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# Distribution of *Agrobacterium tumefaciens* Biovars in Jordan and Variation of Virulence

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**One hundred and nine *Agrobacterium* isolates were recovered from 68 samples (51 plant tumor and 17 soil) that were collected from different habitats in Northern Jordan. The isolated cultures were grouped into 3 biovars based on their biochemical characteristics and biovar I, II, and III comprised a total number of 46, 41, and 22 isolates, respectively. Isolates of biovar I were obtained primarily from the diseased peach, oak and rose plants, whereas isolates of biovar II and III were obtained mostly from apple and grape plants, respectively. Twenty-nine isolates were found to be virulent to at least one of the tested hosts such as carrots, chick-peas, garden peas and tomato plants with a response of tumor formation or tumor with roots induction. Our result suggested that *A. tumefaciens* strains from tumor of various plants and soil of Jordan were diverse and they have a variation in their virulence.**

***Keywords*** : biotype, crown gall disease, plant tumors, pathogenicity

Crown gall is a world wide plant disease of economic significance in nurseries, vineyards and fruit orchards (Abssaoud and Al-Momani 1992; Keane et al., 1970; Ma, et al., 1987; Panagopoulas and Psallidas, 1973; Schroth and Moller, 1976; Sule, 1978). *Agrobacterium tumefaciens* is a soil borne bacterium, its virulent strains infect dicotyledonous plant that belong to about 90 different families and few monocotyledonous plants causing crown gall disease throughout the world (De Cleene and Deley, 1976). Yield loss from the disease occurs primarily at nurseries, where galled plants should be discarded (Al-Momani, 1987; Moore and Cooksey, 1981). Crown gall disease can also cause severe stunting on the mature plants (Agrios, 1978). The tumor induction of *A. tumefaciens* is related to the presence of an extra-chromosomal DNA designated as tumor inducing (Ti) plasmid (Zaenen et al., 1974). During the course of infection, a defined portion of the Ti- plasmid (T-DNA) is stably transferred to plant cell genome where it is integrated and expressed (Thomashow et al., 1980). The expression of the integrated portion leads to the formation of neoplastic cell which forms the crown gall disease. Infected cells produce plant hormones and unusual amino acids called opines, resulting in the continuous cell plant division on hormone free medium (Hooykaas et al., 1994). On the basis of biochemical characteristics, *A. tumefaciens* strains were grouped or clustered into two groups or two biotypes (Keane et al., 1970; Kersters et al., 1973). Kerr and Panagopoulas (1977) reported a third biotype isolated from grapevines. Three biotypes were reported world wide (Abssaoud and Al-Momani, 1992; Kersters and Deley, 1984; Ma et al., 1987; Perry and Kado, 1982; Sawada and Ieki 1992; Sule, 1978). Isolation, characterization and identification of the *A. tumefaciens* strains were based on their biochemical characteristics and virulence as reported by Beregey's Manual of Determinative Bacteriology (1984). Pathogenicity of the pathogenic isolate was due to the presence of a conjugative plasmid (Ti), which could be transferred from pathogenic strain to non-pathogenic strain. Recently, molecular phylogenetic analysis using bacterial genomic DNA were adapted to classify *Agrobacterium* strains. The plasmid typing using specific primers has been also widely applied to classify the bacteria based on the specific plasmid DNA amplification. It has been reported that there were some variation of bacterial strains isolated from different habitats or tumor from different plant.

In this study, we isolated *Agrobacterium* strains from different habitats of Northern Jordan and from tumor of various plants. The bacterial strains were subjected biochemical characterization, virulence analysis and idiotyping. This study reports the diversity of *A. tumefaciens* strains from Jordan and their virulence.

## Materials and Methods

**Isolation of bacterial strains.** Tumor samples from infected hosts were randomly collected from planted areas in Northern Jordan. Tumor samples were washed under tap water for 10 minutes, surface sterilized in 10% sodium hypochlorite for 10 minutes and macerated in an electric blender. Soil samples from the same planted areas were

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randomly collected from the top 20 cm after removing the upper 3-5 cm layer. Soil samples were dried at room temperature and sieved to remove particulates larger than 2 mm. One gram of dried soil particles were suspended in 100 ml of sterile distilled water. After vigorous shaking, serial dilutions were prepared and 0.1 ml of the appropriate dilution was spread by an L-shaped glass rod on the selective medium (Kado and Heskett, 1970). The cultures were incubated at 27°C for 48h. Colonies of different morphology were further purified into pure cultures and identified based on Beregey's Manual of Determinative Bacteriology (Kersters and Deley, 1984).

