventional techniques. One, the number of embryos is much higher than in continuous culture or any other technique tried. Secondly, since suspended pollen grains develop into embryos, the material should have nearly all the advantages expected of an *ab initio* pollen culture. It is to be noted that our finding of a high response in liquid cultures, is at variance with that of WERNICKE and KOHLENBACH (1976), who were unable to obtain embryo formation in liquid medium with *Datura*. They assumed that this is probably due to sinking of anthers in their experiments. These authors also did not try cold treatment.

Finally, as to the explanation of the higher efficiency of the "serial culture" technique as compared to continuous culture. It seems very likely that the anther wall or the anther sac contains inhibitor(s), this was already postulated by SUNDERLAND and WICKS (1971). Transfer to fresh medium probably dilutes out the inhibitor(s) and thus allows successive fractions of pollen grains to develop more readily into embryos. In continuous culture, however, pollen would remain in contact with the supposed inhibitor(s) resulting in lower induction frequencies. Leaching of such inhibitory factor(s) in liquid culture has also been suggested in *Nicotiana* (WERNICKE and KOHLENBACH 1976). However, we should point out that NITSCH (1974 a) has shown that boiled water extracts of 7-day-precultured anthers were promotive for the induction of pollen embryos in *Datura*. Likewise, SUNDERLAND and ROBERTS (1977) postulate a stimulatory effect of the anther wall. The promotory role of anther wall is also apparent by the inability reported by most investigators, including us, to induce the development of embryos in pollen freshly isolated

from anthers. In the light of these findings it seems that the anther wall has both promotory and inhibitory substances for formation of pollen embryos. But, the real identities of these substances remain to be resolved and this will obviously require much more work.

Acknowledgement

This study was aided by grants from University Grants Commission and Indian Council of Agricultural Research.

References

GUHA, S., MAHESHWARI, S. C., 1964: *In vitro* production of embryos from anthers of *Datura*. Nature **204**, 497.

— — 1966: Cell division and differentiation of embryos in the pollen grains of *Datura in vitro*. Nature **212**, 97—98.

HEBERLE, E., REINERT, J., 1977: Factors of haploid production by isolated pollen cultures. Naturwissenschaften **64**, 100.

NITSCH, C., 1974 a: Pollen culture—a new technique for mass production of haploid and homozygous plants. In: Haploids in higher plants—advances and potential (KASHA, K. J., ed.). Univ. of Guelph, pp. 123—135.

— 1974 b: La culture de pollen isolé sur milieu synthétique. C. R. Séanc. Acad. Sci. (Paris) **278**, 1031—1034.

— NORREEL, B., 1973: Effet d'un choc thermique sur le pouvoir embryogène du pollen de *Datura innoxia* cultivé dans l'anthère ou isolé de l'anthère. C. R. Séanc. Acad. Sci. (Paris) **276**, 303—306.

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

REINERT, J., BAJAJ, Y. P. S., HEBERLE, E., 1975: Induction of haploid tobacco plants from isolated pollen. Protoplasma **84**, 191—196.

SANGWAN-NORREEL, B. S., 1977: Androgenic stimulating factors in the anther and isolated pollen grain culture of *Datura innoxia* Mill. J. exp. Bot. **28**, 843—852.

SOPORY, S. K., MAHESHWARI, S. C., 1976: Development of pollen embryoids in anther cultures of *Datura innoxia*. 1. General observations and effects of physical factors. J. exp. Bot. **27**, 49—57.

SUNDERLAND, N., ROBERTS, M., 1977: New approach to pollen culture. Nature **270**, 236—238.

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

WERNICKE, W., KOHLENBACH, H. W., 1976: Investigations on liquid culture medium as a means of anther culture in *Nicotiana*. Z. Pflanzenphysiol. **79**, 189—198.

— — 1977: Experiments on the culture of isolated microspores in *Nicotiana* and *Hyoscyamus*. Z. Pflanzenphysiol. **81**, 330—340.