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Incidence and Severity of Crown Gall Disease of Cherry, Apple and Apricot Plants Caused by *Agrobacterium tumefaciens* in Nagar Valley of Gilgit-Baltistan, Pakistan

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Abstract: The crown gall is a world wide tumor forming disease of the plants and are a major problem for plant nursery industries. This disease is caused by pathogenic species of soil borne bacteria *Agrobacterium* and cause great economic loss in fruit plants. From July to November in 2008 an extensive survey was conducted in five villages (Chalt, Skindarabad, Gulmit, Askurdas and Nagarkhas) of the Nagar valley of Gilgit-Baltistan randomly by walking in a zigzag pattern and examined the plants for typical curly top symptoms to determine the incidence and severity of crown gall on cherry, apple and apricot plants. A total of 6100 cherry, 6900 apple and 8000 apricot plants were inspected and the mean incidence of crown gall on cherry plants was found to be 5360 (87.87%), apple plants 6069 (87.96%) and in apricot plants 00 (00.00%). In cherry plants, the severity of the disease observed varied from 33.80-40% in mild, 20-34.49% in moderate and 27.85-40% in severe. While in the apple plants it varied from 36.81-42.5% in mild, 29.14-34.49% in moderate and 27-30.31% in severity. There was no infestation of crown gall in apricot plants. A total of 35 samples (15 soil samples; 1 from each orchard of each village and 20 tumor samples; 2 from each orchard of cherry and apple infected plants) from each inspected village were cultured on modified selective medium (3-Ketolactose agar). The growth of *Agrobacterium tumefaciens* was identified on the basis of colony characteristics and biochemical tests based on Bergey's Manual of determinative Bacteriology. A 100% infestation of *A. tumefaciens* was observed from both the plant and soil samples except for the soil sample cultures from the apricot orchard. All the 30 *A. tumefaciens* strains isolated from the soil specimens and infected cherry and apple plant tumors were tested against six different antibiotics by disc diffusion method. All the strains were resistant against Lincomycin, Amoxycillin, Ampincillin and Cloxacillin while Cephradine, Tetracycline and Dioxycycline, showed intermediate sensitivity.

Key words: Crown gall diseases, crown gall in cherry, apple and apricot trees, incidence of *A. tumifaciens*

INTRODUCTION

Crown gall is a worldwide plant disease of economic significance in nurseries, vineyard and fruit orchards (Abussaoud and Al-Momani, 1992; Keane *et al*., 1970; Wang *et al*., 1991; Panagopoulas and Psallidas, 1973; Schroth and Moller, 1976; Sule, 1978).

In fruit plants, crown gall is caused by a rod shaped flagellated, gram negative soil borne bacterium, *Agrobacterium tumefaciens* (Smith and Townsend, 1907).

The galls provide a nutrient rich environment for the growth of *A. tumefaciens* and it returns to the soil as the galls decompose (Gillman, 2005). In the soil, survival of the organism is well documented (Burr and Katz, 1983). From the soil, with the help of flagella they swim towards photoassimilates that accumulate in the rhizosphere around roots. Some strains may chemotactically move towards chemicals that indicate a wounded plant cell, such as acetosyringone, where they colonize on the plant's wounded tissue. These wounds may be made by use of agriculture tools, nematode, insect feeding and/or frost injury. The attachment of the bacteria to the plant is a two step process; following an initial weak and reversible attachment, the bacteria synthesize cellulose fibrils that anchor them to the wounded plant cell. *A. tumefaciens* manages to survive in the rhizosphere on materials that leak from wounded host plant stems and roots, these are sugars and phenolic compounds and attract the motile crown gall bacteria. The bacterium primarily affects the plant by attachment to the cell and insertion of the plasmid (Ti plasmid) with genes that code for production of plant growth-regulating hormones by horizontal gene transfer. The bacterial plasmid genes induce the production of higher than normal concentrations of plant hormones (auxins and cytokinins) that favor bacterial growth at the expense of the plant. Thus, the crown gall genes induce the plant cells to grow more profusely and to a larger size than they would normally grow, thereby forming galls.

Galls appear within weeks at above 70°F temperature and latent infection typically develops into galls in a later growing season.

Agrobacterium is a causal agent producing crown gall disease in over 600 species of trees (Wang *et al*., 2000). Its virulent strains infect dicotyledonous plants that belong to about 90 different families and few

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monocotyledonous plants causing crown gall disease throughout the world (De Cleene and Deley, 1976).
The infected plants become weakened, stunted and unproductive. 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 of the mature plants (Agrios, 1978).

