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Full Length Research Paper

Explant autonomy in Indian tobacco cultivars under *in vitro*

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Hormonal autonomy in leaf explants of 15 Flue-cured Virginia tobacco varieties grown under Indian conditions was studied. Most of the varieties responded in 19-30 days on MS basal media. Multiple shoot initiation was observed in cultivars Hema, FCV Special and Rathna, while rhizogenesis was observed in Kanakaprabha, VT 1158, CM 12 and Kanthi. These results indicated the hormonal autonomy of the tobacco cultivars. Most of the varieties that showed different types of organogenesis on MS basal media showed callus initiation on callus media indicating that hormone supplement in the *in-vitro* media regulating the endogenous hormone levels in the explants or the growing tissues. The study suggested that varieties, Jayasri, VT-1158 and Kanakaprabha have inherited hormonal autonomy from their ancestral variety Delcrest. Hema leaf explants that gave multiple shoots on basal media showed callus induction on auxin (NAA) supplemented MS media indicating cytokinin autotrophy of its explants and their balance through the addition of auxin. Jayasri that gave rooting on MS basal media, produced hairy roots on MS media supplemented with NAA suggesting the synergic effect of supplemented NAA on the already available free auxin in its explants. Cultivars adapted to reduced moisture conditions showed hormonal autonomy indicating the possibility of using this trait for the selection of stress tolerant lines.

Key words: Hormonal autonomy, tobacco, tissue culture.

Abbreviations: MS media; Murashige and Skoog media, **FCV**; Flue-cured Virginia.

INTRODUCTION

Organogenesis in the form of shoot or root from somatic cells of an explant under tissue culture is a complex phenomenon involving the subtle synergistic interaction of several factors. Culture conditions like composition of the nutrient media, level of phyto-hormones and physical factors like temperature, humidity, light, aeration etc are the major factors that control organogenesis. When all the physical and chemical factors remain constant, the ratio of cytokinins and auxins play a key role in determining the type of organogenesis response. Hormones are chemical substances that induce differentiation and growth of plantlets under *in vitro*. Higher concentrations of auxins compared to cytokinines promote rooting and its contrary results in shoot initiation and their equal amounts lead to unorganized growth of tissue (callagenesis). As plants can naturally synthesize

hormones, the combined effect of internally available and externally supplemented hormones determine the type of organogenic response under *in-vitro*.

The genus *Nicotiana* is an ideal test system for studying the basic mechanism of hormonal autonomy of explants (Kumari, 2002). The word hormonal autonomy refers to the ability of the plant to produce callus and organs under *in vitro* conditions without hormonal supplement. Several interspecific hybrids of *Nicotiana* producing genetic tumors were reported (Kehr and Smith, 1957). Abnormal phytohormone relationships have been considered as the cause to such genetic tumors in interspecific hybrids of *Nicotiana* as they have inherent tendency to synthesize and accumulate greater than regulatory amounts of growth hormones (Smith, 1972). Such timorous hybrids reported to show autonomy for hormones under culture condition. Although a number of studies have so far been conducted on the hormonal autonomy of interspecific hybrids (Schaeffer and Smith,1963; Kumari 2002), hormonal autonomy of the explants of cultivated tobacco has not been probed so far. In view of this an attempt has been made to investigate the hormonal autonomy of cultivated *N. tabacum* lines.

In the present investigation, hormonal autonomy of leaf explants of 15 Flue-cured Virginia (FCV) tobacco cultivars grown under Indian conditions was studied. Source of autonomy was investigated in a cultivar, Jayasri. Interacting influence of varying concentrations of Murashige and Skoog (basal) media along with the phytohormone NAA, a major root inducing auxin on the leaf explants of tobacco varieties that showed hormone autonomy (different types of organogenesis) was studied to under stand the type of hormonal autotrophy. Such study helps to understand the explant autonomy and phytohormone role in organogenesis.

MATERIALS AND METHODS

Biological material

Leaf expalnts of 15 Indian FCV tobacco varieties; namely Chatham, Kanakaprabha, Dhanadayi, Hema, VT-1158, Gauthami, Kanthi, FCV Special, Swarna, Bhavya, Kanchan, Rathna, Mc Nair 12, 16/103 and CM-12 were used in the present study. First seven varieties were grown in Vertisols of Andhra Pradesh under conserved soil moisture conditions. The rest of the varieties were being grown in Alfisols under irrigated or assured rain fall areas of Andhra Pradesh and Karnataka.

