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MORPHOLOGICAL VARIATIONS AND TUMORIGENESIS IN *N. GLAUCA* X *N. LANGSDORFFII* HYBRID AND ENHANCED GROWTH RESPONSE UNDER DNA HYPERMETHYLATED CONDITIONS

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Neoplastic growth was reported at the time of flowering from *N. glauca* x *N. langsdorffii*, the interspecific genetic tumorous hybrid of *Nicotiana*. The progeny of the hybrid plants behaved differently under different set of environmental conditions i.e., they showed segregation for tumorous and nontumorous plants when grown under green house conditions but developed variable levels of morphological aberrations in roots, leaves, stem and reproductive behavior under field conditions. Further, the percentage of plants that produced tumors are more after flowering than the plants before flowering and as the plants grew older many of the plants turned tumorous. Since variations are caused by changes in the levels of DNA methylation due to changes in the environment, the response of the seed and leaf discs to cefotoxime, an antibiotic known to cause genome wide hypermethylation in *Nicotiana tabacum* was studied. Seed treatment resulted in early tumor expression and led even to death of the plants whereas the treated leaf discs showed early growth response than their controls under culture conditions. These results coupled with 5-azacytidine treatment experiments were considered for explanation. The changes in regulation pattern of DNA methylation in hybrids when compared to parents may be responsible for tumor induction and phytohormone independent growth.

Keywords: Callus; Cefotaxime; DNA hypermethylation; Morphology; Tumorigenesis.

Introduction

In plants, the tumors usually develop because of the infection caused by viruses, microorganisms or insects but they arise spontaneously in some interspecific hybrids of *Nicotiana*. The potential to develop a tumor depends on the genetic constitution of the plant. Kostoff reported the genetic tumors of *Nicotiana* for the first time[1], later extensive investigations and thorough studies were done in this area[2-7]. Some species of *Nicotiana* like *N. langsdorffii, N. glauca, N. rustica, N. alata* were considered to be most powerful tumor producers[8]. Naf[9] divided the parents of interspecific hybrids into plus and minus groups. A cross between a plus and a minus group would be tumorous rather than between minus and minus group or plus and a plus group. Exposure to certain chemicals like turpentine, mercaptoethanol, nucleic acid analogs like azauracil[10], 5-azacytidine[11] caused early expression of tumors and severe morphological aberrations. Further, high temperature 24-27°C[12] and seedling over crowding enhanced tumorigenecity[13].

DNA methylation plays an important role in the maintenance of transcriptional activity. Altered methylation patterns both hypo and hypermethylation result in inappropriate gene expression or silencing and this phenomenon is common in cancers. Increase in cytosine methylation is generally responsible for transcriptional quiescence[14] but sometimes it can also be associated with increased gene activity[15].

Present study is focused on the environment influenced morphological variations and tumorigenesis in the *N. glauca* x *N. langsdorffii* hybrid and the consequences of cefotoxime induced DNA hypermethylation on seed and leaf disc treatments of the hybrid.

Material and Methods

The material used are seeds of *N. glauca, N. langsdorffii*, their hybrid and Izard mutant (provided by Dr. G.R.K.Sastry, University of Leeds, U.K from the plants grown under green house conditions i.e., in pots at 25°C with 16 hr light/8 hr dark photoperiod). The parents, advanced generations of the hybrid plants and Izard (the nontumorous mutant of the hybrid) were grown in pots at experimental farm, Andhra University, Visakhapatnam, India.

Hybridization between N. glauca and N. langsdorffii: The parents flowered between November and January. Hybridization was done by dusting the pollen of *N. langsdorffii* on to the stigma of *N. glauca*. Fifty five

crosses were done, the capsules were dried and the seed were collected. The seeds were kept for germination.

Morphological variations and tumorigenesis: They were studied for fresh hybrid plants (one generation) and advanced generations of hybrid (five consequent generations) grown under field conditions. The fresh hybrid plants flowered but did not set seed hence rest of the study was continued with the advanced generations of the hybrid.