MATERIALS AND METHODS

Survey area: This extensive survey of fruit trees (cherry, apple and apricot) was conducted in five villages of Nagar valley by walking in a zigzag pattern from July to November, 2008 and the samples were collected for culture in sterilized polythene bags.

Disease assessment: Plants were randomly inspected for the assessment of crown gall disease using two parameters; disease incidence and its severity level as shown below:

$$\text{Disease incidence} = \frac{\text{Number of infected plant}}{\text{Total number (Healthy and infected)}} \times 100$$

Severity level was assessed as:

Size of Infection	
Mild	1-3 cm
Moderate	3-6 cm
Severe	above 6 cm

Sample cultures: Twenty tumor samples (2 from cherry plants, 2 from apple plants of each orchard inspected) from each village and fifteen soil samples (1 sample from each orchard of cherry, apple and apricot) were cultured on modified selective medium (3-Ketolactose agar) and incubated at 28°C for 48 hours.

Preparation of tumor samples for culture: In the laboratory, the surface of the tumor tissue was disinfected by dipping for 1-3 min (depending on the sensitivity of tissue) in 10% household bleach (1 part bleach: 9 parts water). A 10 to 20 gram piece of tissue was cut with a sterilized surgical blade, homogenized in 10 ml sterile distilled water using mortar and pestle, mixed by vortexing and 100 µl of the sample was cultured on the modified selective medium (3-Ketolactose agar).

Preparation of soil sample for culture: The soil samples from different orchards of each surveyed village were collected from the top 20 cm, sieved to remove particles larger than 2 mm. One gram of the soil particles were suspended in 100 ml of sterile distilled water. After vigorous shaking, serial dilution up to 10^5 was prepared and 0.1 ml of the appropriate dilution was spread on the selective medium plate and incubated at 28°C for 48 hours.

Identification of *Agrobacterium tumefaciens*: Bacterial colonies with smooth, glistening, translucent, convex, circular and colony colour ranging from light blue to olive green were selected. Further identified by Gram staining, motility, oxidase, catalase, urease, citrate utilization tests and alkaline and acid production from Tripple Sugar Iron (TSI) and H_2S production from cystine, based on Bergey's Manual of Determinative Bacteriology (Kersters and Deley, 1984) were performed.

Sensitivity against antibiotics: Two to three isolated colonies were picked and mixed in 2 ml nutrient broth, vortexed to resuspend the cells and spread onto the sensitivity test agar surface of Mueller-Hinton agar plates (MHA, Difco) by disc diffusion method (Bauer *et al*., 1966).
The *Agrobacterium tumefaciens* strains were subjected to sensitivity tests against seven antibiotics at varying concentrations by placing sterile discs with Lincomycin 10 µg, Amoxycillin 10 µg, Tetracycline 30 µg, Cephradine 30 µg, Ampincillin 10 µg, Cloxacillin 5 µg and Doxycycline 30 µg obtained from Oxoid/Difco suppliers and sensitivity was monitored after overnight incubation of the plates at 28°C.

RESULTS

Disease incidence: Table 1 shows that 6100 cherry plants from different villages of Nagar valley were randomly inspected for the incidence of crown gall and 5360 plants were found infected and the mean infestation of the disease in cherry plants was 87.87%. Of 1500 cherry plants inspected from Chalt, 1200 from Skindarabad, 700 from Gulmit and Askurdas each and 2000 from Nagarkhas, the highest infestation of the disease was found in Chalt 1370 (91.34%) followed by

Table 1: Incidence of crown gall on cherry, apple and apricot in different villages of Nagar Valley

	Name of village					
	Chalt	Sikandarabad	Gulmit	Askurdas	Nagarkhas	Total
Cherry plants inspected	1500	1200	700	700	2000	6100
Cherry plants diseased	1370	988	600	580	1822	5360
Disease incidence %	91.34	82.34	85.72	82.86	91.1	87.87
Apple plant inspected	1400	1200	900	1000	2400	6900
Apple plants disease	1270	1000	777	800	2222	6069
Disease incidence %	90.72	83.34	86.34	80.00	92.59	87.96
Apricot plants inspected	1600	1400	800	1200	3000	8000
Apricot disease	00	00	00	00	00	0000
Disease incidence %	00	00	00	00	00	00.00

Nagarkhas 1822 (91.1%), Gulmit 600 (85.72%), Askurdas 580 (82.86%) and Skindarabad 988 (82.34%). Similarly, 6900 apple plants were randomly inspected and 6069 were found infected with a mean infestation of the disease on the apple plants of 87.96%. Of 1400 apple plants from Chalt, 1200 from Skindarabad, 900 from Gulmit, 1000 from Askurdas, and 2400 from Nagarkhas, the highest infestation was observed in Nagarkhas 2222 (92.59%) followed by Chalt 1270 (90.72%), Gulmit 777 (86.34%) and Sikandarabad 1000 (83.34%).

