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Rhizosphere competence of native *Rhizobium rhizogenes* strain and its use in management of crown gall

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Abstract: Native *Rhizobium rhizogenes* strain UHFBA-212 [141/1A (NCBI: KC488174)]was isolated from rhizosphere soil of peach nursery plant of wild peach collected from Himachal Pradesh. In addition to this,159 isolates were also collected and were screened *in vitro* for their biocontrol potential against *Agrobacterium tumefaciens*. Out of these strain, UHFBA-212 showed maximum zone of inhibition i.e. 4.16 and 3.57cm without and after exposure to chloroform against C58.Sequence analysis (16SrDNA) of the strain showed nucleotide homology similar to *Rhizobium* sp. Amplification of total genomic DNA of the strain with *Vir D2* and*ipt* primers didn't showed amplification with these virulence genes suggesting the absence of tumorigenic factors. In the field conditions, maximum population (329.33x10^6 cfu/g of soil) was observed in antibiotic resistant mutant of *R. rhizogenes* strain K84 applied on cherry rootstock Colt followed by 285.33 (x 10^6) cfu/g of soil in UHFBA-212 after 9 months at the time of uprooting of plants when applied alone as root dip. Minimum incidence of crown gall (2.00%) was observed in strain UHFBA-212 co inoculated with strain C58 as seed treatment on behmi seeds. The data on population indices in rhizosphere and incidence of crown gall further suggested that for better management of disease *R. Rhizogenes* isolates should be either equal or more in population than that of *A. tumefaciens* isolates. Strain UHFBA-212 controls crown gall as effectively as strain K84 and can be exploited against tumorigenic isolates under field conditions.

Keywords: Colonization, Disease incidence, Resistant, *Rhizobium rhizogenes*

INTRODUCTION

The area under stone fruit cultivation has shown an upward swing from the last few years in Himachal Pradesh. The natural genetic engineer *Agrobacterium tumefaciens* (Smith and Townsend, 1907) Conn. (1942) is a ubiquitous soil inhabitant and causes crown gall of various stone and pome fruits nurseries (Bouzar *et al.*, 1991 and Thakur *et al.*, 2007). During infection process the T-DNA of*Agrobacterium tumefaciens* gets integrated in host genome, resulting in over expression of the biosynthesis of phytohormones such as auxins and cytokinins and other low molecular weight opines, leading to abnormal cell division and proliferation (Zambryski, 1998). The galled seedlings become unfit for production and need to be disposed off. These problems have resulted in lower productivity and increased susceptibility of infected plants to biotic and abiotic stresses.

The pathogen belongs to family Rhizobiaceae and taxonomically designated as *Agrobacterium tumefaciens* for the tumorigenic isolates and non pathogenic strains as *A. radiobacter*. Sawada *et al.* (1993) proposed *A. radiobacter* in place of *A. tumefaciens* to include both pathogenic and non pathogenic*A. tumefaciens*. Further different agrobacterial genus (*A. larrymoorei*, *A. radiobacter*, *A. tumefaciens*, *A. vitis*, *A. rubi* and *A. rhizogenes*) were amalgamated in a single genus '*Rhizobium*' (Young *et al.*, 2001).On the basis of 16S rRNA sequencing the pathogen showed unique phenotypic genetic circumscriptions and was found similar to *Allorhizobium* and *Rhizobium* (Costechareyre *et al*, 2010;Lindstrom and Young 2011). Based on the multilocus sequence analysis (MLSA) of protein coding housekeeping genes among different agrobacterial taxa the taxonomic nomenclature of *Agrobacterium* as *Rhizobium rhizogenes* was considered as proper name (Mousavi *et al.*, 2014).

Successful biological control of crown gall by using *R. rhizogenes* strain K84 has been reported worldwide after its discovery by Kerr (1972) from Australia. However, this particular strain is not commercially available in India nor as effective as reported in other countries. In spite of the success of K84, some potential problems can arise from its application (Moore and Canfield, 1996). Under field conditions the biocontrol efficiency of strain K84 fails due to the conjugal transfer of agrocin plasmid (pAgK84), resulting in breakdown of the biocontrol capacity, because the recipient becomes resistant to agrocin 84 thus remaining pathogenic (Penalver and Lopez, 1999; Stockwell *et al.*, 1996, Raio *et al.*, 2009).

