Journal of Al Azhar University-Gaza (Natural Sciences), 2012, 14 : 77-84

Isolation and detection of *Agrobacterium tumefaciens* from soil

Rola Jadallah[1] and Nasser Sholi[2]

1 Biology and biotechnology Department, AAUJ
2 National Agriculture Research Center, Jenin

Received 4/9/2011 Accepted 20/9/2012

Abstract:
Palestinian agriculture sector is on thrive, especially stone fruit plantation and mainly almond, but this is threatened by pest and diseases. Agrobacterium tumefaciense that causes crown gall diseases is one of the major problems affecting stone fruit production. Uncontrolled and irresponsible practices by nurseries and farmers allowed the spread of this disease at alarming rate. Bacterial culture on selection medium followed by PCR amplification with specific primers directed towards ipt gene was found to be very useful and efficient in detection of Agrobacterium tumefaciense both in soil and root samples. Direct detection from soil and root samples diluted with water was not possible at this time.
Prevalence of Agrobacterium tumefaciense in soil sample was found to be 12.5 % and 5% in root samples. From results obtained, there is a need to follow very strict quarantine methods and follow up by respective authority in order to minimize infection and spreading of this disease. In addition to that, the time for sample collection should be considered.

1. Introduction:

Agrobacterium tumefaciens is a rod shaped, flagellated, soil borne pathogen, gram negative bacteria include pathogenic and nonpathogenic strains, the problem lies on pathogenic strains that induce crown gall formation as it is tumor formation causing bacteria, this tumor forming disease consider destructive economical problem affects the plants on vast areas as it remains undetectable for years in soil of location of injury (Matthew and Abhay, 2003).

Agrobacterium tumefaciense can infect a wide range of plant species and it can survive in plants, in nurseries, vineyard, and fruit plants as well as in soil (Abussaoud, and Al-Momani, 1992, Ponsonnet, and Nesme, 1994). This bacteria have both pathogenic and non pathogenic strains and have strains share common features, that contains one at least but large (Ti plasmid : tumor-inducing plasmid),with two

regions together determine the bacteria virulence : the transferred DNA (T-DNA) and the virulence genes (Vir) genes (mediate transfer T-DNA) (Ponsonnet, and Nesme, 1994, Van Larebeke, et al, 1974, Watson, et al , 1977,Chilton, et al, 1980)

Agrobacterium is a causal agent producing tumor forming disease called crown gall at the site of wounding due to using agriculture tools , frost injury , nematode, or/and insect feeding. The phenolic compound work on attract the Agrobacterium that leak from wounded roots and stems. Where the bacteria uses its flagella to move towards wound and colonize either the wound or the rhizosphere on material sugar. (Wang,etal, 1991,Burr, and Katz, 1983). The infected plant at the end become unproductive, weakened, stunted result in economical losses for farmers and at business level of the area in general (Agrios, 1978).

Different PCR methods have already been used for Agrobacterium detection targeting Bacterial sequences (Wei etal. 2011), nested PCR (Francesca et al., 2010) and real time PCR (Federica et al., 2008).

In Palestine, uncontrolled distribution of plants by nurseries especially almond and other stone fruit led recently to spread of the *Agrobacterium tumefaciens* at alarming rate especially in irrigated area. This ultimately led to that Palestinian Ministry of Agriculture to interfere and made it obligatory for any nurseries to test the soil every year for the presence of Agrobacterium. So far there is no documented reports or research has been done so far in Jenin area where production of stone fruit is in thrive. On the other hand, there is no report regarding the prevalence of Agrobacterium in Jenin soil itself. For these reason we sought to carry out this research to:

1- To detect and isolate *Agrobacterium tumefaciens* from Jenin soil and determine its prevalence.

2- To test plant roots for the presence of *Agrobacterium tumefaciens*.

2. Materials and Methods:

2.1 Sample collection:

Soil and stone fruit root samples were collected from nurseries and soil belongs to farmers that cover irrigated land in Jenin district in

November and were recollected from the same sites in December and January. This time was selected because nurseries and farmers started planting their stone fruit at November.