Serial dilutions of each macerated tumor sample were prepared and 0.1 ml of appropriate dilution was cultured as mentioned above. The isolates were biotyped according to Kerr and Panagopoulas (1977). Virulence of the isolates was tested under *in vitro* conditions as reported by Al-Momani (1991). Garden peas and chickpea seedlings were then wounded aseptically by a flame sterilized needle, inoculated with a loop-full of active bacterial cultures (24 h) under aseptic conditions. The seedlings were grown on Murashige and Skoog medium (1962). Inoculated cultures were kept at room temperature and the pathogenecity results were recorded after 15 days. As a negative control, 5 seedling from each host were wounded without inoculation with bacteria.

**Identification and virulence analysis.** Bacterial colonies grown on the selective medium were subjected to bacterial identification by following the Bergey's manual. The biotype of the bacterial isolates was determined as previously described by Kerr and Panagopoulas (1977). Virulence of the isolates was tested under *in vitro* conditions as reported by Al-Momani (1991). Briefly, Garden peas and chickpea seedlings were wounded aseptically using a flame sterilized needle and subsequently inoculated with a loop full of active bacterial cultures under aseptic conditions. Four different host plants were used in this study to test the virulence of the isolates. The seedlings of tomato plants, carrot plants, Garden peas, and chickpea were grown on Murashige and Skoog medium (1962). Inoculated plants were kept at room temperature and the pathogenicity results were recorded 15 days after inoculation by the appearance of tumors in the seedlings. As a negative control, 5 seedlings from each host were wounded without inoculation with bacteria.

## Results

**Isolation and identification.** Bacterial colonies with characteristics of *Agrobacterium,* such as smooth, glistening, translucent, convex, circular, and from light blue to olive green colonies, were selected. Microscopy and biochemical analysis of many of the selected colonies showed the following characteristics: i.e. gram-negative, motile, acids production from simple sugars (glucose, lactose, mannitol, xylose, arabinose and sucrose), $H_2S$ production from cystine, catalase positive and urease positive. All isolates failed to utilize gelatin, casein and starch. No fluorescent pigment after 2 days of incubation on King's B medium (1954) were detected. Therefore, selected isolates with the above-mentioned characteristics were identified as *Agrobacterium* isolates according to Kerstes and Deley (1984). From 68 collected samples from both soil and tumor, 201 selected colonies were picked from the selective medium designed by Kado and Heskett (1970) with the above colony morphology. A total of 109 isolates were identified as *Agrobacterium* based on the description of the Bergye's Manual of Determinative Bacteriology (1984).

**Biotypes of *Agrobacterium* and virulence analysis.** Biotyping of the 109 *Agrobacterium* isolates was performed as described previously by Kerr and Panagopoulas (1977). Among 109 isolates, 46, 42 and 22 isolates were classified as biovar I, II and II, respectively (Table 1). Virulence analysis of the 109 isolates on the plant host revealed that only 29 isolates produced apparent tumor or tumor with root at the inoculation site. This result indicated that 29 isolates are virulent strains (Table 1). Biovar I was the most frequently detected from the isolates of plant tumor origin, whereas biovar II was the most frequently detected from the isolates of soil origin (Table 1). Among the 29 virulent isolates, the 21 isolates from plant tumor were proven to be virulent to one f the tested host under *in vitro* conditions either by the induction of tumor or tumor with roots, whereas 8 virulent isolates from soil sample were virulent.

**Table 1.** The Number of recovered *Agrobacterium* isolates from plant tumors and soil sample, their biotypes and pathogenicity

| Number of collected Samples | | Number of selected colonies | Number of *Agro bacterium* isolates | Biovar | | | Number of pathogenic isolates |
|---|---|---|---|---|---|---|---|
| | | | | I | II | III | |
| Tumor samples | 51 | 133 | 70 | 32 | 23 | 15 | 21 |
| Soil samples | 17 | 68 | 39 | 14 | 18 | 7 | 8 |
| Total | 68 | 201 | 109 | 46 | 41 | 22 | 29 |