Treatment of seed with cefotaxime: The surface sterilized seeds of *N. glauca, N. langsdorffii* and the hybrid (300 each) were placed on sterile filter papers soaked with cefotaxime (500 mg/l), an antibiotic known to induce genome wide hypermethylation in *Nicotiana tabacum* cultures[16] in different petridishes. They were kept at 25 ± 2°C in dark for 5 days and then transferred to 16 hr light/8 hr dark photoperiod. The seeds were transferred every day to a fresh filter paper containing a fresh cefotaxime solution. After germination the seedlings were carefully transferred to 250 ml conical flasks containing 50 ml of MS basal medium[17] supplemented with cefotaxime. The plantlets were transferred to fresh cefotaxime medium every week. After four weeks half of the cefotaxime treated plants of each of *N. glauca* and *N. langsdorffii* and the hybrid were continued to grow on MS supplemented with cefotaxime and the rest were grown under field conditions. The observations were recorded for 3 months in culture and 8 months in field. The experiment was repeated thrice.

Treatment of leaf discs with cefotaxime: The surface sterilized leaf discs were collected from fresh young leaves of *N. glauca* (400 discs), *N. langsdorffii* (400 discs), and the hybrid (400 discs) plants. Out of these for each plant 100 discs were incubated on MS basal medium, 100 discs on MS basal medium supplemented with phytohormones, (NAA 1 mg/l and kinetin 0.1 mg/l) and 200 discs on MS supplemented with cefotaxime (500 mg/l). The leaf discs were incubated at 25± 2°C in dark for a week and then they were transferred to 16 hr light/8 hr dark photoperiod. The leaf discs on MS supplemented with cefotaxime were changed to a fresh medium every week and the discs on MS basal medium and MS supplemented with phytohormones were subcultured for every 21 days. The observations were recorded for 60 days. The experiment was repeated thrice.

Result and Discussion

The morphology of the parents and the advanced generations of hybrid plants grown under field conditions were shown in Fig. 1. The shape of the leaf and that of the flower, the size and shape of the pod are intermediate between the two parents.

Hybridization between N. glauca and N. langsdorffii: 55 crosses were done and 20 were successful. Only 10% (50 out of 500) of the hybridized seeds germinated. They were allowed to grow on MS basal medium for propagation but it was observed that all the plants developed tumors within 45 days after germination (Fig. 2a). Fifteen plants were grown in the field but they too developed tumors, flowered but did not set seed (Fig. 2b). Thus the hybrid developed could not be maintained.

Morphological variations of the hybrid: The plants grown under green house conditions in UK segregated for tumorous and nontumrous plants, did not show so divergent morphological behavior and tumorigenesis was observed mostly at the time of or after flowering. When the progeny of these segregated plants and the fresh hybrid plants were grown under field conditions of Botany Experimental farm, Andhra University the following abnormal morphological behavior was observed. Plagiotropic bulbus roots grown above the ground (Fig. 3a), dwarf stems, very short swollen internodes, sudden wilting of the terminal bud, necrosis of young leaf (Fig. 3b) and development of lateral buds into multiple shoots after necrosis of terminal bud (Fig. 3c). Leaf morphology also changed, showing very thick leaves with distortions (Fig. 3d) like wrinkling, curling, waving and blister like structures on the leaf lamina (Fig. 3e). Other changes include floral variations like distorted flower shapes with fused stamens and stigma, physiological variations like variations in plant height ranging from 0.5 ft to 8 ft in the same generation (Fig. 3f), wilting of the whole plant (Fig. 3g) and partial to complete sterility of the flowers resulting in less or no seed set. These variations were not expressed to the same extent in all the generations but they differed from generation to generation and even from plant to plant within the same generation (Quantitative data not shown).

Tumorigenesis: The plants did not show segregation as tumorous and nontumorous plants. The nontumorous mutant of these hybrids called lzard when grown under field conditions neither developed tumors nor set seed and hence cannot be maintained. Out of three plants that survived till maturity, one produced three shoots showing loss of apical dominance flowered but failed to set seed, one died by necrosis and abrupt wilting and the other flowered but did not set seed. Among the advanced generations of the hybrid plants the tumorigenecity increased and the capacity to set seed and seed viability were greatly decreased. The proportion of tumorous plants increased from 25% to 86% and the rate of flowering decreased from 70% to 18%, from 1st generation to 5th generation studied (Table 1). The tumors developed in the hybrids were teratomatous - mass of tissue with multiple shoots (Fig. 4a). Tumors were developed on many parts of the plant like hypocotyl region (Fig. $4b_1$), roots (Fig. $4b_2$), stem, surface of the leaf lamina (Fig. $4c_1$), axil of

Table 1. Data on tumorigenesis and flowering of the hybrid plants grown under field conditions for the five generations.

