Biological Control of Crown Gall on Peach and Cherry Rootstock Colt by Native *Agrobacterium radiobacter* Isolates

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Abstract: Crown gall caused by *Agrobacterium tumefaciens* is the major limiting factor in raising healthy stone fruit plants in nurseries. The incidence of crown gall varies from 4 to 97.5 per cent at different locations resulting in out right rejection of an average of 30 per cent stone fruit plants in nurseries. *Agrobacterium radiobacter* strain K-84 has been reported to provide nearly complete control of this disease, however this strain is not available in India. Therefore, the present work was aimed to evaluate the antagonistic activity of twelve native *A. radiobacter* isolates from peach cv. Redhaven, July Elberta and cherry cv. Stella rhizosphere soil against *A. tumefaciens*. Agrocin production was detected in three isolates of *A. radiobacter* UHFBA-8, UHFBA-11 and UHFBA-12, and in cross inoculation test against *A. tumefaciens*, all the three isolates completely inhibited gall formation into tomato stems, whereas inoculation of pAg^- variants of UHFBA-8 and UHFBA-11 resulted in 87.6 per cent less number of galls per wound and 96 per cent reduced gall size into inoculated tomato stems. Root dip treatment of peach plants with isolate UHFBA-11 resulted in 5.37 per cent incidence of crown gall in contrast to more than 54 per cent galled plants that had not received the treatment. In cherry rootstock Colt, isolate UHFBA-8 as root dip minimized crown gall incidence to 22.22 per cent as compared to 71.11 per cent incidence in untreated plants. Rifampicin resistant mutants of *A. radiobacter* isolate *viz*., UHFBA-8 and 11 efficiently colonized the root system of peach and Colt and their populations remained in the tissues of treated plants throughout the growing season. The data suggest that other mechanisms such as efficient colonization of root system, binding and physical blockage of infection sites are involved in biological control by *A. radiobacter* in addition to production of agrocin.

Keywords: Biological control, crown gall, peach, cherry rootstock-Colt, *Agrobacterium tumefaciens*, agrocin producing *Agrobacterium radiobacter*.

INTRODUCTION

Crown gall disease caused by *Agrobacterium tumefaciens* is an economically important disease, which infect dicotyledonous plants from almost 100 different families [1] including stone fruit, grapevines, roses and some ornamental species. *Agrobacterium tumefaciens* is considered a quality pathogen in the European countries, USA and a quarantining pathogen in others countries including India. The disease which is considered the disease of nursery plants also affect the grown up stone fruit trees of peach, almond, apricot, plum and cherry. The incidence of crown gall varies from 4 to 97.5 per cent at different locations causing an estimated loss of Rs. 6 million, annually. However, peach and cherry are worst affected by crown gall. Almost in every block of Rajgarh and Sangrah known as peach bowl of India, the incidence on peach is as high as 97.5 per cent. Although there is strict domestic quarantine for the movement of crown gall easpreviously free from this disease, presumably caused by the movement of apparently looking healthy plants carrying incipient crown gall infection from diseased area to disease free area [2, 3]. *Agrobacterium* is divided in three biovars. Biovar 1 and 2 are generally found in tumours from various plants of family Rosaceae comprising the most important pome and stone fruits, while the biovar 3 is restricted to grapevine [4]. In India occurrence of biovar-1 and 2 of *A. tumefaciens* has been reported in stone fruit nurseries [5-8]. Management of crown gall disease attracted many management strategies including chemicals, pre-plant application of soil sterilents, soil solarization, herbicides, soil amendments [9-11] or rhizobacteria like *Pseudomonas fluorescens*, *Bacillus subtilis* [11] that provide 30 to 40 per cent control of crown gall. Integration of soil solarization with *Bacillus subtilis* [12] although provide 84 per cent control of crown gall

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in cherry rootstock Colt, is cumbersome as the nursery growers have to leave nursery sites bare for complete one year for carrying soil solarization from April to June and only in next year nursery can be raised in solarized plots.

In 1972, New and Kerr [13] published the first report of biological control of crown gall by using strain K-84 of *Agrobacterium radiobacter*. It was the first biological control successfully used against pathogenic strains of *Agrobacterium* on different hosts in countries all over the world for almost 30 years [14, 15]. The strain K-84 supplied commercially on agar plates or in peat substrate is used by suspending the bacterial cells in water, then dipping seeds, seedlings or cuttings in this suspension before planting. The strain K-84 harbours pAgK84 plasmid [16, 17] which codes of production of an inhibitor agrocin (bacteriocin). However, pAgK84$^-$ variants also provide some degree of protection when co-inoculated with *A. tumefaciens* into tomato stems [17]. The efficient colonization and persistence on roots is at least conferred by the chromosomal genes, because many studies have shown that transfer of agrocin plasmid into other *A. radiobacter* strains does not make them as efficient as strain K-84 [18]. In spite of the success of K-84, some potential problems could be associated with its application [19]. The principal cause of failure of efficacy of strain K-84 is related to the pAgK84 transfer because the genes controlling agrocin 84 production and resistance can be transferred from strain K-84 to a pathogenic *Agrobacterium* [20, 21] becoming resistant to agrocin 84. In order to avoid this transfer and safeguard the biocontrol of crown gall, the genetically modified strain K 1026 was successfully developed in the frame of an Australian and USA cooperation [22]. The plasmid pAgK1026 is incapable of conjugal transfer at a detectable frequency in the laboratory [22]. However, due to restrictions of the use of genetically modified organisms, K1026 is currently not used in many countries. Besides, crown gall biocontrol using K1026 could also breakdown via the transfer of Ti plasmid from a pathogenic *Agrobacterium* donor to K1026, which thus become pathogenic.

