Pakistan J. Zool., vol. 52(5), pp 2019-2021, 2020 DOI: https://dx.doi.org/10.17582/journal.pjz/20181021061044

Short Communication

Isolation and Characterization of *Agrobacterium tumefaciens* Strains from Malakander Farm, University of Agriculture, Peshawar

Shaheen Rahman[1], Kafeel Ahmad[1]*, Niaz Ali[2], Muhammad Idrees[3] and Waqar Ali[4]

[1]*Centre of Biotechnology and Microbiology, University of Peshawar, Peshawar*
[2]*Department of Life and Sport Sciences, University of Greenwich, London, UK*
[3]*Department of Microbiology and Biotechnology, Abasyn University, Peshawar*
[4]*Department of Biotechnology, University of Malakand, Chakdara, Khyber Pakhtunkhwa, Pakistan*

Article Information
Received 10 February 2018
Revised 11 May 2019
Accepted 11 September 2019
Available online 29 May 2020

Authors' Contribution
SR and NA conducted the experiments and compiled the data. SR wrote the manuscript. KA designed and supervised the project. MI, KA and WA peformed data analysis and proof reading the manuscript.

Key words
***Agrobacterium tumefaciens*, Biochemical test, Morphological identification, Pathogenicity, YEMA medium**

ABSTRACT

Agrobacterium tumefaciens is a plant pathogen that causes devastating Crown Gall disease. Current studies were aimed at the isolation of *A. tumefaciens* from local soil. Five soil samples were collected from research farm of Agricultural University Peshawar, Pakistan. Soil samples were processed for the isolation of *A. tumefaciens*. Various biochemical and morphological tests were conducted to confirm the identity of *A. tumefaciens*. The pathogenicity of the isolates was confirmed by in vitro carrot and potato discs inoculation assays. A total of seven strains of *A. tumefaciens* were isolated on yeast extract mannitol agar (YEMA) medium. The isolated strains were confirmed as *A. tumefaciens* on the basis of morphological, biochemical and pathogenicity tests. The bacterial cells were rod shaped having rounded ends. The strains were Gram positive. Results confirmed the pathogenicity of the isolated strains as shown by development of tumours on carrot and potato discs. The pathogenic potential of the isolated strains demand for proper management and control measurements to spread and prevent infections of *A. tumefaciens*.

Agrobacterium tumefaciens is aerobic, Gram negative, rod shaped and motile bacterium that belongs to the family Rhizobiaceae. These bacteria do not form endospores (Davoodiand Hajivand, 2013). *A. tumefaciens* enters plant tissues through wounds and causes infection producing tumours on a broad range of plants including many dicots, few monocots and some gymnosperms. The most favourable temperature for *A. tumefaciens* is 28°C. Many strains of *A. tumefaciens* and the non-pathogenic *A. radiobacter* are capable to grow on simple carbon sources and on minimal media having salt. Ammonium salts and nitrate are utilized as nitrogen sources by *A. tumefaciens* and *A. radiobacter* but not used by A.*rubi*and *A. rhizogen* (Wise *et al*., 2006). Throughout the world *A. tumefaciens* can be isolated from both cultivated and uncultivated soils. It particularly establishes affiliation with rhizosphere. Virulent strains are normally only isolated from galls and soils around the infected roots. In soil, the population of avirulent strains is particularly much higher than the virulent strains. It was declared that the loamy soil due to its water retention capacity and acidic pH has increased number of pathogenic strains (Marashi, 2000). The virulent and non-virulent strains have common characteristics. They have at least one but large Ti plasmid (tumor-inducing plasmid) with two important regions i.e. transferred DNA (T-DNA) and the virulence genes (Vir genes). These two regions control bacterial virulence (Watson *et al*., 1975).

In 1897, Fridiano Cavara for the first time described that *Bacillus ampelopsorae* a flagellated bacterium induces grown gall in grape (Cavara, 1897). This organism is now called *Agrobacterium vitis* which is the causative agent of neoplastic tumours. It produces tumours on the crown and stem of grapevine and induces necrotic lesion of the roots of grape. *A. vitis* can live in intercellular spaces of tissues of grape without causing disease but on wounded tissues, most commonly due to frost injury, it causes tumour formation. After ten years of the discovery of *A. vitis*, Smith and Townsend (1907) reported that bacterium tumefaciens which is now called *A. tumefaciens* was the cause of crown gall in Paris daisy. This organism can induce tumour at wound site particularly on crown, stem and roots of dicots.