In order to identify the source of hormonal autonomy trait in *N. tabacum* cv. Jayasri, leaf explants of Jayasri and its tobacco mosaic virus resistant version Jayasri (MR) along with its parents were used. Delcrest, Hicks and CTRI special are the main parents involved in the pedigree of Jayasri. Hence, leaf explants from these three lines were used in identifying the hormonal autonomy trait in Jayasri.

Nutrient medium

Basal nutrient medium formulated by Murashige and Skoog (1962) commonly known as MS was used to study the hormonal autonomy. The MS callus medium was prepared by supplementing MS basal media with NAA (1.5 mg L^{-1}), IAA (1.5 mg L^{-1}) and 2, 4-D (0.25 mg L^{-1}) for callus induction. Both the culture media had 3% sucrose as the carbohydrate source and 0.8% agar as solidifying agent. The pH of the medium was adjusted to 5.8 using 1 M potassium hydroxide solution or hydrochloric acid solutions. The flasks containing culture media were autoclaved at 121°C under 15 lb pressure for 20 min.

MS basal media of full, half and quarter strength, with or without NAA @ 2 mg L^{-1}, were used to study the response of tobacco varieties; namely Hema and Jayasri to the interacting effect of NAA with various concentrations of MS basal media.

Inoculation of explants

Well growing leaves from healthy plants were collected from 60 days old nursery. The leaves were initially washed thoroughly with running tap water and subsequently sterilized with 0.1% $HgCl_2$ for l to 2 min. Traces of the sterilant were washed off by thorough rinsing of the leaves with double distilled water. The leaves were then cut into 1 cm^2 pieces with the help of a sterilized scalpel and inoculated on to the appropriate medium placing their ventral or dorsal sides in contact with the medium. All culture vessels with inoculum were incubated in the culture room at 25±10°C, 16/8 h day and night cycles and 80% relative humidity (RH). Observations recorded on number of days taken for response and type of response.

RESULTS

Response of different tobacco varieties to basal media

The leaf explants showed response in 19-30 days (Table 1). Among the varieties, the response was faster in Kanthi (19 days) and slower in FCV Special and CM 12 (30 days). Multiple shoot initiation was observed from the leaf bits of Hema (Figure 1A), FCV Special and Rathna (Figure 1B). Rooting was observed in Kanakaprabha, VT-1158 (Figure 1C), CM 12 (Figure 1D) and Kanthi, and less amounts of callus in Chatham and Dhanadayi. In most of the explants the roots emerged from veins and

Table 1. Response of tobacco varieties to hormone free MS medium.

S.No	Varieties	Number of days taken for response	Type of response observed from margins of explants after 30 days of inoculation
1.	Chatham	23	Less amount of callus
2.	Kanakaprabha	24	Root initiation
3.	Dhanadhayi	24	Less amount of callus
4.	Hema	23	Multiple shoot initiation
5.	Gauthami	24	Less amount of callus
6.	VT-1158	22	Root initiation and later complete plantlet developed.
7.	FCV Special	30	Multiple shoot initiation
8.	Swarna	NR [a]	--------
9.	Bhavya	NR	--------
10.	Kanchan	NR	--------
11.	Rathna	22	Multiple shoot initiation and faster multiplication of shoots
12.	Mc Nair 12	NR	--------
13.	16/103	NR	--------
14.	CM-12	30	Hairy root Initiation and later shoot initiation
15.	Kanthi	19	Hairy root initiation and later multiple shoot initiation at the site away from root initiation

