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DEVELOPMENT OF TOBACCO SOMACLONES FROM LEAF CURL VIRUS INFECTED LEAVES AND THEIR CHARACTERIZATION

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Kanchan is a popular Flue-cured Virginia tobacco variety grown in the light soils of Andhra Pradesh and Karnataka. It is a high yielding variety but susceptible to leaf curl virus. An attempt was made to develop high yielding and/or leaf curl resistant somaclones from leaf curl infected leaf explants of Kanchan. Promising somaclones thus developed were characterized. Somaclones recorded higher leaf curl virus tolerance (100%) than control (7-75%) under artificial condition. Somaclones showed 27-33% increase in cured leaf yield over control Kanchan in a replicated trial. Genetic similarity estimated based on RAPD polymorphism among the clones varied from 74 to 96% indicating that the clones are genetically different. In the cytological analysis, neither numerical nor structural chromosome variation observed among the somaclones. These results indicated that somaclones of economic value were successfully developed.

INTRODUCTION

Flue-cured Virginia tobacco is an exportable cigarette tobacco type grown in the states of Andhra Pradesh and Karnataka. The tobacco grown in Northern Light Soil areas of West Godavari, Andhra Pradesh is considered as semi-flavourful to flavourful quality. Kanchan is a popular variety of these areas and occupies 95% of the 1.03 lakh ha of area. Though it is a high yielding (~2000 kg/ha) and produces good quality leaf, it is susceptible to leaf curl virus. Due to lack of resistant donors for the development of resistant varieties through conventional breeding, an attempt was made to develop Kanchan somaclones resistant to leaf curl virus.

The potential of somaclonal variation has yet to be fully exploited by breeders although a few somaclone cultivars have been reported in *Brassica juncea*, rice and others (Larkin *et al.*, 1989). Virus pressure in the leaf tissue was assumed to increase the chance of occurrence of mutations in the infected cells. Regeneration of shoots from such cells may result in isolation of commercially useful clones. In order to exploit such mutations, shoots were regenerated from leaf curl infected Kanchan explants and somaclones thus developed were characterized.

MATERIALS AND METHODS

Development of Somaclones

Leaf curl infected leaves of Kanchan were surface sterilized and inoculated on MS medium supplemented with Kinetin (2 mg l^{-1}) and IAA (0.25 mg l^{-1}) during 2001-02. Along with direct organogenesis, callus formation was observed in few explants, such callus was again used for the regeneration of the shoots on the same medium. Directly regenerated shoots and callus derived shoots that are free from leaf curl symptoms, were rooted on MS basal medium and transferred to pots for further screening during 2002-03.

Characterization of promising Kanchan somaclones

Testing for leaf curl resistance

As tobacco leaf curl virus is transmitted by white flies, R_0 generation clones were inoculated with white flies fed on the leaf curl infected plants. Acquisition feeding time allowed for white flies on leaf curl infected plants was 24 h and inoculation feeding time was 48 h and 10 viruliferous white flies/plant were released Observations were recorded on leaf curl infection and the tolerant plants were selected for further studies. Tolerant clones in R_2 to R_4 generation were planted under field condition along with Kanchan. White fly population in the screening plot was increased by releasing viruliferous white flies on the brinjal and

sunflower plants that were grown around the plot. Based on degree of infection, the curl plants were classified as resistant (no leaf curl symptoms), mildly infected (with few symptoms and not much damage to economic leaf) and severely infected (Vidavisky and Czosneki, 1998). Number of resistant and mild plants were combined to obtain the per cent tolerant plants. In each generation, resistant plants were selected, selfed and advanced to next generation.

Evaluation of somaclones under field condition

Nine somaclones were tested in progeny row trial under irrigated Alfisols at CTRI Research Station, Jeelugumilli during two seasons (2004-05 and 2005-06). Among them six R_4 generation clones found promising and were tested in replicated trial for three seasons (R_4-R_6 generations) during 2006-09 along with a field selection, Natural Mutant (NM), from Kanchan variety, two low tar advanced breeding lines (JS 116-1 and JS 124) and check variety, Kanchan. Data were recorded on green leaf, cured leaf and grade index. The leaf yield data, thus, collected was statistically analyzed for individual years and pooled for three seasons. Various morphological characters and chemical quality characteristics *viz.*, nicotine and reducing sugars in cured leaf (both 'X' and 'L' positions) were estimated (Harvey *et al.*, 1969) during 2008-09 season.

