

FLOWER INDUCTION IN *NICOTIANA TABACUM* CV. VIRGINICA IN STERILE CULTURE

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Abstract

Flower buds were induced *in vitro* on whole stem explants of *Nicotiana tabacum* cv. Virginia and also on thin epidermal layers excised from stem segments. The explants supported flower bud induction and growth, the number of induced flower buds was more on the epidermal explants than on the stem segments. The flower buds thus produced contained viable pollen grains, a greater proportion of which resembled "E" grains of the *in vivo* produced flower buds. All the stages of meiotic and mitotic sequences were recorded.

Introduction

Flower induction in tissue culture is important from commercial point of view since it provides a suitable method of inducing variability in a given plant genome with almost full control on environmental factors. *In vitro* flower induction in different cultivars of *Nicotiana tabacum* has been achieved by Aghion-Prat (1965), Wardell & Skoog (1969a) and Tran Thanh Van (1973), but there is no report of the *in vitro* flower induction in cv. Virginia. Experiments were, therefore, carried out on *in vitro* flower induction in cv. Virginia with the object of using flower buds as source of anthers for pollen embryogenesis.

Material and Method

Cultivation of donor plant: Seeds of *Nicotiana tabacum* cv. Virginia were obtained from Thompson and Morgan, Ipswich Ltd., and were grown in "Levington Universal" and "Levington Potting" compost in pots in the glasshouse, under a light regime of 18 hrs day and 6 hrs night. In winter, the plants were provided with supplementary light from 400 W mercury vapour lamps (MBFR/U lamps) set at 2 feet distance from the plant to give 16 hrs daily photoperiod. The plants were given liquid feed everyday (Vita feed 301 (NPK), Vitax Ltd.). New plants were sown at 2-3 week intervals. The plants were sprayed with Pirimor (2.5 ml/gallon) or Permisect (1 ml/gallon) from time to time to control aphid and white fly infestation.

Physiological stages of the donor plant: For investigating the effect of the physiological stage of the donor plant on the *in vitro* flower producing potential of epidermal

explants, the following five physiological stages in the development of the donor plants were distinguished.

1. Top fruit of the inflorescence green; branches flowering. Main stem of the plant unbranched.
2. Most floral branches fruiting; their tips still flowering. Shoots just emerging from axillary buds.
3. Flowering on all floral branches finished; fruits set. All fruits on all branches green; none brown.
4. Fruits at the base of the branches brown. Axillary shoots well developed.
5. All fruits on the inflorescence brown. The internodes at the tips of the branches turning brown.

Source of explants: The floral branches at various developmental stages were excised from the plants, surface sterilized with NaOCl solution (4% available chlorine) for 10-15 mints, washed three times with sterile distilled water and the basal internode from each branch used for excising epidermal explants or stem segments. Each epidermal explant was 2-4 mm wide x 1 cm long. (Fig. 1 A & B). In some experiments the morphological base of each explant was marked to note the morphogenetic difference between the two ends in culture.

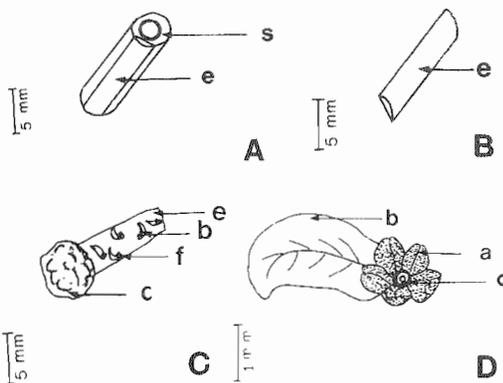


Fig. 1. A. A piece of stem from the floral branch. Note position of the cut for the excision of epidermal explant (e) on the piece of stem (s).
 B. An epidermal explant after excision.
 C. Epidermal explant (e) after 2 weeks of culture. Note callus at one end (c) and flower primordia (f) arising from the axils of bracts (b).
 D. Closer view of one flower primordium. Note well developed bilobed anthers (a) surrounding ovary (o) and bract (b).

Explants from the top, middle and basal branches of the inflorescence were used at each physiological stage to investigate the variation in flower bud forming ability within the inflorescence of a plant. In each case only the basal internode was utilized for excision of the explants.

