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MORPHOGENESIS IN THE SUSPENSION CULTURE OF *NICOTIANA TABACUM* CV. VIRGINICA: THE EFFECT OF KINETIN

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Abstract

Suspension cultures of *Nicotiana tabacum* cv. Virginica established from epidermal tissue-derived callus on medium supplemented with $5x10^{-6}$M NAA alone or with $5x10^{-7}$ M kinetin when subcultured on auxin lacking morphogenetic medium containing kinetin, exhibited morphogenesis. The mode of regeneration was dependent on the concentration of kinetin. Low concentrations of kinetin favoured root and embryo-like structure development while high concentration of kinetin induced tightly aggregated clusters of shoots. The regenerated structures showed a general tendency towards a gradual increase in the number of shoots with a concomitant decrease in their size at increasing concentrations of kinetin. Histological study of the embryo-like structures revealed their resemblance with the zygotic embryos.

Introduction

Plant regeneration via somatic embryogenesis in tissue culture was first described in carrot by Reinert (1958) and Steward *et al*., (1958). Since then a large number of plants regenerating through this process have been reported (Tisserat *et al*., 1979; Sharp *et al*., 1980; Ammirato, 1983; Raghavan, 1986; William & Maheshwaran, 1986). The technique allows the production of a large number of true to type individuals in a relatively short time and its use in the synthetic seed industry confers its agricultural application. It also provides a shortest possible route for obtaining transformed plants from genetically modified cells.

The phenomenon involves two phases the induction phase and the morphogenetic phase (Khatoon, 1982; Stamp & Henshaw, 1987; Meijer & Brown, 1987; Baker & Wetzstein, 1992). The requirement of a relatively high concentration of auxin appears to be essential for the acquisition of totipotency during the first phase (Terzi & Lo-Schiavo, 1990), however, during the second phase it is either inhibitory for the embryogenic expression or allows it at a relatively low concentration (Fujimura & Komamine, 1975; Reynolds, 1986, Kageyawa *et al*., 1991). The role of cytokinins is still not very well defined. Experiments were therefore, carried out to study the effects of kinetin during both induction and morphogenetic phases, on cell proliferation and regeneration from somatic tissues of *Nicotiana tabacum* cv. Virginica.

Material and Methods

Plant material: The batch of *Nicotiana tabacum* cv. Virginica seeds, conditions of cultivation and isolation of epidermal explants were same as described by Khatoon (1985).
Culture medium: Murashige & Skoog (1962) medium with 30 g/l sucrose at pH 5.5, was used throughout the experiments. Growth hormones were mixed with the medium before autoclaving.

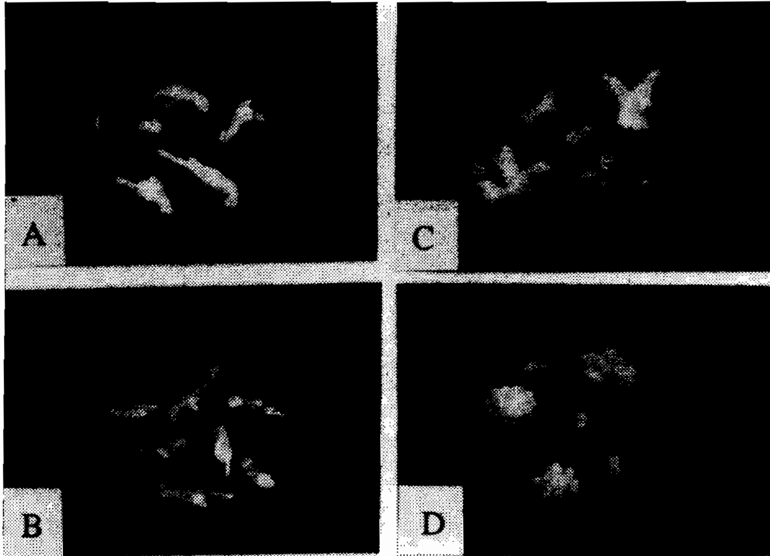

Fig.1 A-D. Development of roots, embryo-like structures and shoots from stock suspension in which induction medium contained only NAA ($5x10^{-6}M$). Morphogenetic medium contained kinetin. A= $5x10^{-7}M$, B= $10^{-6}M$ C= $2.5x10^{-6}M$, D= $5x10^{-6}M$. r, roots; e, embryo-like structure; s, shoot.