Interestingly, the 8000 apricot plants from Nagar valley inspected for the infestation of crown gall showed no infestation throughout this study.

Table 2 shows that from Chalt, 1370 cherry and 1270 apple plants were inspected; in cherry plants the incidence of the disease was mild with 548 (40%) plants, moderate in 274 (20%) plants and severe in 548 (40%) plants. While, in apple plants it was mild in 515 (40.55%), moderate 370 (29.14%) and severe in 385 (30.32%) plants.

From Skindarabad, 988 cherry and 1000 apple plants were inspected; in cherry plants, the incidence of the severity level of the disease was mild in 380 (38.46%), moderate in 318 (32.19%) and severe in 290 (29.35%) plants. While, in apple plants it was mild in 425 (42.5%), moderate in 300 (30.0%) and severe in 275 (27.5%) plants.

In Gulmit, 600 cherry and 700 apple plants ware inspected and the incidence of the disease in cherry plants was mild in 214 (35.66%), moderate in 204 (34%) severe in 182 (30.34%) plants, whereas, in apple plants, out of 777 inspected plants, 286 (36.81%) with mild, 256 (32.95%) moderate and 235 (30.25%) were observed.

From Askurdas, 580 cherry and 800 apple plants were inspected; in cherry plants, mild incidence of the disease was found in 196 (33.80%), moderate 200 (34.49%) and severe in 184 (31.73%). In apple plants, the mild incidence was in 298 (37.25%), moderate in 274 (34.25%) and severe in 228 (28.5%) plants.

From Nagarkhas, 1822 cherry and 2222 apple plants were inspected; in cherry plants, the incidence was mild in 720 (39.52%) plants, moderate in 594 (32.57%) and severe in 508 (27.85%) plants. While, in apple plants, mild incidence in 926 (41.68%), moderate 696 (31.33%) and severe in 600 (27%) plants was recorded.

Table 3 shows the number of colonies of *A. tumefaciens* from the soil of cherry, apple and apricot orchards. The infestation in the soil of cherry orchards is high in all the villages as compared to apple orchards. There is no observed infestation of *A. tumefaciens* in the soil from the apricot orchards in all the villages.

Table 4 shows the growth of *A. tumefaciens* from tumors of cherry and apple plants from each village of the Nagar valley. All the cultured samples show growth of *A. tumefaciens*.

Table 2: Severity level of crown gall disease in cherry apple and apricot in different villages of Nagar valley

	No. of Plants infected	Disease severity		
		Mild	Moderate	Severe
Chalt				
Cherry	1370	548	274	548
Percentage (%)		40%	20%	40%
Apple	1270	515	370	385
Percentage (%)		40.55%	29.14%	30.32%
Apricot	0	0	0	0
Skindarabad				
Cherry	988	380	318	290
Percentage (%)		38.46%	32.19%	29.35
Apple	1000	425	300	275
Percentage (%)		42.5%	30.0%	27.5%
Apricot	0	0	0	0
Gulmit				
Cherry	600	214	204	182
Percentage (%)	-	35.66%	34.0%	30.34%
Apple	777	286	256	235
Percentage (%)	-	36.81%	32.95%	30.25%
Apricot	0	0	0	0
Askurdas				
Cherry	580	196	200	184
Percentage (%)	-	33.80%	34.49%	31.73%
Apple	800	298	274	228
Percentage (%)	-	37.25%	34.25%	28.5%
Apricot	0	0	0	0
Nagarkhas				
Cherry	1822	720	594	508
Percentage (%)	-	39.52%	32.57%	27.85%
Apple	2222	926	696	600
Percentage (%)	-	41.68%	31.33%	27%
Apricot	0	0	0	0

Table 3: Number of colonies grown from soil samples of orchards of cherry, apple and apricot from different villages of Nagar Valley

Name of villages	Number of colonies		
	Cherry	Apple	Apricot
Chalt	480	300	00
Sikandarabad	430	300	00
Gulmit	410	280	00
Askurdass	400	390	00
Nagarkhas	450	380	00

Table 5 shows the antibiotic sensitivity pattern of *A. tumefaciens* isolated from the plant tumors and soil samples. All the isolated strains are resistant to lincomycin, Amoxacillin, Ampincillin and Cloxacillin. Only 22 (73.34%) strains showed sensitivity to Cephradine, 28 (93.33%) strains to Tetracycline and 26 (86.66%) strains were sensitive to Doxycycline.

