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HOST RANGE SPECIFICITY OF *AGROBACTERIUM TUMEFACIENS* STRAINS ISOLATED FROM CROWN GALL TUMORS ON FRUIT TREES IN PAKISTAN

Principal Investigator:

PROFESSOR SHEIKH RIAZUDDIN

Grantee Institution:

Centre for Advanced Molecular Biology
University of the Punjab,
Canal Bank Road,
Thokar Niaz Beg, Lahore

Collaborator:

PROFESSOR EUGENE W. NESTER

Institution:

Microbiology and Immunology Department
University of Washington
Seattle, WA 98195, USA

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A.I.D. Grant Project Officer:	Curtis Nissley
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EXECUTIVE SUMMARY

The main goals of the project were to a) collect a variety of *Agrobacterium* isolates from plant tumors growing in local environment b) characterize the local isolates for biotyping, opine synthesis, host range properties, presence of Ti-plasmids, and c) study DNA homology of locally isolated plasmid DNA with standard *Agrobacterium* strains.

As part of the program 122 galls on different plants growing in all the four provinces of Pakistan have been collected and processed to yield 183 *Agrobacterium* isolates which have been fully identified by different biochemical and biophysical tests. The local *Agrobacterium* isolates have also been biotyped and investigated for the identity of opine production and pathogenic properties on 10 dicotyledonous and 3 monocotyledonous plants.

It has been demonstrated that local *Agrobacterium* isolates invariably contain multiple plasmids of varying sizes; synthesize predominantly but not exclusively nopaline; exhibit differing levels of drug sensitivity when tested against 10 antibiotics. DNA homology studies with *tmr, tms* and T_B-DNA probes revealed that most but not all isolates showed some degree of homology with *tmr, tms* and T_B-DNA. Restriction digestion and southern blot hybridization studies have suggested some homology between CAMB 2648, CAMB 2649 CAMB 2653, in the *vir*B region.

Works carried out under this program, and described in the following pages are in preparation/submitted for publication in the form of 2 research papers. One M.Phil. and one Ph.D. degree was awarded on this work by University of the Punjab, Lahore.

RESEARCH OBJECTIVES

The following goals were approved under the PSTC program:

1. Field survey of crown gall disease/tumors in Pakistan.

2. Isolation of *Agrobacterium* from the tumorous tissues.

3. Characterization of the isolated *Agrobacterium* for pathogenicity, opine synthesis, biotyping and host range properties on selected plants.

4. Establish the presence of Ti-plasmids in the newly isolated *Agrobacterium* strains.

5. Analyse the Ti-plasmid, if present, for DNA sequence homologies with the previously characterized Ti-plasmids.

Proposed Objectives	Progress Made
Field survey of crown gall disease/ tumors in Pakistan.	Achieved
Isolation of *Agrobacterium* from the tumorous tissues.	Achieved
Characterization of the isolated *Agrobacterium* for pathogenicity, opine synthesis, biotyping and host range properties on selected plants.	Achieved
Establish the presence of Ti-plasmids in the newly isolated *Agrobacterium* strains.	Partially Achieved
Analyse the Ti-plasmid, if present, for DNA sequence homologies with the previously characterized Ti-plasmids.	Partially Achieved

METHODS AND RESULTS

Goal 1: Field Survey to Identify Crown Gall Tumors

During a field survey, one hundred and twenty two, crown galls were located on cherry, rose, plum, peach, apple, grape, almond, poplar, abies, walnut, oak, eucalyptus, quircus, lemon, mango, loquat, malburry, pear sunflower, apricot and shina in different regions of Pakistan (Fig. 1). On the basis of occurrence of crown galls on different plants, it is estimated that incidence of the crown gall disease varied from 1 to 21% in different hosts. It was further observed that the disease was not confined to a particular region in Pakistan, instead, it was spread throughout Pakistan (Table 1), with the highest incidence in poplar trees in Lahore. When results of these surveys are compared with observations made in Europe or USA (Seigler and Bowman, 1940; Levine,1936), the incidence of crown gall disease in Pakistan does not appear very severe, yet it causes considerable economic losses in the nurseries where cuttings are used as source material for the propagation of plants.

Goal 2: Isolation and Identification of Agrobacteria from Crown Gall Tumors

Crown gall tumors were hand collected from source plants, thoroughly washed in running tap water with continuous scrubbing on the surface of the tumors, surface sterilized by immersion for 10 minutes in 5% sodium hypochlorite and washed thrice with sterile distilled water. A small portion (2 gm) of the tumorous tissue was macerated in 5 ml of sterile distilled water in a pestle and mortar and an aliquot (0.1 ml) of the filtered macerate was plated on selective medium (Schroth et al., 1965; New and Kerr, 1971). On the basis of morphology of colonies appearing after three days at 28°C, three hundred and ninety-five colonies were hand picked and successively cultured to obtain seemingly *Agrobacterium* isolates. The isolates were identified upto the genus level by the various biochemical tests (Allen & Holding, 1974). About one-half of the hand picked colonies (183) were identified as *Agrobacterium*. It was surprising to note that some crown gall tumors yielded no *Agrobacterium*. Out of 122 tumors, we were able to separate Agrobacteria from only 84 samples. It is possible that the surface bacteria were killed during sterilization or the galls were caused by

organisms other than *Agrobacterium*, or the *Agrobacterium* lost the plasmid/virulence after infection of plant tissues.