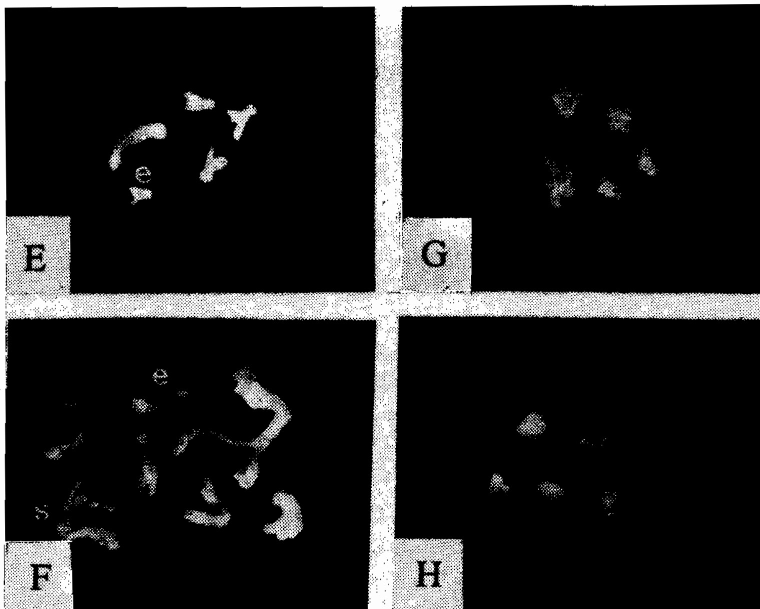

Fig.1 E-H. Development of embryo-like structures and shoots from stock suspensions in which kinetin ($5x10^{-7}M$) was present in the induction medium in addition to NAA ($5x10^{-6}M$). Morphogenetic medium contained kinetin. E= $5x10^{-7}M$, F= $10^{-6}M$, G= $2.5x10^{-6}M$, H= $5x10^{-6}M$, e, embryo-like structures; s, shoot.

Culture conditions: Stock suspensions were maintained in 250 ml Erlenmeyer flasks/containing 70 ml medium and test cultures in 100 ml flask containing 25 ml medium, unless otherwise stated, Inoculated flasks were mounted on a refrigerated shaker adjusted to 100 r.p.m. at 25°C, under 2.6 W/M^2 light from white fluorescent tubes.
Callus culture: Callus was induced on excised epidermal explants inoculated on 1% agar gelled medium containing 10^{-5} M NAA (Naphthaleneacetic acid). This callus was utilized as shown in the Flow sheet 1.
Suspension cultures: Stocks of primary suspension cultures were initiated from 6-8 week old actively growing green calli bearing roots. Roots were removed and cells chopped with a sterile scalpel before inoculation into the liquid medium. Two series of experiments were run from suspensions established in MS induction medium supplemented with 5×10^{-6}M NAA or with 5×10^{-6}M NAA plus 5×10^{-7}M kinetin. Test cultures established in media containing 5×10^{-7}M, 10^{-6}M, 2.5×10^{-6} and 5×10^{-6}M kinetin were each inoculated with 2 ml packed volume of cells and cell aggregates, collected by centrifuging the stock suspensions at 1000g for 10 minutes. Pellets were thoroughly washed prior to culture with fresh auxin free morphogenetic medium containing 5×10^{-7}M kinetin, for the induction of morphogenesis. Control cultures lacked both NAA and kinetin. To quantify regeneration, a standard culture period of 4 weeks was employed and observations were recorded under a stereo microscope. Regenerated structures were placed on filter paper and washed with water. Excess of water was removed by applying suction and total fresh weight recorded.

For histological study the somatic embryos were fixed by immersing them in formalin: glacial acetic acid: 50% ethanol (FAA, 5: 5: 90) for 24 hrs. Following dehydration in ethanol series the material was infilterated and embedded in commercial paraplast (Monoject Scientific, Saint Louis, MO., U.S.A.) in a hot air oven at 55°C. Microtome sections were cut (10 μm) and mounted on glass slides using Haupt solution (Johansen, 1940). The sections were stained sequentially in 1% w/v safranin dissolved in 50% alcohol for 1 h and 0.1% w/v fast green (Sigma Chemical Co., St. Louis, MO., U.S.A.) dissolved in 95% alcohol for 20-30 sec., and mounted in Canada balsam.

Results

Finely dispersed cells and cell aggregates were produced in stock suspensions established in the medium containing 5×10^{-6}M NAA. Incorporation of 5×10^{-7}M kinetin in a similar suspension resulted in 50% increase in fresh weight of the cellular units and stimulated increased aggregation. Cell aggregates were rounded compact organised structures.

