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POLLEN EMBRYOGENESIS IN THE CULTURED ANTHERS OF *NICOTIANA TABACUM* CV. VIRGINICA OBTAINED FROM *IN VIVO* AND *IN VITRO* PRODUCED FLOWER BUDS

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Abstract

The performance of anthers from in vitro produced flower buds and those derived from glasshouse-grown plants was compared. In liquid medium anthers of all types exhibited some degree of browning. In vivo produced anthers were moderately prone to browning irrespective of the presence or absence of hormones in the culture medium. Browning in anthers derived from in vitro produced flower buds was far more in hormone-free media. The anther response based on the number of anthers incubated was much higher in hormone free media than in the media containing hormones. In vitro produced anthers exhibited a strong dependence of anther response to the stage of pollen development at excision The productivity of the in vitro produced anthers was greater in the hormone supplemented media whereas the in vivo produced anthers did so in the basal medium.

Introduction

Nicotiana species are well known plant material for the induction of pollen embryogenesis *in vitro*. *Nicotiana tabacum* offers a particular advantage that a large proportion of anthers produce embryoids (Bajaj *et al.,* 1977) which are directly derived from pollen grains without the intervention of an intermediate callus stage. The response is so consistent that the species is utilized as a model for studying the phenomenon in other plant species.

In *Nicotiana* inadequate yield of embryoids and batch to batch variation in embryo yield is a constant problem in anther culture, especially in plants grown throughout the year in the glasshouse. In the past attempts have been made to manipulate growth conditions of donor plants by varying light, temperature and giving additional mineral nutrition (Dunwell, 1976; HeberleBors & Rienert, 1979) or by applying stress of nitrogen starvation (Sunderland, 1978). Excised anthers have also been subjected to various treatments either prior to culture (Sunderland & Roberts, 1979; Dunwell, 1981) or after putting them in culture (Imamura & Harada, 1980; Harada *et al.,* 1988). Although yield of plantlets has increased but still a large proportion of pollen grains degenerate in culture and do not produce embryoids. In this study, therefore, an alternative approach was adopted. Flower buds were induced *in vitro* as described by Khatoon (1985) and anthers excised from such buds were used for the induction of pollen embryogenesis.

Anthers from glasshouse-grown plants of Virginica cultivar of tobacco tested in culture for pollen embryogenesis show that the anthers containing mitotic and binucleate stages of pollen grains give maximum yield of plantlets and cold pretreatment to such anthers increases both the number of anthers producing plantlets and the number of plantlets per anther (Khatoon, 1987). *In vitro* produced anthers of this cultivar do not seem to have been tested for pollen embryogenesis in culture. *In vitro* produced anthers of N. tabacum cv. Wisconsin-38 have been used for inducing pollen embryogenesis and haploid plant production (Tran Thanh Van, 1977). In the present study the performance

of both *In vivo* and *in vitro* produced anthers of *N. tabacum* cv. Virginica for the induction of pollen embryogenesis was compared.

Material and Methods

Cultivation of donor plants and flower induction in vitro: The batch of seeds of *N. tabacum* cv. Virginica, the conditions of cultivation of donor plants, the method of excision of epidermal explants and their culture conditions were the same as described by Khatoon (1985).

Relationship between bud length and stage of pollen development: *In vitro* produced flower buds of different lengths ranging from 3 to 35 mm were excised from the cultured explants. Anthers were squashed in acetocarmine and the stage of pollen development in anthers at each bud length was recorded. In *in vitro* produced flower buds petal length could not be used as a guide for the determination of stage of pollen development because of the emergence of corolla through calyx at a late stage of pollen development. When petals were first visible externally, the pollen grains were already at binucleate stage of pollen development. Therefore, measurements were restricted to the overall bud length, which proved to be fairly reliable. Staging of *in vivo* produced anthers was done as described by Khatoon (1987).

Cytology: *In vitro* and *in vitro* produced fresh anthers and root tips excised from regenerated plants were subjected to cytological investigations. Fresh anthers were utilized for the quick determination of the developmental stage of anthers before anther culture. Root tips of regenerated plantlets were used for counting the number of chromosomes. Fixation of the root tips was carried out in a 1:3 mixture of acetic acid/alcohol at 4°C for 24 h. For long term storage, fixed root tips were transferred to 70% aqueous alcohol until required. For staining, fresh anthers were directly squashed in one drop of 4% acetocarmine, which was prepared by refluxing 4 g of carmine in 100 ml of 45% acetic acid for 24 h. Fixed root tips were first taken through a series of alcohol to water and were then squashed in acetocarmine.