Therefore, research of other effective *Agrobacterium radiobacter* is currently of interest for rapid selection and augmentation of superior strains in controlling crown gall disease.

Thus, within the framework of the present work, we explored the efficiency of twelve native *A. radiobacter* isolates which have better colonization and persistence, besides producing agrocin for antagonism against *A. tumefaciens* and their potential to control crown gall *in vitro* and *in planta*.

MATERIALS AND METHODOLOGY

Isolation of *A. tumefaciens and A. radiobacter*

Agrobacterium tumefaciens was isolated from young tender and milky galls on diseased peach plant cvs. Redhaven, July Elberta and cherry rootstock Colt collected from peach and cherry rootstock nurseries at University Research farm at Nauni and Regional Horticultural Research station, Bajaura (Kullu) in the month of September, 2005. This material was washed under non-chlorinated tap water for 24 h, with the help of a sharp sterilized razor, the galls were separated from the infected plants, necrotic tissues trimmed off from the periphery and then galls were diced into small cubes (2mm approx.). These cubes were surface sterilized in $HgCL_2$ (0.1%) for 30 seconds and were rinsed in sterilized distilled water three times to remove traces of mercuric chloride. Five cubes were crushed in 1 ml of sterilized distilled water with the help of sterilized glass rod in a sterilized Petri plates to form the suspension, which was kept undisturbed for 10 minutes. A loopful of bacterial suspension was then streaked on yeast extract mannitol agar (YEMA) medium. A single colony was picked up after incubation of plates at 27 ± 1℃ in a BOD incubator for 5 days and further re-streaked in fresh medium and incubated for another 24h. This exercise was repeated three times to get single cell bacterial colony, which was transferred to YEMA slants. The bacteria growth in each slant was checked after incubation of slants at 27±1℃ for 5 days in a BOD incubator. These slants were then stored at 4℃ in a frost-free refrigerator for further use.

For isolation of *A. radiobacter*, loosely attached soil were collected in sterilized polythene bag from roots of five randomly selected uprooted nursery plants of peach cvs. Redhaven, July Elberta and cherry cv. Stella during the month of September and October, 2005. These samples were mixed in a separate sterilized polythene bags, respectively to make a composite soil sample. Each composite soil sample was then shade dried for 24 h over sterilized butter papers and filtered through sterilized muslin cloth. One g soil from each sample was added to 99 ml of sterilized distilled water in 250 ml conical flask and then kept in an orbital shaker at 100 rpm for 15 minutes to make homogenous suspension.

The suspension was then diluted to 10^{-3} by serial dilution method [23]. A loopful of bacterial suspension was streaked on YEMA supplemented with 25 ppm Congo red. Single bacterial colony, which stained red in the center, was picked up after incubation of plates at 27±1℃ in a BOD incubator for 5 days and further re-streaked on the same medium in a fresh sterilized plate. The re-streaking after picking a single bacterial colony was repeated three times to get a pure single cell colony. Individual colony of each isolate was further transferred to slants of YEMA, which were incubated at 27±1℃ for 5 days. After checking the growth in each slant, they were stored at 4℃ in a frost-free refrigerator for further use. Periodic sub-culturing of stock culture of all the isolates was done after every three month

Biovar Characterization and Opine Utilization of *A. tumefaciens* and *A. radiobacter*

Biochemical tests *viz*., 3-ketolactose, oxidase, acid production from erythritol and litmus milk were performed for biovar characterization of different isolates into biovar 1,2 and 3. Utilization of opines-nopaline and octopine of isolates of *A. tumefaciens* and *A. radiobacter* was done by the method of Moore *et al*. [24].

In Vitro Antagonistic Activity of *A. radiobacter*

The method of Stonier was modified as per Moore *et al* [24, 25]. The method consists of spot inoculating a test antagonist- *A. radiobacter* isolates on mannitol glutamate agar medium supplemented with biotin (2 µg / l) and incubation of the plates for 3 days at 27±1℃ in a BOD incubator. In one set, the test antagonist was killed by chloroform and

plates were then lightly atomized with *A. tumefaciens* I2 (grown in mannitol agar broth medium supplemented with biotin 2µg / l having 10^{+} CFU^{-ml} after incubation at 27±1℃ for 3 days). In second set, plates were lightly atomized with *A. tumefaciens* without killing the test antagonist. The plates were further incubated for 3 days at 27℃ in a BOD incubator. The presence of zone of inhibition in plates without exposure to chloroform, indicated that isolate was having antagonistic activity against *A. tumefaciens* and zones of inhibition in the plates exposed to chloroform suggested production of bacteriocin-agrocin.