Thoroughly washed stock suspensions initiated in the presence of 5×10^{-6}M NAA produced roots, shoots and embryo-like structures on transfer to the auxin-omitted morphogenetic medium containing 5×10^{-7}M, 10^{-6}M, 2.5×10^{-6}M or 5×10^{-6}M kinetin (Fig.1A-D). Morphogenesis did not occur in kinetin lacking control cultures. Roots always differentiated in the medium containing 5×10^{-7}M kinetin alongwith embryo-like structures. They were unbranched, usually 3-4 mm long and attached to a callus mass. Higher concentrations of kinetin were inhibitory for root differentiation. Embryo-like structures and shoots developed in morphogenetic media containing higher concentra-

Flow sheet 1. General lay-out of the experiments performed with cell suspension cultures established from calli induced on epidermal explants.

Primary callus culture	Calli intiated on epidermal explants in agar medium supplemented with NAA (10^{-5}M), in their first passage of growth.	
Stock suspensions (Induction phase)	Stock cell suspensions initiated in liquid medium supplemented with NAA ($5x10^{-6}$M).	Stock cell suspensions initiated in liquid medium supplemented with NAA ($5x10^{-6}$M) plus kinetin ($5x10^{-7}$M).
Test cultures (Morphogenetic phase)	Washed cells transferred to NAA-free medium containing kinetin as below	Washed cells and cell aggregates transferred to NAA-free medium containing kinetin as below
	$5x10^{-7}$M, 10^{-6}M, $2.5x10^{-6}$M, $5x10^{-6}$M	$5x10^{-7}$M, 10^{-6}M, $2.5x10^{-6}$M, $5x10^{-6}$M

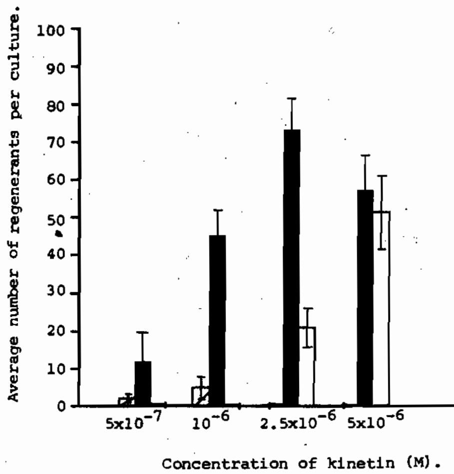

Fig.2. Total number of morphogenetic structures produced in suspensions as shown in Figs.1 A-D. ▨ mean number of roots per culture; ▮ mean number of embryo-like structures per culture; [] mean number of shoots per culture. Counts made after 4 weeks. Each point represents the mean of three cultures. Vertical bars represent the standard error of the mean.

tions of kinetin but their number showed separate peaks at $2.5x10^{-6}$M and $5x10^{-6}$M kinetin, respectively (Fig.2). Embryo-like structures were characterised by the presence of 2-4, usually 2, thick and fleshy when young, and well developed resembling first leaves, when grown up, foliar appendages attached to a long slender, smooth axis, terminating into a rounded basal end, with relatively less developed root pole. Elongation of the root was suppressed. Shoots were generally produced in clusters with individual shoots oriented to the exterior of the cluster. Elongation of embryo-like structures and shoots was inhibited at high levels of kinetin (Fig.1CD), as compared to the low levels (Fig.1AB) or in cultures which had been grown in the presence of kinetin during induction phase than their respective controls.

Stock suspensions which were initiated in the presence of $5x10^{-7}$M kinetin (also containing $5x10^{-6}$ NAA), produced embryolike structures and shoots on transfer to the auxin lacking morphogenetic medium containing $5x10^{-7}$M, 10^{-6}M, and $2.5x10^{-6}$M kinetin (Fig.1E-G). Roots were not produced. Kinetin $5x10^{-6}$M was inhibitory for morphogenesis and very few shoots differentiated (Fig.1H). The optimum concentrations of kinetin for the formation of embryo-like structures and shoots in their series, were lower than in the previous series (Fig.2) and their number was much greater (Fig.3).

Total fresh weight of the regenerated structures recorded after 3 weeks in the first series showed a maximum with 2.5^{-6}M kinetin. Doubling this concentration lead to decline in the number of regenerants. In four week old cultures fresh weight increased further in all treatments and particularly in those growing in medium containing $5x10^{-6}$M kinetin, where the increase was several fold (Fig.4). Maximum fresh weight of regenerated structures recorded after 3 week of culture was obtained in treatment containing 10^{-6}M kinetin. Higher concentrations yielded less fresh weight. During 4th week total fresh weight increased immediately owing to rapid development of shoots and the maximum was observed with $2.5x10^{-6}$M kinetin (Fig.5).

