Punjab Univ. J. Zool., Vol. 15, pp. 1-14, 2000

INDIGENOUS *AGROBACTERIUM TUMEFACIENS*: GROWTH RESPONSES TO METALLIC SALTS, ANTIBIOTICS, TEMPERATURE AND pH

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Abstract: Sixty two *Agrobacterium tumefaciens* strains were isolated from local plants of Punjab, Pakistan. Their metallic salts and antibiotics resistance/sensitivity profiles, growth response at two different temperatures and at two different temperatures with various pH levels were investigated. The results of these experiments revealed that isolates shared many physiological characters. Majority of strains could tolerate different metallic salts but all of them could grow in the presence of $MnSO_4$, $PbNO_3$ and $ZnSO_4$ and all were sensitive to Cr salt. These strains could be divided into 15 groups on the basis of metallic salts resistance/ sensitivity profiles. While for different antibiotics these strains showed different behaviour and could be grouped into 10 clusters on the basis of their antibiotics resistance/sensitivity profiles. Results of bacterial growth experiments at 28° and 37°C and at different levels of pH (4, 5, 6, 7, 8, 9) were almost similar in some aspects. Most of the strains, initially, showed better growth rate at 37°C except some strains isolated from colder areas which exhibited better growth at 28°C. The experiments performed at different levels of pH revealed that all strains showed maximum growth rate at pH 6. However pH affect on the growth rate exhibited that all strains could grew better in extreme alkaline pH (9) as compared to extreme acid pH (4).

Key words: *A. tumefaciens*, metallic salts, antibiotics, growth rate, temperature, pH.

INTRODUCTION

The soil phytopathogenic bacterium *Agrobacterium tumefaciens* causes crown gall disease on a variety of plants (Hooykaas and Schilperoort, 1992; Shaw *et al.*, 1991). The soil dwelling phytopathogenic *A. tumefaciens* belongs to family Rhizobiaceae (Krieg and Holt, 1984; Lippincott *et al.*, 1986). The disease caused by *A. tumefaciens* can be recognized by the presence of tumors (galls) of different sizes, shapes and forms on stem, crown (at root-shoot junction) and roots of the infected plants. *A. tumefaciens* is found abundantly in soil as a soil inhabitant and the soil surrounding the infected plants is highly populated by the genus *Agrobacterium* (New and Kerr, 1972; Kerr, 1969; Bouzar and Moore, 1987). The number of the genus *Agrobacterium* around the roots of the infected plants is thousand fold more than the near by soil (Bouzar and Moore, 1987). The higher number of *Agrobacterium* around roots of infected plants is due to a highly sensitive chemotaxis system, responsive to a variety of sugars, amino acids and phenolics exuded by the plants roots and wounds (Ashby *et al.*, 1987; Loake *et al.*, 1988). For chemotaxis the organism must be motile

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Table 1: **List of the parent plants, from which *Agrobacterium tumefaciens* strains were isolated and the different ecological and temporal regions of Punjab, Pakistan, in which these plants were growing.**