A. tumefaciens has the ability to cause devastating diseases in a number of crops. Some strains of agrobacterium are pathogenic while others are not harmful to crops. Hence, it is desirable to analyse the presence of pathogenic *A. tumefaciens* strains in local soil to devise/

* Corresponding author: kafeelpbg@gmail.com
0030-9923/2020/0005-2019 $ 9.00/0

implement proper prevention and control strategies.

The objectives of this project were to isolate, characterize and evaluated the pathogenicity of *A. tumefaciens* from university campus fields.

Materials and methods

Soil samples were collected from Agricultural University field. Five samples were collected from topsoil and subsoil. The samples were collected in a sterile polythene bag with the help of sterile spatula. The samples were brought to the laboratory and kept at 28˚C for further use.

Soil stock solution was made by mixing 10 g soil with 90 ml saline solution. Serial dilutions of the soil sample were made. Soil solution sample (0.1 ml) was inoculated onto Yeast Extract Mannitol Agar (YEMA) medium (Mannitol 10g/l, Yeast extracts 1g/l, Dipotassium phosphate 0.5g/l, sodium chloride 0.2g/l, $CaCl_2$ 0.2g/l, $MgSO_4.7H_2O$ 0.2g/l, $FeCl_3$ 0.01g/l, Agar 15g/l, pH 7.0). Petri dishes were incubated upside down for 48 h at 28˚C. Pure cultures were isolated and bacteria were preserved in glycerol cultures.

Isolated bacteria were identified as *A. tumefaciens* using Gram staining and biochemical tests like Benedict test for production of 3-ketolactose, catalase, oxidase, citrate, motility and 2% NaCl tolerance tests.

For pathogenicity test potato (*Solanum tuberosum*) and carrot (*Daucus carota*) discs were used for tumour induction properties of *Agrobacterium*. Potato tubers and carrot roots were washed to remove soil. Both were cut into small slices on a sterile marble tile with the help of sterile knife under sterile conditions. The discs were sterilized with 15% commercial bleach for 15 min. The discs were then rinsed with sterile water three times. The discs were placed on Murashige and Skoog medium. Each disc was inoculated with 100 µl inoculum. The plates were sealed with parafilm and incubated in growth chamber 25˚C for 3 weeks. After 3 weeks, the discs were checked for tumours development.

Results

Colonies on YEM medium were observed as pink, pinkish red and brick red in colour. These colonies were circular in shape. The isolated bacteria (7 isolates) were rod shaped and red in colour (Gram negative) as given in Table I. Yellow ring was observed around the colony in about 1-2 h when 3-ketolactose production occurred. All the seven isolates were positive for 3-ketolactose production (Table I). In citrate test, growth was observed on the slant surface and the medium colour was changed from green to dark greenish blue colour indicating positive results (Table I). Dispersed growth was observed from line of inoculation in motility test (Table I). Black precipitate was also observed, indicating H_2S production. The selected seven isolates were positive for H_2S production (Table I). The isolated bacteria were positive for 2% salt in the growth medium (Table I). In catalase test, bubbles were formed on the slide after adding H_2O_2. The isolated bacteria were positive for catalase test (Table I). The inoculated filter paper turned violet immediately after adding the reagent in oxidase test showing positive results (Table I).

All the isolates were positive for pathogenicity test under *in vitro* bio-assays (Table II). Tumours were developed around the central vascular stem.

Table I. Morphological and biochemical characteristics of *Agrobacterium tumefaciens* isolates (+ sign indicates positive result and – sign indicates negative result.

Test	*A. tumefaciens* isolates						
	Sp1	Sp2	Sp3	Sp4	Sp5	Sp6	Sp7
Gram staining	–	–	–	–	–	–	–
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
2% salt tolerance	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+	+
H_2S production	+	+	+	+	+	+	+

Table II. Pathogenicity Test: + sign indicates tumor production.