2.2 Bacterial culture

Five grams of soil samples were added to 30 ml of sterile distilled water in 100 ml capacity beakers and shaked for 30 minutes at 70 rpm. The samples were allowed to settle down for another 10 minutes and 50µ of supernatant was inoculated to semi selective medium prepared according to Kerr, 1983. The culture was kept at 28˚C for 5-7 days till the appearance of colonies.

For root samples, 3 gram of tissue was grinded using mortar and pestle and the same above procedure was followed.

2.3 Colony count:

Presence of whitish with dark center was calculated according to the following formula:

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL) x total dilution used}}$$

The results will be divided by 5 to give per gram of tissue.

2.4 Colony PCR analysis

Colony PCR were performed as following: single colony were picked and added to 100 µl of sterile distilled water, boiled for 5 minutes and 5 µl of supernatant were used as DNA templates.

Reaction mix contains 4 µl of DNA templates, Reaction mixtures (25 ul) contained primer oligonucleotides at 10 picomole each, deoxynucleoside triphosphates at 200 mM each, 1 U of thermostable DNA polymerase (hylabs), reaction cocktail supplied by the manufacturer (Perkin-Elmer, 10 mM Tris [pH 8.3 at 258C], 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin [Sigma G2500]; Epicenter, 50 mM Tris [pH 9.0 at 258C], 20 mM ammonium sulfate, 1.5 mM MgCl2). PCR is started by the denaturation at 94°C for 5 minutes,

then annealing at 50°C for 1 minutes and extension at 72 for 1 minutes and final extension at 72 for 10 minutes. This was repeated for 35 cycles. The PCR products were separated on 2% agarose gel via electrophoresis, stained with ethedium bromide and observed under UV light.

The *ipt* primer sequences come from a somewhat less conserved region, are used as universal primers to detect different Agrobacterium isolates and the sequence of the sense-strand primer was 59-GAT CG(G/C) GTC CAA TG(C/T) TGT-39 (coordinates 8867 to 8884 in reference 3), and the sequence of the antisense-strand primer, CYT9, was 59- GAT ATC CAT CGA TC(T/C) CTT-39 (coordinates 9293 to 9276 in reference 3). This primer pair yields a 427-bp PCR product (Jerry et al., 1995)

3. Results and discussion:

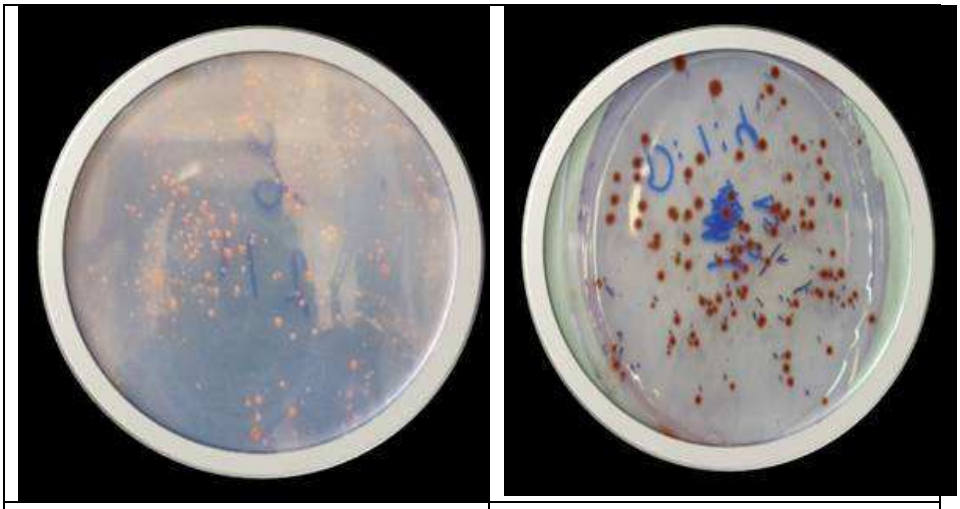
The crown gall caused by *A. tumefaciens* is becoming a major threat to nursery and stone fruit production. A sensitive and specific detection method is needed for early pathogen detection especially for the symptomless host plant. PCR methods have effectively been employed for sensitive and rapid detection and identification of phytopathogenic bacteria (Louws et al., 1999). For early detection either from infected plant or soil, PCR primers specific for and sensitive to phytopathogenic bacteria have been used widely (Tsai and Olson, 1992; Louws et al., 1999).