Sr. No.	Parent plant	Family	Strain	Locality
1.	*Acacia nilotica*	Mimosaceae	AN1, AN2	Murree
2.	*Acacia modesta*	Mimosaceae	AM1	Rai Wind
3.	*Bombax ceiba*	Bombacaceae	BC1, BC2	Lahore
4.	*Broussonetica papyrifera*	Moraceae	BP1, BP2	Islamabad
5.	*Cassia fistula*	Caesalpinanceae	CF1, CF2, CF3	Lahore
6.	*Cedrela toona*	Meliaceae	CT1	Lahore
7.	*Cedrus deodara*	Piaceae	CD1, CD2	Bhoor Ban
8.	*Cedrus deodara*	Piaceae	CD3	Islamabad
9.	*Celtis caucasia*	Ulmaceae	CC1, CC2	Lahore
10.	*Diospyros embryopteris*	Ebenaceae	DE1	Lahore
11.	*Erythrina suberosa*	Fabaceae	EI1, EI2, EI3	Lahore
12.	*Ficus virens*	Moraceae	FV1	Kala Shah Kaku
13.	*Fraxinus hookeri*	Oleaceae	FH1, FH2	Bhoor Ban
14.	*Hamelia patens*	Rubiaceae	HP1	Lahore
15.	*Mangifera indica*	Anacardiaceae	MI1	Lahore
16.	*Mangifera indica*	Anacardiaceae	MI2, MI3, MI4	Islamabad
17.	*Melia azedarach*	Meliaceae	MA1, MA2, MA3, MA4	Renala Khurd
18.	*Melia azedarach*	Meliaceae	MA5, MA6	Murree
19.	*Morus alba*	Moraceae	Ma1, Ma2	Charra Pani
20.	*Morus alba*	Moraceae	Ma3, Ma4, Ma5, Ma6	Murree
21.	*Morus serrata*	Moraceae	MS1, MS2	Murree
22.	*Phyllanthus emblica*	Euphorbiaceae	PE1	Lahore
23.	*Pinus roxburghii*	Pinaceae	PR1, PR2	Rawalpindi
24.	*Polyalthia longifolia*	Euphorbiaceae	PL1	Lahore
25.	*Pongamia pinnata*	Papilionaceae	PP1, PP2, PP3	Lahore
26.	*Psidium guajava*	Myrtaceae	PG1	Rai Wind
27.	*Psidium guajava*	Myrtaceae	PG2	Manga Mandi
28.	*Punica granatum*	Myrtaceae	Pg1, Pg2	Rawalpindi
29.	*Putrajiva roxburghii*	Euphorbiaceae	Pr1	Lahore
30.	*Salix tetrasperma*	Salicaceae	ST1, ST2, ST3, ST4	Lahore
31.	*Sapindus mukorossi*	Sapindaceae	SM1	Wah Cantt
32.	*Suaeda fruticosa*	Chenopodiaceae	SF1	Manga Mandi
33.	*Termanalia catappa*	Meliaceae	TC1, TC2	Rawalpindi

Antibiotics resistance profiles

For the selection of the antibiotic resistance markers, after autoclaving, medium was supplemented with 50 $\mu g\ ml^{-1}$ kanamycin (Km), 5 $\mu g\ ml^{-1}$ chloramphenicol (Cm), 300 $\mu g\ ml^{-1}$ ampicillin (Ap), 100 $\mu g\ ml^{-1}$ carbenicillin (Cb), 25 $\mu g\ ml^{-1}$ tetracycline

(Tc), after autoclaving the medium. Bacterial strains were streaked on these selective plates and incubated at 28±1°C. Results were recorded after 48 hours of incubation.

Effect of temperature on bacterial growth rate

A. tumefaciens strains were normally grown at 28±1°C. The growth rate of *A. tumefaciens* strains was determined at 28° and 37°C after different intervals of time. From overnight cultures inoculum was given to 50 ml of potato dextrose broth and incubated at 28° and 37°C with 150 rpm (revolution per minute) shaking. Samples were drawn periodically (after 2, 4, 6, 8, 12, 24, 28, 32 hours) and optical density was monitored on spectrophotometer (Model S200D, R&M Marketing, England) at 600 nm.

Effect of pH on bacteria

From overnight culture in potato dextrose broth (pH 6.5) inoculum was given to 50 ml of prewarmed potato dextrose broth, adjusted to different pH levels (pH levels 4, 5, 6, 7, 8 and 9), and incubated at 28±1°C with 150 rpm shaking. After 24 hours bacterial growth was monitored at 600 nm (spectrophotometer S200D, R&M Marketing, England).

RESULTS

Strains were characterized physiologically by exploring the metallic salts and antibiotics resistance/ sensitivity profiles, by comparing growth curve at different temperatures (28° and 37°C) and by monitoring growth rate at two different temperatures (28° and 37°C) with various pH levels (4, 5, 6, 7, 8, 9). All strains exhibited individual as well as similar responses reflecting its characteristics and attributes.

