FLOWER INDUCTION IN *NICOTIANA TABACUM* CV VIRGINICA IN STERILE CULTURE: EFFECT OF NUTRITIONAL AND ENVIRONMENTAL FACTORS

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Epidermal explants of *Nicotiana tabacum* cv. Virginica excised from fruiting branches of the inflorescence produced maximum number of flower buds when cultured on MS medium containing glycine and vitamins. Presence of GA_3 in the medium was found unsuitable for flower induction. The *in vitro* produced flower buds produced well developed green calyx whereas other organs were either absent or less developed. Adenine at relatively low concentrations permitted flower bud development on epidermal explants but their number was less than the respective control. 10^{-3}M adenine added to the MS medium was toxic for the survival of explants. Flower induction was found best in cultures kept in continuous light.

INTRODUCTION

The growth conditions of the donor plant often affect the *in vitro* responses of tissues derived from them. It is, therefore, advantageous to take explants from *in vitro* grown cultures where the growth conditions can be kept under fairly close control. In tobacco, Chouard and Aghion [3] were the first to demonstrate flower induction on excised stem segments grown in sterile culture. Later on, Tran Thanh Van [16] introduced a simpler system which consisted of thin superficial cell layers, "the epidermal explants", on which different morphogenetic expressions can be achieved directly without intermediate callus formation. The system because of the small size of explant bears a great potential of maintaining a high degree of uniformity in the regenerated structures. The ultimate aim of the continuing investigation is to make a comparison between the embryogenic potential of anthers coming from flower buds induced *in vitro* and those produced on *glasshouse* grown plants, for which a continuous supply of *in vitro* produced flower buds in large numbers with high degree of uniformity in origin is essential.

Successful *in vitro* induction of flower buds an epidermal explants in *Nicotiana* cv. Virginica has already been reported [6]. A more detailed study is now being presented of the factors affecting the formation of flower buds.

MATERIALS AND METHODS

Cultivation of donor plants. Seeds of *Nicotiana tabacum* cv. Virginica were obtained from Thompson and Morgan, Ipswich Ltd., and were cultivated as described by Khatoon [6].

Source of explants. The floral branches terminating in a green fruit were excised in the morning at II A. M. from the palnts in which flowering had been over and were surface sterilized with NaOCl solution (4% available chlorine) for 10–15 min. Surface sterilized branches were washed three times with sterile distilled water and only the basal internode from each branch was used for excising epidermal explants from stem segments. Each epidermal explant was 2 to 4 mm wide, 1 cm long and 3 to 6 cell layers thick.

Culture conditions. Complete MS medium [9] without growth hormones was used as basal medium. It was solidified with 0.7% agar. The growth substances IAA, kinetin and GA_3 were used at 10^{-6}M each. For some experiments, the basal medium was supplemented with 10^{-3}M adenine whereas in other MS mediums, containing IAA and kinetin, was supplied with four different concentrations of adenine (10^{-1}M, 5×10^{-5}M, $2.\times10^{-4}$M and 10^{-3}M). The pH of the medium was adjusted to 5.0 by adding 0.5 M KOH. The contents were autoclaved at 121 °C for 15 min.

The cultures were maintained in 25 X 150 mm boiling tubes which containd 15 ml of medium each and were inoculated with one explant per tube. Tubes were either plugged with cotton wool or with polyurethane bungs which were externally covered with aluminium foil and were stored at 25°C under continuous light at 10 W/m^2 from white fluorescent tubes.

RESULTS

Influence of the composition of the medium. Throughout the present investigations MS medium without growth hormones was used as the standard basal medium, viriations of which were tested for the induction of flower buds *in vitro.*

Omission of Glycine and Vitamins. Tran Thanh Van [16] was able to induce flower buds on epidermal explants of *Nicotiana tabacum* cv. Wisconsin 38 on a medium devoid of glycine, nicotinic acid and pyridoxine. In the experiments, the results of which are presented in Table I, the MS medium was tested with and without these three compounds. Omission of these three substances from the medium considerably reduced the response in terms of both percent of explants producing flower buds and the number of flower buds per explant. Their addition has a favourable effect on flower bud formation on the epidermal explants in *Nicotiana tabacum* cv. Virginica.