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Therefore, a search for other antagonists for controlling crown gall is currently under way all over the world (Raio *et al*., 2009; Gupta *et al*., 2010; Kawaguchi, 2014; Kawaguchi, 2015). Thus, in the present work we explored the efficacy of native agrobacterial strain for its potential to control *A. tumefaciens in vitro* and *in planta* and also determined the comparative colonization efficacies and persistence of *R. rhizogenes* **(**native strainUHFBA-212 and standard strain K84) and *A. tumefaciens* isolates (native isolate I_1 and C58) in the rhizosphere soil of stone fruit plants.

MATERIALS AND METHODS

Samples collection and isolation of *Rhizobium* isolates from infected seedlings: Actively growing galls on diseased peach plants were collected in sterilized polyethylene bags from nurseries of different locations of Himachal Pradesh (Fig.1) in the month of October-November. The fresh galls were detached carefully and washed in running water to remove soil. Small sections of living tissues chopped with a sterilized scalpel and immersed in sterilized distilled water and were left standing for 30 min at room temperature. A loopful of the suspension was streaked on two selective media namely Yeast Extract Mannitol Agar (YEMA) and D1 medium and plates were incubated for 2-3 days at 25± 1ºC (Murugesan *et al.*, 2010). The colonies that were representative of *Agrobacterium* were restreaked on D1 medium for further purification. The purified colonies were then stored at 4°C on YEMA medium supplemented with 0.5% $CaCO_3$.

***In vitro* evaluation for antagonism and agrocin production:** The isolates were evaluated for their antagonism against *A. tumefaciens* strain C58 as per method described by New and Kerr (1972). Antagonistic isolates were spot inoculated on mannitol glutamate agar medium supplemented with biotin (2µg/ml) and the plates were incubated for 3 days at 27°C in a BOD incubator. In one set, the test antagonist was killed by chloroform and plates were then lightly misted with *A. tumefaciens*. In second set, plates were lightly misted with *A. tumefaciens* without killing the test antagonist. The presence of zone of inhibition (diameter measured in cm) in plates without exposure to chloroform, indicated that isolate was having antagonistic activity against *A. tumefaciens* and zone of inhibition in the plates exposed to chloroform suggested production of bacteriocin.

***In vitro* evaluation for inhibition of gall developments by antagonistic isolate (s) on tomato plants:** Tomato plants var. Solan Gola were wounded by causing 3mm deep injuries at crown, middle and tip portion of stem with the help of sterilized blunt steel rod of 2mm diameter, with the help of sterilized micropipette, 0.004 ml of test

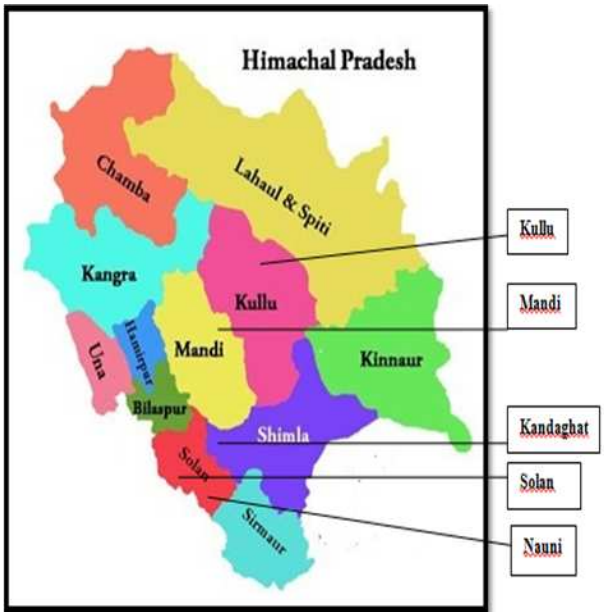

Fig. 1. *Location map indicating the different sampling sites in Himachal Pradesh.*

antagonist suspension (10^8 CFU/ml) was inoculated in each wound. Wounds were then wrapped with sterilized non-absorbent cotton. After 24 h, cotton was removed from the wounds and the pathogenic strain (*A.tumefaciens* strain C58*)* was applied (0.004 ml having 10^8 CFU /ml) to the same wounds. The wounds were again wrapped with fresh sterilized non-absorbent cotton. Each test antagonist was again inoculated on nine tomato plants representing three replication (three plants/ replication). Plants inoculated with only *A.tumefaciens* (C58,I_1 and I_2) and *R.rhizogenes* K84 served as positive and negative control, respectively. The observation on gall development on tomato seedlings were made after four weeks of inoculation (New and Kerr, 1972).