DISCUSSION

The main fruits of Gilgit-Baltistan are apple, almond, apricot, cherries, peaches, grapes, pomegranate and pears, which are infected by many pests and by infectious diseases. Yet many of these diseases have not been reported or recognized and they pose a

Table 4: Infestation of *Agrobacterium tumefaciens* from tumor of crown gall

Name of villages of Nagar valley	Name of plants and number of specimens cultured				
	Cherry specimens cultured (10)		Apple specimen cultured (10)		Apricot specimens cultured (10)
	Growth	No growth	Growth	No growth	Not processed
Chalt	02 (100%)	00 (00%)	02 (100%)	00 (00%)	Not processed
Skindarabad	02 (100%)	00 (00%)	02 (100%)	00 (00%)	Not processed
Gulmit	02 (100%)	00 (00%)	02 (100%)	00 (00%)	Not processed
Askurdass	02 (100%)	00 (00%)	02 (100%)	00 (00%)	Not processed
Nagarkhas	02 (100%)	00 (00%)	02 (100%)	00 (00%)	Not processed

Table 5: Antibiotic resistance pattern of *Agrobacterium tumefaciens* from the soil and plant samples

Antibiotics	No. of specimen investigated	Resistant	Sensitive
Lincomycin (10 µg)	30	30 (100%)	00 (00%)
Amoxycillin (10 µg)	30	30 (100%)	00 (00%)
Tetracycline (30 µg)	30	02 (6.67%)	28 (93.33%)
Cephradine (30 µg)	30	08 (26.66%)	22 (73.34%)
Ampincillin (10 µg)	30	30 (100%)	00 (00%)
Cloxacillin (5 µg)	30	30 (100%)	00 (00%)
Doxycycline (30 µg)	30	04 (13.34%)	26 (86.66%)

potential threat to plants in the horticultural industry in this region.

In Gilgit, the occurrence of crown gall disease in the local nurseries of (Jalalabad) in almond seedling was first reported (Anonymous, 1991) and on the bases of this report conducted a (symptom based) survey of existing fruit trees nurseries in some selected localities of Northern Areas (Gonar farm, Jalalabad, Skarkoi, Thasuit, Murtazabad, Singal, Gakutch, Pokara, Yasin and Skardu and found 90% nurseries are infected with as high as 80% infestation (FAO report, 1992).

The present investigation was conducted during July to November 2008 in fruit nurseries of different villages of Nagar Valley in the District Hunza-Nagar to record the incidence, severity level and causative agent of the Crown gall disease in stone fruits especially cherry, apple and apricot. The cherry and apple plants were severely affected. In Algeria, Bouzar and his colleagues in (1991) conducted a survey and found 99% of the plant nurseries were infected with crown gall. The results of this study in Gilgit-Baltistan nurseries are not unexpected. Crown gall is considered universal in its distribution, occurring wherever stone fruits are grown. The cultivation of stone fruits in the area for centuries and the propagation and distribution of the plants from these nurseries throughout the area for more than 40 years would have provided ample opportunity for the introduction and dissemination of pathogen in the area as the disease spreads by transplantation and grafting of the infected section of the plants (Hafiz, 1986). In India Sharma *et al*. (2002) conducted a survey in Sirmour, Solan, Shimla, Kullu and Mandi districts of Himachal Pradesh during 2001-2002 to record the prevalence of crown gall disease in stone fruit nurseries. They also found the cherry and peach rootstock Colt, were worst affected with the disease incidence of 100% on cherry plants of Palsehar in Mandi.

Seventeen nurseries in England were surveyed for crown gall (Agrobacterium tumefaciens). The disease was detected in nine of 101 apple root-stock beds examined: five were on the only nursery surveyed that was on peat soil (pH 5.9) and 49-84% of these beds were galled; 1-4% was recorded in single beds on four other nurseries. It was found on five of seven nurseries with F12/1 cherry rootstock beds: three of four beds examined had 6, 6 and 43% of stools affected and two of three layer beds had 4 and 12 gall clusters/20 yd (18.3 m) run of bed. Slight infection (one gall cluster in 20 yd of bed) was found in one of 17 plum beds on seven nurseries and 16% of stools affected were in one of 14 quinces A beds on 11 nurseries. The presence or amount of crown gall could not be related to type of bed, to age of bed, to soil pH or to type of loam or silt soil (Lelliott, 2007).

In our study the sensitivity of the isolated strains of *A. tumefaciens* is very low. Only the tetracycline has 94% sensitivity and the cephradine and doxycycline is 73-86%. The lincomycin, amoxicillin, ampincillin and cloxacillin are 100% resistant. In a study conducted by Hafiz (1986) proved quite effective the treatment of terramycin and venomycin against tumor formation.

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