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PROGRESS REPORT OF THE PROJECT

Entitled

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

NO. 8.275

in progress

at

CENTRE FOR ADVANCED MOLECULAR BIOLOGY
UNIVERSITY OF THE PUNJAB,
NEW CAMPUS, LAHORE
P A K I S T A N

PRINCIPAL INVESTIGATOR

Professor Sheikh Riazuddin
Centre for Advanced Molecular Biology
University of the Punjab,
New Campus, Lahore
PAKISTAN

US Counterpart Scientist:

Professor Eugene W. Nester
Chairman
Microbiology and Immunology,
University of Washington,
Seattle, WA 98195, USA.

SUMMARY

A research programme entitled "Host Range Specificity of Agrobacterium tumefaciens isolated from Crown gall tumors on fruit trees in Pakistan" is being pursued in the laboratories of the Centre for Advanced Molecular Biology (CAMB), University of the Punjab, Lahore, as part of the PSTC programme of the Agency for International Development, Washington, USA (Project No.8.275).

During the period under report, the previously collected one hundred and forty five isolates of Agrobacterium were characterized and biotyped by different biochemical and biophysical tests. Pathogenicity of selected isolates has been tested on chick pea, sweet peas, broad beans, tomato, tobacco, sunflower, datura, maize and rice.

GENERAL INTRODUCTION

Agrobacterium tumefaciens can infect a remarkably wide range of plants (1-2). Bacterial cells enter plant tissue through fresh wounds and stimulate adjacent living cells to proliferate (3-4). In the process, a plant gall or tumor is formed most commonly at or near the junction of the root and the stem (crown). The affected portion of an infected plant is distinguishable by its ability to form an overgrowth when grafted onto a healthy plant.

All virulent Agrobacterium strain harbour extra chromosomal Ti-plasmid DNA which carries oncogenic characters (5). It has been established that Agrobacterium irreversibly transfers a dis-

tinct portion of the plasmid called T-DNA, into infected plant cells where it gets stably integrated into plant nuclear genome and expressed to initiate crown gall formation (6-8).

Crown gall disease of plants causes considerable economic losses particularly in the stone fruit orchards, vineyards, as well as in ornamental plants. Since the first report by Smith & Townsend (1907), who identified A. tumefaciens as the causative agent of the disease, a large number of plant pathologists have engaged in intensive research which has yielded a wealth of valuable information about the process of tumor formation. The transfer and integration of T-DNA from the Ti-plasmid into the plant nuclear genome requires an intact vir region which functions in trans to the T-DNA (9,10). The vir region spans approximately 35 Kb of Ti-plasmid DNA encompassing at least six distinct transcriptional loci designated as vir A, B, C, D, E and G. Vir A and vir G control the induction of the remaining vir loci (9), and in some strains vir H, vir F, and tzs locus have been localized (Milton P. Gordon, Personal Communications). A portion of the vir D operon encodes a site-specific endonuclease which cleaves at unique and identical locations within the border sequences delineating the T-DNA (11). A model describing how vir A and vir G may interact with plant derived phenolic compounds to result in the expression of the remaining vir genes, has recently been proposed (9).

Host range specificity is an important aspect of plant bacterial interactions, and is particularly interesting in the interaction between Agrobacterium and host plants. Most Agrobacterium research has employed wide host range (WHR) strains which are capable of tumor formation on a wide variety of

dicotyledonous and some monocotyledonous plants (1-2). Some Agrobacterium strains such as of biotype III, however, are host specific and confer virulence on a few limited plant species. Host range is primarily determined by the T-DNA and vir regions (11) while chromosomal factors rarely contribute to host specificity.

Although recent years have seen a substantial growth in our knowledge and understanding of the various factors controlling plant bacterial interactions and initiation of the process of plant tumorogenesis, a complete understanding of the physiological functioning of various Ti-plasmid genes, their mechanism of entry and integration into the plant nuclear genome and subsequent expression of the incoming genes is lacking. There is obviously a need for a more detailed analysis of the various factors involved in plant DNA interaction. Future prospects of plant genetic engineering will ultimately depend on an improved understanding of the factors controlling host range specificity and the functioning of Ti-plasmid genes involved in plant genetic transformation, the development of an increased repertoire of gene transfer methods and systems, as well as the identification of plant genes that are agriculturally significant targets for manipulation. This is especially true since the commercially important crop plants, the monocots, are generally resistant to Ti-plasmid mediated transformation either due to inability of Agrobacterium to infect monocots or the inability of monocots to respond to infection by Agrobacterium.