In- *Planta* Evaluation of *A. tumefaciens* and *A. radiobacter* Isolates for their Pathogenicity and Antagonism on Tomato Plants

Screening of three isolates of *A. tumefaciens* and 12-test antagonists- *A. radiobacter* was done by inoculating 4-week old potted tomato plants-hybrid KS-16. The 20 cm diameter plastic pots were filled with sterile substrate containing soil, sand and compost in a 1:1:1 ratio. Minimum and maximum temperatures recorded were 20 and 26℃, respectively, and the relative humidity fluctuated between 60% and 90%. Three tomato seedlings raised in each pot, were fertilized every 15 days with a solution of 15-10-5 (N-P2O5-K2O) with a final concentration of 1.4 g of fertilizer per day. YEMA slants of 72 h old of *A. tumefaciens* and *A. radiobacter* were suspended separately in 10 ml sterile distilled water and shaken vigorously to give bacterial suspensions of 10^8 $CFUml^{-1}$. Each *A. radiobacter* isolate was tested following the New and Kerr's method [13] on 4-weeks old tomato plants (five replicates of three plants / pot). Tomato plants were wounded with a blunt cylindrical sterilized steel rod of 2 mm diameter, at three different portions of stem to a depth of 3mm. In one set, each wound was inoculated with 0.004 ml suspension of each *A. tumefaciens*, second and third set were inoculated with 0.004ml of *A. radiobacter* and immediately wrapped with sterilized non-absorbent cotton. In third set after 24h of incubation, 0.004 ml suspension of pathogenic *A. tumefaciens* containing 10^8 $CFUml^{-1}$ was inoculated on the same wounds after removing the cotton [26]. The wounds were again wrapped immediately with fresh sterilized non-absorbent cotton. A control of uninoculated tomato plants was also kept for comparison. Wounds were examined for the presence or absence of galls after 4 weeks. The galls developed on tomato plants, were separated from the stem with the help of a sharp edged rajor and cut into two halves through its center to measure the average diameter of 15 galls. Further experiments were conducted with *A. tumefaciens* I2 as it resulted in development of maximum number of galls per wound and maximum gall size into inoculated tomato stem.

Curing of pAg Plasmid from *A. radiobacter* Isolates with Mtomycin C

Agrobacterium radiobacter isolate UHFBA-8, UHFBA-11 and UHFBA-12 cells grown on YEMA slants were suspended in sterile distilled water, washed once, and resuspended to 10^6 cells/ml in 100 ml volumes of mannitol-glutamate (MG) broth. Mitomycin C was then added to 0.0, 0.1, 1.0 and 2.0 µg/ml. The cultures were incubated at 27 ± 1^0 C on a orbital shaker for 18 h and diluted to 10^2 cells/ml and spread on MG agar plates. Individual colonies appearing on MG agar were transferred to fresh MG plates and were screened for agrocin production as per Moore *et al*. method and were also tested for development of galls into tomato stems preinoculated 24 h prior to inoculation of *A. tumefaciens* I2.

Field Trials

Research trials were carried out during 2006 and 2007 in a nursery site, with a history of high incidence of crown gall (30 to 90 per cent) in peach and cherry rootstock-Colt, at Dr.Y.S.Parmar University of Horticulture and Forestry Regional station, Bajaura (Kullu). In December 2005 and 2006, peach seeds were stratified for three months, also 1-year old peach seedlings and Colt suckers were obtained for research trials in dormant bare root conditions from the commercial nursery of Progeny-cum-Demonstration orchard, Kwagdhar (Sirmaur) and Temperate fruit Research Station, Kotkhai (Shimla) respectively, both nursery sites almost free from crown gall infection. Peach seedlings and Colt suckers were held in cold storage (4℃) until 3 days before treatment or transplanting which were tied in bundles of 36 plants and were acclimatized to outdoor conditions. In a randomized block design experiment with five replications in each treatment, each *A. radiobacter* isolate *viz*., UHFBA-8, UHFBA-11 and UHFBA-12 found effective against *A. tumefaciens* under *in-vitro* conditions, were tested as seed and root dip treatment on peach and on Colt as root dip treatment. For this continuous growth of individual *A. radiobacter* isolate as a lawn was obtained by spreading a loopful of test antagonist (*A. radiobacter*) with the help of L-Shaped spreader on YEMA in a plate incubated at 27±1C in a BOD incubator for 5 days. The growth of individual plate was scrapped with the help of a sterilized blade and suspended in 4 l non-chlorinated water. Alternatively, the bacteria were cultured on YEMA, and then washed cell suspension from agar surface was diluted to the desired concentration in sterile distilled water. Final concentration of 10^8CFU^{-ml} (OD = 0.2 at 600nm) was adjusted by further diluting the suspension with non-chlorinated water. In seed treatment, stratified seeds were soaked for 30 minutes in suspension of individual *A. radiobacter* isolate and later were shade dried for 1h before sowing. In root dip treatment, the roots of 1 year-old healthy peach seedlings and Colt suckers were washed in running non-chlorinated water for 1h to remove adhering soil particles. One-third roots of each plant were removed to create fresh wounds. Immediately after pruning plants were soaked in 10 l suspension of individual *A. radiobacter* isolate (36 plants/ treatment) for 30 minutes and later shade dried for 1h prior to transplanting them in the field.