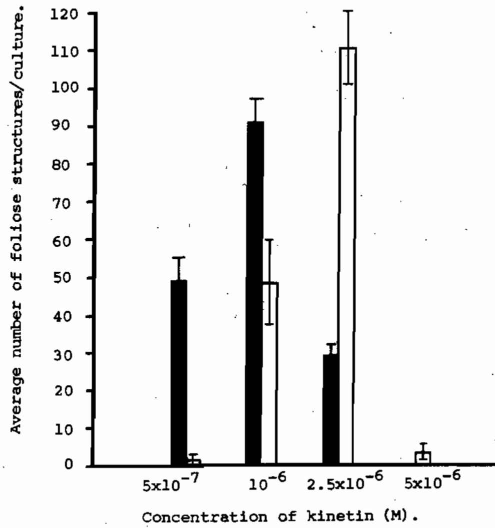

Fig.3. Total number of morphogenetic structures produced in suspensions as shown in Figs.1 E-H ▮ mean number of embryo-like structures; [] mean number of shoots per culture. Counts made after 4 weeks of culture. Each point represents the means of 3 cultures. Vertical bars represent the standard error of the mean.

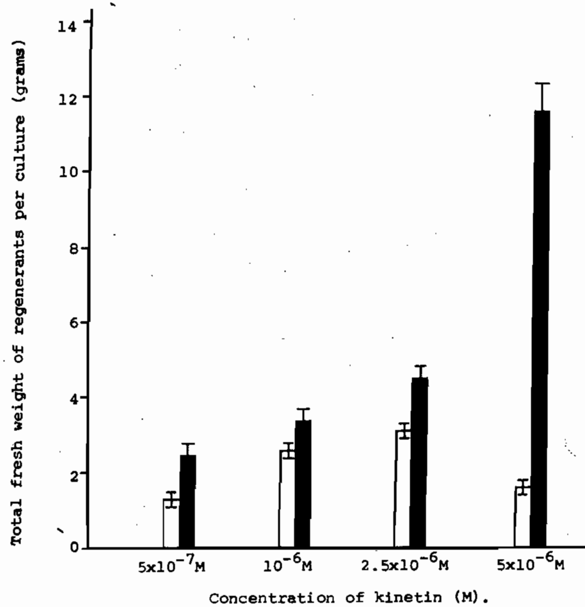

Fig.4. Total fresh weight of the regenerated structures produced in the experiment depicted by the data as shown in Fig.2. Each value represents the mean of 3 replicates. The vertical bars represent the standard error of the mean. [] 3 week old cultures: ▮ 4 week old cultures.

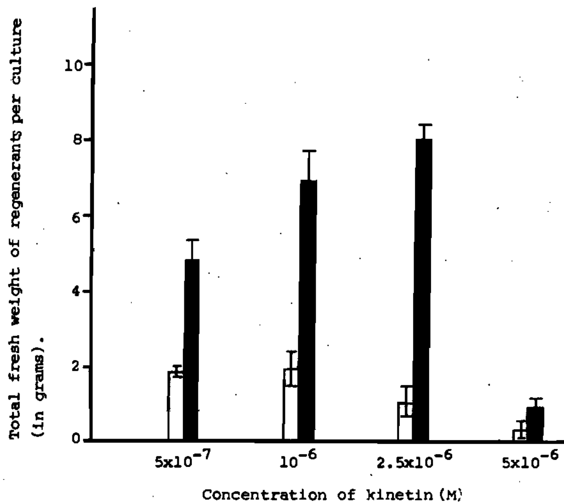

Fig.5. Total fresh weight of the regenerated structures produced in the experiment depicted by the data as shown in Fig.3. Each value represents the mean of 3 replicates. Vertical bars represent the standard error of the mean. [] 3 week old cultures; ▮ 4 week old cultures.

Longitudinal sections of the *in vitro* produced embryo-like structures revealed a distinct outer layer of the cells forming epidermis followed by a conspicuously vacuolated cortex and a well defined procambium, ends of which extended from the embryonic axis to the cotyledon primordia, characteristic of globular and more advanced stages of zygotic embryo development. They did not show a vascular connection with the surrounding tissues and possessed a rounded radicular end (Fig.6). Regenerated shoots showed prominent vascularization. Their basal ends were connected to the neighbouring shoots in the cluster through vascular tissues (Fig.7).