Culture conditions: The cultures were maintained in 25 x 150 mm boiling tubes which contained 15 ml of medium each and were inoculated with one explant per tube. Tubes were either plugged with cotton wool or with polyurethane bungs externally covered with aluminium foil and stored at 25°C in continuous light at 10 W/m² from white fluorescent bulbs.

Complete M.S. medium (1962) without growth hormones and solidified with 0.7% agar was used as basal medium. IAA and K at 10⁻⁶M were used as growth substances. The hormone supplemented medium was adjusted to pH 5.0 and contents were autoclaved at 15 lbs./in² for 15 minutes.

Cytology: For examination of different stages of meiosis and mitosis anthers were fixed in a 1:3 mixture of acetic acid: alcohol at 4°C for 24 hrs. For long term storage, fixed anthers were transferred to 70% aqueous alcohol. For staining fixed anthers were taken through a series of alcohol to water and were squashed in acetocarmine prepared by refluxing 4g of carmine in 100 ml of 45% acetic acid for 24 hrs. The tissue on the slides was warmed gently for a few seconds and examined under the microscope.

Viability of pollen grains was estimated by the method of Heslop-Harrison & Heslop-Harrison (1970). Slides were examined under a Leitz "Orthoplan" microscope. A combination of exciting filter UGI 2mm and barrier filter K 400 were used with an additional barrier filter slide K 430.

Results

Influence of the nature of the explant:

A comparison was made between the flower induction on epidermal explants and on whole stem segments, of the same length, taken from equivalent positions on the inflorescence. Epidermal explants provide a suitable source for the quick production of flower buds *in vitro* (Table I). In two week old cultures, 69% of the epidermal explants produced flower buds. The newly induced buds were very small in length (0.5 mm) and their floral nature was confirmed by the presence of bracts. During the second half of the culture period some more explants produced flower buds and after 30 days, 88% of the explants produced flower buds; in addition some of them also produced a few vegetative buds.

Table 1. Effect of the nature of explant on the *in vitro* production of flower buds on *Nicotiana tabacum* cv. Virginia explants, grown on M.S. medium supplemented with IAA and kinetin 10^{-6} M each and 3% sucrose.

Nature of Explant	After 15 days of culture			After 30 days of culture				
	Morphological characters	No. of explants initially cultured	Explants producing flower buds No. %	Size of the biggest bud	Morphological characters	Explants producing flower buds No. %	Mean no. of flower buds per explant	Mean size of flower buds
Epidermal explants	Explants with distinct polarity. Callus at the morphological base. Many flower primordia.	16	11 69%	0.5 mm	Floral buds along with few vegetative buds. Callus at the morphological base.	14 88%	6	3.5 mm
Stem explants	Plenty of callus all round the explant. More at the morphological base. No primordia.	16	— —	—	Many vegetative buds all round the explant. No distinct polarity. Very few floral buds.	5 31%	3	1 mm

The stem explants produced callus all around the explant during the first two weeks of cultures. More callus was formed at the morphological base of the explant than at the apical end. They increased in their diameter, presumably both due to cell proliferation and extension but no primordia of any kind were visible to the naked eye. During the second half of the culture period many vegetative buds arose on the callus which, after producing one or two leaves, produced flower buds. After 30 day culture period, only 31% of the explants produced flower buds, ranging from 1 to 3 in number and 0.5 – 2 mm in length. The biggest flower bud produced from the epidermal explants at the end of 30 days of culture was 6 mm in length and their number ranged from 2 – 11 per explant.

