J. Sci. Soc. Thailand, 4 (1978) 139-145

VEGETATIVE DEVELOPMENT OF FIELD BEAN POLLEN GRAIN CULTURED *IN VITRO*

THIPMANI PARATASILPIN

Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand

(Received 31 May 1978)

Summary

*Experiments have been undertaken with the objective of inducing vegetative growth of pollen grains in cultured anthers of field bean (**Vicia faba**). Line 14 was a responsive cultivar which produced pollen capable of forming the proembryoids when its anthers cultured on the basic medium supplemented with 0.2 mg/l kinetin, 0.1 mg/l α-naphthaleneacetic acid and 5.0 mg/l 2,4-dichlorophenoxyacetic acid. The finding was also discussed in relation to current views on the origin and pathway of development of multicellular pollen.*

Introduction

A study of gametophytic tissues derived from the male gametophyte of a seed plant was first reported by Telucke[1]. He obtained callus growth originated from the tube cell of *Ginkgo* pollen when cultured on a suitable medium. Similar works were also reported on other gymnosperm pollen[2-4]. A very important step was achieved when Guha and Maheshwari[5] showed that haploid plants were produced by *Datura* anthers grown *in vitro*. There has been a worldwide interest in developing this method for a wide variety of crop plants. However, only limited success has been obtained in producing haploid development involving not more than 9 families of angiosperms[6-9]. For legumes, no anther culture has yet been reported. A review of the literature suggests that legume tissue is in this respect somewhat intractable. This paper deals with the investigation on a vegetative development in culture of pollen of field bean (*Vicia faba* L.), an important legume crop, showing the promising development toward androgenesis. Attention was also paid to the pathway of an early embryoid development of the pollen grain.

Materials and Methods

The basic medium used contained the inorganic salt solution of Murashige and Skoog (MS)[10] and the vitamin-amino acid solution of Nitsch (H)[11]. The concentra-

Abbreviations: NAA, α-naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

tion of sucrose employed was 30 g/l and that of agar was 0.6%. Growth substances tested at various concentrations and combinations were kinetin, α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2, 4-D). The liquid nutrient medium was adjusted to pH 5.8 by using 1 N NaOH and sterilized by filtration through a Millipore filter. The agar solution was, on the other hand, sterilized by autoclaving. Finally, the two solutions were mixed together, 1:1 in proportion, just before the agar reached a setting point.

Inflorescences were obtained from greenhouse grown plants of *V. faba* : Line 14, Line 59 and two hybrids, H963 and H2597. Three groups of flower buds were employed; 1-2 mm long (group I) usually contains pollen at tetrad stage, 2-3 mm long (group II) uninucleate stage and 4 mm long onward (group III) binucleate stage. Inflorescences were sterilized by soaking in a freshly prepared 5% sodium hypochlorite for 5 min, then freely washing twice with sterile distilled water to ensure removal of the sterilant. Dissection of anthers was done with the aid of fine forceps and a dissecting microscope. Nine anthers from a bud were placed on the surface of solid medium in one culture bottle and one anther was examined to assess the stage of pollen development. The culture bottles after assessment were incubated at 28°C. The photoperiod used was 8 h dark and 16 h light supplied by cool white fluorescent tubes with light intensity of 3000 lux.

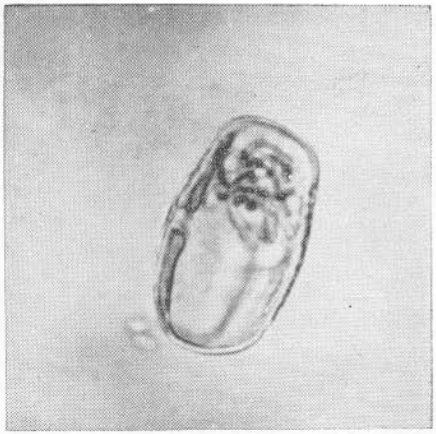
Examination of anthers was done at 4, 5, 6, 7 and 14 days after culturing. Feulgen squash technique recommended by Darlington and La Cour[12] was used to assess the pollen development both before and after culturing.