[a] NR—No response

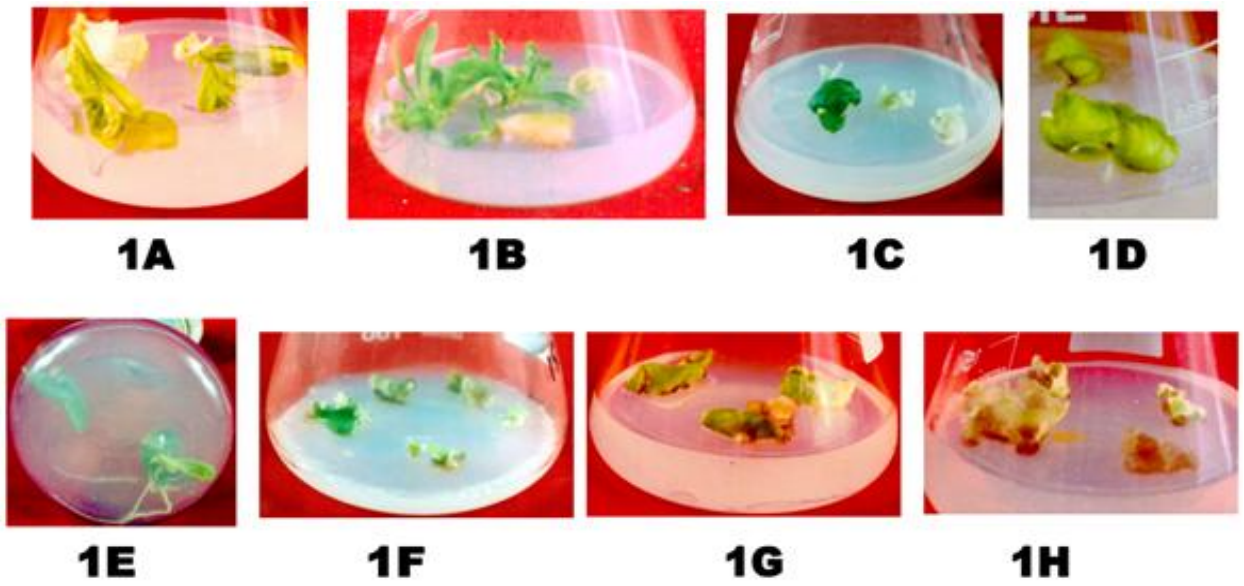

Figure 1. Response of leaf explants of tobacco varieties to hormone free MS media (1A -1E) and MS callus media (1F-1H). 1A and 1B shows shoots developing from the explants of Hema and Rathna on MS (b), respectively. 1C and D shows rooting from the explants of VT 1158 and CM 12 on MS (b). 1E shows development of roots and shoots in the explants of Kanthi from different regions on MS (b). 1F-H shows callus development from leaf explants of Hema, Swarna and Rathna on MS (callus), respectively.

the growth of roots was fast. Initial rooting followed by shoot induction observed in VT 1158, CM 12 and Kanthi (Figure 1E). In VT 1158 a complete plantlet with shoot and root was observed.

Response of different tobacco varieties on Callus media

The time for callogenesis in different varieties (Figure 1F-

Table 2. Response of tobacco varieties to MS callus medium having NAA (1.5 mg l^{-1}), IAA (1.5 mg l^{-1}) and 2, 4-D (0.25 mg l^{-1}).

S.No	Varieties	Number of days taken for response	Type of organogenesis observed after one month of inoculation
1.	Chatham	18	Callus
2.	Kanakaprabha	19	Callus
3.	Dhanadhayi	19	Callus
4.	Hema	18	Callus
5.	Gauthami	16	Callus
6.	VT-1158	17	Callus
7.	FCV Special	17	Callus
8.	Swarna	8	Callus
9.	Bhavya	11	Callus
10.	Kanchan	22	Callus
11.	Rathna	16	Callus
12.	Mc Nair 12	NR[a]	--
13.	16/103	NR	--
14.	CM-12	17	Callus
15.	Kanthi	14	Callus

[a]NR—No response

Table 3. Response of *N. tabacum* cv. Jayasri, its derivative Jayasri (MR) and parental leaf explants to hormone free MS medium and MS callus medium having NAA (1.5 mg l^{-1}), IAA (1.5 mg l^{-1}) and 2, 4-D (0.25 mg l^{-1}).