No. of generations	No. of Plants	Tumor appearance					Flowering		
		Yes	No	% of tumorous plants	Time		Yes	No	% of flowered plants
					Before flowering	After flowering			
1st	150	38	112	25	9	29	106	44	70
2nd	200	84	116	42	22	62	145	55	72
3rd	300	179	121	60	38	141	202	98	67
4th	250	180	70	72	54	126	63	187	25
5th	250	216	34	86	80	136	46	204	18

Table 2. Effect of cefotaxime on tumor expression of *N. glauca, N. langsdorffii* and the hybrid.

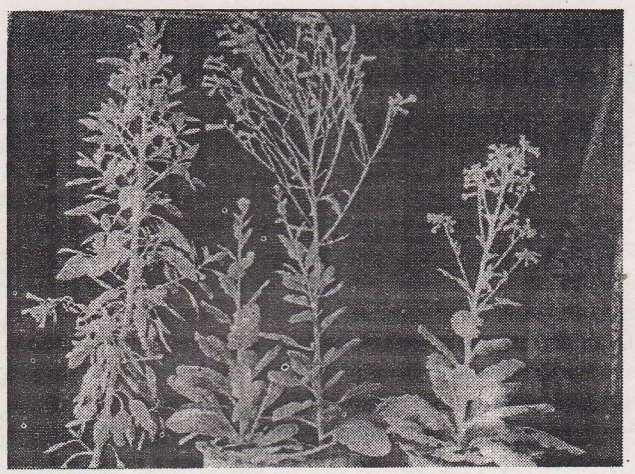
Name of the Species	Control plants		No. of seed treated with cefotaxime (500mg/l)		Plants grown under culture conditions				Plants grown under field conditions			
	Total	% of T	Sown	Germinated	Total	NT	T	% of T	Total	NT	T	% of T
N. glauca	86	0	300	240	120	120	Nil	0	120	120	Nil	0
N. langsdorfii	94	0	300	280	140	140	Nil	0	140	140	Nil	0
GGLL hybrid	150	24	300	210	105	95	10	90	105	62	43	41

NT : Nontumorous plants
T : Tumorous plants

Table 3. Effect of cefotaxime on callus induction and growth of the leaf discs of *N. glauca, N. langsdorffii* and their hybrid.

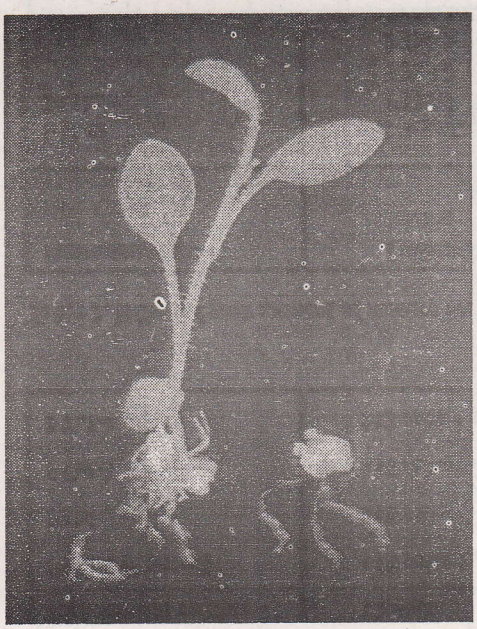
Type of the medium used	No. of discs from each plants	Growth response of the leaf discs		
		N. glauca	*N. langsdorffii*	Hybrid
MS basal medium	100	No callus	No callus	Teratoma
MS with cefotaxime (500 mg/l)	200	No callus	No callus	Teratoma
MS + H*	100	Callus	Callus	Callus