Results

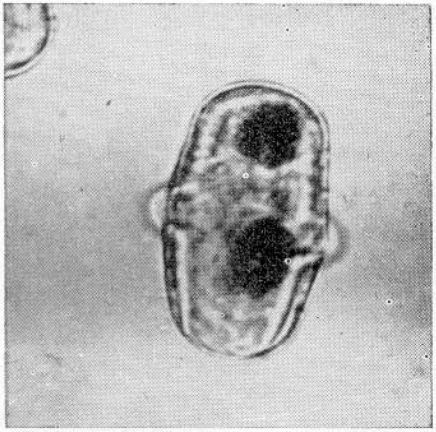
The response of the cultured anthers on the different media varied greatly and depended on the developmental stage of pollen, the plant variety and the type and level of growth substances present in the culture media.

All anthers of all the bean varieties except that of Line 14 failed to grow and rapidly became brown and necrotic. Anthers from group II buds of Line 14 were found to respond well in culture and their pollen could be induced to form multinucleate pollen grains. Few of pollen from group III buds also divided to form trinucleate grains but eventually degenerated. None of anthers from group I buds showed any response on any medium. This most responsive stage of uninucleate pollen grain of *V. faba* was found similar to that of other plant species[6].

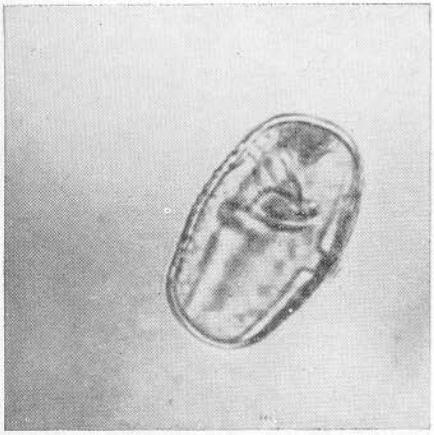
The experiments showed that among the tested growth substances kinetin (0.2 mg/l) was the key substance for a vegetative growth of *V. faba* pollen (See Table I). On addition of auxin, either NAA or 2,4-D at the concentrations of 0.1 mg/l or 5.0 mg/l respectively to the medium, the production of multinucleate grains increased considerably. The most effective medium for the differentiation of the proembryoid up to a 7-cell stage was the basic medium supplemented with 0.2 mg/l kinetin, 0.1 mg/l NAA and 5.0 mg/l 2,4-D. Although the level of 2,4-D at 5.0 mg/l appeared to be very high but the intermediate concentrations ranging between 0-5.0 mg/l ex-