S.No	Varieties	Hormone free MS medium	MS callus medium
1.	Jayasri	Rooting and callus	High quantities of callus
2.	Jayasri (MR)	High amounts of callus	Less quantity of callus
3.	Delcrest	Rooting and callus	Less quantity of callus
4.	Hicks	Less quantities of callus	High quantities of callus
5.	CTRI special	High amounts of callus	High quantities of callus

1H) varied from 8 to 22 days after inoculation, while two varieties Mc Nair 12 and 16/103 did not show callusing (Table 2). Callusing efficiency was highest in Swarna followed by Bhavya, Rathna, FCV Special, CM-12 and Kanchan.

Hormonal autonomy trait in N. tabacum cv. Jayasri, its parents and derivative

On basal medium, rooting was observed in most of the leaf explants of Jayasri (Figure 2A) and Delcrest (Figure 2B) (Table 3). In both these lines, roots observed from veins of leaf explants. Fast growing callus observed from the explants of CTRI Special (Figure 2C) and Jayasri (MR) (Figure 2D), and slow growing callus from Hicks.

All the varieties recorded callogenesis in the callus medium. However, the amount of callus developed on MS(c) varied with varieties. The callus production was extensive in Jayasri, Hicks and CTRI Special while it was low in Jayasri (MR) and Delcrest.

Response of Hema and Jayasri to varying concentrations of MS basal media supplemented with NAA

Hema that gave multiple shoots (may be due to higher endogenous cytokinin levels) and Jayasri rooting (may be due to higher endogenous auxin levels) on MS basal medium were selected to study the effect of NAA (2 mg L^{-1}) with varying concentrations of MS basal (full, half and quarter) media. Explants of both the varieties showed no response on half-basal and quarter-basal media (hence, data not presented). In the media supplemented with NAA, the lines showed callus induction and hairy root formation (Table 4). However, Hairy root formation was more in Jayasri (Figure 3A-3C) and callus induction was

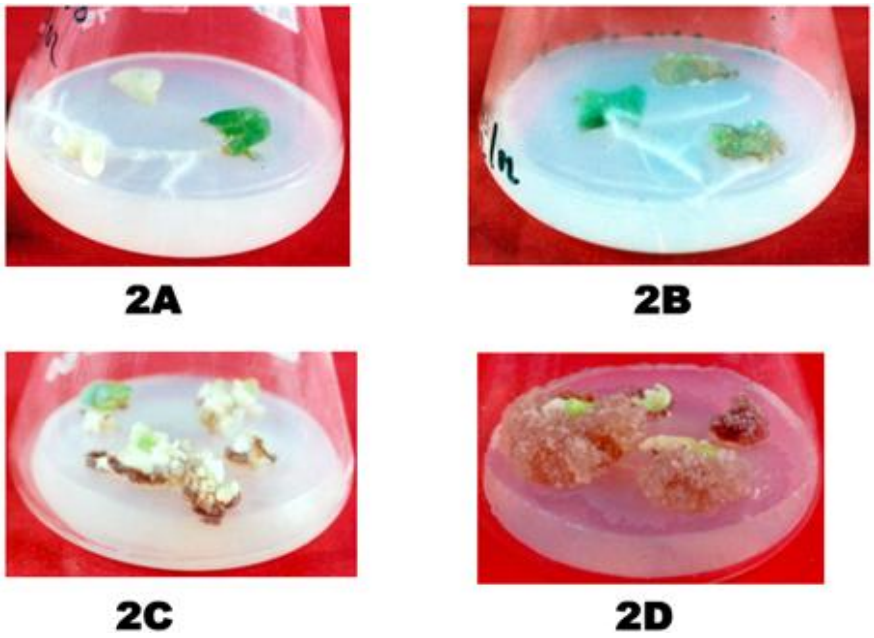

Figure 2. Response of Jayasri (2A), its parents Delcrest (2B) and CTRI Spl (2C), and derivative Jayasri (MR) (2D) on hormone free MS medium. 2A and 2B shows leaf explants of Jayasri and its parent Delcrest developing roots on MS (b) medium. 2C and 2D shows leaf explants of CTRI Spl. and Jayasri (MR) developing callus on MS (b) medium.

Table 4. Response of Hema and Jayasri to varying concentrations of MS basal media supplemented with NAA.