Direct amplification of supernatant yielded no positive results. This may be attributed to low number of Agrobacterium and or to inhibitor that may affect PCR reaction. Lim et al, reported that detection of *Agrobacterium vitis* strains could be only possible if Agrobacterium reaches 10^{2-3} CFU/g soil or plant tissue (Lim et al., 2009). In our study we found that culturing step is very important to get positive results

3.1 Agrobacterium colony:

The used medium is semi selective and other colonies appeared in the medium and these ranged from dark and light orange (distinctive for

pseudomonas spp.) and whitish with dark-red center (distinctive for ***Agrobacterium tumefaciens***) (**Fig 1. A and B).**



A	B

Fig1 A and B: Bacterial colony on semi selective medium. A. Agrobacterium tumefaciens as white colony and B Pseudomonas after 5 days of culture.

The total colonies count for *Agrobacterium tumefaciens* is about 1 x10 ^{6} colony per gram of soil and 1x 10 ^{4} for root samples.

3.2 PCR analysis:

PCR procedure allows differentiation between pathogenic and nonpathogenic *Agrobacterium tumefaciens*. Out of 120 soil samples tested covering 7 nurseries and 20 locations for the presence of the pathogen, 15 samples gave positive results and 6 of these samples were found to belong to one nursery. The presence of 427 bp band represented the presence of *ipt* gene **(Fig. 2).** The same results were obtained by Jerry Hass et al, 1985).

There is no correlation between sample collected in November, December and January and the level of Agrobacterium presence. Samples that were tested positive in November, also tested positive in December and January.

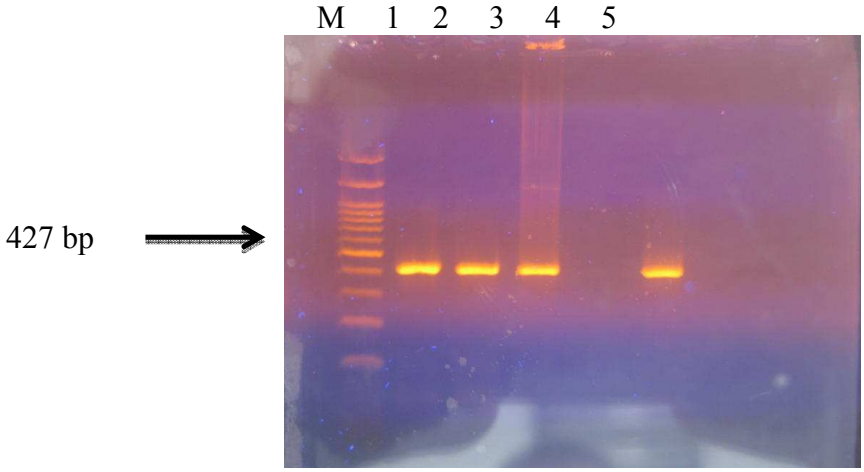


Fig 2: PCR product. Lane 1-3 positive PCR, lane 4, negative control, lane 5 positive control and M 100 bp DNA ladder.

For root samples, out of 120 samples tested, only 6 samples were tested positive and out of that 4 samples with their corresponding soil tested positive, but the remaining 2 samples, their corresponding soil tested negative in November, but later on tested positive with their corresponding soil in December and January. This may indicate that these samples were planted recently and there is less time for Agrobacterium to transfer into soil. Furthermore, testing soil samples alone is not sufficient and plant samples must be tested for detecting the presence of *Agrobacterium tumefaciens* to be certified as clean samples.

3.3 Conclusion:

Detecting Agrobacterium directly from soil and root samples is not possible at this moment, more work need to be done to standardize the procedure. Using Bacterial culture, then colony PCR was very helpful to detect *Agrobacterium tumefaciens*

There is need to test root sample especially for newly planted stone fruit.

4. Future work:

Soil samples and root samples will be collected though out the year and the best time to collect the sample will be determined.