Molecular characterization of somaclones

The six somaclones and variety Kanchan were characterized using seventeen random primers (Operon Technologies, USA), that found to amplify tobacco genome (Sarala and Rao, 2008).

For RAPD study, DNA was extracted from thirty days old seedlings of each clone and quantified on 0.8% agarose gels. The isolated DNA was amplified (Williams *et al.*, 1990) using Thermal cycler (Eppendorf, Germany) in a 25 l reaction mixture containing 30 ng template DNA, 0.5 units of Taq polymerase, 0.2 mM dNTPs and 30 ng of each primer. PCR cycles consisted of initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 37°C for 1 min and 72°C for 2 min with final primer extension cycle of 7 min at 72°C. Amplified RAPD products were separated on agarose gels (0.8% w/v) having ethedium bromide (0.5 mg/ml) and gel images captured using Gel Doc-2000 (Biorad, Australia). Percentage of polymorphic bands is defined as the percentage of the number of polymorphic bands amplified by a single primer to that of the total number of bands produced by that primer. The binary data was recorded depending on the presence (1) or absence (0) of the bands. Using this data, genetic variation and Jaccard's (1908) similarity coefficients among the tobacco lines were analyzed using the NTSYS-pc software version 2.02 (Rohlf 1998). Based on UPGMA and SAHN clustering, a dendrogram depicting the genetic relationship among the lines was prepared.

Cytology of Kanchan somaclones

To study the chromosomal stability of somaclones, flower buds of appropriate size were fixed in Modified Carnoys fixative (1 acetic acid: 3 alcohol: 1 chloroform) and transferred to 70% alcohol after 24 h. Meiosis of the pollen mother cells was studied by smearing technique using acetocarmine stain. The slides were observed under microscope (Labomed, India) and photographed.

RESULTS AND DISCUSSION

Development of Somaclones

Tissue culture regenerants often display altered phenotypes, termed somaclonal variation (Larkin and Scowcroft 1981). Heritable somaclonal variation was sporadically employed in various plant improvement programmes (Larkin and Scowcroft, 1981). In order to develop the leaf curl tolerant Kanchan lines, somaclones were developed from leaf curl virus infected leaf explants of tobacco variety, Kanchan. Shoot regeneration from explants was slow and taken about 20-25 days. Around 10 shoots were regenerated from each of the responding explants and a total of around 500 plantlets were regenerated. Most of the cases the shoots regenerated from virus infected leaf explants showed viral symptoms and stunted growth. Among the regenerants, 14 plantlets with no apparent viral symptom were observed. These plantlets were inoculated on to MS basal medium for rooting. Rooted plantlets were transferred to pots and screened against leaf curl virus.

Regeneration of mutant cells from the virus infected explants might be the reason for the development of disease symptom free plantlets. Presence of virus in the explants may be acting as a selection environment for virus tolerance.

Characterization of promising Kanchan somaclones

Testing for leaf curl resistance

Out of 14 somaclones plants tested in the pots, eight plants were free from leaf curl infection. Screening of these resistant clones in field during 2004-07 revealed higher leaf curl virus tolerance (100%) in somaclones than control (7-75%) (Table 1). Over the years the mean percent of resistant plants among somaclones were found to increase from 36 in R_2 generation to 97 in R_4 generation. This increase may be due to the continuous selection pressure for resistant plants in each generation. Heath-Pagliuso *et al.* (1988) developed resistance to *Fusarium oxysporum* f. in celery and found that the developed somaclonal variation was heritable and conditioned by more than one locus.

Testing the somaclones under field condition

The above selected eight clones and NLCR 7(K), a morphological variant isolated in NLCR 7 was tested in the row trial, during 2004-06. Among the nine somaclones tested, clones *viz.* NLCR-1, NLCR 4, NLCR 5, NLCR 7, NLCR 7(K) and NLCR 10 that gave higher leaf yields (Data not presented) were selected for further field testing.