**Table 2.** Distribution of *Agrobacterium* isolates recovered from plant tumors, their biovars and the number of pathogenic isolates

| Tumor Sample | Locations | Number of collected samples | Number of selected colonies | Number of *Agrobacterium* isolates | Biovar | | | Number of Pathogens |
|---|---|---|---|---|---|---|---|---|
| | | | | | I | II | III | |
| Cherry | JUST | 4 | 14 | 9 | 5 | 1 | 3 | 3 |
| Peach | JUST | 3 | 13 | 7 | 5 | 2 | 0 | 2 |
| | Ajlun | 2 | 7 | 4 | 3 | 1 | 0 | 1 |
| | BK | 2 | 8 | 4 | 4 | 0 | 0 | 2 |
| Apple | JUST | 4 | 12 | 7 | 0 | 6 | 1 | 2 |
| | Ajlun | 4 | 10 | 6 | 1 | 5 | 0 | 2 |
| | BK | 1 | 4 | 2 | 0 | 2 | 0 | 1 |
| Olive | Jerash | 7 | 10 | 4 | 1 | 3 | 0 | 1 |
| | Ajlun | 2 | 6 | 2 | 1 | 0 | 1 | 0 |
| | BK | 4 | 7 | 3 | 1 | 0 | 2 | 1 |
| Oak | Ajlun | 3 | 12 | 5 | 5 | 0 | 0 | 1 |
| Grape | Ajlun | 8 | 17 | 10 | 2 | 1 | 7 | 2 |
| Rose | JUST | 7 | 13 | 7 | 4 | 2 | 1 | 3 |
| Total | | 51 | 133 | 70 | 32 | 23 | 15 | 21 |

BK=Bani Kananah.
JUST=Jordan University of Science and Technology

**Table 3.** Distribution of soil samples, *Agrobacterium* isolates, their biovars and pathogenic isolates

| Soil Sample | Location | Cultivated plant | Number of selected colonies | Number of *Agrobacterium* isolates | Biovar | | | Number of Pathogenic isolates |
|---|---|---|---|---|---|---|---|---|
| | | | | | I | II | III | |
| 1 | JUST | Peach | 2 | 0 | 0 | 0 | 0 | 0 |
| 2 | JUST | Cherry | 3 | 2 | 0 | 0 | 2 | 0 |
| 3 | Ajlun | Peach | 6 | 5 | 3 | 2 | 0 | 1 |
| 4 | BK | Peach | 4 | 1 | 1 | 0 | 0 | 1 |
| 5 | BK | Apple | 6 | 3 | 0 | 2 | 1 | 0 |
| 6 | JUST | Peach | 6 | 4 | 2 | 2 | 0 | 1 |
| 7 | JUST | Peach | 4 | 2 | 0 | 1 | 1 | 0 |
| 8 | Ajlun | Peach | 1 | 1 | 0 | 1 | 0 | 1 |
| 9 | JUST | Peach | 4 | 3 | 1 | 1 | 1 | 0 |
| 10 | Ajlun | Apple | 6 | 2 | 0 | 2 | 0 | 1 |
| 11 | JUST | Uncultivated | 3 | 1 | 1 | 0 | 0 | 0 |
| 12 | JUST | Cherry | 4 | 3 | 2 | 0 | 0 | 1 |
| 13 | Ajlun | Peach | 4 | 2 | 0 | 2 | 0 | 1 |
| 14 | Ajlun | Apple | 6 | 6 | 0 | 4 | 2 | 1 |
| 15 | Ajlun | Cherry | 4 | 1 | 0 | 1 | 0 | 0 |
| 16 | Ajlun | Peach | 3 | 3 | 3 | 0 | 0 | 0 |
| 17 | BK | Peach | 2 | 0 | 0 | 0 | 0 | 0 |
| Total | | | 68 | 39 | 14 | 18 | 7 | 8 |

**Distribution of *Arobacterium* isolates.** The distribution isolated from plant tumor is shown in Table 2. Seventy *Agrobacterium* isolates were identified and grouped into three biovars biovar I, II and III with the following number of isolates 32, 23 and 15, respectively. Twenty one of them were virulent to one of the tested host plants under *in vivo* or *in vitro* conditions. The virulent isolates expressed their virulence by the formation of either tumors or tumor with roots Distribution and biotyping of soil isolates were indicated in Table 3. Out of the 17 collected samples, 39 *Agrobacterium* isolates were identified and 14, 18 and 7 of them were grouped into biovar I, II and III, respectively. Biovar II was the predominant among them, the virulence of the soil isolates on the tested host plants under *in vitro* conditions indicated that only 8 of them were virulent.