It is the purpose of these studies to collect a variety of Agrobacterium tumefaciens strains from tumors on apricot, beet, cherry, conifers (Cedar & Juniper), grapevine, pear, potato,

rose, tobacco, tomato, turnip and walnut plants growing in Quetta, Gilgit and the North West Frontier of Pakistan. The isolated strains, presumably showing variations in their host range will be identified with respect to biotype, synthesis of opines in the host tumor tissues, and their host range properties on native host, beet, cherry, chick pea, grapevine, potato, rose, strawberry, sweet potato, tobacco, tomato and turnip plants. Interesting strains, selected on the basis of host range properties, will be characterized and their plasmid DNA will be identified in order to determine homology, if any, to previously characterized T-DNA and vir loci.

Main objectives of the approved programme:

1. Field Survey of fruit orchards in Balochistan and North West Frontier of Pakistan to make a search for crown gall tumors.
2. Collection of crown gall tumorous tissues and their analysis for the isolation of Agrobacterium.
3. Characterization of the isolated bacteria for biotyping, opine synthesis and host range properties in native hosts.
4. Characterization of isolates for the presence of Ti-plasmid and analysis of the plasmid DNA for homologies, if any, with the previously characterized LHR and WHR plasmids.

PROGRESS REPORT

One hundred and forty five Agrobacterium isolates were separated from crown gall tumors appearing on cherry, rose, plum, peach, apple, grape, almond, poplar, abies, walnut, oak, quircus, shina and ash, growing in Blochistan and North West Frontier of

Pakistan. These results were reported in Report-I. During the period of present report, the Agrobacterium isolates were biotyped by different biochemical and biophysical tests as described in the Materials and Methods. Based on these studies, forty-two isolates were classified as biotype I, 63 as biotype II and 40 as biotype III (Table-1).

Pathogenicity of the selected isolates was tested on chick pea, broad beans and sweet peas. Twenty six isolates produced tumors on chickpea, thirty eight isolates showed pathogenic response on broad beans, and thirty isolates exhibited pathogenicity on sweet peas (Table-2. Fig-1: A, B, C, D, E, and F) Isolates are being investigated for the presence of Ti-plasmids, and host range characteristics. We got four isolates from grapewine which are producing tumors on their original hosts while they are giving roots on the leguminous plants. This may be due to the physiological response of the host. Studies on one of the selected isolate are in progress for its molecular characterization.

Opines in the tumorous tissues of infected hosts with twenty-two selected isolates were detected by paper electrophoresis. Opine detection for some of the isolates is shown in Fig-2. Out of twenty-two tumors incited by selected isolates, nine showed the presence of nopaline , 1 represented the presence of octopine, while the rest of the fourteen tumors neither showed the presence of nopaline nor octopine. They may contain another type of opines or the quantity of opines was so less that it could not be detected by paper electrophoresis following the method of Otten and Schilperoort (12).

MATERIALS AND METHODS

The following methodologies were used:

a) Biotyping:

Pure cultures were further characterized and biotyped by the following biochemical and biophysical tests.

1) Growth on selective media for different biotypes.
2) Oxidase activity
3) Catalase activity
4) Growth at 37°C
5) Growth in 3% NaCl
6) Alkali Production from malonate
7) Alkali production from tartarate
8) Alkali production from propionate
9) Alkali production from mucic acid
10) Acid production from erythritol
11) Acid production from melezitose
12) Litmus milk reaction
13) Pellicle formation from ferric ammonium citrate
14) 3-Ketolactose production

b) Pathogenicity:

Seedlings were grown in MS medium under sterilized conditions. Fifteen day old seedlings were wounded and 0.1ml of overnight culture of different isolates was inoculated on the wounded sites. The seedlings were incubated at 22°C. Results were recorded one month after inoculation.

c) Opine detection:

The synthesis of opines in plant tissues was detected by a modifications of the method of Otten & Schilperoort (12). Transformed tissues were homogenized by grinding in a mortar & pestle and the samples were centrifuged in Eppendorf tubes. 5ul of the supernatant from each samples was spotted on a 34 3 28cm Whatmann 3MM filter paper and dried. Standard solutions contained 1mg/ml of the opine and 2% methyl green. The materials were electrophoresed at 300V for 1 hour in a running buffer consisting of formic acid: acetic acid: water 5:15:80. The electrophorogram was dried, stained with a solution containing 1 vol. 0.02% (W/V) phenantherenequinone/ethanol, 1 vol. 10% (W/V) 366nm) UV light.

Table-1

BIOTYPE DISTRIBUTION OF THE ISOLATES FROM DIFFERENT HOSTS.