Goal 3: Characterization of Agrobacterium Isolates

a) Biotyping

The isolates were classified into one of the three biotypes (Table 1) on the basis of following biochemical tests: utilization of meso-erythritol, melezitose, malonate, citrate, D(-) tartarate, propionate, mucic acid; growth at 28°C, reaction in litmus milk; NaCl tolerance; and production of 3-ketolactose. On these basis 59 isolates were classified as biotype 1, 79 as biotype 2 and 45 as biotype 3.

All the isolates, categorized into Biotype 1, were 3-ketolactose positive; failed to grow on meso-erythritol and New & Kerr (1971) medium; grew well on Schroth et al. (1965) medium, and gave alkaline reaction in litmus milk. The isolates were further categorized (Table 2) according to their biochemical characteristics. Some of the isolates did not correspond to the grouping of Keane et al. (1970), Kersters et al. (1973), Kerr & Panagopoulos (1977), Sule (1978).

All isolates of biotype 2 group gave positive response during growth on meso-erythritol, malonate, mucic acid, and New & Kerr (1971) medium. Further they grew neither on the medium by Schroth et al. (1965) nor did they produce 3-ketolactose. The members of this group generally gave acidic reaction in litmus milk. Biotype 2 isolates were further classified into six sub-groups (Table 3).

All isolates of biotype 3 utilized malonate and tartarate; gave alkaline reaction in the litmus milk, and failed to produce 3-ketolactose. Other biochemical tests were variable. Biotype 3 isolates were further classified into six subgroups (Table 4).

b) Pathogenicity

All isolates were tested for pathogenicity on one week old seedlings of chickpea (*Cicer arietinum*), broad bean (*Phaseolus vulgaris*) and peas

(*Pisum sativum*). The choice of host plants was based on availability in the laboratory and future plans to work on leguminous plants were stem wounded and inoculated with various *Agrobacterium* isolates. After three weeks of incubation at 25°C, 36 isolates induced formation of tumor/root or both, at the wounded site (Table 1). It was surprising that the remaining *Agrobacterium* isolates did not exhibit pathogenic behaviour on the experimental host plants. The response was variable; being highest in *Agrobacterium* isolated from peach as native host and gradually decreased in the order of grape> plum> rose> cherry> ash> apple> shina> poplar. The observed variations in pathogenicity or failure to induce tumor or root formation by different isolates may be attributed to host specificity (Anderson & Moore; 1979), or inability to induce phytohormones (Lippincott and Lippincott; 1975), or the loss of Ti-plasmid as reported by Kerr (1969).

c) Opine Utilization and Synthesis

The abilities of various agrobacterium isolates to utilize opines were investigated by their growth on Perry & Kado (1982) medium supplemented with either octopine or nopaline as carbon/nitrogen source. Other opines such as argopine could not be used mainly because of their non-availability in Pakistan (the scope of there studies was therefore, limited). The results show that 57 isolates utilized octopine; 32 utilized nopaline; 78 utilized both octopine and nopaline, whereas 16 isolates utilized neither octopine nor nopaline.

The synthesis of opines in the tumorous tissues was also studied by a modification of the method of Otten & Schilperoort (1978) which detects guanidine based opines. 1-2 g of the tumorous tissue was homogenized in an eppendorf tube with a plastic rod and centrifuged at 12000 rpm for 10 minutes to obtain a clear supernatant. A 5 ul aliquot of the supernatant was spotted on a Whatman 3 MM paper, 3.5 cm from the anodal end and 1.5 cm apart alongwith controls and electrophoresed for 2 hours at 300 V in a buffer medium containing formic acid : acetic acid : water (5:15:80). The electrophorogram was dried and stained with phenanthrene quinone and observed under 360 nm UV light. All the

isolates except CAMB 2650 synthesized nopaline in the tumor lines, whereas CAMB 2650 synthesized octopine.