MS+H* : MS with hormones (NAA 1.0 mg/l and kinetin 0.1 mg/l)

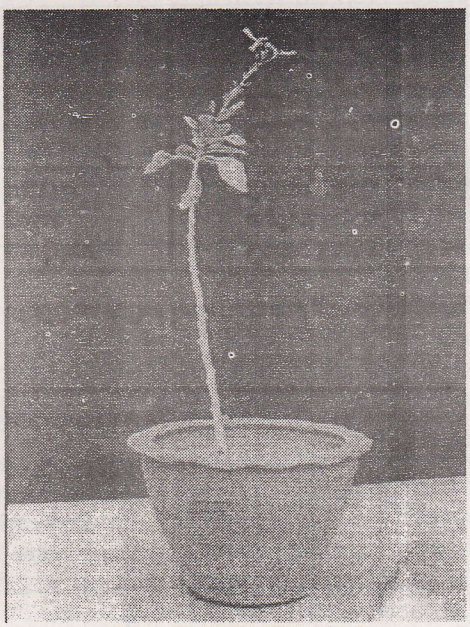


(a) (b) (c)

Fig 1. Morphology of parents and the advanced generations of the hybrid (a) *N. glauca* (b) *N. glauca x N. langsdorffii* Hybrid (c) *N. langsdorffii*.



(a)



(b)

Fig 2. Fresh hybrid produced between *N. glauca* and *N. langsdorffii*
(a) Plants grown under culture conditions on MS basal medium (Hypocotyl tumors are evident)
(b) Plants grown under field conditions (flowered but no seed set)