Interestingly, the plant response to the tested bacterial

**Table 4.** Host range specificity and morphology response of infection

| Isolate Number* | Source | Virulence analysis on | | | |
|---|---|---|---|---|---|
| | | Tomato | Carrot slices | Garden peas | Chick peas |
| 1.2 | cherry | + | + | − | ++ |
| 2.1 | peach | + | + | ++ | ++ |
| 3.1 | apple | − | + | − | − |
| 3.2 | apple | − | + | ++ | ++ |
| 4.2 | apple | − | + | − | + |
| 5.2 | apple | + | + | ++ | + |
| 8.2 | apple | − | + | − | ++ |
| 9.1 | cherry | + | + | − | ++ |
| 9.2 | cherry | + | + | − | ++ |
| 11.2 | peach | + | + | + | + |
| 13.2 | grape | − | + | ++ | ++ |
| 14.1 | grape | − | + | − | ++ |
| 15.1 | rose | + | + | ++ | + |
| 18.1 | rose | + | + | ++ | ++ |
| 19.1 | peach | + | + | + | ++ |
| 19.3 | peach | + | + | + | + |
| 2.5 | peach | + | + | + | + |
| 12.1 | rose | − | + | − | ++ |
| 6.2 | Olive | − | + | − | − |
| 7.3 | Olive | − | + | − | ++ |
| 10:4 | Oak | − | + | ++ | − |
| 3.4 | soil | + | + | − | + |
| 4.1 | soil | + | + | − | + |
| 6.3 | soil | + | + | − | + |
| 8.1 | soil | + | + | − | − |
| 10.2 | soil | + | + | ++ | − |
| 12.3 | soil | − | + | ++ | − |
| 13.1 | soil | + | + | ++ | ++ |
| 14.3 | soil | − | + | ++ | ++ |

*XY: X is the sample No., Y is the isolate No.
+, tumor only; ++, tumor with root; −, no response

isolates was varied and showed some host plant specific virulence (Table 5 and Fig. 1). We should further characterize the host specific virulence response in different isolates.

## Discussion

In Bergey's Manual (1984), the genus *Agrobacterium* is divided into four species mainly on the basis of their pathogenicity and symptoms induced on the host plants. Pathogenicity of the genus *Agrobacterium* depends mostly on the presence of a conjugative plasmid which could move from one cell to another. Two major groups were designated as biotype I and II (Keane et al., 1970) and the third group, biotype III was recognized by Kerr and Panagopoulas (1977) and Sule (1978). Later the biotypes were changed into biovars (Kersters and Deley, 1984). The most frequent appearance of biovar I among our isolates was coincident with the previous report (Keane et al., 1970; Panagopoulas and Psallidas, 1973). Most of our isolates were originated from tumor samples or soil samples of stone plants such as cherry plants and peach plants. Most of biovar II were isolated from apple plant or soil around apple plants. It was previously reported that biovar II was dominant among *Agrobacterium* isolates originated from apple plants (Kerr and panagopoulas, 1977; Perry and Kado 1982; Süle, 1978). Low number of biovar III may be due to the less samples of grape tumor or soil cultivated with grape. Previously, the dominance of biovar III among grape plant tumor samples or soil cultivated with grape were recognized (Abssaoud and Al-momani, 1992; Ma et al., 1987; Perry and Kado, 1982). Low percentage of virulent isolates among biovar III may be due to host range specificity of the isolates as previously reported (Al-momani, 1991; Yanofsky et al., 1985). Variation on the virulence of

**Table 5.** Biochemical characteristics of the *Agrobacterium* isolates

| Biochemical test | Biovar I | | Biovar II | | Biovar III | |
|---|---|---|---|---|---|---|
| | Tumor (26)* | Soil | Tumor (10) | Soil (18) | Tumor (6) | Soil (7) |
| 3-Ketolactose production | 26 | 14 | 0 | 0 | 1 | 0 |
| Litmus milk: Acid | 0 | 0 | 10 | 18 | 1 | 1 |
| Base | 16 | 14 | 0 | 0 | 5 | 6 |
| Pellicle formation in ferric ammonium citrate | 26 | 14 | 0 | 0 | 0 | 0 |
| Growth on New and Kerr medium | 0 | 0 | 10 | 18 | 1 | 0 |
| Acid from : Melaezetoze | 26 | 14 | 7 | 12 | 0 | 0 |
| Meso-erythritol | 0 | 0 | 10 | 18 | 0 | 0 |
| Alkali from : Malonate | 0 | 0 | 10 | 18 | 5 | 6 |
| Propionate | 26 | 14 | 0 | 0 | 6 | 7 |
| L-tartate | 0 | 0 | 10 | 18 | 4 | 6 |
| Mucic acid | 0 | 0 | 10 | 18 | 0 | 1 |

*Numbers between parentheses represent the total number of isolates.

different host plants to the same isolates could be due to different concentrations of endogenous auxins or cytokinin in host plants (Yanofsky et al., 1985). Variation between plant response to different isolates could be due to their host range specificity (Yanofsky et al., 1985), so some of the isolates showed wide host range while others had limited host range.

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