d) Host Range Properties

Host range properties of ten selected isolates were studied on thirteen different host plants including chickpea (Cicer arietinum), peas (Pisum sativum), broad beans (Phaseolus vulgaris), datura (Datura stramonium), kalanchoe (Kalanchoe daigremontiana), tomato (Lycopersicon esculentum), tobacco (Nicotianatobaccum rustica), sunflower (Helianthus annuus), grape (Vitis venefera), poplar (Populus tremuta), maize (Zea mays), rice (Oryza sativa), and wheat (Triticum indicum). Data in Table 5 show that two isolates CAMB 2648 and 2649, were wide in their host range and showed pathogenic response on all the dicotyledonous hosts. Three isolates, CAMB 2651, 2652, and 2655 were limited in their host range to only leguminous plants. None of the isolates exhibited infection symptoms on monocotyledonous test plants.

e) Drug Sensitivity

To investigate resistance to common antibiotics, 10^6 cfu of the various Agrobacterium isolates were layered on LB plates supplemented with ten different drugs at several different concentrations. After 48 h incubation at 28 ^oC the concentrations of various drugs which resulted in 50% loss of survival were calculated. The results in Table 6 show that the *Agrobacterium* isolates were relatively more resistant to carbenicillin and streptomycin and least resistant to tetracycline, gentamycin and nalidixic acid when compared with other antibiotics.

Goal 4: Presence of Ti-Plasmids

Bacterial culture were grown at 28^oC in 250 ml YEP medium to a final cell density of $5x10^7$. The Cells were harvested and analyzed for the presence of Ti-plasmid by two independent methods [Wheatcroft et al. (1990) and Currier & Nester (1976)]. Bacterial cultures were washed in 500 uL of 0.1% sarkosyl solution in TE buffer (0.05 M Tris, 0.02M EDTA at pH 8.0) and resuspended in 40 uL of lysis buffer consisting of 7500 units/mL lysozyme, 10 ug/mL RNase I,

0.05% bromophenol blue, and 40% Ficol in tris-borate buffer. The bacterial suspension was rapidly transferred to the slot in 0.7% agarose gel in TBE buffer. After ten minutes at room temperature 40 uL of 0.2% SDS solution in TBE containing 10% Ficol was gently overlayed. Finally 100 uL of 0.2% SDS and 5% Ficol 400 in TBE was gently overlayed as the third layer and the slots were sealed and electrophoresed at 40 mA for 4 hours. The results of analysis are presented in Fig. 1. The gel pictures clearly demonstrate that all the isolates contained at least one or more plasmids of different molecular sizes. CAMB 2648, 2649 and 2654 exhibited the presence of at least five plasmid bands (lanes 1, 2 and 4 in Fig. 1) whereas, CAMB 2647, 2652, and 2651 showed four plasmid bands and CAMB 2655 and 2650 appear to have only two plasmid bands (lane 7 & 8).

Secondly, harvested cells were resuspened in 1 mL TE buffer followed by the addition of 3 mL of lysis buffer (Tris 0.05M, Na_2EDTA 0.02M pH 12.4 and 2.0% SDS). After incubation at 37°C for 30 minutes, the pH was adjusted to 8.3 by the addition of 140 mL of 2M Tris pH 8.0 and content were gently mixed. The samples were reincubated at 37°C for 1 hour after the addition of 200 mL of predigested proteinase K and analyzed for the content of plasmid DNA by electrophoresis at 40 V for 4 hours (Fig. 2). The results show that CAMB 2653, 2647 and 2651 contained two plasmid bands (lane 4, 5 and 7); CAMB 2655 and 2652 contained five plasmid bands each (lane 2 and 6); whereas CAMB 2654 contained four bands (lane 1 and 3).

A comparison of figures 1&2 shows a distinct discrepency in the number of plasmid DNA bands observed with either of the two methods which may be attributed to shearing of DNA by different methods.

Goal 5: Sequence Homology of Ti-Plasmids

Isolated Ti plasmids were studied for sequence homology with previously well characterized *Agrobacterium* plasmids by a) dot blot hybridization, and b) southern blot analysis.

a) Dot Blot Hybridization

The isolated plasmid DNA was denatured in 0.2 N NaOH for 10 minutes, and quickly chilled on ice for 15 minutes. The denatured DNA was dot blotted on Biotrans^{TM} membrane, dried at 40°C for 10 minutes, and baked at 80°C for 2 hours. The blot was prehybridized in prehybridization solution overnight at 42°C. The blot was used for hybridization in a solution (5xSSC, 50% deionized formamide, 25 mM K_2HPO_4 + KH_2PO_4 pH 7.0, 10 x Denhardt's solution, 50 ug/mL denatured Salmon Sperm DNA, 0.5% SDS and 10% dextran sulphate) with nick translated denatured probe for overnight at 42°C. The blot was washed twice in 2xSSC and 0.1% SDS for 5 minutes at room temperature. Final washes were given with 0.1xSSC and 0.1% SDS. The membrane was dried at room temperature and exposed to X-Ray film.