Addition of GA₃. Results presented in Table 2 show that both vegetative and floral buds developed under the influence of GA₃. The buds were not produced directly on the epidermal explants as it happens in GA₃ lacking controls but were formed on the vegetative shoots which produced I-2 leaves, the flower buds being in terminal position. Some buds were so compact that it was difficult to assign them to a particular type and therefore were named as undifferentiated buds.

Addition of GA₃. in the medium was not found suitable as it not only decreased the percentage of explants producing flower buds but also reduced the number of flower buds per explant (Fig. I). The amount of callus produced at the morphological base of the explant was also less than the respective controls. The pedicels of the flower buds were elongated and thin. The bracts were narrow and elongated. Each flower bud produced a well developed green calyx. Other parts were either absent or weekly developed.

Addition of Adenine. Adenine at a range of concentrations was tested for its effect on the formation of flower buds on thin epidermal explants grown in sterile culture. Results from Table 3 show that adenine does not favour the formation of flower buds on the epidermal explants of *Nicotianá tabacum.* The mean number of flower buds and their size decreased with the increasing concentration of
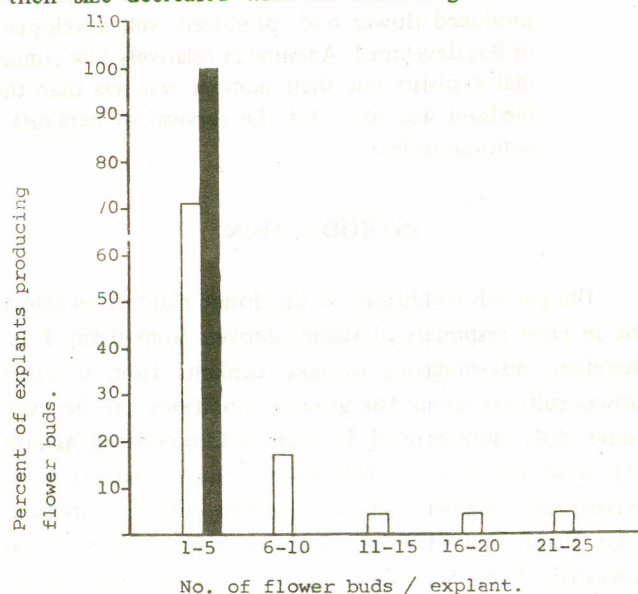

Fig. 1. The effect of GA_3 on the production of flower bunds solid bar – treatment ($+ GA_3 \ 10^{-6}$ M); open bars – control (GA_3).

Table 1. The influence of the type of culture containers and of culture media on flower production *in vitro* on the epidermal explants of *Nicotiana tabacum* (Virginica) in culture for four weeks.

Type of culture medium used used	PETRI DISHES				FLASKS				BOLLING TUBES			
	No. of explants initially cultured	Explants with floral buds No. %	Mean No. of floral buds/ explant	Other mophological characters	No. of explants initially cultured	Explants with floral buds No. %	Mean No. of floral buds/ explant	Other morphological characters.	No. of explants initially cultured	Explants with floral buds No. %	Mean No. of floral buds/ explant	Other morphological characters
Complete MS	26	2 8%	1	Some undifferentiated primordia.	13	10 77%	4	Formation of few leaves	16	14 88%	6	Floral buds along with few vegetative buds.
MS without glycine, nicotinic acid and pyridoxine-HCl.	24	2 0	––	Either dead or greenish brown	20	9 45%	3	With some vegetative buds	–– –– ––		––	––

adenine. Maximum number of flower buds were produced on media lacking adenine. Adenine at 10^{-3}M was toxic for the thin layer explants as they senesced at this concentration. IAA and kinetin appeared to be essential for the survival of explants and also for the production of flower buds. In the absence of IAA and kinetin explants gradually turned brown and died.