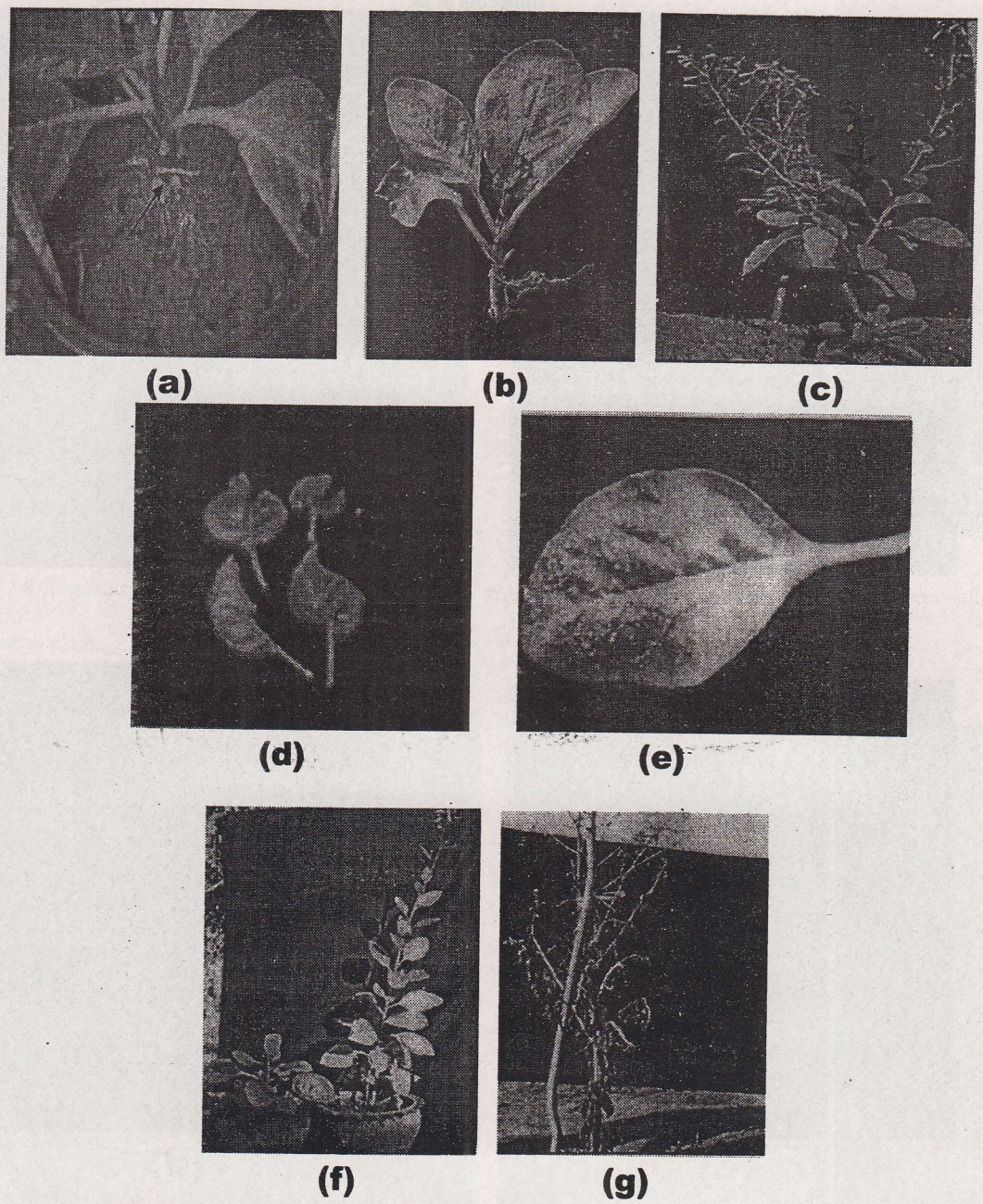


Fig 3. Morphological variations of *N. glauca x N. langsdorffii* hybrid
(a) Plagiotropic roots above the ground (↑) (b) Necrosis of terminal bud (loss of apical dominance)
(c) Development of three shoots (d) Leaf distortions (e) Blister like structures on the leaf (f) Plant height variations (extremes represented) (g) Wilting of the whole plant.