Reuslts of Dot blot hybridization with JM83 labelled DNA probes containing tmr region of T-DNA of *Agrobacterium* strain A348 are shown in Fig 3. All the selected isolates except CAMB 2653 (lane 3) exhibited some degree of homology with the *tmr* region. CAMB 2649, 2651, 2652, 2655 and 2647 (lanes 4,7,8,10 & 11) indicated strong similarity. CAMB 2648, 2646 and 2654 (lanes 2, 5 & 9) indicated only moderate homology. The observed homology indicates the presence of *tmr* region of T-DNA in the locally isolated *Agrobacterium* strains.

In order to determine the presence of tms like loci in local isolates, the blots in Fig. 3 were again probed with SupA3 labled DNA containing the tms loci of *Agrobacterium* strain A348. The results in Fig 4 show that only CAMB 2646 and 2647 (lane 3 and 7) showed some degree of homology with the tms region, other isolates were not homologous with the tms region of A348. These data indicate the presence of a small number of conserved sequences in the vicinity of tms region of T-DNA in the local isolates.

Hybridization with pVCK431 labled probe, harboring the T_B-DNA of *Agrobacterium* strain A856, showed (Fig. 5) that CAMB 2646, 2647, 2654, 2650 and 2652 (lanes 1,2,3,7 & 8) have a 30% homology of the probe. In CAMB 2649, no homology was observed. The results lead to the suggestion that the observed homology may be due to the presence of

conserved sequences in this portion of T-DNA, as is the case with previously characterized Ti-plasmids.

b) Southern Blot Analysis

In order to confirm the data obtained by dot blot analysis we decided to analyse the local isolates by southern blot analysis. The isolated Ti-plasmids were digested with different restriction enzymes according to the manufacturer's recommendation. The digested DNA fragments were separated by agarose gel electrophoresis and transferred to Biotrans^{TM} membrane by capillary action in 0.4 N NaOH transfer solution. The membrane was subsequently washed with 2xSSC (0.3 M NaCl and 0.03 M Na-citrate), dried, placed in a bag containing 10 ml of 1% SDS/prehybridization solution (25mM phosphate buffer pH 7.4, 5xSSC, 5xDenhardt's solution, 50 ug/mL Salmon Sperm DNA and 50% formamide) and incubated at 42°C overnight. The membrane was hybridized with the nick translated probe in the hybridization solution (same as prehybridization solution supplemented with dextran sulphate at 10%) at 42°C overnight. Finally, the membrane was washed gently with 2xSSC/0.1% SDS for 5 minutes, with 0.5 x SSC/0.1% SDS for 15 minutes at room temperature and with 0.1 x SSC/0.1% SDS for 15 minutes at 42°C. The membrane was dried and exposed to X-Ray film.

When Hind III digested Ti-plasmid DNA was hybridized with {^{35}S} dCTP labelled probe pVCK431, the results show that only isolates CAMB 2652, 2646, 2654 and 2647 (lane 1,4,5 & 9 respectively) showed homologous bands of varying sizes (Fig. 7). The fuzzy bands in lane 4 and 9 were attributed to incomplete digestion of plasmid DNAs. These data confirm the sequence homology of T_B-DNA region of local isolates with that of previously characterized Ti-plasmids. These results are in agreement with dot blot analysis discussed in the preceding paras.

Data from a similar experiment with HindIII digested lambda DNA as marker (lane 7) and pTZ containing vir B region as positive control (lane 6) are presented in Fig 8a. Autoradiogram of southern blot hybridization of the same gel (Fig 8b) showed that the various isolates exhibited very little homology with vir B region. CAMB 2654 (lane 2) showed 5 homologous

bands of various sizes; CAMB 2649 (lane 3) 4 bands; CAMB 2648 (lane 4) 3 bands; and CAMB 2653 and 2655 (lane 1 & 5) showed only one band near the well which is homologous to the probe. Such insignificant homology, in different size fragments, suggests either differences in the restriction enzyme pattern of Ti-plasmid or presence of more than one plasmid in the isolates or similarity with some other vir loci of the same Ti-plasmid. Further studies are needed to resolve these issues.