Detection of *virD2* and *ipt* genes in antagonistic strains: Genomic DNA of *Rhizobium rhizogenes* strain (UHFBA-212 and K84) and *A.tumefaciens* (C58,andI_1) was isolated using total DNA isolation Kit (real genomic isolation kit as per manufacturer's instructions). The isolated DNA was finally suspended in 100µl of elution buffer and quantified on one per cent agarose gel.

PCR reaction mixtures (50µl) contained primer oligonucleotides at 0.4 µM each, deoxynucleotide triphosphates at 200µM each, 1U of thermostable DNA polymerase (Perkin-Elmer [Taq] or Epicenter technologies [Tft], reaction cocktail supplied by the manufacturer (Perkin- Elmer, 10mM Tris [pH 8.3 at 25ºC], 50 mMKCl, 1.5mM $MgCl_2$, 0.001% gelatin [sigma G2500]; Epicenter, 50mM Tris [pH 9.0 at 25ºC], 20mM ammonium sulphate, 1.5mM MgCl2), and 50 to 250 ng of purified template DNA. Amplification was initiated by incubation at 94ºC for 1 min followed by 40 cycles at 94, 50 and 72ºC for 1 min at each temperature.

PCR based virulence assay was carried out based on *virD2* and *ipt* genes as previously described by Haas *et al.* (1995). Amplification of *virD2* gene was carried out using, one sense-strand oligonucleotide, primer A, and two antisense-strand oligonucleotides, primer C' and primer E' (Table 1). These primers were used in two different pairs to produce PCR products of 338 bp (A-E') and 224 bp (A-C'). The *ipt* gene of 427bp was amplified using primers sense-strand primer, CYT, and antisense-strand primer, CYT' (Table 1).

Development of mutants of *Rhizobium rhizogenes* and *A. tumefaciens*: The most effective native *Rhizobium rhizogenes* strainUHFBA-212 along with standard strain K84, *A. tumefaciens* isolate $I_1$and C58were selected for further studies. *Rhizobium rhizogenes* (UHFBA-212 and K84) resistant to rifampicin (2500ppm) and *A. tumefaciens* (C58 and I_1)resistant to streptomycin (2500ppm) were developed by repeated culturing in YEM broth amended with desired concentration of antibiotics (upto 100 generations with no selection pressure) as per method described by Moore and Allen (1977). The stability of mutants was evaluated after growth at 2500 ppm concentrations of antibiotics for over 100 generations by streaking on antibiotic amended medium. Antibiotic resistant mutants were also compared with parent strain (s) for agrocin production i.e. in strain K84 and UHFBA-212 and for pathogenicity of *A. tumefaciens* strain C58 and $I_1$by artificial inoculation on 4 weeks old potted tomato plants.

Application of resistant mutants as seed and root dip treatment: The antibiotic resistant mutants of *Rhizobium rhizogenes* and *A. tumefaciens* were evaluated as seed treatment on bitter almond and root dip treatment on cherry rootstock Colt as per method described by Vicedo *et al.* (1993) in the followings sets:

i) Seed treatment/root dip with *R. rhizogenes* strains.

ii) Seed treatment/root dip with *A. tumefaciens* isolates.

iii) Co-inoculation of seed /roots with *R. rhizogenes* and *A. tumefaciens*.

iv) Un-inoculated seeds/roots dipped in non chlorinated water to serve as control.