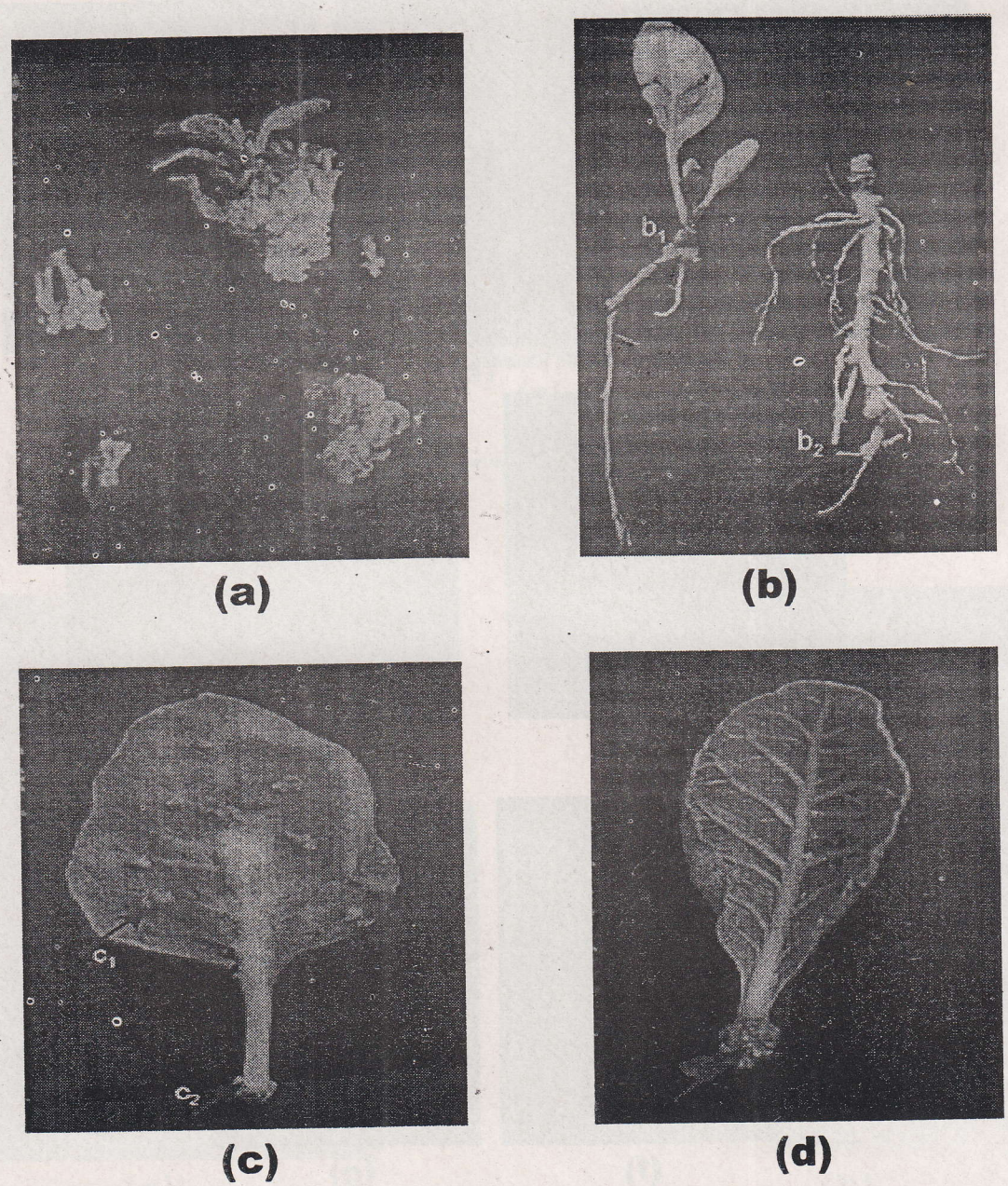
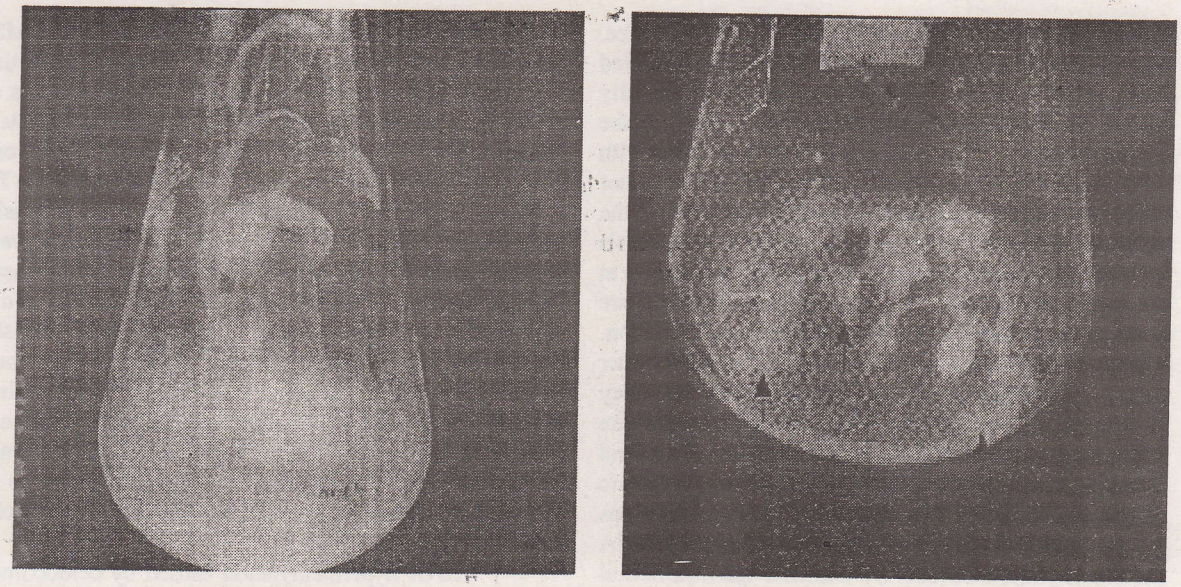
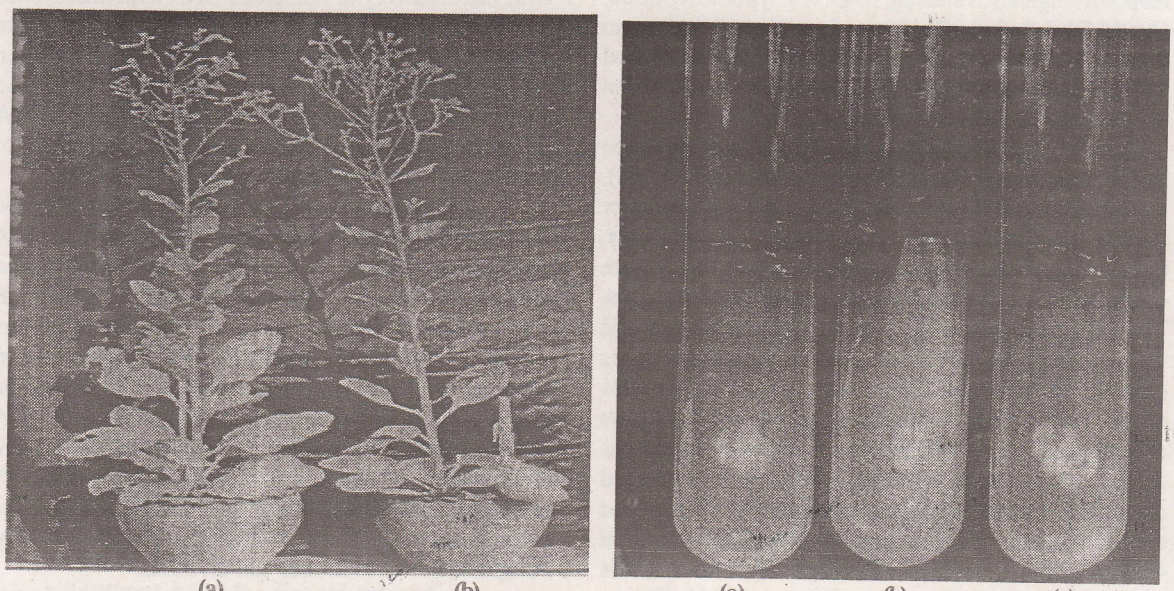