Metallic salts resistance/sensitivity profiles

The metallic salts resistance/sensitivity profiles revealed that majority of strains could tolerate metallic salts. All strains fall into 15 groups or clusters, isolated either from the same or different biotopes, depending upon their metallic salts resistance/sensitivity (Table II). The metallic salts profiles showed that all isolates could tolerate Mn (500 μg ml^{-1}, Pb (250 μg ml^{-1}) and Zn (350 μg ml^{-1}) in the medium, while all strains showed sensitivity to Cr (50 μg ml^{-1}) salt (Table II). After these Ba (61 strains), Mo (59 strains) and Fe (55 strains) salts were tolerated by majority of strains. Out of 62, 26 strains showed resistance to majority of (10 out of 11) salts tested and showed sensitive behavior to Cr salt. Next to Cr, most of the strains showed sensitive behavior for Ni (27 strains) and Co (21 strains) salts (Table II).

Antibiotics resistance/sensitivity profiles

Antibiotics (Ap, Cb, Km, Sm and Tc) resistance/sensitivity spectrum of all the strains was worked out. The strains which were exhibiting similar antibiotic resistance/sensitivity profiles were associated together. These strains fall into 10 groups on the basis of specific resistance/ sensitivity attributes for different antibiotics (Table III). The antibiotics resistance/ sensitivity spectrum revealed that different strains showed similarities as well as differences in the antibiotics resistance markers. The experimental data revealed that most of the strains could tolerate ampicillin (39 strains)

Table II: **Results of metallic salts resistance/sensitivity profiles of *Agrobacterium tumefaciens* isolates.**

Metallic salts concentrations			Strains														
			AN1 AN2	AM1,BC1,CF2,CT1, CC1,CC2,DE1,EI1, EI3,FV1,HP1,MI1, MI3,MA4,Ma1,Ma2, PR1,PR2,PL1,PP1, PG1,Pg2,Pr1,SM1, SF1	BC2,CF1, CF3,MI2 MI4,MA5, MA6,PE1, ST4,TC1, TC2	BP1	BP2 MA1 MA2	CD1	CD2 PG2	CD3 Ma6 ST1 ST2	FH1	FH2 ST3	MA3 PP2 PP3	Ma3 Ma5	Ma4	MS1 MS2	Pg1
1.	$Ba(BaSO_4)$	250μg ml^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
2.	$Co(CoCl_2)$	250μg ml^{-1}	-	+	+	-	-	-	-	-	-	+	-	-	-	-	+
3.	$Cr(Cr_2O_3)$	50μg ml^{-1}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4.	$Cu(CuSO_4)$	200μg ml^{-1}	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-
5.	$Fe(FeCH_5O_7)$	250μg ml^{-1}	+	+	+	-	+	+	+	+	+	+	-	-	-	+	-
6.	$Mn(MnSO_4)$	500μg ml^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7.	$Mo(Na_2MoO_4)$	500μg ml^{-1}	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+
8.	$Ni(NiSO_4)$	200μg ml^{-1}	+	+	+	-	-	+	+	+	-	+	+	-	-	-	-
9.	$Pb(PbNO_4)$	250μg ml^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10.	$Sn(SnCl_2)$	200μg ml^{-1}	+	+	-	-	-	-	-	+	-	-	-	-	+	+	-
11.	$Zn(ZnSO_4)$	350μg ml^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = Resistant
- = Sensitive

carbenicillin (41 strains) and kanamycin (41 strains) (Table III). While most of the strains were sensitive to streptomycin (48 strains) and tetracycline (53 strains) (Table III).

Temperature effects on bacterial growth

The results of the experiment reflect that in majority of the cases *A. tumefaciens* strains, initially, showed better growth response at 37° as compared to growth rate at 28°C (Fig.1). In few cases, the strains, AN1, AN2, FH1, MA5, MA6, MS1 and MS2, isolated from colder areas showed better growth response at 28°C (Fig.1). The experimental data showed that growth rate of the strains, in majority of cases, was almost equal after 24 hours of incubation. The strains isolated from colder areas had slow growth rate, except Ma1 and Ma2, and generally showed similar growth response at 28° and 37°C (Fig.1). Some of the strains isolated from different ecological and temporal regions showed similar growth rate. However some of the strains showed minor differences in growth rate, i.e., some strains showed almost equal growth rate at 28° or 37°C, while some strains showed very rapid growth rate at 37°C from 4 to 12 hours as compared to 28°C (Fig.1).