Anther Culture: For culture of anthers from *in vitro* produced flower buds, buds were first removed aseptically from the culture container and collected in a sterile Petri dish. Buds from glasshouse-grown plants were surface sterilized with NaOCl solution (4% available chlorine) for 10 min., followed by washing 3 times with sterile distilled water before dissection. One anther from each *in vitro* and *in vivo* produced flower bud was used for the determination of the stage of anther development at excision. The other four anthers from each bud were floated on the surface of liquid media in 5 cm diam., plastic Petri dishes, each containing 5 ml medium. This medium was same as the one used for the induction of flower buds. All Petri dishes were sealed with Parafilm and stored in transparent plastic boxes containing a thick layer of moist paper tissue. The boxes were made airtight by sealing the lids with sticky tape. All the boxes were externally covered with aluminium foil to exclude light and were stored at 25°C for 15 days. The aluminium foil was then removed and the cultures exposed to a light regime of 16 h day and 8 h night at 20 w/m^2 for the rest of the experimental period. Observations of cultures were made under a stereomicroscope at low magnification. Number of brown anthers was counted after 10 days of culture. The number of plantlets arising from the anthers was counted after 6 weeks. All experiments were repeated 3 times. From the number of anthers incubated the percentage of anthers producing plantlets (anther response) and average number of plantlets produced per anther (anther productivity) was evaluated.

Results

Staging of flower buds produced in vitro: Unlike *in vivo* produced anthers of *N. tabacum* cv. Virginica and other cultivars of *N. tabacum* which show a correlation between the stage of pollen development and the length of the petals of flower buds (Sunderland & Wicks, 1971; Horner & Street, 1978; Misoo & Mitsubayashi, 1981; Khatoon, 1987) in the *in vitro* produced flower buds consistent reults were obtained when overall bud length was used as a guide for the determination of the stage of pollen development. Table 1 shows that up to a length of approximately 3 mm, *in vitro* produced buds contained anthers in which all the sporogenous tissues were at pollen mother cell stage. Meiosis was observed in these cells in the buds between 3-4.5 mm in length. Microspores were observed in buds between 5-5.5 mm in length. The first pollen mitosis occurred in anthers of buds which ranged from 6-7 mm in length. Buds between 7-19 mm in length contained early bicellular grains and above 19 mm, pollen grains almost always showed deposition of starch.

Influence of the presence of IAA and kinetin in the medium: The anthers produced on glasshouse grown plants were more vulnerable to browning in culture than *in vitro* produced anthers at all stages of development (Table 2). This effect was particularly noticeable at the uninucleate stage where 67% of the *in vitro* produced anthers remained green after 10 days, while only 6% of the *in vivo* produced anthers showed browning.

The *in vitro* produced anthers at the uninucleate and mitotic stages produced callus at the cut end of the filament. Uninucleate anthers produced more callus than older anthers. Control anthers produced *in vivo* rarely gave rise to callus.

Anther response was generally low in the two types of anthers under the prevailing culture conditions. In *in vitro* produced anthers, any response was restricted to the early binucleate stage, whereas the *in vivo* produced anthers showed some response in both the early and late binucleate stages (Fig.1A). Uninucleate and mitotic anthers did not produce plantlets whether they were produced *in vivo* or *in vitro*. The plantlets produced from the *in vitro* produced anthers were greater in number (Fig.1D) and were similar to those arising from the *in vivo* produced control anthers. Their sequence of development was also similar to that of the control anthers.

Table 1. Relationship between bud length and stage of pollen development in *in vitro* produced flower buds.

Stage of pollen development	Bud length (mm)
Pollen mother cells	2 to 3
Meiosis	3 to 4
Tetrads	4.1 to 4.5
Uninucleate microspores	5 to 5.5
Mitotic	6 to 7
Early binucleate	7.1 to 19
Late binucleate (starch containing)	20 and above

Table 2. Influence of the presence of IAA and kinetin in the culture medium on the extent of browning of anthers induced *in vitro* and taken directly from glasshouse-grown plants. Duration of experiment six weeks.