S.No	Name of the Variety	Media	No.of days taken for response	Response
1	**Hema**	MS(b) + NAA @ 2mg l^{-1}	18	Fast growing callus and few hairy roots.
		½ MS(b) + NAA @ 2mg l^{-1}	15	Low callusing; less number of hairy roots.
		¼ MS(b) + NAA @ 2mg l^{-1}	21	Low callusing; less number of hairy roots.
2	**Jayasri**	MS(b) + NAA @ 2mg l^{-1}	13	Normal callusing and hairy root formation.
		½ MS(b) + NAA @ 2mg l^{-1}	12	Very little callus and clusters of fast growing thin hairy roots.
		¼ MS(b) + NAA @ 2mg l^{-1}	21	Less callusing and robust hairy roots. Number of roots is lesser then above

more in Hema (Figure 3D-3F). Both the varieties produced more number of hairy roots on half-basal media supplemented with NAA and growth of these roots was also faster compared to basal and half-basal hormone supplemented media.

DISCUSSION

In the present study, ten out of fifteen varieties showed organogenesis or callogenesis response on hormone-free MS basal media, thereby indicating the hormonal

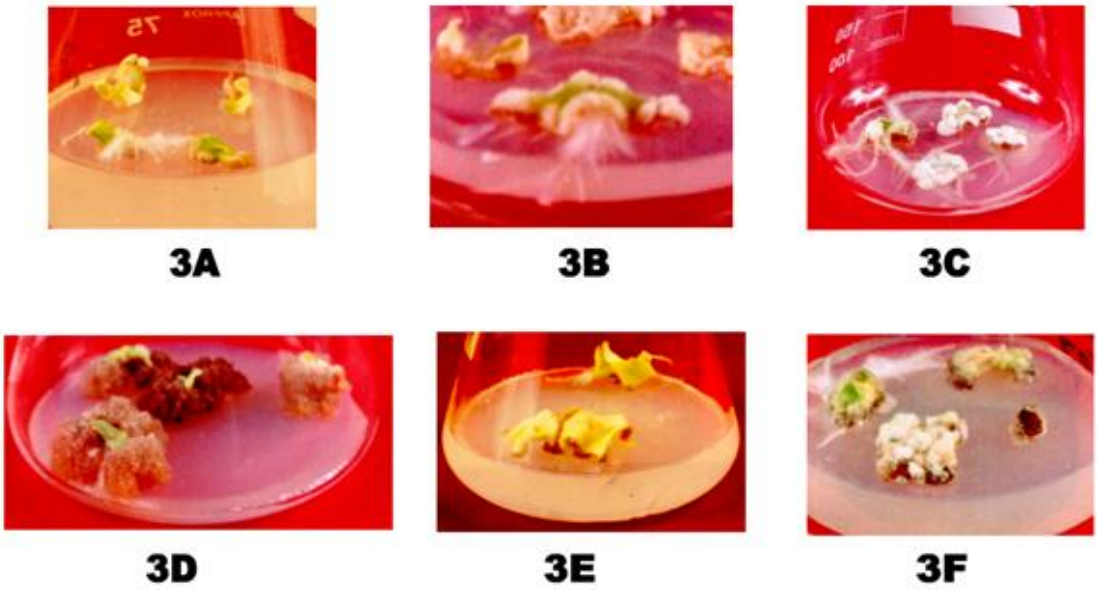

Figure 3. Response of Jayasri (3A-3C) and Hema (3D-3F) leaf explants to three different strengths of MS media supplemented with NAA (2 mg/l). 3A shows development of callus and hairy roots from Jayasri explants on NAA supplemented MS media. 3B shows development of callus and hairy roots from Jayasri explants on NAA supplemented ½ strength MS media. 3C shows development of callus and hairy roots from Jayasri explants on NAA supplemented ¼ strength MS media. 3D shows development of callus & hairy roots from Hema explants on NAA supplemented MS media. 3E shows development of callus and hairy roots from Hema explants on NAA supplemented ½ strength MS media. 3F shows development of callus and hairy roots from Jayasri explants on NAA supplemented ¼ strength MS media.