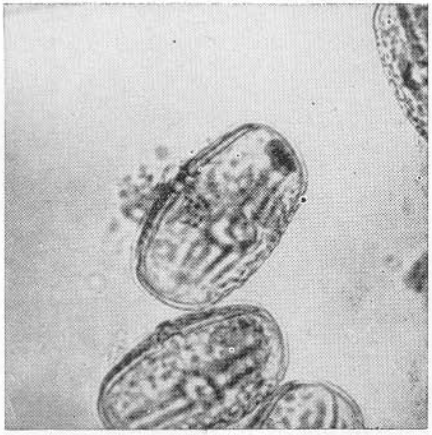
1



2

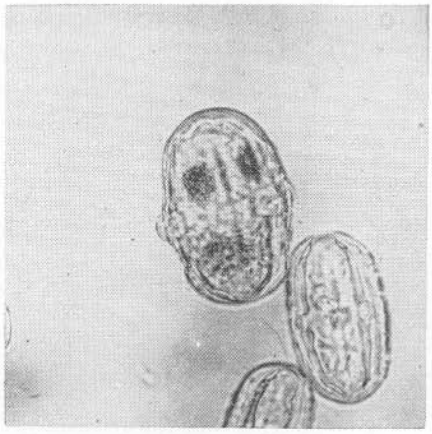


3

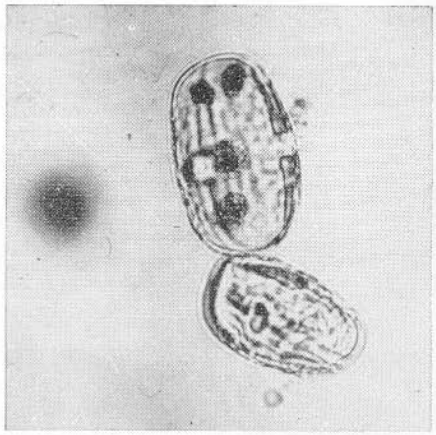


4

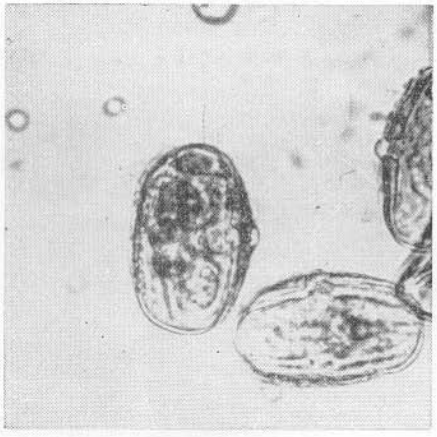
Fig. 1. Uninucleate grain at prophase, × 500
Fig. 2. Symmetrical binucleate grain, × 700
Fig. 3. Asymmetrical binucleate grain of which the vegetative nucleus at prophase, × 500
Fig. 4. Trinucleate grain with two vegetative and one generative nuclei, × 500



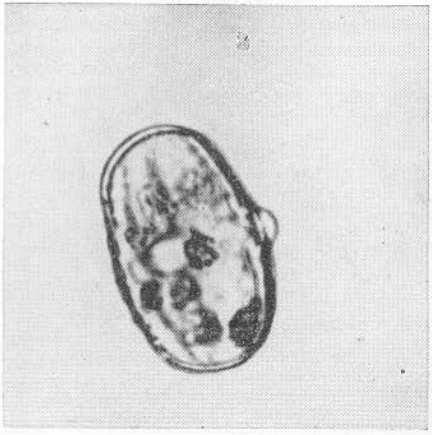
5



6



7



8

Fig. 5. Trinucleate grain two generative and one vegetative nuclei, × 500
Fig. 6. Tetranucleate grain, × 500
Fig. 7. A proembryoid containing 7 cells, × 500
Fig. 8. A coenocytic 8-nucleate grain, × 700

perimented as a source of growth factor alone or in combination with kinetin (0.2 mg/l) and NAA (0.1 mg/l) showed no effect on pollen growth.

TABLE I: POLLEN RESPONSE OF LINE 14 TO LEVELS AND COMBINATIONS OF GROWTH SUBSTANCES

Kinetin (mg/l)	NAA (mg/l)	2, 4–D (mg/l)	Pollen response
0.01	–	–	–
0.1	–	–	–
0.2	–	–	+
0.2	0.1	–	++
0.2	–	5.0	++
0.2	0.1	5.0	+++
–	0.1	–	–
–	–	5.0	–
0.4	–	–	–

The response was measured as the percentage of multinucleate grains per anther produced within 7 days in culture and was graded of follows:–
– = no pollen growth, + = 1–10%, ++ = 15–20%,
+++ = 30–40% with proembryonic development