Stage of pollen development.	Growth hormones					
	IAA + kinetin		kinetin		Basal medium	
	a(%)	b(%)	a(%)	b(%)	a(%)	b(%)
Uninucleate	33	94	58	50	100	87
Mitotic	21	59	67	42	69	31
Early binucleate	31	50	50	50	100	35
Late binucleate	50	44	-	-	100	81
Percentage of total anthers cultured	33	63	58	47	93	57

a = in vitro induced anthers; b = in vivo produced anthers

Influence of the presence of kinetin as the sole hormone in the medium: There was little difference between the extent of browning in anthers produced *in vivo* and *in vitro* (Table 2). The anther response was significantly improved in the absence of IAA from the medium (Fig.1B), however, a shift in the stage at which the anthers are responsive was observed for both type of anthers. In the presence of kinetin only, the responsive stages were the mitotic and binucleate ones, with the optimum at the mitotic stage. No response was observed at the binucleate stage in the *in vitro* produced anthers (Fig.1B), whereas in the presence of IAA (Fig.1A) all response occurred at that stage only. Anther productivity was not significantly changed (Fig.1E).

Influence of the absence of both IAA and kinetin in the medium: Absence of IAA and kinetin from the medium did not affect browning of the *in vivo* produced anthers (Table 2). The uninucleate and late binucleate stages were more affected than the mitotic and early binucleate stages. The latter are important from the point of view of anther culture. However, over 90% of the *in vitro* produced anthers showed browning after 10 days of culture where the uninucleate and binucleate stages were more affected than the mitotic stages (Table 2).

Anther response and anther productivity of the *in vitro* induced anthers was restricted exclusively to the mitotic stage, however, the anther response and productivity of *in vivo* anthers was found in all other stages. The average yield of plantlets was rather same for the *in vitro* produced anthers as in the previous experiments but it increased considerably for the *in vivo* produced anthers (Fig.1C).

Pollen derived plantlets grown in pots were short, with narrow pale green leaves, exhibiting profuse flowering. The flowers were similar in morphology to diploid flowers but were sterile and did not produce seeds. The chromosome counts of the root tip cells confirmed their haploid nature.

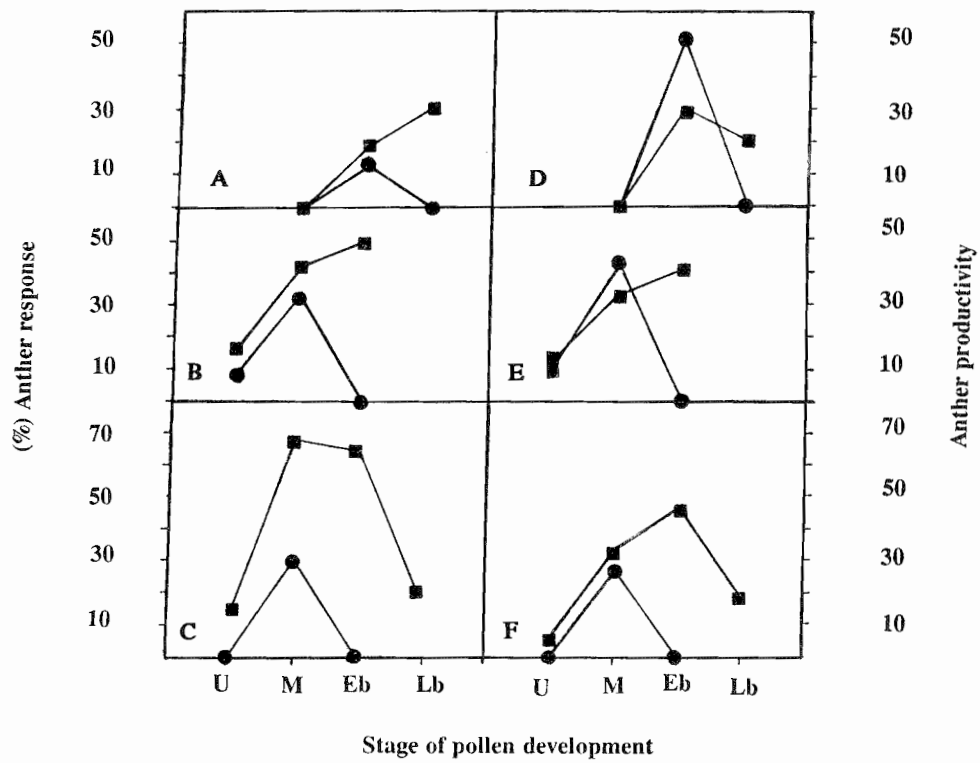

Fig.1A-F. Anther response and anther productivity in cultures established from in vivo (■) and in vitro (●) produced anthers of Nicotiana tabacum cv. Virginica containing uninucleate (U), mitotic (M), early binucleate (Eb) and late binucleate (Lb) pollen grains.
A. Anther response in medium containing IAA and kinetin.
B. Anther response in medium containing kinetin alone.
C. Anther response in medium lacking both hormones.
D. Anther in medium containing IAA and kinetin.
E. Anther productivity in medium containing kinetin alone.
F.Anther productivity in medium lacking both hormones.