Discussion

Auxin omission from the cellular units was used for the induction of somatic embryogenesis. The process was divided into two phases; the induction phase, during which the cells and cell aggregates were grown in the presence of auxin and the morphogenetic phase during which the auxin was removed. Removal of auxin initiated organized growth and induced morphogenesis. Addition of different concentrations of kinetin to the morphogenetic medium influenced morphogenesis. Root development occurred only in those cultures which contained kinetin at low levels, whereas higher levels of kinetin favoured embryo-like structures and shoot morphogenesis (Fig.2). In the absence of kinetin, morphogenesis did not occur.

The cellular units which had been exposed to kinetin and auxin, during induction phase, exhibited a complete suppression of root development. However, a large number of embryo-like structures differentiated in the medium supplemented with low concentrations of kinetin and still higher concentrations favoured shoot development. Kinetin $5x10^{-6}$M suppressed morphogenesis (Fig.1H&5). The results suggest that sufficient kinetin was perhaps carried over despite prolonged and thorough washing of the cellular

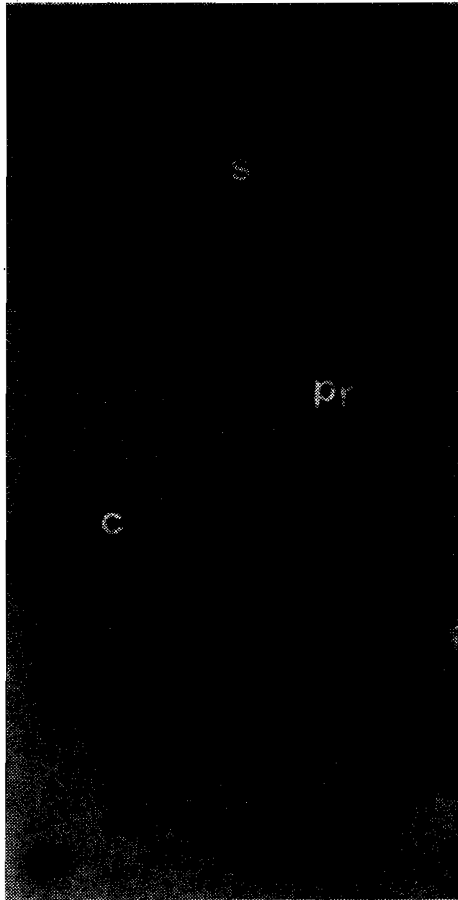

Fig.6. Section through a shoot cluster showing two shoots connected through a common vascular supply (vs). The other shoots are out of the plane of the section.

units before transfer to morphogenetic medium. This carry over of kinetin could also account for the observed shift in the optimal kinetin concentration for the production of embryo-like structures and shoots (Fig.3).

The embryo-like structures and shoots appear to constitute two distinct populations as is exhibited by two peaks formed by these structures (Fig.2&3). The formation of embryo-like structures and shoots under similar culture conditions may possibly be attributed to the heterogenity of cells of the epidermal explants. The present study has also indicated that the addition of kinetin to the induction medium is not essential for the induction of somatic embryogenesis in the suspension cultures of *Nicotiana tabacum* cv. Virginica. However, its addition to the medium increases their number and this may be due to increased growth and aggregation of the suspensions in the presence of kinetin as also reported for *Atropa belladonna* (Thomas & Street, 1970).

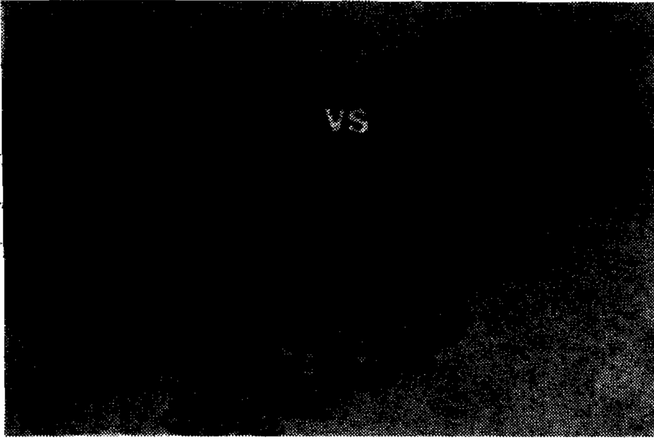

Fig.7. An embryo-like structure with well-defined procambium (pr) and cortex (c) made up of highly vacuolated cells. r, root pole; s, shoot pole.

Histologically, *in vitro* produced embryo-like structures possessed tissue zonations characteristics of zygotic embryos. Premature vacuolation of cortical cells is suggestive of the onset of precocious germination of *in vitro* produced embryos. The absence of vascular connection with the surrounding tissues and the presence of closed radicular end confirms their embryogenic nature (Terzi & LoSchiavo, 1990).

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