Origin	No.of tumor samples	No.of isolates	Biotype 1	Biotype 2	Biotype 3
Cherry	4	15	11	3	1
Rose	5	11	6	1	4
Peach	3	9	0	4	5
Shina	1	8	0	2	6
Walnut	1	1	0	1	0
Almond	1	1	0	1	0
Grape	2	6	4	2	0
Ash	2	5	2	1	2
Abies	3	2	1	1	0
Poplar	6	19	8	5	6
Apple	7	31	4	18	9
Plum	5	21	2	18	1
Quircus	2	5	4	1	0
Oak	2	5	0	5	0
Ornamental plant	1	6	0	0	6
Total:	**45**	**145**	**42**	**63**	**40**

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Table-2

DISTRIBUTION OF PLANT TUMORS, NUMBER OF ISOLATES AND NUMBER OF PATHOGENS

Origin	Location	No.of samples tested	No. of expected Agro-bacteria	No. of Agroba-bacterium Isolates	No.of Pathogens on		
					Chick pea	Broad beans	Peas
Cherry	Quetta	4	25	15	3	5	5
Rose	Quetta	3	10	7	0	3	2
	Lahore	2	10	4	0	2	2
Plum	Quetta	4	19	19	4	4	4
	Murree	1	4	2	3	3	3
Peach	Quetta	2	6	4	4	3	3
	Peshawar	1	7	5	2	2	2
Apple	Quetta	4	30	22	2	2	2
	Peshawar	2	10	6	2	2	2
	Murree	1	7	3	2	2	1
Grape	Quetta	2	12	6	1	5	2
Shina	Quetta	1	15	8	0	2	2
Ash	Quetta	2	7	5	1	1	0
Almond	Quetta	1	4	1	0	0	0
Poplar	Lahore	6	30	19	0	1	0
Abies	Murree	3	10	2	1	0	0
Walnut	Murree	1	4	1	0	0	0
Oak	Murree	2	8	5	1	1	0
Quircus	Murree	2	9	5	0	0	0
Ornamen-al plant	Lahore	1	6	6	0	0	0
Total:		45	233	145	26	38	30

Fig-1-A: Tumor formation on Chick pea seedlings after infection with Agrobacterium isolate.

Fig 1-B: Root formation on Chick pea seedlings after infection with Agrobacterium isolate.

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Fig 1-C: Tumor formation on peas seedlings after infection with Agrobacterium isolate.

Fig 1-D: Root formation on peas seedlings after infection with Agrobacterium isolate.

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Fig 1-E: Tumor formation on Broad beans seedlings after infection with Agrobacterium isolate.

Fig 1-F: Root formation on Broad beans seedlings after infection with Agrobacterium isolate.

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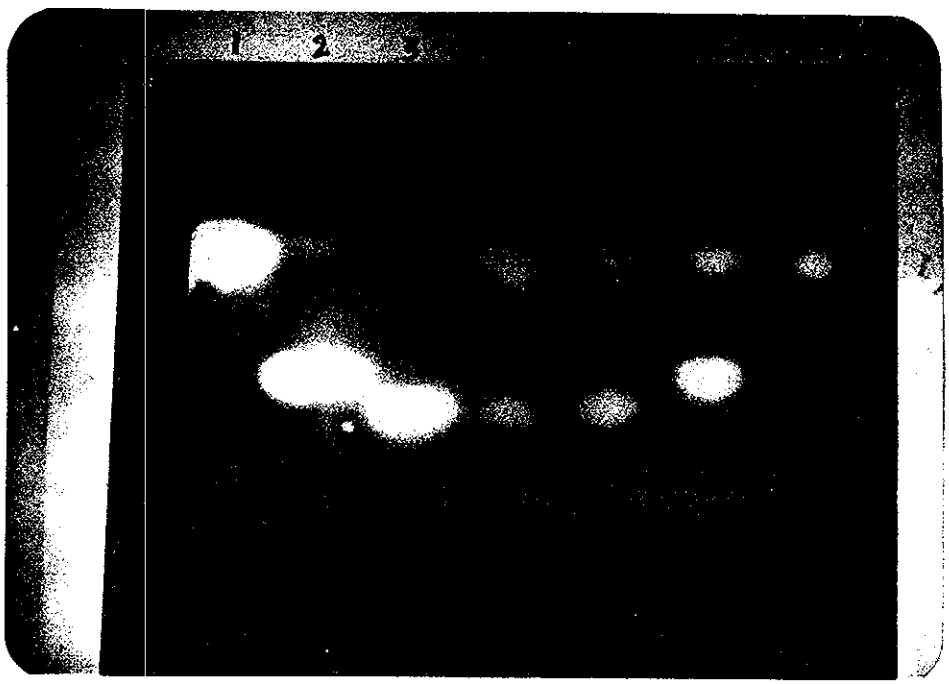

Fig 2: Opine production in the tumor lines induced by different isolates of Agrobacterium.

Lane No.	Opines
1.	Arginin (standard)
2.	Octopine (standard)
3.	Nopaline (standard)
4.	3P1
5.	4P4
6.	Eb7
7.	Untransformed tissue

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