In the replicated trial (2006-09), mean leaf yields found to be significant among the lines (Table 2). Seasons were significant for leaf yields and season x entries interaction was non-significant. All the somaclones and NM consistently performed superior in all seasons with significantly higher yields. All the somaclones and NM recorded higher mean green leaf (26-32%), cured leaf yields (27-33%) and grade index (32-55%) than Kanchan. Most of the somaclones recorded higher leaf number, leaf length and leaf width values than parent Kanchan (Table 3). NLCR 7 recorded maximum leaf length (67 cm) and leaf width values (28 cm). NLCR 7(k) recorded maximum number of leaves (33) after topping. The observed increase in leaf number, leaf length and leaf width values in somaclones were responsible for significant increase in leaf yields than control, Kanchan. Phenotypic variation among plants regenerated from tissue culture has been reported in *Solanum tuberosum* (Landsman and Uhrig, 1985), wild barley (Breiman *et al.*, 1987), cotton (David *et al.*, 1991), rice (Godwin *et al.*, 1998) and reed canary grass (Gyulai *et al.*, 2003).

Acceptable chemical quality characteristics of Kanchan somaclones (Table 4) observed during 2008-09 crop season clearly indicate that these clones can be commercially exploited. Reduction in mean seed yields, increase in lint percentage and certain fiber properties in somaclones were reported in cotton (David *et al.*, 1991). Lower mean neutral-detergent and acid-detergent fibre concentrations were observed in somaclones of reed canary grass (Gyulai *et al.*, 2003) and a gradual increase in the mean content of hypericins in field grown *Hypericum perforatum* somaclonal plants of R_1-R_4 generations (Koperdakova *et al.*, 2007).

Molecular diversity of Kanchan somaclones

Thirteen primers produced polymorphic bands and four primers monomorphic bands with an average of 3.6 bands per primer (Table 5). Of the 61 bands detected, 24 were polymorphic (39 %). Genetic similarity values among somaclones ranged from 0.74 to 0.96 (Table 6). NLCR-10 found to show lower similarity values with other clones (0.74 to 0.79). Kanchan recorded lower similarity value with NLCR-1(0.78) and maximum with NLCR-7 (0.90). The dendrogram revealed two major clusters (Fig 1). NLCR-1 and NLCR-4 formed one cluster, NLCR-5, NLCR-7, Kanchan, NLCR-7(K) second cluster and NLCR-10 independently linked to main cluster. These results indicate that somaclones are genetically divergent from parent, Kanchan. Many reports of phenotypic variation among plants regenerated from tissue culture suggested underlying alterations at DNA level (Landsman and Uhrig, 1985; Breiman *et al.*, 1987). RAPD technique was successfully used to detect somaclonal variation in garlic (Al-Zahim *et al.*, 1999), rice (Godwin *et al.*, 1998), reed canary grass (Gyulai *et al.*, 2003) and banana (Tui Ray *et al.*, 2006). However, some of the DNA level variation