Strains AN1, AN2, FH1, MA5, MA6, MS1 and MS2 exhibiting better growth response at 28°C. These strains were isolated from colder areas (Table I), whereas none of the strain isolated from warmer areas gave better growth response at 28°C (Fig.1). AN1, AN2, MA5 and MA6 had a higher growth rate at 28°C as compared to 37°C even after 32 hours of incubation. While FH1, MS1 and MS2 showed initially better growth rate at 28°C which later on succeeded by the growth rate at 37°C and after twelve hours of incubation these strains showed better growth at 37°C (Fig.1). Some strains, CD1, CD2, FH2, Ma1, Ma2, Ma3, Ma4, Ma5 and Ma6, which were also isolated from colder areas did not show better growth rate at 28°C (Fig.1). However better growth rate was observed in some strains (Ma1, Ma2, Ma4, FH2 and Ma6) at 28°C at different time intervals. In case of Ma1, Ma2 and Ma4 better growth rate was observed at 28°C after 12 hours of incubation, which was again superseded by growth response at 37°C in strains Ma2 and Ma4. While FH2 and Ma6 gave better growth rate after 32 hours of incubation at 28°C (Fig.1). BC1, BC2, HP1, Ma1, Ma2, PL1 and PG2 showed better growth rate at 37°C, approximately, from 4 to 8 hours of incubation and later on these strains showed higher growth rate at 28°C after 8 hours of inncubation. However in Ma2 and PG2 growth rate at 28°C was again dominated by the growth response at 37°C after 32 hours (Fig.1). In majority of cases, after 32 hours, the growth rate was almost equal or even in some cases, i.e., AM1, CF2, CT1, CC2, FV1, FH1, MI4, MA1, Ma6, PE1, PR2, PG1, Pg2, Pr1 and TC1, was better at 28°C (Fig.1). Generally growth at 28°C and 37°C, increased rapidly upto 12 hours, except MA5, MA6 annd Pg1 which started growing after 8 hours (at both temperatures) and Ma1 which showed slow growth rate at 28°C (Fig.1). These results depict that majority of *A. tumefaciens* strains initially showed rapid growth rate at 37°C but after 24 to 32 hours this growth rate is almost equal at both temperatures (28°C and 37°C) and even in nsome cases it was better at 28°C (Fig.1).

Effects of pH on bacterial growth

The bacterial growth with different pH levels at 28° and 37°C showed that all *A. tumefaciens* strains grow better at pH 6 and 7 and with maximum growth was observed at pH 6. All strains, with few exceptions, gave higher population density at pH 8 and 9

Table III: Results of antibiotics resistance/sensitivity profiles of *Agrobacterium tumefaciens* isolates.

Antibiotic concentrations	Strains									
	AN1.CD2.EH1. EH2.MI1.MA2. Ma3.Ma5.Ma6. MS1.MS2.PE1 PR2.PL1.PG1. SF1	AN2.BC1. CT1.HP1. MA4.PP3. SM1	BP2.CD1. EI2.EI3. Ma4.TC1	BC2.CD3.CC1. CC2.PG2.Pg1. ST1.ST2	EI1	CF1.Ma1 Ma2	CT2	AM1.BP1.CF3. MI2.MI3.MI4. MA1.MA3.MA5. MA6.PR1.Pg2. ST3.ST4	DE1.FV1. PP1.PP2 Pr1	TC2
1. Ampicillin 300 μg ml^{-1}	+	+	+	-	+	+	-	-	+	-
2. Carbenicillin 100 μg ml^{-1}	+	+	-	+	-	+	+	-	+	+
3. Kanamycin 50 μg ml^{-1}	+	+	-	+	-	+	+	-	+	+
4. Streptomycin 300 μg ml^{-1}	-	+	-	-	+	-	+	-	+	-
5. Tetracycline 25 μg ml^{-1}	-	-	-	-	+	+	-	-	+	-