The behaviour of the epidermal explants in culture:

Thin epidermal explants were excised from the stems of apical floral branches of the inflorescence of plants at physiological stage 4 or sometimes 3 with the terminal bud in green fruit stage. When grown on the basal medium of Murashige & Skoog (1962) supplemented with IAA and kinetin at 10^{-6} M each and 3% sucrose, under continuous light, signs of proliferation became visible within 4 days of culture. The thickness of the explants increased considerably within one week of culture. Callus formation occurred at one end of the explant and a distinction between the two poles was established (Fig. 1C). The floral or vegetative buds first appeared near the callus-free end. Marking the morphological base of the explants at the time of culture revealed that it was the basal end which formed profuse callus. This pattern of development was observed in the explants which were grown in light and were green. Flower buds visible to the naked eye first appeared on explants which were kept under continuous light (10W/m^2) on the 10th to 12th day of culture (Fig. 3A & B). *In vitro* produced flower buds invariably appeared directly on the surface of the explant without preceding callus formation. The first sign of the development of the flower buds was the appearance of a small bract which bore a floral bud in its axil (Fig. 1C). As the flower bud grew the pedicel elongated and held the bract and the flower bud above the surface of the explant. The newly initiated flower buds were easily recognizable while still small (0.5 mm in diam.) by their anther primordia which were prominent and appeared to be growing directly on the surface of the explant; the other floral parts were reduced and developed later (Fig. 1D). In three week old cultures, the floral buds were 3 – 4 mm in diam., and closely resembled young flower buds on the intact plant, having a well developed 4 to 5 toothed green calyx enclosing the other organs completely. They required a further 1 – 2 weeks to reach the mitotic to bicellular stage of pollen development (Fig. 3C). A further 10 – 15 days extension of the culture period lead to fully developed flower buds (Fig. 3D, E and F).

Fully open flowers produced *in vitro* were 4.1 – 4.4 cms in length under the experimental conditions used. Each anther on an average contained about 33000 pollen grains (Fig. 3F).

The influence of physiological stage of the donor plants on the production of flower buds:

There is some evidence indicating the importance of physiological stage of the donor plants and of the effect of location from which the explants are taken on the efficiency of *in vitro* flower induction (Aghion-Prat, 1965; Tran Thanh Van, 1973, Tran Thanh Van *et al*, 1974). This was examined in *N. tabacum* cv. Virginia. Explants coming from top branches of the inflorescence (type A in Fig. 2) showed an optimum in the efficiency of flower induction at physiological stage 3, with a decline on either side, i.e., in physiological stages 2 and 1, and 4 and 5, respectively (Fig. 2). Although there were some occasional differences in flowering response, there seemed to be no effect on flower induction efficiency of either the physiological stage of the donor plants or of the position of the explants on the inflorescence prior to excision.