A control was also maintained for peach seed, peach seedlings and Colt suckers where stratified seeds, seedlings and Colt suckers were soaked in non-chlorinated water and shade dried for same duration before sowing or transplanting in beds. Twelve stratified peach seeds, 1-year-old peach seedlings and Colt suckers were sown or transplanted in each bed of $1m^2$ size with plant to plant distance of 20 cm and row to row distance of 25 cm in the first *week* of February, 2006 and 2007. The recommended package of practices (Farmyard manure @ 60 MT/ ha, P at 30kg/ ha, K at 50 kg/ ha at the time of planting and N at 90 kg/ ha applied in two split doses of 45kg/ ha; first dose applied during last week of February

and second dose applied in last week of August, irrigation of nursery beds to field capacity after every 4th day and weeding after every 15 days) were adopted throughout the growing season. The plants were uprooted in last week of December in each year to record the incidence of crown gall, shoot length, root length and stem girth of healthy plants in each treatment.

Colonization of Peach and Colt Roots by Drug Resistant Mutants

The survival of isolates viz., *A. tumefaciens* I2 and *A. radiobacter* UHFBA-8, UHFBA-11 and UHFBA-12 on field planted peach seed and root dip treated Colt sucker, and subsequent colonization of roots of seedling and Colt sucker, was followed by means mutants of *A. tumefaciens* I2 resistant to streptomycin (2.5 mg/ml) and *A. radiobacter* resistant to rifampicin (2.5 mg/ml). The resistant mutants were obtained by repeated culturing in liquid media containing increasing concentrations of the antibiotics [27]. The resistant mutants grew on YEMA containing 2.5 mg/ml of streptomycin or rifampicin. Pathogenicity of *A. tumefaciens* and agrocin production in *A. radiobacter* isolates were the same to that of parents. Peach seeds were dipped in 4 l suspension of $3x10^8$ CFU/ml of individual resistant mutants of different isolates, drained, and planted in first week of February, in one set with individual *A. tumefaciens* I2, in second with individual *A. radiobacter* isolate, in third set in combination with individual *A. radiobacter* and *A. tumefaciens* I2, and fourth set of uninoculated seed dipped in 4 l non-chlorinated as control. Each treatment consisted of three groups of 12 seeds randomly planted in field. Nine months later, intact crowns and root systems were cut from two seedlings and assayed individually by macerating the tissue in a Waring blendor in 100 ml of water for 5 minutes, diluting the suspension serially, and spreading three 0.1 ml aliquant directly from the suspension and from selected dilutions on the selective medium containing streptomycin and rifampicin (2.5 mg/g).

To determine if the drug –resistant mutants could colonize uninoculated parts of the root system of growing plants, $3x10^8$ CFU/ml were sprayed over the crown of 1-year old Colt sucker in first week of February, and the inoculated and uninoculated suckers (36 plants/treatment in three replicates of 12 plants each randomly planted in the field) were harvested 2 h and 9 months later. In addition to assaying the total root system for the drug resistant mutant, the tap root system (30-33 cm from the crown), lateral root tips, and cylinders of the epidermal surface 2.3 to 3 cm long (peeled from the tap root at the crown and 13 to 14 cm below the crown) were assayed separately.

Statistical Analysis

The actual data on zone of inhibition without and after exposure to chloroform as indicated in Table **1**, and number of wounds inoculated, wounds showing galls, number of galls per replication and gall size as indicated in Table **2** were analyzed in a CRD. The data on percentage of incidence of crown gall in different treatments from field experiments were subjected to statistical analysis in a RBD as described by Gomez and Gomez [28]. Critical differences ($CD_{0.05}$) to determine the effectiveness of different treatments at 5% level of significance were also calculated by multiplying critical 't' value with standard error (SE±).

Table 1. ***In Vitro*** **Evaluation of** ***Agrobacterium radiobacter*** **Isolates for their Antagonism against** ***Agrobacterium tumefaciens*** **Isolate I 2 without and after Exposure to Chloroform**

Isolate	Zone of Inhibition (cm)	
	Without Exposure	After Exposure
UHFBA-1	0.00	0.00
UHFBA-2	0.00	0.00
UHFBA-3	0.00	0.00
UHFBA-4	0.00	0.00
UHFBA-5	0.00	0.00
UHFBA-6	0.00	0.00
UHFBA-7	0.00	0.00
UHFBA-8	3.17	1.77
UHFBA-9	0.00	0.00
UHFBA-10	0.00	0.00
UHFBA-11	3.13	2.37
UHFBA-12	2.07	0.90
SE±	0.10	0.21
$CD_{0.05}$	0.29	0.48

RESULTS

Isolation of *A. tumefaciens and A. radiobacter*

Agrobacterium tumefaciens could be isolated from young tender galls appeared on crown gall infected uprooted plants. The colonies were circular to oblong, squishy-squashy, convex shaped with smooth margin ranging from 0.3 to 1.1 cm in size. In all three isolates of *A. tumefaciens*-I1 from crown gall affected peach plants cv. Redhaven collected from main campus Nauni, I2 from peach plant cv. July Elberta and I3 from Colt suckers form Bajaura could be isolated. In addition to this, twelve non-pathogenic isolates of test antagonist-*A. radiobacter* were isolated from rhizosphere soil with accession numbers UHFBA-1 to 5 from peach cv. Redhaven, UHFBA-6 and 7 from peach cv. July Elberta and UHFBA-8 to12 from cherry cv. Stella and were maintained in YEMA slants for further studies.