Plant tissue	*A. tumefaciens* isolates						
	Sp1	Sp2	Sp3	Sp4	Sp5	Sp6	Sp7
Potato discs	+	+	+	+	+	+	+
Carrot discs	+	+	+	+	+	+	+

Discussion

The aim of this project was to isolate and characterize *A. tumefaciens* strains from soil from Agricultural university field and to confirm their characteristics by morphological, biochemical and pathogenicity tests. On the basis of colour development, some pink colour colonies were observed on YEM agar medium as reported previously (Kumar *et al*., 2013). However, in our results pinkish red and brick red coloured colonies were also observed on YEM agar medium. The colonies were smooth, circular and translucent. The bacterial cells were rod shaped in Gram staining as described previously (Setti and Bencheikh, 2013). For further confirmation of *A. tumefaciens*, various biochemical tests were performed,

and the isolates were found negative in Gram test and positive in motility, catalase, oxidase, 3-ketolactose production, H_2S production and 2% salt tolerance.

The native soil bacterium, *A. tumefaciens,* is famous for plant diseases. *A. tumefaciens* initiates crown gall disease in many dicotyledonous plants, particularly in members of Rosaceae like almond, cherry, pear, peach, apple, roses and raspberry. Crown gall disease produces tumor like swellings (galls) that usually arise at the plant crown just above the ground. The bacterium transfers a piece of plasmid DNA into the plant through infection. This T-DNA incorporates into the genome of the plant and produces tumors in the host plant and alters the metabolism of the host plant. The infected plant produces new plant metabolites such as opines. Opines are specific by-products of amino acids that are used as a source of carbon and nitrogen by the tumor causing bacteria. Agrobacteria are famous for utilizing a broad range of substrates (Koivunen *et al*., 2004). The ability to form tumours by the selected isolates on potato and carrot confirmed them as pathogenic strains of *A. tumefaciens* as suggested previously (Sarker *et al*., 2011).

The isolates possessed pathogenic characteristics that indicate the disease-causing ability of the isolated *A. tumefaciens* strains. These virulence characteristics which are due to the presence of Ti plasmid in these strains are potential threats to cultivated crops and could be disseminated to other localities through transportation, human and farming practices, and germplasm exchange.

Conclusion

Current study confirmed the prevalence of pathogenic strains of *A. tumefaciens* in local soil samples. These findings highlight the need for proper management and control strategies to avoid or minimize crop damages in case of infections and prevent the spread of pathogenic strains of *A. tumefaciens* through quarantine measures.

Statement of conflict of interest

The author has no conflict of interest.

References

Cavara, F., 1897. *Sperim. Agrar. Ital.,* **30**: 483-487.

Davoodi, A. and Hajivand, S., 2013. *J. Ornam. Hort. Pl.*, **3**: 117-124.

Koivunen, M.E., Morisseau, C., Horwath, W.R. and Hammock, B.D., 2004. *Can. J. Microbiol.,* **50**: 167-174. https://doi.org/10.1139/w04-001

Kumar, A., Bhawsar, N.G., Badnagre, P., Panse, U., Gayakwad, S.R. and Khasdeo, K., 2013. *Int. J. Adv. Res.*, **1**: 1-4.

Marashi, S.H., 2000. *Identification and characterisation of behavioural genes of* Agrobacterium tumefaciens. Doctoral dissertation, Durham University.

Sarker, A.Q., Palash, C. M., ISLAM, S., and ALAM, M. F., 2011. *Agric. conspec. Sci.,* **76:** 147-152.

Setti, B. and Bencheikh, M., 2013. *Eur. Sci. J.,* **9**: 1857-7431.

Smith, E.F. and Townsend, C.O., 1907. *Science,* **25**: 671-673. https://doi.org/10.1126/science.25.643.671

Watson, B., Currier, T.C., Gordon, M.P., Chilton, M.D. and Nester, E.W., 1975. *J. Bact.*, **1**: 255-264.

Wise, A.A., Liu, Z. and Binns, A.N., 2006. Agrobacterium *protocols.* Humana Press. pp. 3-14.