TABLE-1

AGROBACTERIUM ISOLATES SEPARATED FROM CROWN GALLS LOCATED IN DIFFERENT ECOLOGICAL ENVIRONMENTS IN PAKISTAN

Location	Source Plant	Number of Agrobacterium Isolates sparated	BIOTYPE I	II	III	Pathogenic Isolates	
QUETTA, CHITRAL	CHERRY	17	13	3	1	5	
QUETTA, LAHORE ISLAMABAD, KARACHI	ROSE	18	10	4	4	6	
QUETTA CHITRAL SWAT	PLUM	21	2	18	1	7	
QUETTA SWAT PESHAWAR	PEACH	9	0	4	5	5	
QUETTA PESHAWAR CHITRAL SWAT	APPLE	52	11	29	12	7	
QUETTA LORALAI CHITRAL SWAT	ALMOND	1	0	1	0	0	
CHITRAL ISLAMABAD QUETTA SWAT	APRICOT	2	1	1	0	0	
MURREE CHITRAL SWAT	WALNUT	5	2	1	2	0	
SWAT CHITRAL	PEER	2	1	0	1	0	
QUETTA SWAT CHITRAL	GRAPE	6	4	2	0	3	
ISLAMABAD CHITRAL	MALBURRY	1 3	2	0	0	2	0
MURREE	ABIES	3	2	1	1	0	0
MURREE	QUIRCUS	1	5	4	1	0	0
MURREE	OAK	3	5	0	5	0	0
QUETTA	SHINA	3	8	0	2	6	1
LAHORE	POPLAR	21	19	8	5	6	1
SIBBI	LEMON	2	3	0	0	3	0
TURBAT LAHORE	MANGO	1 1	1	0	1	0	0
QUETTA	ASH	4	5	2	1	2	1

TABLE-2

BIOCHEMICAL CHARACTERIZATION OF BIOTYPE I ISOLATES

Sub-groups	1A	1B	1C	1D	1E	1F	1G	1H	1I	
Growth on:										
Schroth medium		+	+	+	+	+	+	+	+	+
New & Kerr medium		+	+	-	-	-	-	-	-	-
5% NaCl	+	+	-	-	-	-	-	+	+	
Malonate	+	+	-	+	+	+	+	-	-	
Tartarate	-	+	-	+	-	-	-	-	-	
Propionate		-	+	+	-	-	+	-	-	-
Mucic acid		-	+	+	-	-	+	-	-	-
Citrate	+	+	+	+	+	+	+	+	+	
Litmus Milk	B	B	B	B	B	B	B	B	B	
Erythritol	-	-	-	-	-	-	-	-	-	
Melezitose	+	+	+	+	+	+	-	-	-	
Growth at 37°C		+	-	+	+	+	+	+	+	+
3-Ketolactose		+	+	+	+	+	+	+	+	+

Note:

+ = Positive test.
- = Negative test.
B = Alkaline reaction.

TABLE-3

BIOCHEMICAL CHARACTERIZATION OF BIOTYPE II ISOLATES

Sub-groups	IIA	IIB	IIC	IID	IIE	IIF
Growth on:						
Schroth medium	-	-	-	-	-	-
New & Kerr medium	+	+	+	+	+	+
5% NaCl	+	+	-	+	-	+
Malonate	+	+	+	+	+	-
Tartarate	+	+	+	+	+	+
Propionate	-	+	-	-	-	-
Mucic acid	+	+	+	+	+	+
Citrate	+	+	+	-	+	+
Litmus Milk	A	A	A	A	B	B
Erythritol	+	+	+	+	+	+
Melezitose	-	+	-	-	+	+
Growth at 37°C	+	+	-	-	+	+
3-Ketolactose	-	-	-	-	-	-

Note:

+ = Positive test.
- = Negative test.
A = Acidic reaction.
B = Alkaline reaction.

TABLE-4

BIOCHEMICAL CHARACTERIZATION OF BIOTYPE III ISOLATES

Sub-groups	IIIA	IIIB	IIIC	IIID	IIIE	IIIF
Growth on:						
Schroth medium	-	+	+	+	+	+
New & Kerr medium	-	+	-	-	-	+
5% NaCl	+	-	-	-	-	+
Malonate	+	+	+	+	+	+
Tartarate	+	+	+	+	+	+
Propionate	-	-	+	-	+	-
Mucic acid	±	±	±	±	±	-
Citrate	-	-	+	+	+	+
Litmus Milk	B	B	B	B	B	B
Erythritol	-	+	-	-	-	-
Melezitose	-	+	+	-	+	-
Growth at 37°C	+	-	+	+	-	+
3-Ketolactose	-	-	-	-	-	-

Note:

+ = Positive test.
- = Negative test.
A = Acidic reaction.
B = Alkaline reaction.

TABLE-5

PHENOTYPIC RESPONSE OF SELECTED ISOLATES ON DIFFERENT HOSTS

CAMB 2648	PEACH	I	T	T	T	T	T	T	T	T	T	T	-	-	-
CAMB 2649	PEACH	I	T	T	T	T	T	T	T	T	T	T	-	-	-
CAMB 2647	GRAPE	I	R	R	R	-	-	-	-	-	T	-	-	-	-
CAMB 2646	GRAPE	I	R	R	R	-	-	-	-	-	T	-	-	-	-
CAMB 2650	GRAPE	I	R	R	R	-	T	-	-	-	T	-	-	-	-
CAMB 2651	CHARY	II	R	R	R	-	-	-	-	-	-	-	-	-	-
CAMB 2652	APPLE	II	R	R	R	-	-	-	-	-	-	-	-	-	-
CAMB 2653	PEACH	II	T+R	T+R	T+R	-	-	T	-	-	-	T	-	-	-
CAMB 2654	PEACH	III	T	T	T	-	-	T	T	-	-	T	-	-	-
CAMB 2655	APPLE	II	R	R	R	-	-	-	-	-	-	-	-	-	-

Note:
T = Tumor
R = Root
TR = Tumor with root.