Discussion

In *in vivo* produced anthers, the degree of browning is largely independent of the exogenous hormone content of the medium. About half of such anthers turned brown during the first 10 days of culture. The extent of browning of the *in vitro* induced anthers, on the other hand was fairly sensitive to the presence of exogenous hormones. With IAA and kinetin, both at 10^{-6}M, the extent of browning of these anthers was relatively low (about 1/3 turned brown in 10 days) whereas with kinetin alone browning reached about the same level as it did for *in vivo* produced anthers, about 1/2 of these anthers turned brown. In the absence of both the exogenous IAA where and Kinetin, browning was rather severe affecting over 90% of the *in vitro* induced anthers (Table 2).

In vivo anthers appeared to be better buffered against hormone induced changes in the extent of browning than *in vitro* anthers. Browning of organs and tissues is a

widespread phenomenon in plant tissue cultures and has been suggested to occur as a result of accumulation of phenolic compounds. Regulation of accumulation of phenolics has been studied in detail in suspension cultures of *Haplopappus* (Constabel *et al.*, 1971), Pauls scarlet rose (Davies, 1972) and *Acer pseudoplatanus* (Westcott & Henshaw, 1976) and has been shown to be stimulated by a reduction in the level of auxin present in the culture medium. Reduction in the frequency of brown *in vitro* produced anthers cultured in medium containing IAA might have been due to inhibition of synthesis of phenolics (Table 2). The behaviour of *in vivo* anthers with respect to browning cannot be explained in this way.

Fig.1A shows addition of IAA favoured androgenesis in binucleate anthers. In *in vitro* produced anthers the response was restricted to the early binucleate class whereas in *in vivo* produced anthers it was spread to both the early and late binucleate types. Anther response increased with elimination of hormones from the medium for both *in vivo* and *in vitro* anthers, irrespective of the stage of pollen development (Figs.1B and 1C). This observation appears to be in line with the view that pollen embryogenesis in anther culture of *N. tabacum* proceeds well in the absence of growth hormones and does not require an exogenous supply of growth hormones in the medium (Sunderland & Dunwell, 1977; Sunderland, 1979).

There are conflicting reports in the literature regarding the influence of exogenous growth hormones on anther response and anther productivity in *N. tabacum* anther culture. In *N. tabacum* cultivars Bright Yellow and Red a decrease in the anther response has been observed as a result of addition of either hormones alone (IAA and Kinetin) or hormones plus vitamins to the medium (Nitsch, 1971), however, an increase in anther response and anther productivity was observed in the cultivar Wisconsin-38 (Niizeki, 1973) and in *N. tabacum* L., by Kasperbauer & Collins (1974). In the present work one concentration of IAA and kinetin was used. It is possible that this concentration was supraoptimal either for induction or post induction process. Premature bursting of the embryoids from the pollen grain wall and their subsequent degeneration after only a short period of development was also observed by Sunderland & Wicks (1971) in the anthers of *N. tabacum* cv. White Burley cultured on a medium supplemented with NAA (1 mg/1). Since in the present work the anthers were not usually checked for the presence of aborted or degenerated embryoids, it is therefore, difficult to say whether the presence of IAA or both IAA and Kinetin in the medium did influence the induction or the post induction process.

Comparison of the productivity of anthers shows that the *in vitro* produced anthers gave maximum yield of plantlets per anther in medium containing both IAA and kinetin (Fig.1D), whereas the *in vivo* ones did so in the absence of these hormones (Fig.1F). From these results it appears that the two types of anthers differ in their endogenous level of growth regulators or growth inhibitors or both. The determination of these factors in fresh anthers taken from galsshouse grown plants of *N. tabacum* cv. Badischer Burley has shown that they contain high concentration of endogenous IAA (Dollmantel & Reinert, 1980). The anthers of cv. Virginica may also contain considerably high concentration of endogenous growth regulators and culturing such anthers in medium supplemented with IAA and kinetin rendered their level supraoptimal for the process of androgenesis and hence reduced the plantlet yield. The *in vitro* anthers either may not contain high level of such growth promoting substances or may contain relatively greater

amount of inhibitors so that their interaction allows greater number of plantlet development. Whether these substances are present in the anthers or some other factors are involved in the process can only be conclusively demonstrated after the estimation of these substances in the two types of anthers.

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