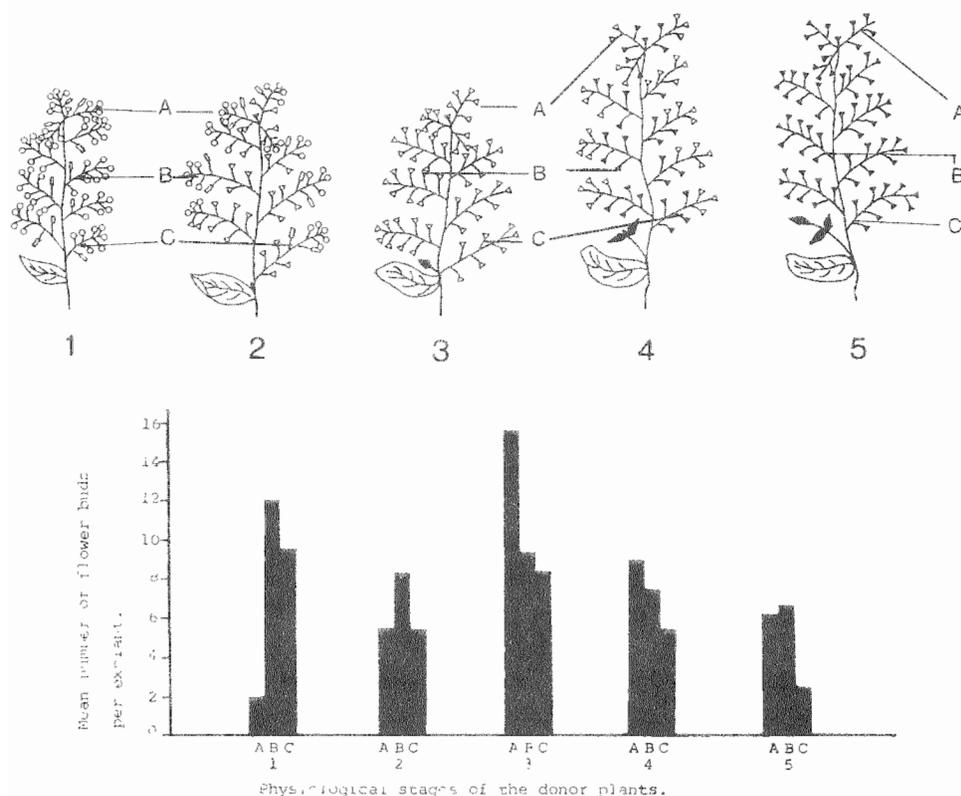


Fig. 2. Different physiological stages of the donor plant (1-5). A, top branch of an inflorescence; B, middle branch of the inflorescence; C, basal branch of the inflorescence; (o), floral buds; (□), open flowers; (▽), green fruits; (♥), brown fruits and (●), axillary shoots. Histogram shows the number of flower buds produced by the respective explants after four weeks of culture.

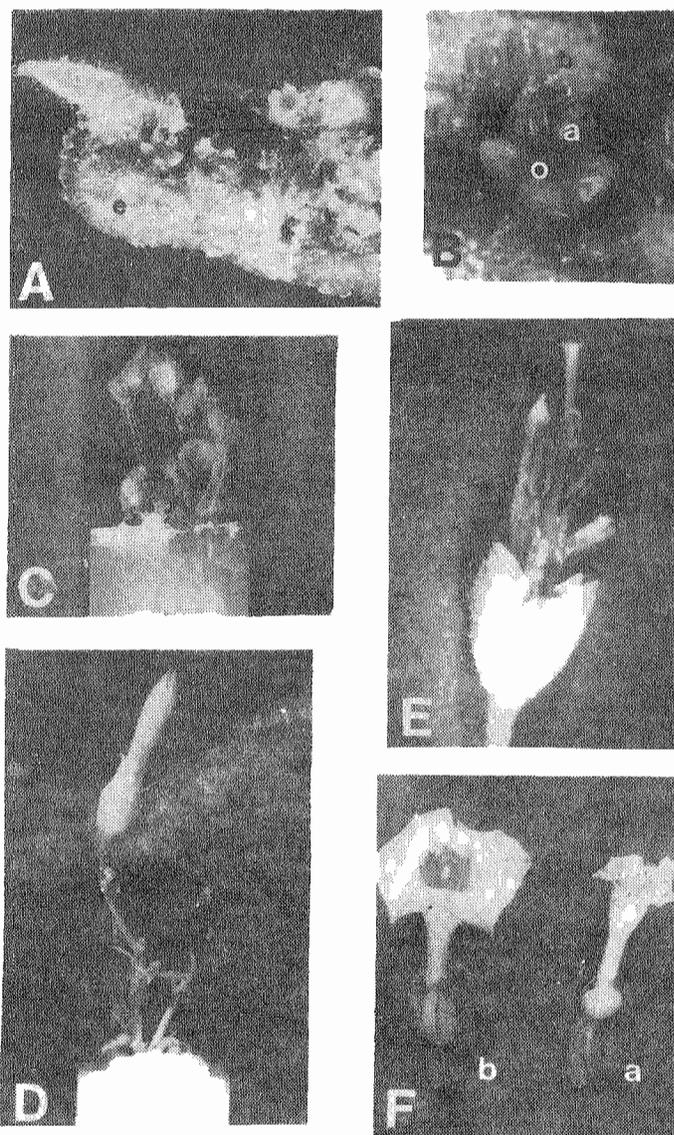


Fig. 3. A. An epidermal explant (e) after two weeks of culture.
 B. Closer view of one flower primordium. Note well developed bilobed anthers (a) surrounding ovary (o) and bract (b).
 C. *In vitro* produced flower buds in a culture tube.
 D. Fully developed flower bud before opening into flower.
 E. Corolla partly removed from the flower bud formed *in vitro*.
 F. An *in vitro* (a) and an *in vivo* (b) induced flower buds at the comparable stage of development.