A small proportion of anthers of group II buds examined after 4 days in culture contained numerous pollen grains undergoing mitoses (Fig. 1). Most of the mitoses appeared to represent normal stages in the formation of binucleate pollen with one generative and one vegetative nuclei which was also found in a large number in the preparation. Some mitoses, however, could have deviated from the normal developmental pattern since a few anomalous symmetrical, vegetative-type binucleate grains (Fig. 2) was sporadically observed. The classification of the nuclear type stated here is based on the size and the affinity for Feulgen stain. The vegetative nucleus was relatively larger and more diffuse than the generative nucleus, showing poor affinity for the stain resulting in a pale pink colour while the generative one was dense and darker in consequence. The division of a vegetative nucleus (Fig. 3) could lead to the production of a trinucleate grain containing two vegetative and one generative nuclei (Fig. 4). The occurrence of these trinucleate grains were observed in anthers after 5 days in culture together with those containing a vegetative and two generative nuclei (Fig. 5) and many of tetranucleate grains with two derivatives of both vegetative and generative nuclei (Fig. 6). The most advanced vegetative development of pollen observed in culture after 7 days was the formation of a proembryoid containing 7 cells (Fig. 7). This proembryoid contained six derivatives of vegetative cell and one was the original quiescent generative cell which remained attached to the wall. The coenocytic 8-nucleate grains (Fig. 8) were also found sporadically in the same preparation. During the week-examination, it was noted that the num-

ber of abortive and dead grains increased with time and the examination done after two weeks showed that no pollen growth but only dead grains was generally observed.

Discussion

The requirements for pollen growth of each plant species varied greatly[1-11, 13-16]. It was evident that pollen of *V. faba* responded well when cultured on the basic medium supplemented with kinetin (0.2 mg/l). NAA (0.1 mg/l) or 2,4-D (5.0 mg/l), which produced negative result when supplied alone, enhanced the effect of kinetin. It was suggested[13] that the balance of hormones needed to be explored to switch pollen development toward vegetative growth, although many species such as *Nicotiana* and *Datura* could form androgenic embryoids without hormones or at very low levels[6, 11]. Clearly, families differ in the ease with which pollen of their component species can be cultivated to yield embryoids[1-11, 13-16]. It appears that requirements for species of Leguminosae, such as *V. faba*, are complex and need extended enquiry for complete haploid formation.

Line 14 seemed to be the most promising cultivar to produce pollen capable of dividing vegetatively. Plant genotype directly affected the ability of pollen to form embryoids[14]. Nitsch[11] studied a number of species of *Nicotiana* and a number of strains of *N. tabacum* and suggested that the levels of ploidy, self-incompatibility including the presence of lethal genes are the factors involving the ability of a plant to produce gametophytic embryoids. Compared with other lines, pollen of Line 14, a nominally homozygous cultivar, responded more readily in culture—a result almost certainly dependent on its genetic make up. However, slightly different environmental factors of the pollen grains before collection for *in vitro* culture could play a role in the observed growth differences.

The uninucleate stage of pollen of *V. faba* Line 14 was observed to be the most plastic stage which divided within 4-7 days after culturing. The lag period of 4 days was the important inductive period during which a presumed switch mechanism inaugurating development via the vegetative nucleus could come into operation. The result indicated that the earliest development of vegetative growth of pollen passing through the formation of asymmetrical rather than symmetrical binucleate grains since the production of the latter was low and among those produced, only one further division was observed. This route toward androgenesis of a microspore was also described in cultured pollen of *Hordeum vulgare*[15], *Triticum aestivum*[16] and *N. tabacum*[6]. The haploid cells of *V. faba* divided repeatedly within the framework of the exine to form a multinucleate structure up to 8-nucleate stage with no cell enlargement unlike such species as tobacco or *Datura*[6] where cell enlargement accompanied cell division. It could be the very thick exine of *V. faba* pollen that was a physical restraint upon further development of the proembryoids.

The result of this work showed that among all legume crops, pollen growth of field bean *(V. faba)* could be initiated when its anthers are cultured on the suitable medium. By exploring a suitable hormonal balance of a medium and giving

the right choice of plant genotype, it may also be possible to induce haploid growth from cultured anthers of other economic legume crops.