Isolation and detection of Agrobacterium tumefaciens from soil

Standardization of direct sampling from soil and or root will be determined and strains will be identified

5. References:

Matthew, A.E.; and Abhaya M. D. *Agrobacterium tumefaciens* as an agent of disease. TRENDS in Plant Science 2003, 8 (8), 380-386.

Abussaoud, M.J.I.; and Al-Momani, F. Microbial survey of the genus Agrobacterium in grapevine nurseries in Jordan. Arab Gulf J. Scient. Res. 1992, 10, 121-131.

Ponsonnet, C.; and Nesme, X. Identification of Agrobacterium Strains by PCR-RFLP Analysis of pTi and Chromosomal Regions. Archive Microbiology 1994,6, 300-309.

Van Larebeke, N., Engler, G.; Holsters, M.; Van den Elsacker, S.; Zaenen, I. ; Schilperoort, R. A; and Schell, J. Large plasmid in *Agrobacterium tumefaciens* essential for crown gall inducing activity. Nature (London) 1974, 252,169–170.

Watson, B., Currier, T. C.; Gordon, M. P. ; Chilton, M.-D ; and Nester, E. W. Plasmid required for virulence of *Agrobacterium tumefaciens.* J. Bacteriol. 1975. 123, 255–264.

Chilton, M. D., Drummond, M. H.; Merlo, D. J.; Sciaky, D.; Montoya, A. L; Gordon, M. P.; and Nester. E. W. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell 1977,11, 263–271.

Chilton, M. D., Saiki, R. K.; Yadav, N.; Gordon, M. P. and Quetier, F..T-DNA from Agrobacterium Ti plasmid is in the nuclear DNA fraction of crown gall tumor cells. Proc. Natl. Acad. Sci. USA 1980 77,4060–4064.

Wang, H.M., Liang, Y.J. ; Yun, T.; and Di, Y.B.. Biological control of crown gall with introduced K84 in China. I. Investigation and identification of *Agrobacterium tumefaciens*. Acta. Agri. Univ. Pekin. 1991, 17, 91-94.

Burr, T.J.; and Katz, B.H. Isolation of *Agrobacterium tumefaciens* Biovar 3 from grapevine, galls, sap and from vineyard soil. Phytopathol. 1983, 73,163-165.

Agrios, G.N., 1978. Plant Pathology. Academic press, New York, pp: 703.

Wei, Y.L. J.; Li-Rong, T.; Shi-Mo, L.; Yan, W.; Hong-Xia, L.; and Yu-Ming, L. Sensitive and specific detection of *Agrobacterium tumefaciens* in soil using a rapid polymerase chain reaction (PCR). African Journal of Microbiology Research 2011, 5(6), 708-713.

Francesca, P.; Guido, M.; and Giuseppe S. Indexing *Agrobacterium vitis* in symptomatic Grapevine Propagation Material by Two Nested PCR Assays. Am. J. Enol. Vitic. 2010, 61(1),102-112.

Federica, B. ; Klaus; G.; and Carlo, B. Detection of *Agrobacterium vitis* by PCR using novel *virD2* gene-specific primers that discriminate two subgroups. European Journal of Plant Pathology 2008, 122 (3), 403-411.

Kerr, A. ; and Brisbane, P.G. ***Selective media*** for three ***biovars*** of Agrobacterium. Journal of Applied Bacteriology 1983, 54 (3),425- 431.

Jerry, H. H. ; Larry W. M. ; Walt, R.; and Shula, M. Universal PCR Primers for Detection of Phytopathogenic Agrobacterium Strains. Applied and Environmental Microbiology 1995, 61(8), 2879–2884.

Louws, FJ; Rademaker, J.L.W.; de Bruijin, F.J. The three ds of PCR-based genomic analysis of phytobacteria: diversity, detection, and disease diagnosis. Annu. Rev. 1999, 37, 81-125.

Tsai, Y.L.; and Olson, B.H. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl. Environ. Microbiol. 1992, 58, 754-757.

Lim, S. H.; Jong G. K.; and Hee W. K. Novel SCAR primers for specific and sensitive detection of *Agrobacterium vitis* strains. Microbiological Research 2009, 164 (4) , 451-460.