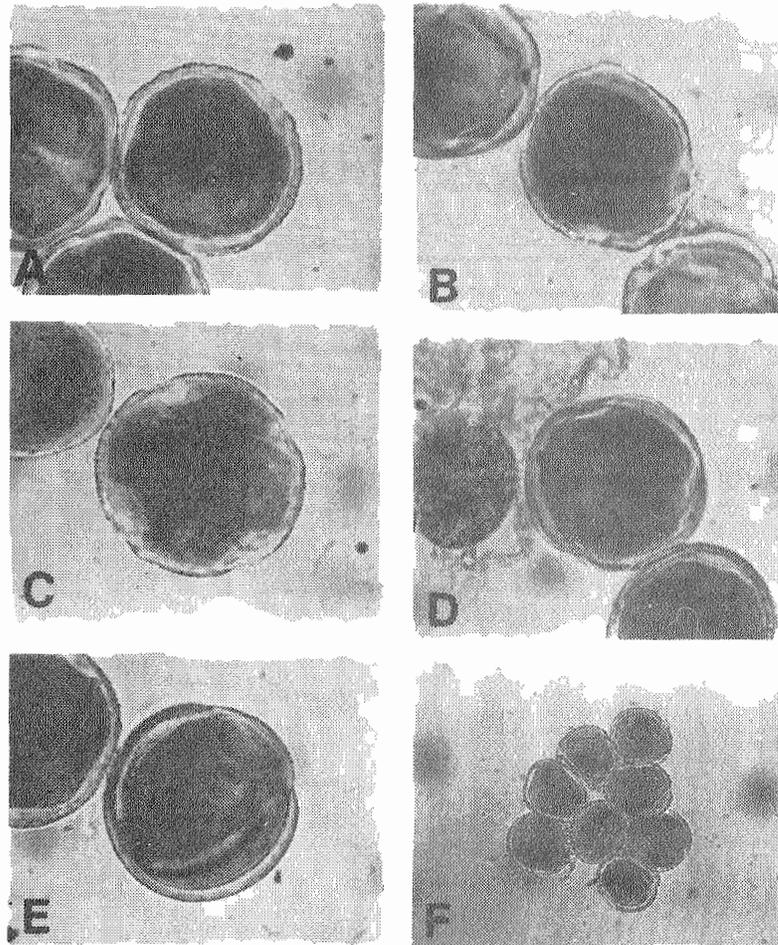


Fig. 4. Cytology of the pollen grains from buds induced on epidermal explants. Microspores showing interphase (A); early prophase (B); metaphase (C); anaphase (D); telophase (E) and (F), mature bicellular pollen grains. Note the condensed generative and diffuse vegetative nucleus.

Cytology of the in vitro produced pollen grains:

The microspores produced in the anthers taken from the *in vitro* induced flowers (Fig. 4A) were very similar in morphology to the microspores produced by the intact plants. The *in vitro* induced microspores went through the normal stages of mitosis (Fig. 4B-E). The early bicellular grains of the *in vitro* produced anthers were mainly of the unequal celled type ("A grains") (Fig. 4E) with large cells containing a large, light staining diffuse nucleus and a smaller cell containing a small, dark staining condensed nucleus very similar to the bicellular grains produced in the anthers taken from plants grown in the glass house. However, 0.1 to 0.5% contained two equal nuclei resembling "B" grains produced by the anthers taken from the plant

The late bicellular stage anthers showed two types of pollen grains. A large proportion contained starch grains and were stained darker with acetocarmine, whereas a small number was devoid of starch and stained light. There was no noticeable size difference between the two types of grain. In anthers taken from glasshouse grown plants, the light staining grains are usually smaller than the dark staining ones. The smaller light staining grains are considered to be the ones involved in the formation of embryoids in cultured anthers and were named "E" grains by Sunderland & Wicks (1971) and "S" grains by Horner & Street (1978b). Results from the viability determination experiments showed that no significant difference in the number of viable grains in the two types of anthers was apparent.

Discussion

The two types of explants have been used for the *in vitro* induction of flower buds in *N. tabacum*: stem segments (Aghion-Prat, 1965) and thin cell layers (Tran Thanh Van, 1973). The present study showed that thin cell layers excised from the inflorescence of flowering tobacco constitutes a better system for studying the physiology of induction and development of flowers *in vitro*. The establishment of polarity in the explants in culture prior to any sign of flower induction is indicative of the function of the explant as a unit.