Fig 4. Tumors developed on different parts of *N. glauca* x *N. langsdorffii* hybrid
(a) Teratomatous tumors (Mass of tissue with multiple shoots)
(b_1) Hypocotyl tumor[↑] (b_2) root tumors [↑]
(c_1) Tumors on leaf lamina [↑] (c_2) Tumor on leaf axil[↑]
(d) Tumor on leaf base [↑]



(a) (b)

Fig 5. Morphological variations of cefotaxime treated *N. glauca* x *N. langsdorffii* hybrid seed grown under culture conditions
(a) Control plants
(b) Cefotaxime treated (white root tumors [↑])



(a) (b) (a) (b) (c)

Fig. 6. Morphological variations of cefotaxime treated hybrid seed grown under culture conditions
(a) Control plants (b) Cefotaxime treated

Fig. 7. Response of hybrid leaf discs to cefotaxime treatment
(a) On MS basal medium (b) On MS with Cefotaxime (500 mg/l)
(c) On MS with hormones (NAA 1.0 mg/l + Kinetin 0.1 mg/l),

the leaf (Fig. 4c$_2$), leaf base (Fig. 4d) and inflorescence. The time and position of appearance of tumors varied from plant to plant. Tumorigenesis predominantly observed at the time of flowering and it increased with the age of the plant. These changes may be associated with DNA methylation of promoter at CpG islands of tumor suppressor genes. Similar results were observed with the genes such as P16, RB1 etc., enhancing tumorigenesis with the increase in the age[18]. Since, tumorigenesis is at the time of flowering, we propose that these genes are activated by the conditions favorable for floral induction. The tumors were also observed at vegetative phase either induced by wounding or external stress conditions. They can also be developed by mechanical injury, insect bite and external stress like over crowding of seedlings and high temperature, which could be due to the epigenetic effects on gene expression. The morphological variations and early tumorigenesis could be due to changes in DNA hypomethylation caused by external environmental conditions. DNA hypomethylation caused by 5-azacytidine treatment of the hybrid plants further severed the morphological variations and caused early expression of tumors[11]. Further, Wada *et al.*,[19] provided evidence that in tobacco external stress causes activation of stress responsive genes resulting in DNA hypomethylation. Hence, DNA hypomethylation of some genes leading to their expression may be responsible for morphological variations and tumorigenesis as changes in methylation do not occur in uniform pattern.