TABLE-6

LD_{50} VALUES OF VARIOUS ANTIBIOTIC DRUGS

Isolate	Tet	Gent	Nal	Rif	Kan	Neo	Amp	Cm	Strep	Cb
CAMB 2648	7.5	7.5	12.5	20	20	25	30	35	190	500
CAMB 2649	5	10	10	15	20	25	40	45	180	400
CAMB 2647	10	7.5	12.5	15	20	25	40	25	210	250
CAMB 2646	7.5	2.5	12.5	15	25	30	40	25	180	350
CAMB 2650	7.5	5	12.5	15	30	30	40	60	290	500
CAMB 2651	2.5	7.5	10	15	30	25	35	30	210	250
CAMB 2652	7.5	7.5	12.5	15	30	25	30	60	200	500
CAMB 2653	10	5	12.5	15	20	20	30	40	200	500
CAMB 2654	7.5	7.5	12.5	15	25	20	40	45	250	300
CAMB 2655	10	5	10	15	35	20	40	40	250	350

Tet = Tetracycline
Rif = Rifampicin
Gent = Gentamycin
Cm = Chloramphenicol
Nal = Nalidixic acid

Kam = Kanamycin
Strep = Streptomycin
Neo = Neomycin
Amp = Ampicillin
Cb = Carbenicillin.

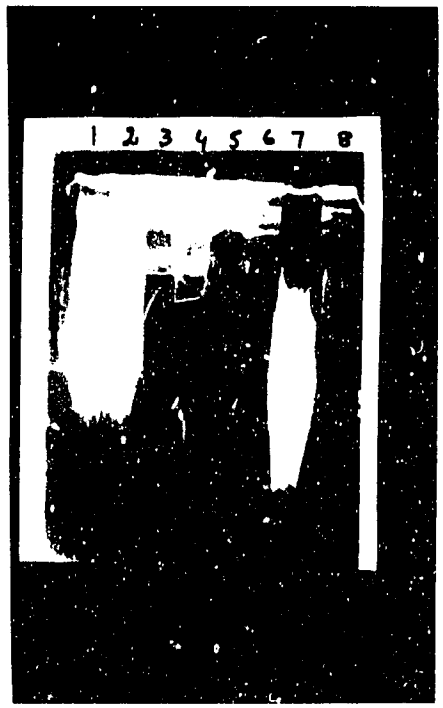

Fig 1: Agarose electrophoresis of various selected isolates showing the presence of multiple Plasmids.

Lane No.	Isolate
1	CAMB 2648
2	CAMB 2649
3	CAMB 2647
4	CAMB 2654
5	CAMB 2652
6	CAMB 2651
7	CAMB 2655
8	CAMB 2650

Fig. 2: Resolution of plasmids isolated by alkaline denaturation procedure.

Lane No.	Isolate No.
1	CAMB 2654
2	CAMB 2655
3	CAMB 2654
4	CAMB 2653
5	CAMB 2647
6	CAMB 2652
7	CAMB 2651

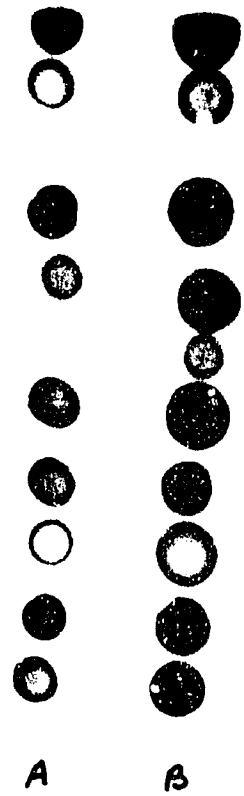

Fig. 3: Dot blot hybridization of different plasmids with tmr region of T-DNA.Columns. A and B represent 5uL and 10uL DNA concentration respectively.

Lane No.	Isolate No.
1	Probe
2	CAMB 2648
3	CAMB 2653
4	CAMB 2649
5	CAMB 2646
6	CAMB 2650
7	CAMB 2651
8	CAMB 2652
9	CAMB 2654
10	CAMB 2655
11	CAMB 2647

Fig. 4: Dot blot hybridization of different plasmids with tms region of A348 Agrobacterium strain.