+ = Resistant
- = Sensitive

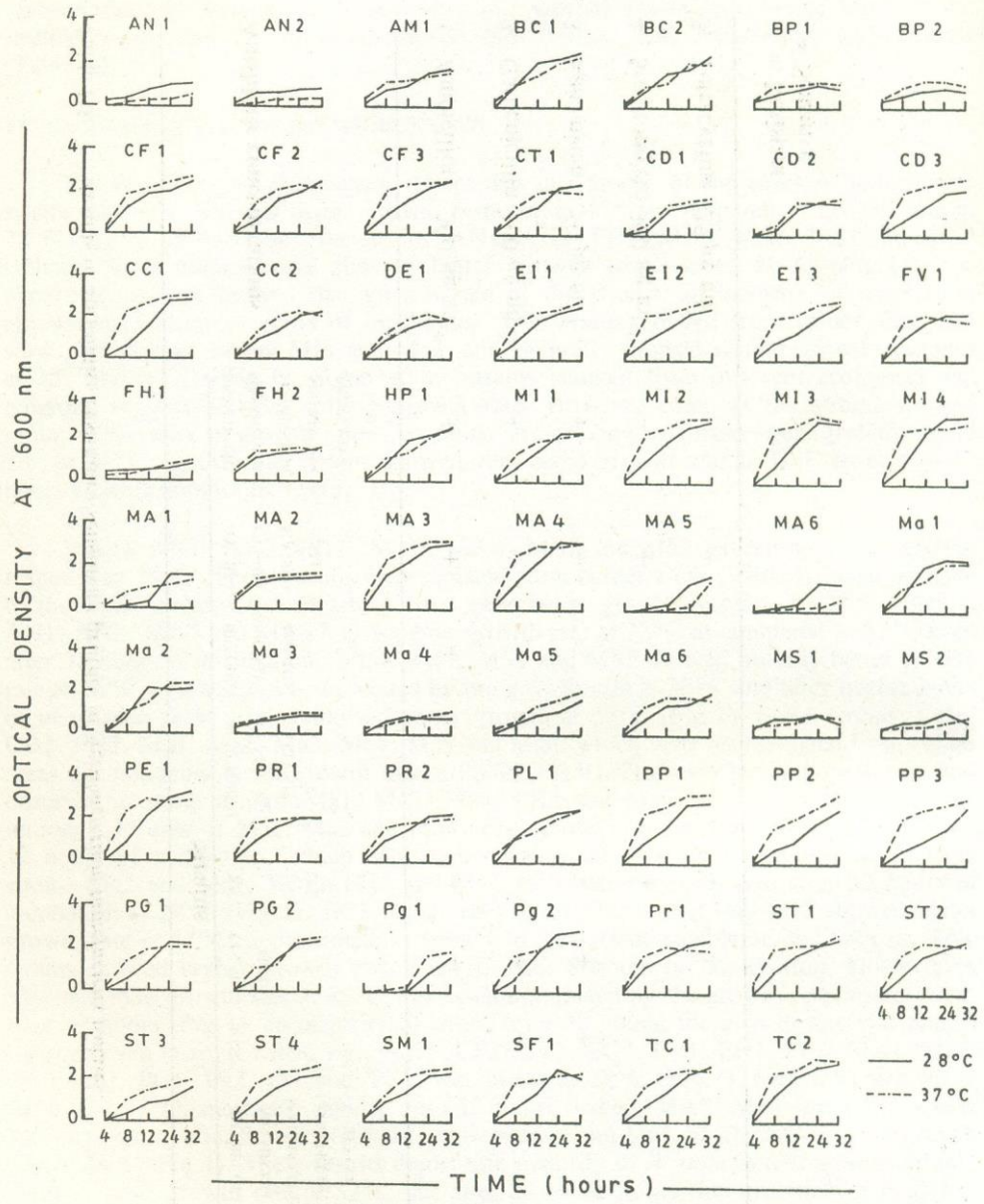

Fig. 1: Growth curves of *Agrobacterium tumefaciens* strains at 28° and 37°C.

as compared to cell density at pH 4 and 5. This shows that mostly *A. tumefaciens* survived better in extreme alkaline pH as compared to extreme acidic pH (Fig.2). Similar results were recorded as were observed when bacterial strains were grown at two different temperatures (28°C and 37°C) i.e., higher growth density was observed in all pH levels at 37°C as compared to 28°C, except for some strains (AN1, AN2, MA5, MA6, Ma1, Ma2, MS1 and MS2) which were isolated from colder areas as well as few strains (AM1, BC1, MI1, MA1, Pg2, SM1 and SF1) isolated from warmer areas (Table I, Fig.2).