Fig. 1: Dendogram of Kanchan somaclones

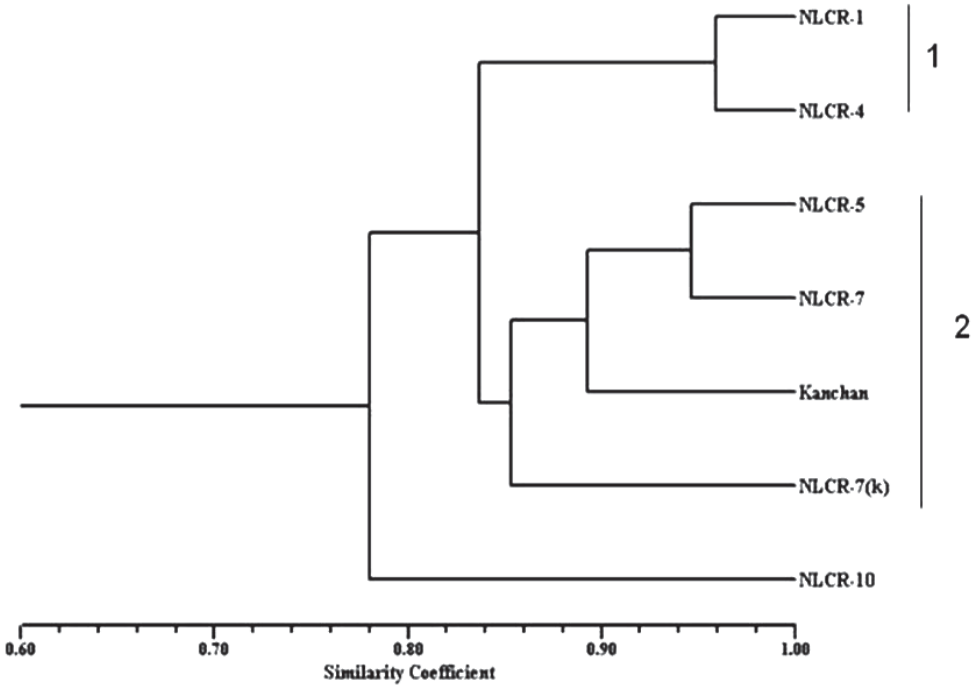

Table 1: Reaction of Kanchan somaclones (R_2-R_4 generation) to leaf curl virus under artificial conditions (2004-07)

S No	Line/Entry	R_2 (2004-05)			R_3 (2005-06)			R_4 (2006-07)		
		Resistant (%)	Mild(%)	Tolerant (%)	Resistant (%)	Mild (%)	Tolerant (%)	Resistant (%)	Mild (%)	Tolerant (%)
1	NLCR-1	44	56	100	94	6	100	100	-	100
2	NLCR-4	22	78	100	100	-	100	100	-	100
3	NLCR-5	39	61	100	94	6	100	100	-	100
4	NLCR-6	28	72	100	100	-	100	89	11	100
5	NLCR-7	56	44	100	100	-	100	89	11	100
6	NLCR-8	47	53	100	87	13	100	100	-	100
7	NLCR-10	50	50	100	100	-	100	100	-	100
8	NLCM-8	44	56	100	100	-	100	100	-	100
	Mean of somaclones	36	64	100	96	4	100	97	3	100
	Kanchan (Control)	0	7	7	0	75	75	22	22	44

Table 2: Mean leaf yields (kg/ha) of Kanchan somaclones in a replicated trial (2006-09)

S. No.	Line / Variety	Green leaf	Cured leaf	Grade index
1	NLCR-1	18296*(32)	3023*(32)	2091*(51)
2	NLCR-4	17569*(27)	2918*(28)	1884*(36)
3	NLCR-5	17394*(26)	2933*(28)	1991*(44)
4	NLCR-7	17875*(29)	3017*(32)	2039*(47)
5	NLCR-7 (K)	17796*(29)	2998*(31)	1968*(42)
6	NLCR-10	18277*(32)	3043*(33)	2147*(55)
7	NM	17319*(26)	2896*(27)	1828*(32)
8	JS-116-1	14634(6)	2455(7)	1595(15)
9	JS-124	13829	2346	1258
10	Kanchan	13838	2285	1383
	Mean	16683	2792	1818
	SEm±	**566**	**110**	**77**
	CD (P=0.05)	**1570**	**304**	**214**
	CV (%)	**10.18**	**11.79**	**12.71**
Seasons				
	2006-07	15112	2748	1592
	2007-08	17190	2954	2053
	2008-09	17746	2672	1810
	SEm±	**432**	**79**	**59**
	CD (P=0.05)	**1495**	**NS**	**205**
	CV (%)	**14.19**	**15.43**	**17.84**
Seasons X Entries				
	SEm±	**981**	**190**	**133**
	CD (P=0.05)	**NS**	**NS**	**NS**

Table 3: Morphological characterization of Kanchan somaclones (2006-07)

S. No	Line/ Entry	Plant height (cm)	No. of leaves after topping	Leaf length (cm)	Leaf width (cm)	Internodal length (cm)
1	NLCR-1	187	30	57	23	2
2	NLCR-4	215	28	64	25	3
3	NLCR-5	196	30	61	26	4
4	NLCR-7	182	26	67	28	2
5	NLCR-7(k)	200	33	63	27	2
6	NLCR-10	186	27	65	26	2
7	NM	177	23	61	27	3
8	JS-116-1	186	27	55	23	2
9	JS 124	191	23	58	23	2
10	Kanchan	163	25	60	22	2
	Grand mean	188	27	61	25	2
	SEm±	**10**	**2**	**1.79**	**1.27**	**0.2**
	CD (P=0.05)	**—**	**5**	**5**	**NS**	**0.7**
	CV (%)	**8.99**	**11.42**	**5.07**	**8.80**	**16.49**