References

1. Tulecke, W. (1953) A Tissue from the Pollen of *Ginkgo biloba*. *Science* **117**, 599-600.
2. Tulecke, w. and Sehgal N. (1963) Cell Proliferation from the Pollen of *Torreya nucifera*. *Contrib. Boyce Thompson Inst.* **22**, 153-163.
3. Bonga, J.M. and Fowler, D.P. (1970) Growth and Differentiation in Gametophyte of *Pinus resinosa* Cultured *In Vitro*. *Can. J. Bot.* **48**, 2205-2207.
4. Razmologov, V.P. (1973) Tissue Culture from the Generative Cell of the Pollen Grain of *Cupressus* spp. *Bull. Torrey Bot. Club* **100**, 18-22.
5. Guha, S. and Maheshawari, S.C. (1964) *In vitro* Production of Embryo from Anthers of *Datura*. *Nature (Lond)* **204**, 497.
6. Sunderland, N. (1974) Anther Culture as a Means of Haploid Production, in *Haploids in Higher Plants. Advance and Potential*. (Kasha, K. J, ed) pp. 91-122. Proc. 1st. Int. Symp. Univ. of Guelph, Canada.
7. Corduan, G. and Spix, C. (1975) Haploid Callus and Regeneration of Plants from Anthers of *Digitalis purpurea* L. *Planta (Berl)* **124**, 1-11.
8. Hughes, K.W.; Bell, S.L. and Componetti, J.D. (1975) Anther-derived Haploids of the African Violet. *Can. J. Bot.* **53**, 1442-1444.
9. Rosali, P., Devreux, M. and Laneri, K. (1975) Anther Culture of Strawberry. *Hortscience* **10**, 119-120.
10. Murashige, T. and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. *Physiol. Plant.* **15**, 473-497.
11. Nitsch, J.P. (1970) Experimental Androgenesis in *Nicotiana*. *Phytomorphology* **19**, 389-404.
12. Darlington, C.D. and La Cour, L.F. (1970) *The Handling of Chromosomes*, 5th ed., George Allen and Unwin Ltd., London.
13. Sunderland, N. (1973) Pollen and Anther Culture, in *Plant Tissue and Cell Culture* (Street, H.E., ed) pp 205-239. Blackwell Scientific, Oxford.
14. De Fosard, R.A. (1974) Summation : Methods for Producing Haploids, in *Haploids in Higher Plants. Advance and Potential*. (Kasha, K. J., ed) pp 145-150. Proc. 1st Int. Symp. Univ. of Guelph, Canada.
15. Clapham, D. (1971) *In Vitro* Development of Callus from the Pollen of *Lolium* and *Hordeum*. *Z. Pflanzenzucht*, **65**, 285-292.
16. Wang, C.C., Chu, C.C., Sun, C.S., Wu, S.H., Yin, K.C. and Hsu, C. (1973) The Androgenesis in Wheat *(Triticum aestivum)* Anthers Cultured *In Vitro*. *Sci. Sin.* **16**, 218-222.

บทคัดย่อ

จุดประสงค์ของการทดลอง ก็เพื่อจะกระตุ้นให้ละอองเกสรตัวผู้ของถั่วปากอ้า *Vicia faba* มีการเจริญเป็นเนื้อเยื่อ โดยการนำเอาอับเกสรไปเลี้ยงในหลอดทดลอง ได้พบว่า ถั่วพันธุ์เบอร์ 14 เป็นพันธุ์ที่เหมาะสมในการศึกษา ทั้งนี้เพราะสามารถผลิตละอองเกสรตัวผู้ซึ่งสามารถเจริญเป็นคัพภะในระยะต้นซึ่งประกอบไปด้วยเซลล์ถึง 7 เซลล์ได้ เมื่อนำเอาอับเกสรไปเลี้ยงในอาหารที่มี kinetin 0.2 mg/l, α-naphthaleneacetic acid 0.1 mg/l และ 2,4-dichlorophenoxyacetic acid 5.0 mg/l นอกจากนี้ ได้มีการอภิปรายถึงจุดกำเนิดและวิถีทางของการเจริญของละอองเกสรที่มีหลายเซลล์เหล่านี้ด้วย