AN1, AN2, MA5 and MA6 showed better growth in all pH levels at 28°C, whereas at 37°C very poor growth rate was recorded (Fig.2). The strain, MI3, showed almost similar growth pattern at 28°C and 37°C, however higher growth rate was observed at 37°C. MI3 showed slightly higher growth rate at 28°C with pH 7 as compared to pH 7 at 37°C, but at pH 8 and 9 growth rate again increased at 37°C (Fig.2). Some of the strains which were isolated from warmer areas (Table I) also showed higher growth rate at 28°C in all pH levels as compared to growth rate at 37°C. Whereas some strains, CD1, CD2, FH1, FH2, Ma3, Ma4, Ma5 and Ma6, isolated from colder areas (Table I) gave better growth response at 37°C at all pH levels (Fig.2). At pH 4 all strains showed either poor or no growth response, while at pH 9 somewhat better growth rate was noticed. These results reflect that these *A. tumefaciens* strains could grew better in extreme alkaline pH (9) as compared to extreme acidic pH (4). However these strainns gave maximum growth rate at pH 6 at both temperatures (28°C and 37°C).

DISCUSSION

The physiological characterization revealed that strains shared many aspects and attributes even though isolated from different ecological and temporal regions (Table I). The metallic salt resistance/ sensitivity profile revealed that many isolates showed similar characters, however some strains exhibited individual attributes which were different from the rest of the strains. Differences were observed in strains which were isolated from same parent trees growing in same ecological and temporal region (Table I), and vice versa i.e., similarities were exhibited by strains isolated from differet parent plants growing in different biotope (Table II, III). It has been observed that metallic salts effect the metabolism and growth of the bacterium (Jonas *et al.*, 1984) and decreased growth at elevated levels of metabolic salts have been attributed to impaired metabolic activities and reduced cell expansion (Wood and Wang, 1987). Some bacteria which survive the higher concentration of metallic salts could be genetically adapted or their survival could be due to the composition of the medium, because metallic salt toxicities to microbes also depends on the growth medium (Hughes and Poole, 1989). Bacterial growth medium components form complexes with metallic salts and remove them from solution, thus reduces its apparent concentration in the medium (Guffanti and Hicks, 1991). For Cr (50 μg ml^{-1}) metallic salt all strains showed negative growth response which might be due to impaired metabolic activities and reduced cell expansion of the strains. While all strains exhibited tolerance for metallic salts of Mn (500 μg ml^{-1}), Pb (250 μg ml^{-1}) and Zn (350 μg ml^{-1}) in the growth medium (Table II). All strains tolerated these metallic salts which might be due to the presence of resistance markers on the plasmids of these strains. Lead and zinc are toxic salts and tolerance of all strains to these salts indicate the presence of these salts in the environment, and is of serious health concern. Out of 62 strains, 26 strains exhibited resistance to majority of salts tested. Similarly for antibiotics resistance/ sensitivity spectrum different strains