Lane No.	Isolate No.
1	CAMB 2648
2	CAMB 2649
3	CAMB 2646
4	CAMB 2653
5	CAMB 2655
6	CAMB 2651
7	CAMB 2647
8	CAMB 2654

A B

Fig. 5: Dot blot hybridization of different plasmids with pVCK 431 harboring T_B-DNA of A856 Agrobacterium strain. Column A & B are different amounts of DNA blotted.

Lane No.	Isolate No.
1	CAMB 2646
2	CAMB 2647
3	CAMB 2654
4	CAMB 2653
5	CAMB 2651
6	CAMB 2649
7	CAMB 2650
8	CAMB 2652
9	CAMB 2648
10	CAMB 2655
11	pVCK 431(Probe)

Fig. 6: Electrophoretic mobility of different plasmids digested with Hind III.

Lane No.	Isolate No.
1	CAMB 2652
2	CAMB 2649
3	CAMB 2648
4	CAMB 2646
5	CAMB 2654
6	CAMB 2653
7	CAMB 2655
8	CAMB 2650
9	CAMB 2647
10	CAMB 2651

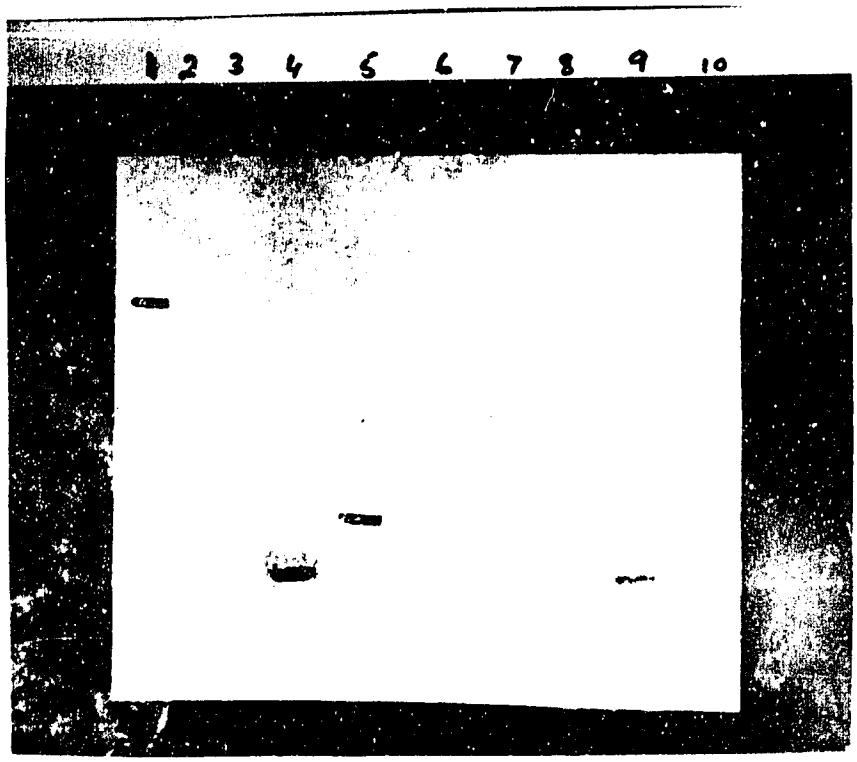

Fig. 7: Southern blot of plasmid DNA hybridized with pVCK 431.

Lane No.	**Isolate No.**
1	CAMB 2652
2	CAMB 2649
3	CAMB 2648
4	CAMB 2646
5	CAMB 2654
6	CAMB 2653
7	CAMB 2655
8	CAMB 2650
9	CAMB 2647
10	CAMB 2651