Biovar Characterization and Opine Utilization of *A. tumefaciens* and *A. radiobacter*

All *A. radiobacter* isolates showed negative reaction in 3-Ketolactose test, acid production from erythritol, acidic reaction in litmus milk test. A positive oxidase reaction was observed in UHFBA-1, 10, 11 and 12, delayed positive reaction in UHFBA-5, 6 and negative reaction in UHFBA-2, 3, 4, 7, 8

Table 2. Evaluation of *Agrobacterium tumefaciens, Agrobacterium radiobacter* Isolates for their Pathogenicity and further Screening of *Agrobacterium radiobacter* Isolates for their Antagonism against *Agrobacterium tumefaciens* Isolate I2 on Tomato Plants

Isolate	Number of Wounds inoculated*	Number of Wounds showing Galls*	Number of Galls per Wound*	Gall Size (cm)*
A .tumefaciens				
I1	9.0	5.67	1.90	1.00
I2	9.0	7.00	2.67	2.50
I3	9.0	6.00	2.33	1.50
A. radiobacter				
UHFBA-1	9.0	0.00	0.00	0.00
UHFBA-2	9.0	0.00	0.00	0.00
UHFBA-3	9.0	0.00	0.00	0.00
UHFBA-4	9.0	0.00	0.00	0.00
UHFBA-5	9.0	0.00	0.00	0.00
UHFBA-6	9.0	0.00	0.00	0.00
UHFBA-7	9.0	0.00	0.00	0.00
UHFBA-8	9.0	0.00	0.00	0.00
UHFBA-9	9.0	0.00	0.00	0.00
UHFBA-10	9.0	0.00	0.00	0.00
UHFBA-11	9.0	0.00	0.00	0.00
UHFBA-12	9.0	0.00	0.00	0.00
A. radiobacter + *A. tumefaciens* I2				
UHFBA-1	9.0	1.00	0.33	0.10
UHFBA-2	9.0	1.00	0.66	0.10
UHFBA-3	9.0	1.00	0.66	0.10
UHFBA-4	9.0	2.00	0.88	1.50
UHFBA-5	9.0	2.67	1.00	1.50
UHFBA-6	9.0	2.67	1.33	1.50
UHFBA-7	9.0	2.33	1.33	1.50
UHFBA-8	9.0	0.00	0.00	0.00
UHFBA-9	9.0	1.00	0.66	0.10
UHFBA-10	9.0	2.67	1.00	1.00
UHFBA-11	9.0	0.00	0.00	0.00
UHFBA-12	9.0	0.00	0.00	0.00
SE±	0.97	0.46	0.58	
$CD_{0.05}$	1.68	0.98	1.12	

*Average of five replications.

and 9. *Agrobacterium tumefaciens* isolates showed negative reaction in 3-ketolactose test, acid production form erythritol and acidic reaction in litmus milk test and negative reaction in oxidase test except isolate I3 that showed delayed positive reaction in oxidase test . All these *A. tumefaciens* and *A. radiobacter* isolates were nopaline utilizing. On the basis of these biochemical tests, all these isolates were identified as belonging to biovar-2.

In Vitro Antagonistic Activity of *A. radiobacter*

Agrobacterium radiobacter isolates UHFBA-8, 11 and 12 showed 3.17, 3.13 and 2.07 cm zone of inhibition without exposure to chloroform and 1.77, 2.37 and 0.90 cm zone of inhibition after exposure to chloroform, respectively (Table **1**). These results indicated the isolate UHFBA-8, 11 and 12 were producers of agrocin with variability in the quantity of agrocin produced.

In- *Planta* Evaluation of *A. tumefaciens* and *A. radiobacter* Isolates for their Pathogenicity and Antagonism on Tomato Plants

Agrobacterium tumefaciens isolates screened for their pathogenicity on tomato plants indicated that all these isolates typically produced gall on stem portions of inoculated tomato plants, whereas none of the *A. radiobacter* isolates could produce gall on artificially inoculated tomato plants. *Agrobacterium radiobacter* isolates UHFBA-8, 11 and 12 when tested for their antagonism against *A. tumefaciens* on tomato plants by cross inoculation method completely controlled gall development, whereas in isolates UHFBA-1, 2, 3, 4, 5, 6, 7, 9 and 10, galls per wound produced were 0.33, 0.66, 0.66, 0.88, 1.00, 1.33, 1.33, 0.66 and 1.00 in number, respectively. The gall size was 0.10 cm for UHFBA-1, 2, 3 and 9, and 1.50 cm for isolates UHFBA-4, 5, 6 and 7. All the pathogenic isolates produced galls ranging from 1.0 to 2.5 cm in diameter. However, *A. tumefaciens* isolate I2 produced maximum galls (2.67) per inoculated wound and out of 9 inoculated wounds seven wounds developed galls (Table **2**). Further screening of *A. radiobacter* isolates was therefore done against *A. tumefaciens* isolate I2.