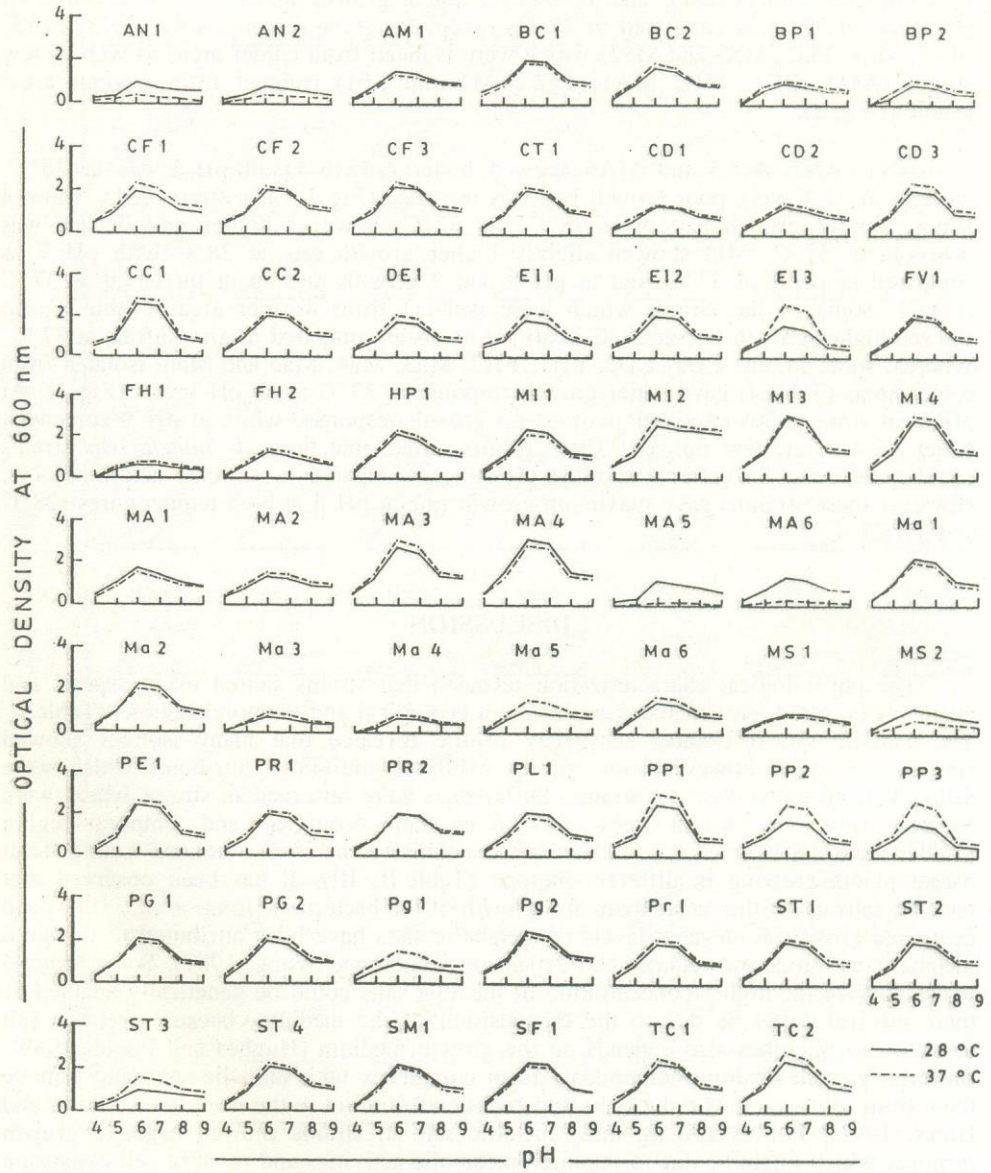

Fig. 2: Effects of different pH levels on the growth response of *Agrobacterium tumefaciens* strains.

exhibited individual as well as similar and common responses (Table III). Antibiotics resistance determinants may play a role in cellular metabolism other than the protection of the host. Majority of strains showed sensitive attribute to Tc (53 strains) and Sm (48 strains). Whereas many of them could bear Ap (39 strains), Cb (41 strains) and Km (41 strains). Fourteen strains showed sensitive behavior to all the antibiotics tested in the experiments (Table III). Expression of the antibiotics resistance genes some times may depend on the cytoplasmic background i.e., in some cases the gene is not expressed because it lacks a functional promoter (Davies, 1992). Therefore it might be possible that strains which showed sensitive behavior for some antibiotics might had resistance marker for that particular antibiotic but due to lack of functional promoter that gene was not expressed. However these results showed that many strains had resistance markers for various metallic salts and antibiotics, and these results could be utilized in selecting the transformed plants or tissues with that particular strains.