Induction of flowers in culture from excised stem segments of *N. tabacum* cv. Wisconsin 38 took 40-48 days (Aghion-Prat, 1965). In the present study flower induction on stem segments was fast but was not as quick as it was from epidermal explants which took 10-14 days. The induced flowers were normal on both types of explants and capable of producing seeds in culture.

It is increasingly realized that flowering stimulus is a complex one and involves flower inducing substances which constitute the floral stimulus and one or more flower inhibitory substances (Cleland, 1978). The explants taken from the inflorescence segments carry these substances with them in culture. The influence of these substances may be reduced by their diffusion into the medium thus lowering endogenous level in the explant. In the epidermal explants the reduction in the endogenous level of the putative substances is possible more quickly and effectively than in the stem segments because of their thinness and larger cut surface in direct contact with the medium. The hormones or other constituents of the medium may also interact with them and modify their effect. The induced callus at one end of the epidermal explant may have a sufficient level of endogenous flower inducing stimulus which may be translocated later to the point of inception of flowers or it may retain the ability to synthesize such substances.

Explants excised from all floral branches produced floral buds in culture irrespective of their position on the inflorescence and the flowering stage of the donor plant. This appears to contradict the findings of Tran Thanh Van (1976) and Dien & Chlyah (1974). These workers did not observe flower induction on cultured epidermal explants taken from the inflorescence of *N. tabacum* cv. Wisconsin 38 in full bloom which corresponds to the physiological stage I of the *N. tabacum* cv. Virginia (Fig. 2). The variation in response may be due to genetic difference between the cultivars. In the *N. tabacum* cv. Virginia, used in this study, the explants taken from green fruiting stage of the inflorescence produced optimum number of flower buds which confirms the finding of Tran Thanh Van (1973) and Tran Thanh Van, Chlyah & Chlyah (1974) and Tran Thanh Van, Dien & Chlyah (1974).

References

- Aghion-Prat, D. 1965. Neoformation de fleurs in vitro chez *Nicotiana tabacum* L. *Physiol Veg.*, 3: 229-303.
- Cleland, C.F. 1978. The flowering enigma. *Bioscience*, 28: 265-269.
- Heslop-Harrison, J and Y. Heslop-Harrison. 1970. Evaluation of pollen viability by enzymatically induced fluorescence: intracellular hydrolysis of fluorescein diacetate. *Stain Technol.*, 45: 115-120.
- Horner, M. and H.E. Street. 1978b. Pollen dimorphism. Origin and significance in pollen plant formation by anther culture. *Ann. Bot.*, 42: 763-771.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-497.
- Sunderland, N. and F.M. Wicks, 1971. Embryoid formation in pollen grains of *Nicotiana tabacum*. *J. Exptl. Bot.*, 22: 213-226.
- Tran Thanh Van, M. 1973. Direct flower formation from superficial tissues of small explants of *Nicotiana tabacum* L. *Planta*, 115: 87-92.
- Tran Thanh Van, M. 1976. Regulation of morphogenesis. — In: *Plant Tissue Culture and its Biotechnological Applications*. (Eds.) W. Barz, E. Reinhard and N.H. Zenk. pp. 367-385. Springer-Verlag, Berlin, Heidelberg, New-York.
- Tran Thanh Van, M. H. Chlyah, and A. Chlyah, 1974. Regulation of organogenesis in thin layers of epidermal and subepidermal cells. In: *Tissue Culture and Plant Science*. (Ed.) H.E. Street Proc. 3rd Int. Congr. Plant Tissue and Cell Culture. pp. 101-131.
- Tran Thanh Van, M., N.T. Dien and A. Chlyah. 1974. Regulation of organogenesis in small explants of superficial tissue of *Nicotiana tabacum* L. *Planta*, 119: 149-159.
- Wardell, W.L. and F. Skoog. 1969 a. Flower formation in excised tobacco stem segments: I. Methodology and effects of plant hormones. *Plant Physiol.*, 44: 1402-1406.