Table 4: Quality characteristics of Kanchan somaclones and advanced breeding lines (2008-09)

S.No	Entry/ Line	X position			L position		
		Nicotine (%)	Reducing sugars (%)	Chlorides %	Nicotine (%)	Reducing sugars (%)	Nicotine (%)
1	NLCR-1	2.75	9.89	0.83	4.42	7.52	0.77
2	NLCR-4	3.19	11.84	0.76	4.49	7.51	0.79
3	NLCR-5	2.65	12.58	0.77	4.25	8.19	0.70
4	NLCR-7	3.35	9.01	0.69	3.71	9.55	0.76
5	NLCR-7 (K)	2.54	9.84	0.90	4.36	6.88	0.86
6	NLCR-10	2.94	12.55	0.64	3.53	11.72	0.70
7	NM	1.98	12.06	0.73	2.88	8.06	0.86
8	JS-116-1	5.01	5.58	1.29	5.61	4.94	1.10
9	JS-124	3.18	8.65	0.87	4.35	7.30	0.65
10	Kanchan	2.61	8.86	0.63	4.82	5.05	0.47

Table 5: Number of bands scored in Kanchan somaclones with different RAPD primers

S. No.	Primer	Primer sequence	Total no. of bands	No. of polymorphic bands	Polymorphism (%)
1.	OPA-08	5' GTGACGTAGG 3'	4	2	50
2.	OPB-01	5' GTTTCGCTCC 3'	2	0	0
3.	OPD-05	5' TGAGCGGACA 3'	3	1	33
4.	OPD-12	5' CACCGTATCC 3'	5	2	40
5.	OPE-01	5' CCCAAGGTCC 3'	3	1	33
6.	OPE-06	5' AAGACCCCTC 3'	4	3	75
7.	OPE-11	5' GAGTCTCAGG 3'	3	1	33
8.	OPF-16	5' GGAGTACTGG 3'	3	1	33
9.	OPH-01	5' GGTCGGAGAA 3'	6	3	50
10.	OPH-05	5' AGTCGTCCCC 3'	2	0	0
11.	OPL-09	5' TGCGAGAGTC 3'	4	1	25
12.	OPM-13	5' GGTGGTCAAG 3'	2	0	0
13.	OPN-07	5' CAGCCCAGAG 3'	5	2	40
14.	OPN-08	5' ACCTCAGCTC 3'	5	1	20
15.	OPN-15	5' TGGCGTCCTT 3'	3	0	0
16.	OPAY-4	5-AAGGCTCGAC 3'	2	2	100
17.	OPAY-5	5-TGGCTGCGTT 3'	5	4	80
		Total	61	24	39

may be in highly repeated sequences with no phenotypic effects (Godwin *et al.*, 1998). Larkin and Scowcroft (1981) in their study considered a number of possible mechanisms for the origin of somaclonal variation. Lee and Phillips (1988) detected the chromosomal basis of somaclonal variation.

Cytology of Kanchan somaclones

Around 300 cells showing different meiotic stages were studied in each clone. All the meiotic stages in the clones studied found to be normal with 24 pairs of chromosomes. The present study did not detect any numerical or structural chromosome variation among the somaclones, unlike wild barley (Breiman *et al.*, 1987) and garlic (Al-Zahim *et al.*, 1999) which indicated that the clones were stable. Selection of fertile and promising clones in the initial stages of selection in the present study might have eliminated any defective clones.

In the present study, somaclones of economic value could be successfully developed from the leaf curl infected leaf explants of tobacco. Testing the clones under field condition proved that the variation created is not epigenetic but heritable. These clones after their due testing can be released for commercial cultivation.

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Table 6: Similarity coefficients among tobacco somaclones

Entries	NLCR-1	NLCR-4	NLCR-5	NLCR-7	NLCR-7(K*)	NLCR-10	Kanchan
NLCR-1	1.00						
NLCR-4	0.96	1.00					
NLCR-5	0.81	0.85	1.00				
NLCR-7	0.84	0.88	0.95	1.00			
NLCR-7(k)	0.83	0.87	0.84	0.89	1.00		
NLCR-10	0.79	0.76	0.74	0.79	0.75	1.00	
Kanchan	0.78	0.82	0.88	0.90	0.83	0.86	1.00

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