(auxin/cytokinin) autonomy of explants. These results for the first time clearly showed autonomous nature of cultivated tobacco lines to hormones under *in vitro* conditions. The differential response could be explained by differences in levels of endogenous hormones (Schroder, 1985). Skoog and Miller (1957) first observed that the ratio of auxins and cytokinins determines the *in-vitro* organ differentiation in *N. tabacum*. Those varieties showing root initiation may be autotrophic for auxins and those showing shoot initiation are autotrophic for cytokinins. Most of the cases, the tobacco varieties adapted to cultivation in Vertisols under conserved soil moisture condition or drought tolerant varieties grown under rainfed condition found to be autotrophic for hormones. Availability of free auxins in the cells may be making these varieties suitable to rainfed conditions. Increased rooting efficiency may be helping these cultivars to absorb water efficiently from deeper layers. Contrary to this varieties, namely Swarna, Bhavya, Kanchan, Mc Nair 12 and 16/103 that are grown in Alfisol (light soils) either under irrigation or under assured rainfall did not show any response.

The phytohormone autonomy of tobacco lines may be due to presence of different lengths of T-DNA of *Agrobacterium tumefaciens* in their genomes due to random genetic transformation in the course of evolution of the genus *Nicotiana* (Furner et al., 1986). Another reason for free phytohormne levels may be due to expression of certain genomic genes that are highly homologous to those of the T-DNA of Ri-plasmids, with subsequent overproduction of phytohormones and/or to elevation in sensitivity to endogenously supplied phytohormones (Takanari and Kunihiko, 1991). Mechanical wounding may also trigger production of phytohomones in explants (Hagen, 1969). Takanari et al. (1989) also recorded increase in the level of endogenous IAA in the stem segments of seedlings of F_1 (*Nicotiana glauca* × *N. langsdorffii*) seedlings from 3.4 ng g^{-1} fr wt to 96 ng g^{-1} fr wt or more, followed by their subsequent decrease to 9.1 ng g^{-1} fr wt by 11 days. Black et al. (1994) demonstrated that mutations in cytokinin biosynthesis locus (tmr) of *Agrobacterium tumefaciens* induce tumors exhibiting rooty phenotype. Initial rooting followed by shoot induction in VT 1158, CM 12 and Kanthi indicate temporal changes in phytohormone levels during *in-vitro* culture.

The level of plant growth regulators in the callus medium and the explant regulates callus formation. Callus induction occurs, if the ratios of exo and endogenous hormones are balanced under culture condition. Except Mc Nair 12 and 16/103, all the cultivars showed callogenesis on standard callus media. The callusing efficiency was more in varieties, namely Swarna, Bhavya, Rathna, FCV special, CM-12 and

Kanchan that are adopted to Alfisols. Induction of callus on callus media, in most of the varieties having different levels of cytokinis and auxins as inferred based on the response of their explants on MS basal medium, indicates that NAA, IAA and 2,4-D in the media are regulating the endogenous hormone levels in the explants or the growing tissues, through their interactions.

Rooting in Delcrest and its absence in other parents of Jayasri indicated that Jayasri might have acquired this character from Delcrest, while the trait was lost in its tobacco mosaic resistant version Jayasri (MR). In the present study, two other derivatives of the Delcrest, namely VT 1158 and Kanakaprabha seems to have inherited hormonal autonomy trait from it. Jayasri, its parents and derivative responded to *in-vitro* callogenesis, even though the quantities and rate of growth of callus varied among lines. This may be due to interacting effect of exo-and endogenous hormone levels.

On different strengths of MS media suplemented with NAA, Jayasri recorded higher hairy root formation and Hema higher callus induction. Callus induction due to addition of auxin in Hema explants suggests the high cytokinin content of the leaf explants, which might have been balanced through the addition of auxin, NAA. Increased rooting ability in Jayasri in NAA supplemented media may be due to the enhancing effect of NAA on its auxin rich explants. Production of more and fast growing hairy roots on half-basal media supplemented with NAA than full and quarter basal media supports the well-known practice of using MS half basal media for rooting in many crops including tobacco.

Conclusion

Response of tobacco explants to MS basal media in the present study for the first time clearly indicated the hormonal autonomy of some of the tobacco cultivars. The study also suggested the genetic nature of the trait. Response of Hema and Jayasri explants to MS media supplemented with NAA further proved that the cytokinin/ auxin autonomy of tobacco cultivars. Hormonal autonomy of explants of the cultivars adapted to reduce moisture conditions suggests the possibility of using this trait for the *in- vitro* selection of lines suitable to these areas.

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