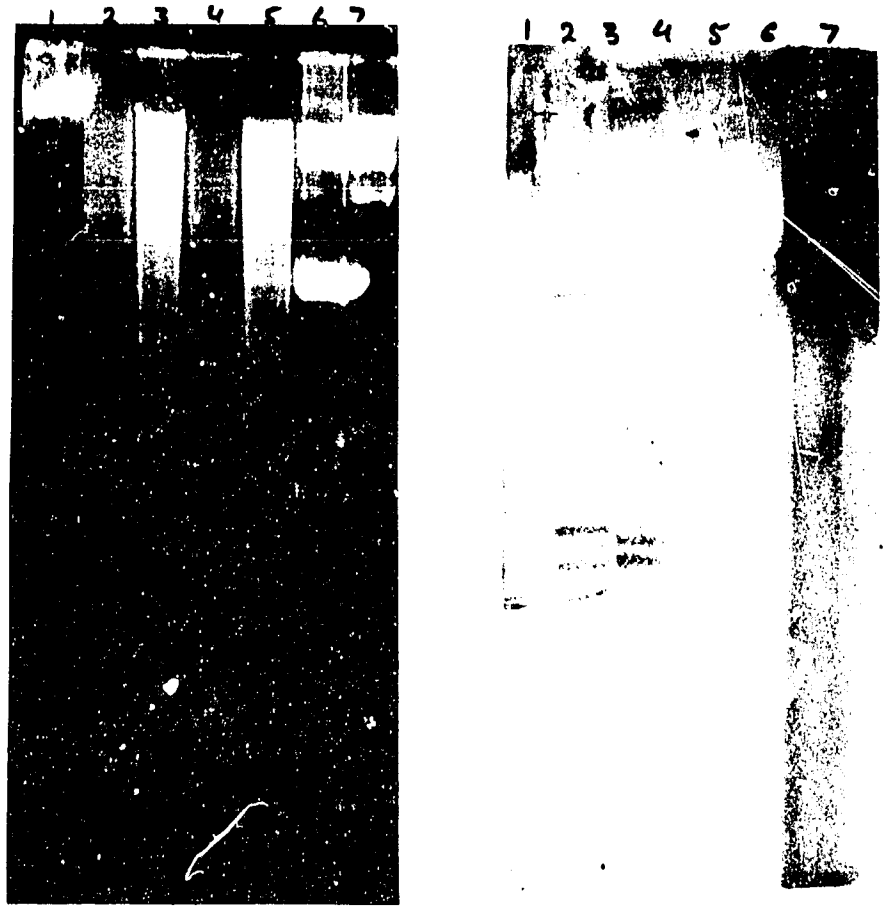

Fig. 8: a) Electrophorogram of selected plasmid digested with HindIII.

b) Southern blot of plasmid DNA hybridized with probe pTZ containing vir B region.

Lane No.	Isolate No.
1	CAMB
2	CAMB 2654
3	CAMB 2649
4	CAMB 2648
5	CAMB 2653
6	pTZ (Probe)
7	Hind III

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

Pakistan's planners and policy makers conceive the utility of transgenic plant technology as a technically useful and otherwise viable strategy to improve agricultural productivity in Pakistan. Agrobacterium mediated transformation plays a very important role in introducing new and novel genes into dicotyledonous plant. Recently, it has been reported (from the lab of the co-principal investigator) that certain Agrobacterium strains can also infect monocots such as, rice and maize. In view of these recent findings, the scope of Agrobacterium mediated transformation looks even brighter than before.

When the project started, the staff and personnel at the Centre were not well equipped to handle Agrobacterium mediated transformation. At the end of the project, work is in progress using established transformation procedures to introduce insecticidal protein genes into rice, chickpea and cotton. The goal is far from being achieved, nevertheless, if the PSTC project had not started, it would not have been possible to initiate the said studies. The project studies have enabled us to create a repository of different Agrobacterium isolates from local environments with varying degree of host range properties. These isolates are being further studied for the construction of vectors for use in plant transformation studies.

When the work started, the sophisticated molecular biological techniques of PCR amplification, southern blotting, nick translation etc. were done by only a handful of researchers at the Centre. These techniques now are routine in the lab. This is attributable largely to the conduct of progress studies and the impact of cooperation with Dr. Eugene Nester's lab.

Execution of the project work has resulted in the acquisition of a large number of standard Agrobacterium strains from Dr. Eugene Nester's lab which are being used in other studies being pursued at the Centre.

PROJECT ACTIVITIES/OUTPUT

The Principal Investigator visited Dr. Eugene Nester/Dr. M.P. Gordon's lab twice in the duration of the project to undertake specific experiments which could

not be carried out in the local setting, discuss previous data and plan future experiments. Such discussions have been extremely helpful in the conduct of proposed studies, needless to mention, it has greatly helped to bring the laboratory personnel upto date in literature information. Dr. M.P. Gordon, a member of the Agrobacterium group at the University of Washington, Seattle, visited this Centre twice during the project period. During his visits to the Centre, he gave lectures to young scientists of the Centre bringing them upto date in literature information and stimulating the excitement that the young researchers harbour at the Centre. Further, one young member of the team visited Dr. M.P. Gordon's lab for a fifteen month training assignment. This assignment exposed him to the latest methodologies and equipped him with latest information in the area.

Two publications are in preparation and will be published within the next year.

PROJECT PRODUCTIVITY

There were five goals approved in the programme. The first four goals have been fully achieved. The last goal has been achieved only 60-70%. We would have liked to extend the scope of hybridization studies to other probes, however, difficulties in the acquisition of radioactively labelled DNA precursors delayed the experiments.

FUTURE WORK

Yes, a repository of Agrobacterium isolates acquired during the conduct of proposed studies will be shared with Dr. Eugene Nester's collection at Seattle.

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As mentioned above, plant transformation group at this Centre is exploiting the transformation properties of local isolates on chickpea, rice and cotton.

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