The influence of the type of culture container. The shape and size of the culture containers determine the volume of the internal gaseous atmosphere available to the tissue. In this experiment the usefulness of the three containers for the induction of flower buds was compared. The results in Table 1 show that the type of culture container and the method of sealing exerts a pronounced effect on the growth and morphogenesis of epidermal explants. In Plastic petri dishes only 8% of the explants produced flower buds and the number of flower buds per explant never exceeded one. In 100 ml conical flasks 45 to 77% of the total explants produced flower buds whereas in boilding tubes 88% did so. The number of flower buds per explant was greatest in boilding tubes. The method of sealing of both conical flasks and boiling tubes was the same; both were plugged with polyurethane bungs which were externally covered with aluminium foil. The 9 cm Petri dishes used in this experiment were sealed with Parafilm.

The effect of light. The explants grown under light, irrespective of the duration of light period, were green and produced callus at one end. Callusing was very obvious in the explants which were grown in complete darkness. Nearly 50% of the dark grown explants produced callus all over their cut surfaces. Others produced callus masses at different places from which floral or vegetative buds made their appearance. All dark grown explants were yellowish white with plenty of callus. The pedicels of the induced buds were long and slender.

Table 4 shows that three types of buds were produced on the explants: floral buds, vegetative buds and undifferentiated buds. Floral buds arose from the axils of bracts and bore anther primordia which were clearly visible in very young buds. In vegetative buds apical dome was surrounded by several leaf primordia. The buds in which apical end was not rounded and well developed as in vegetative buds and in place of anthers or anther primordia 2-3 large foliar structures were present, were regarded as undifferentiated buds. Results show that continuous light was the most effective light regime for the induction of flower buds. It favoured the early emergence of flower primordia (10-14 days) when compared to the cultures which were kept under 16 hr light and 8 hr dark regime (15-20 days). The number of flower buds per explant was also greater on the explants which were kept under continuous light (Fig. 2). A similar effect was observed in the
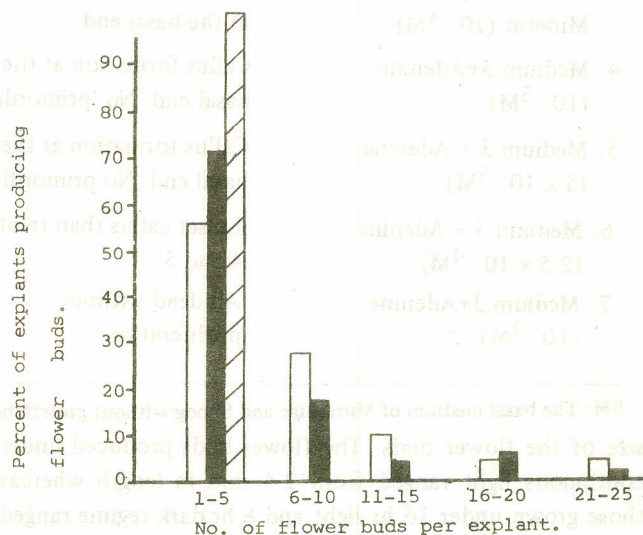

Fig. 2. The effect of light on the production of flower bud on epidermal explants. Solid bars – continuous light; open bars – 16 hours light/8 hours dark and striped bar – total darkness.

Table 2. The effect of GA_3 on the production of flower buds on eqidermal explants of *Nicotiana tabacum* (Virginica) grown under continuous light at 10 W/m² for 20 days.

Growth substances in the medium	Total no. of explants initially used	Total explant survived No.	Total explant survived %	Percent of surviving explants with floral buds No.	with floral buds %	With vegetative buds No.	With vegetative buds %	With undifferentiated buds No.	With undifferentiated buds %
IAA + K	72	69	96%	53	74%	23	32%	31	45%
IAA + K + GA₃	36	31	86%	16	52%	13	42%	7	23%

All growth substances were at 10^{-6}M concentration and were added before autoclaving the medium.

Table 3. The effect of different concentrations of adenine on flower bud formation on the epidermal explants of *Nicotiana tabacum* cv. Virginica grown on different media for 24 days, under continuous light.