Antagonistic Activity of *A. radiobacter* Isolates treated with Mitomycin C

Among mitomycin C treated *A. radiobacter* isolates, variants of UHFBA-8, UHFBA-11 and UHFBA-12 recovered from the 2.0 µg/ml mitomycin C treatment at a frequency of about 10^{-2} mutants per viable cell, and produced zones of inhibition of 1.4, 1.0 and 0.00 cm respectively without exposure to chloroform. However, in all the three variants there was no zone of inhibition after exposure to chloroform. Gall development i.e. 0.33, 0.33 and 1.90 galls per wound in UHFBA-8, 11 and 12, respectively and gall size of 0.10, 0.10 and 1.00 cm in. UHFBA-8, 11 and 12, respectively, were observed in to tomato stem inoculated 24 h prior to inoculation of *A. tumefaciens* I2

Field Evaluation

On the bases of *in vitro* and *in planta* evaluation, *A. radiobacter* isolates viz., UHFBA-8, 11 and 12 were further selected for their evaluation against crown gall as seed and root dip treatment on peach and as root dip treatment on cherry rootstock-Colt. Minimum incidence of crown gall (5.37%) was observed in *A. radiobacter* isolate UHFBA11 applied as root dip on peach plants compared with the 54.17 per cent disease in untreated control plants (Table **3**). Seed treatment with this isolate was also effective as indicated by the 12.22 per cent incidence of crown gall in comparison to 50.37 per cent disease incidence in control (Table **3**). Contrary to this, isolate UHFBA-8 was most effective on cherry rootstock-Colt and it resulted only 22.22 per cent incidence of crown gall as against 71.11 per cent disease incidence in control. Isolate UHFBA-11 as root dip was also effective on Colt and it resulted 23.61 per cent incidence of crown gall statistically at par with UHFBA-8 (Table **4**). *Agrobacterium radiobacter* isolate UHFBA-8, 11 and 12 also enhanced the shoot length, root length and stem girth applied as seed and root dip on peach and as root dip on Colt also. Over all mean values indicate that maximum shoot length (127.30 cm) in UHFBA-8, root length (35.87 cm) and stem girth (1.20 cm) in UHFBA-12 were observed when these isolates were applied as root dip on peach (Table **5**). Seed treatment of peach with isolate UHFBA-11 resulted in maximum (133.20 cm) shoot length, whereas, maximum root length (36.51 cm) was recorded in UHFBA-12. However, maximum stem girth (1.19 cm) was observed in isolate UHFBA-8 followed by 1.18 cm in both UHFBA-11 and 12 (Table **5**). On cherry rootstock-Colt, maximum shoot length (144.30 cm) was observed in UHFBA-12, whereas maximum root length (38.28cm) and stem girth (1.54cm) were observed in isolate UHFBA-11 when applied as root dip (Table **6**). The same results were obtained with washed cell suspension of these isolates.

Table 3. Effect of Different *Agrobacterium radiobacter* Isolates applied as Seed and Root Dip Treatment on the Incidence of Crown Gall on Peach Seedlings

Treatment	Seed Treatment			Root Dip Treatment		
	Disease Incidence (%)			Disease Incidence (%)		
	Year		Mean	Year		Mean
	2006	2007		2006	2007	
UHFBA-8	11.11	26.67	18.89	9.52	14.30	11.86
UHFBA-11	11.11	13.33	12.22	7.40	3.33	5.37
UHFBA-12	48.33	46.67	47.50	19.00	20.00	19.50
Control	40.74	60.00	50.37	33.33	75.00	54.17
Overall mean	27.82	36.67		17.32	28.13	
SE±	10.46	13.05		19.76	17.33	
$CD_{0.05}$	25.61	31.93		48.37	42.40	

Table 4. Effect of Different *Agrobacterium radiobacter* Isolates applied as Root Dip Treatment on the Incidence of Crown Gall on Cherry Rootstock-Colt

Treatment	Disease Incidence (%)		Mean
	Year		
	2006	2007	
UHFBA-8	11.11	33.33	22.22
UHFBA-11	11.11	36.11	23.61
UHFBA-12	50.00	52.78	51.39
Control	55.55	86.67	71.11
Overall mean	31.94	52.22	
SE±	18.07	8.80	
$CD_{0.05}$	44.22	21.54	

Table 5. Effect of *Agrobacterium radiobacter* Isolates applied as Seed and Root Dip on Growth of Peach Seedlings

Isolate	Seed treatment									Root dip treatment								
	Shoot length (cm)			Root length (cm)			Stem girth (cm)			Shoot length (cm)			Root length (cm)			Stem girth (cm)		
	2006	2007	Mean	2006	2007	Mean	2006	2007	Mean	2006	2007	Mean	2006	2007	Mean	2006	2007	Mean
UHFBA-8	133.60	94.53	114.06	28.97	30.67	29.82	1.20	1.19	1.19	123.50	131.10	127.30	27.53	30.73	29.13	1.06	1.26	1.16
UHFBA-11	134.20	132.20	133.20	28.90	28.20	28.55	1.17	1.19	1.18	123.50	109.60	116.55	25.00	35.40	30.20	1.09	0.98	1.03
UHFBA-12	128.40	126.10	127.25	37.10	35.93	36.51	1.16	1.20	1.18	118.60	117.80	118.20	36.47	35.27	35.87	1.21	1.20	1.20
Control	103.20	101.10	102.15	18.23	28.93	23.58	1.05	1.08	1.06	86.27	113.20	99.73	16.98	25.83	21.40	1.11	1.09	1.10
Overall mean	124.85	113.50		28.30	30.93		1.14	1.16		112.90	117.90		26.49	31.81		1.11	1.13	
SE±	5.86	6.13		2.72	5.10		0.08	0.06		9.99	4.25		1.69	5.12		0.07	0.09	
$CD_{0.05}$	14.35	15.00		6.65	12.50		0.19	0.15		24.44	10.40		4.14	12.54		0.17	0.24	