There are environmental factors that affect the physiology and chemistry of the living organism e.g., temperature and pH. Growth and metabolism of bacteria are related with temperature. Either at high (Patterson and Giltespie, 1972) or low (Inniss and Ingraham, 1978) temperature one or more reactions become rate limiting due to inability of cells to adjust to thermally induced changes (Araki, 1991). For growth and each biochemical reaction there is optimum temperature for a bacterial strain. Like temperature or other environmental conditions, pH also exerts noticeable influence on growth of bacteria. Some bacteria can survive in an environment with an extreme acid pH (*Thiobacillus thiooxidants*, *Acetobacter* sp. etc.), while some grow in extremely alkaline pH. For most bacteria, however, the optimum pH for growth lies between 6.5 and 7.5. A sever change in the pH can bring the growth of a microbial population to a halt. Bacterial growth experiments at different temperatures and at different levels of pH were almost similar in some aspects. Optimum temperature for *A. tumefaciens* growth ranges from 25° to 28°C (Holt *et al.*, 1994). The results of these experiments showed that majority of strains isolated from warmer areas showed rapid growth rate at 37°C (Fig.1, 2). According to Hooyakaas (1988) *A. tumefaciens* biotype I strains can grow at 37°C. While some of the strains CD1, CD2, FH2, Ma1, Ma2, Ma3, Ma4, Ma5 and Ma6, which were isolated from colder areas, initially, also showed somewhat better growth at 37°C. These strains were isolated from trees growing along road side in sunny area. This might be a factor in acclimatizing these strains to warmer temperatures. Whereas AN1, AN2, FH1, MA5, MA6, MS1 and MS2, isolated from colder areas showed better growth response at 28°C (Fig.1) and these strains were isolated from trees growing away from road side in a shady area. This showed that not only the temperature of biotope effect the characteristics of a strains but locality of the strain also effects its physiological characters. The experiments performed at different levels of pH also showed that all these strains had a specific affinity for pH 6 irrespective of temperature i.e., all strain showed maximum growth peek at pH 6 when grown at 28° or 37°C. Cell wall components of bacteria are important in determining the pH tolerance of a bacterium. Generally alkalophilic bacteria have more acidic amino acids and sugars in their cell walls (Aono *et al.*, 1993). The strains isolated from warmer areas showed higher growth rate at 37°C except few strains (AM1, BC1, MI1, MI3, MA1, Pg2, SM1 and SF1) which showed better growth response at 28°C (Fig.2). These results were similar to results observed at temperature effect on growth rate. Here the optical density was measured after 24 hours of incubation and growth curves showed that these strains had a higher growth rate at 28°C after 24 hours of incubation (Fig.1). The pH affect on growth rate exhibited that all strains could grew better in extreme alkaline pH (9) as compared to extreme acid pH (4). According to Guffanti and Hicks (1991) bacteria which can grow well in alkaline pH range 9-11, maintain an internal pH 1-2 units lower

than external pH. The bacterial cells also have the capacity to regulate the cytoplasmic pH (pH_{in}) at around neutrality regardless of their external pH (pH_{out}) (Booth, 1985). It can be concluded that bacterial strains were growing in the medium by shifting the pH towards their optima.

The ongoing discussion surmise that *A. tumefaciens* strains from different sources and localities may exhibit similar antibiotic/metallic salt profiles whereas strains from the same source may display variation in resistance/ sensitivity spectrum to antibiotics and metallic salts. *A. tumefaciens* strains from colder areas prefer 28°C for their optimum growth and those belonging to warmer regions prefer 37°C. Previously optimum temperature reported for *A. tumefaciens* growth is 25°-28°C (Holt *et al.*, 1994). Most probably those strains were isolated from colder regions. Reports on tumorigenesity of *A. tumefaciens* state that above 32°C virulence of the strains as well as conjugal transfer of Ti plasmid are hampered (Jin *et al.*, 1993). We anticipate that strains from warmer areas, which grow better at 37°C, might also show virulence as well as conjugal transfer of Ti plasmid at relatively higher temperature and further studies might reveal additional aspects in understanding of this bacterium.

Acknowledgement

University Grants Commission is acknowledged for the award of scholarship to S.H.A. Qazi.

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(Received: August 15, 1999)