Type of medium used	After 10 days of culture Morphological characteristics	After 24 days of culture		
		Mean no. of flower buds/ explant	Size of the biggest flower bud	Other characteristics
1. BM	Explants green. No callus. No primordia. Explants slightly proliferated	— —	— —	All dead
2. BM + Adenine (10^{-3}M)	All dead	— —	— —	All dead
3. BM + IAA (10^{-6}M) Minetin (10^{-6}M)	Cell proliferation. Callus at the basal end.	6.7	7 mm	Few vegetative buds along with floral buds
4. Medium 3+Adenine (10^{-5}M)	Callus formation at the basal end. No. primordia	5.3	0.5 mm	Few vegetative buds along with floral buds
5. Medium 3 + Adenine (5×10^{-5}M)	Callus formation at the basal end. No primordia	6.3	1 mm	Few vegetative buds along with floral buds
6. Medium 3 + Adenine (2.5×10^{-4}M)	Lesser callus than treatment 4 and 5	3.8	1 mm	Very little callus formation
7. Medium 3+Adenine (10^{-3}M)	All dead without proliferation	— —	— —	Very little callus formation

BM: The basal medium of Murashige and Skoog without growth hormones. Sucrose concentration 3% in all media.

size of the flower buds. The flower buds produced under continuous light ranged from 2-4 mm in length whereas those grown under 16 hr light and 8 hr dark regime ranged from 0.5 to 2 mm in length in three-week old cultures.

Rooting was favoured by darkness. 13% of the total surviving explants kept under darkness produced roots. The percentage of rooting explants decreased with the increase of light intensity, so that, under continuous light, less than 1% of the explants produced roots. All types of buds produced under complete darkness were yellowish white. This is also apparent from the results of Table 4 that light favoured differentiation of buds to either vegetative or floral type.

DISCUSSION

To ensure the maximum yield of flower buds *in vitro* two types of media were compared. The medium recommended by Tran Thanh Van [16] was the LS medium [8] which is a modification of the MS medium. In the results of the experiments presented in Table I both MS medium and a modification of it which lacks glycine, and vitamins (nicotinic acid and pyridoxine-HCl) were tested. The results showed that on complete MS medium a large proportion of explants produced flower buds; the number of flower buds per explant was also greater. The elimination of glycine and vitamins from this medium resulted in a marked decrease in flower bud formation. Tran Thanh Van [16] obtained flowering in 100% explants of *Nicotiana tabacum* cv. Wisconsin 38 on LS medium which also lacks glycine and vitamins. The reasons for the discrepancy between the present results and hers are not clear but different *Nicotiana tabacum* cultivars were involved.

There are contradictory reports in the literature about the effect of gibberellins on the formation of flower buds from excised parts of plants in culture. Excised stem segments of *Plumbago indica* show inhibition of flower bud formation as a result of GA_3 application [12], whereas stem segments of *Cichorium intybus* [14] show an increase in the frequency of flower bud initiation. Pierik [15] observed an inhibitory effect of GA_3 at high levels in *Lunaria* but a stimulatory effect at lower levels. In excised stem

segments of *Nicotiana tabacum* cv. Wisconsin 38 gibberellic acid decreased the number of flower bud formation *in vitro* [1]. In the same cultivar, Wardell and Skoog [18] observed a strong inhibition of formation of floral buds as a result of GA_3 application whereas further development of the already induced buds was stimulated.

Results presented in Table 2 show a decrease in the percentage of explants producing flower buds as a result of GA_3 application. Since only one concentration of GA_3 was tested, it is quite possible that the concentration of GA_3 used in the experiment was supraoptimal for the process and incorporation of higher or lower concentrations might have favoured the formation of flower buds. However, a beneficial effect of GA_3 treatment on the formation of vegetative buds is obvious from the results of Table 2.