Colonization of Peach and Colt Roots by Drug Resistant Mutants

Populations of drug resistant mutant of *A. tumefaciens* I2 (4×10^3), *A. radiobacter*- UHFBA-12 (1×10^5), UHFBA-8 (6.5×10^5), UHFBA-11 (1×10^7) per gram of fresh tissue were recovered from roots of seedlings grown from inoculated peach seeds. When *A. radiobacter* and *A. tumefaciens* were co-inoculated, in every sample the population of mutant of UHFBA-8, 11 and 12 were 6.5×10^5 to 8.5×10^5, 3.2×10^6 to 5×10^6 and 0.22×10^3 to 1.5×10^3, respectively. *Agrobacterium tumefaciens* populations varied from 1×10^3 to 1×10^4. No *A. radiobacter* was isolated from roots of seedlings raised from uninoculated seed. The drug resistant mutants successfully persisted on the inoculated seed and subsequently colonized the emerging root system. Populations of drug resistant mutants of *A. radiobacter* isolates (1.2×10^5, $2.6 \times 10^7$and 3×10^7 CFU of UHFBA-12, 11 and 8, respectively and 2.4×10^3 of *A. tumefaciens* I2 per gram of fresh tissue) were detected only on peelings from the crown and tips of lateral roots near the crown of Colt suckers harvested 2 h after inoculation.

When *A. radiobacter* and *A. tumefaciens* were coinoculated, there was no appreciable difference of population to that of individual inoculation of these isolates. At the time of uprooting of Colt suckers after 9 months, populations of *A. radiobacter* (2×10^3 of UHFBA-12, 2×10^4 of UHFBA-11 and 1.2×10^5 of UHFBA-8) and *A. tumefaciens* I2 (2.2×10^4) CFU per gram of fresh tissue were observed only at the crown and tips of lateral roots. In coinoculated plants with *A. radiobacter* and *A. tumefaciens*, populations of drug resistant mutants of *A. radiobacter* isolates (1×10^6, 3.8×10^6 and 4.2×10^7 CFU of UHFBA-12, 11 and 8, respectively per gram of fresh tissue) were detected. *Agrobacterium tumefaciens* populations also varied in different sets of treatment (1×10^5 in plants coinoculated with *A. radiobacter* UHFBA-12, 2.1×10^3 coinoculated with UHFBA-11 and 1×10^3 CFU coinocu-

Table 6. Effect of *Agrobacterium radiobacter* Isolates applied as Root Dip Treatment on Growth of Cherry Rootstock-Colt

Isolate	Shoot Length (cm)			Root Length (cm)			Stem Girth (cm)		
	2006	2007	Mean	2006	2007	Mean	2006	2007	Mean
UHFBA-8	107.50	159.60	133.55	22.90	42.19	32.54	1.32	1.38	1.35
UHFBA-11	96.20	169.10	132.65	33.23	43.33	38.28	1.57	1.51	1.54
UHFBA-12	106.80	181.80	144.30	23.57	44.80	34.18	1.43	1.47	1.45
Control	95.83	147.10	121.46	27.57	36.60	32.08	1.30	1.34	1.32
Overall mean	104.07	164.40		26.81	41.73		1.40	1.42	
SE±	4.60	13.34		2.72	8.40		0.07	0.10	
$CD_{0.05}$	11.27	32.64		6.66	20.57		0.18	0.25	

lated with UHFBA-8). No population of resistant mutants could be observed from roots of uninoculated Colt suckers.

DISCUSSION

After the discovery of *A. radiobacter* strain K-84 by Kerr [29], major emphasis was given on biological control of crown gall by using this strain and it's genetically engineered derivative K-1026 world over for over a decade as till date there is no other management strategy which has been proved effective. However, these strains are ineffective in many instances owing to the transfers of pAgK84 plasmid to *A. tumefaciens* and Ti plasmid from *A. tumefaciens* to strain K-1026. Later it was realized that some biological control activities are independent of agrocin production as $pAgK84^-$ variants of strain K-84 were also able to reduce the gall size Therefore selecting new antagonists on the basis of agrocin production alone has not been very effective [27] as other mechanisms also operate such as ability to colonize the roots, physical blockage and binding to infection sites [30]. Therefore the present investigations were envisaged to find other agrocin producing isolates, which match in its efficacy to that of *A. radiobacter* strain K-84 besides having better colonization and persistence on the root system of stone fruit plants throughout the growing season.

Keeping in view of this, we isolated twelve *A. radiobacter* and three *A. tumefaciens* isolates. Fortunately all our *A. tumefaciens* isolates were 3-Ketolactose negative and were able to utilize nopaline. Out of twelve *A. radiobacter* isolates, only three were able to produce agrocin as detected by varying zone of inhibition after exposure to chloroform and to inhibit gall development into tomato plants.

In pAg^- variants of *A. radiobacter*, zone of inhibition was not to the size as observed in the parent isolates without exposure to chloroform and there was no zone of inhibition after exposure to chloroform which indicate that these isolates have lost pAg plasmid after treatment with mitomycin C. These isolates no longer prevented gall formation 24h prior to inoculation of the pathogen. However, less number of galls per wound and reduced gall size were observed into inoculated tomato plants.