Treatment of seed with cefotaxime: The parents *N. glauca* and *N. langsdorffii* after cefotaxime treatment (500 mg/l), resembled their controls plants in morphology and tumor induction under field and culture conditions. However, hybrid plants showed variations only under culture conditions with the continuous supply of cefotaxime. Tumors started appearing from 15 days old plants and most of the plants developed massive white root tumors (Fig. 5b). 90% of cefotaxime treated plants but only 24% of their controls (Fig. 5a) turned tumorous within 60 days after germination. On the other hand, under field conditions the hybrid plants grown from cefotaxime treated seed mimicked their control plants in morphology (Fig. 6) and only 41% of the plants developed tumors (Table 2) within 4 months after transfer to the field conditions. This difference in response under culture and field conditions towards tumorigenecity could be attributed to the temporary effect of DNA hypermethylation by cefotaxime treatment.

Treatment of leaf discs with cefotaxime: Callus initiation was not observed for leaf discs of *N. glauca* and *N. langsdorffii* cultured on MS basal medium and MS supplemented with cefotaxime. Where as callus initiation was observed from the leaf discs placed on MS supplemented with hormones (NAA 1.0 mg/l and kinetin 0.1 mg/l) after 5 days of incubation. It was grown as a white mass of tissue from all around the disc, which later turned brown. For the leaf discs of the hybrid placed on MS basal medium callus initiation was observed within 7-10 days. The callus was grown from the veins as a greenish white mass of tissue (Fig. 7a) and shoot initiation was observed within 25-30 days. The leaf discs of hybrid when placed on MS supplemented with cefotaxime callus initiation was observed within 3-5 days, grown as greenish white tissue (Fig. 7b) from which shoot development was observed within 20-25 days. When the leaf discs of the hybrid were placed on MS supplemented with hormones (NAA 1.0 mg/l and kinetin 0.1 mg/l) callus initiation was observed within 5-7 days and the callus was grown as a white mass of tissue all around the disc (Fig. 7c), which later turned brown (Table 3).

DNA hypermethylation caused by cefotaxime treatment enhanced tumorigencity and phytohormone independent growth, which could be due to hypermethylation of tumor suppressor genes and/or their promoters accompanied by their silencing, resulting in growth advantage for the cell forming a tumorous tissue. With the discovery of numerous DNA hypermethylated promoters, Jones and Larid[20] stated that tumor suppressor inactivation by DNA hypermethylation results in cancer. Further, Shu *et al.*,[21] from their work on colon cancers and acute lymphoid leukemias showed evidence that DNA hypermethylation of promoters and hypermethylation of CpG islands is a functional consequence in cancers.

Our results of another set of experiment of hybrid seed and leaf disc treatment with 5-azacytidine[11], a DNA hypomethylating agent[22] are different. In the hybrid seed treatment 95-99% of the plants turned tumorous where as only 10-15% of their control plants developed tumors within 45 days after their germination. This increase in tumorigenecity and early expression of tumors could be due to early phase change regulation caused by DNA hypomethylation. 5-azacytidine treated leaf discs failed to show callus induction and teratomatous growth on MS medium. These results of leaf disc treatment support the requirement of DNA hypermethylation of some genes for deregulated or uncontrolled cell divisions.

The hybrid plants grown under green house conditions (University of Leeds, UK) segregated for tumorous and nontumorous plants, but when the progeny of the hybrid seed were grown under field conditions (Andhra University, India) the segregation was lost, several morphological aberrations were observed, tumors developed much earlier than flowering time, the percentage of tumorous plants enhanced generation after generation,

the percentage of flowering, seed set and viability were decreased. This set of variations was considered to be caused by changes in DNA methylation pattern under stress inducing environmental conditions. Further, cefotaxime treated enhancement of tumorigenesis and phytohormone independent growth may be explained as DNA hypermethylation conditions of tumor suppressor genes and/or their promoters resulting in their silencing thus promoting cell divisions and partial morphogenic differentiation rather than normal development.

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