A beneficial effect of purines and pyrimidines on flower induction had been reported by Khailakhyan *et al.,* [5] in *Perilla* stem apices cultured *in vitro*. Adenine which has been found to be essential for the formation of vegetative buds in *Plumbago* [11] also supported the formation of flower buds. A beneficial effect of adenine has also been reported by Nitsch and Nitsch [12]. In the present experiments (Table 3) the addition of adenine to the medium decreased the formation of flower buds on the epidermal explants. $10^{-3}M$ adenine was toxic to epidermal explants. They turned brown within a week and died.

From the results of the experiments (Table 1) on the influence of the type of culture vessel, it appears that the epidermal explants in culture build up an atmosphere which is not suitable for supporting flower bud formation. The high percentage of flower bud initiation on the explants cultured in boilding tubes and in the 100 ml conical flasks

compared with that found in plastic Petri dishes of 9 cm diameter shows that Petri dishes were almost a complete failure. The likely cause of this may be the seal of the Petri dishes which was gas-tight and the volume of the internal atmosphere, which was small. The volume of culture atmosphere in the polyurethane plugged tubes and flasks remains in gaseous contact with the external atmosphere virtually unlimited volume.

Day length is known to have a pronounced effect on the induction of floral buds in some intact plants. Some plants have an absolute photoperiodic requirement for the induction of flowering. Under non-inductive conditions flowering is either inhibited or is markedly decreased. It is of interest to note that even small pieces of excised stem are capable of being influenced by the photoperiodic regime. When excised stem segments in the vegetative phase of growth of *Cuscuta reflex,* a short-day plant, were subjected to different light and dark treatments, the explants behaved as typical short day plants. Flowering was maintained either in continuous darkness or exposed to 14 hr. of daily dark period [2]. Harada [4] also observed the formation of flowers on sections excised from the internodes of flower stalks of *Cichorium intybus,* a cold requiring, long day plant, under long day conditions whereas incubation under short day conditions produced little effect. On incubation of cultures in darkness for more than two weeks the tissues almost lost their ability to form flowers. In the same plant flowering was also induced in the tissues excised from roots and leaves which had been previously vernalized and the cultures were incubated in long days whereas only vegetative buds developed on explants which did not receive any vernalization treatment [13,10].

Table 4. The effect of different light regimes on the production of flower buds on the epidermal explants of *Nicotiana tabacum* (Virginica) after 20 days in culture.

Light regime	Total No. of explants	No. of explants survived	Percentage of surviving explants			
			With floral buds	With vegetative buds	With undifferentiated buds	With roots
Dark	48	(38) 79 %	(10) 26 %	(8) 21 %	(18) 57 %	(5) 13 %
16 hours light/ 8 hours dark	144	(127) 88%	(101) 80 %	(44) 35 %	(51) 40%	(2) 1.5 %
Continuous light	144	(138) 96 %	(117) 85%	(41) 30 %	(15) 1 %	(1) 0.7 %

In the Virginica cultivar of tobacco continuous light produced maximum flower induction in epidermal explants. Reduction in the photoperiod to 16 hr/day decreased the number of flower buds formed but enhanced the formation of vegetative buds, undifferentiated buds and roots. Explants grown in darkness showed a marked increase in the number of undifferentiated buds and roots. It is well known that root formation is favoured by darkness. The increase in the number of undifferentiated buds under a photoperiod of 16 hr/day or in complete darkness shows that in *Nicotiana* continuous light exerts a profound effect on the early determination or the emergence or of both of flower buds. In darkness the majority of the explants produced one or two flower buds. A few produced three and four flower buds per explant.

The formation of flower buds from excised stem segments of *Nicotiana tabacum* cv. Wisconsin 38 has been reported to be independent of any photoperiodic requirement. However, the initiation of meristems required light [1]. In the same cultivar flower induction was shown to be light independent [18], whereas flower development required light. In the present experiments (Table 4) formation of flower buds at all light intensities shows that at least *in vitro*, photoperiod is not an absolute requirement for the induction of flower buds from the stem segments of *Nicotiana tabacum* cv. Virginica. However, the number of explants producing flower buds and the number of flower buds per explant was markedly influenced by the presence of light. Tran Thanh Van [17] also observed a greater percentage of epidermal explants producing flower buds in light than in darkness.

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