Since tumour size is generally proportional to the size of wound [31], competition for attachment and binding to the sites was also attributed to the development of smaller tumours. These results further showed that some biological control activity by these *A. radiobacter* isolates is independent of agrocin production and this has been correlated to binding and physical blockage of infection sites [30].

All the agrocin producing isolates reduced the disease incidence and the amount of agrocin in different *A. radiobacter* isolates (as detected by the zone of inhibition after exposure to chloroform) was correlated with reduction in crown gall incidence. There was a negative correlation between amount of agrocin production and incidence of crown gall both on peach and Colt.

Isolate UHFBA-11 applied as seed treatment on peach resulted in maximum growth whereas no inference could be drawn in inducing variable growth by different isolates both on peach and Colt as root dip. Agrocin which is a fraudulent adenine type nucleotide has been reported to suppress the growth of plants [17]. Results obtained in the present investigations are quite contrary to the earlier findings [17, 19]. Shoot length was found maximum in isolate UHFBA- 8 and 11- producing maximum amount of agrocin whereas in isolate UHFBA-12 (producing minimum amount of agrocin), root length was maximum both in seed and root dip treatments on peach. In root dip treatment on cherry rootstock Colt isolate UHFBA-12 resulted in maximum shoot length and isolate UHFBA-11 resulted in maximum root length. It is stressed upon by various workers [15, 27] that besides the interaction between *A. tumefaciens* and *A. radiobacter*, other interactions may also play crucial role in overall induction of growth of plants. However, the studies related to interactions between the *A. tumefaciens*, *A. radiobacter* and other soil microbes, role of agrocin with plants are still in infancy. When we talk about the silencing of tumour inducing genes by agrocin on Ti plasmid which also carry auxin and cytokinin genes, the field results on growth parameters often are opposite to it. However, the role of agrocin in mitigating the effect of crown gall pathogen can not be ignored and there are quite possibilities that it helps in switch- on the plants own genes responsible for production of growth hormones. Further investigations are needed to conclusively prove it.

Colonization of peach and Colt roots by drug resistant mutants was also studied as this factor also imparts some degree of protection from the invasion of *A. tumefaciens*.

Invariably drug resistant mutants of UHFBA-8 and 11 which drastically reduced the incidence of crown gall efficiently colonized the roots. There was a significant positive correlation with the colonization of roots by *A. radiobacter* isolates and in subsequent decreasing the disease incidence both on peach and cherry rootstock-Colt. These isolates however, behaved differently in inducing the growth of peach and Colt may be due to the production of different antimicrobials.

These mutants were detected from roots of peach seedlings, and only on peelings from the crown and tips of lateral roots near the crown of Colt suckers harvested 2h after inoculation. The present results also confirm the findings of Moore [27]. The inability to detect mutants on other parts of the roots below the crown, as well as the total roots may have resulted (i) much of the inoculums spread over the crown was absorbed to surrounding soil and (ii) the bacteria were diluted below the level of detection. Nine months after inoculation at the time of uprooting, the drug resistant strains had significantly increased in numbers and had spread to other parts of the root system [18, 27].

When *A. radiobacter* isolates were coinoculated with *A. tumefaciens*, initially there was no appreciable difference in the population however, the population of *A. radiobacter* increased manifold whereas *A. tumefaciens* population remained static and concentrated around the tips of the crown and lateral roots. The results suggest that root exudates which have very high concentrations around the tips of roots act as chemo- static receptors directing the movement of *A. tumefaciens* towards it [18] more strongly because of presence of Ti plasmid in them responsible to respond quickly to these receptors. One of the most interesting aspects is that when *A. radiobacter* isolates were co-inoculated with *A. tumefaciens* there was remarkably low population of *A. tumefaciens* as compared to *A. radiobacter*. Surprisingly we did not encounter population of mutants resistant to agrocin either from tomato inoculation experiments or from field experiments, which further prove the work of Kerr and Htay [17], that there is very high correlation between pathogenicity and sensitivity to agrocin. Resistance to agrocin entails loss of pathogenicity and a change from nonpathogenic to pathogenic involves a change from agrocin resistance to sensitivity. We are aware the likely significance of this on the mechanism of tumour induction and this aspect of the work is being investigated by plasmid profile studies and amplification of agrocin producing regions in native *A. tumefaciens* populations or Ti genes in *A. radiobacter* as we did not achieve nearly complete control of crown gall.

CONCLUSION

Among twelve *A. radiobacter* isolates belonging to biovar-2, only three isolates with accession numbers UHFBA-8, 11 and 12 were found agrocin producing and antagonistic both in culture and on artificially inoculated tomato plants against nopaline utilizing *A. tumefaciens*. Minimum incidence (5.37%) of crown gall was observed on peach in *A. radiobacter* isolate UHFBA-11 applied as root dip as compared to 54.17 per cent incidence in control, whereas on cherry rootstock-Colt it resulted in 23.61 per cent incidence statistically at par with 22.22 per cent incidence observed in *A. radiobacter* isolate UHFBA-8 both applied as root dip as compared to71.11 per cent incidence of crown gall in control. These isolates also enhanced the growth of both peach and cherry rootstock-Colt plants.

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