Growth of one antibiotic resistant bacterial culture (late-exponential-phase cells adjusted turbidometrically to densities of approximately 5 x 10^8 CFU/ml) was scrapped in four litre non chlorinated water and dissolved by stirring gradually with the help of sterilized stick. Seeds of bitter almond and roots of cherry rootstock Colt were soaked in the antibiotic resistant bacterial suspension for half an hour and later shade dried for 1 hour before planting in the field. In control, no bacterial culture was used (seeds and roots were dipped in non chlorinated water). Later, the seeds were sown in the field beds in randomized block design layout in 42 beds. In each bed 15 plants of cherry rootstock Colt were planted and 40 seeds of bitter almond per bed were sown with a row to row distance of 25 cm and plant to plant distance of 20 cm in 1 m^2plot size. Initial populations of bacterial culture used for seed and root dip treatments are listed in Table 2.

Colonization of inoculated seeds / roots by antibiotics resistant mutants vis- a-vis incidence of crown gall: The population of antibiotics resistant mutants both from the plants raised from inoculated and un - inoculated bitter almond seeds and root dip treated Colt suckers were estimated from seed surface at the time of sowing and roots of treated plants as per method described by Gupta *et al.* (2010) after 1, 3, 6 and at the time of uprooting of the plants after nine months. The colony forming units/ml was determined by serial dilution method on rifampin and streptomycin supplemented YEMA medium at 2500 ppm after incubation of plates at temp of 25±1^{0}C in a BOD for 3 days. At the time of uprooting of plants, incidence of crown gall was also computed by following formula

Disease incidence (%) = Number of diseased plants / Total number of plants examined ×100 .... (i)

Sequence homology confirmation of *Rhizobium rhizogenes* strain UHFBA-212 by 16S rDNA: Genomic DNA of UHFBA-212 was isolated by growing it at 25^{o}C in YEMA broth at 200 rpm. The cells were harvested and processed for DNA isolation, using total DNA isolation kit (GeNeiGenPro isolation kit as per manufacturer's instructions). The isolated DNA was suspended in 100 µl of elution buffer and quantified on 1 per cent agarose gel. PCR reaction was carried out in 20µl reaction containing 50 ng of template DNA, 20 picomolar of each primers F667-pA-res and F668-pH-res, 0.2 mM of dNTP's and 1U Taq Polymerase (MP Biomedicals, USA) in 1×PCR buffer. The PCR products were analyzed by 1 per cent agarose gel electrophoresis in TEB buffer (90 mM Tris base, 2 mM EDTA, 90 mM boric acid [pH 8.3]) with500 ng of ethidium bromide per ml. PCR product was eluted and purified using PCR purification kit (Real Genomic as per manufacturer's instructions) and sequenced thereafter. The sequences were aligned with those from GenBank database. Phylogenetic trees were inferred using the neighbour-joining (NJ) method. Bootstrap analyses were calculated based on 1000 replications (Felsenstein, 1985). The gene sequence (16S rDNA) determined in this study has been deposited in the NCBI database, and the GenBank accession numberis KC488174. Table 3lists the primers used in this study.

Statistical analysis: Experimental data was analysed using standard analysis of variance (ANOVA) followed by Duncan's multiple comparison tests ($p \leq 0.05$).

RESULTS AND DISCUSSION

Biological control of bacterial plant diseases is of increasing interest and importance. Crown gall caused by *Agrobacterium tumefaciens*is one of the great challenge and the main limiting factor in raising quality nursery plants (Gupta *et al.*, 2005). Various integrated disease management practices have been tested world over (Kapshuk, 1933; Escobar and Dandekar, 2003; Pulawska, 2010) and were found ineffective. *Rhizobium rhizogenes* strain K-84 and it's genetically engineered derivative K1026, were found most effective in controlling the crown gall in stone fruit nurseries (Kerr, 1972; Kerr and Htay, 1974). Later, it was rewgnized that some biological control activities are independent of agrocin production as some of pAgK84 ¯ variants of strain K-84 were also able to reduce the gall size. Hence, other mechanisms such as rhizosphere colonization, blockage and binding to infection sites, production of antibiotics etc. also operate depending upon the genetic makeup of antagonist (Lippincott *et al*., 1977;Gupta *et al*., 2010). Therefore, the present investigations were envisaged to find other agrocin producing isolates, exhibiting rhizosphere persistence and detracting incidence of crown gall in comparison to standard strain K-84.

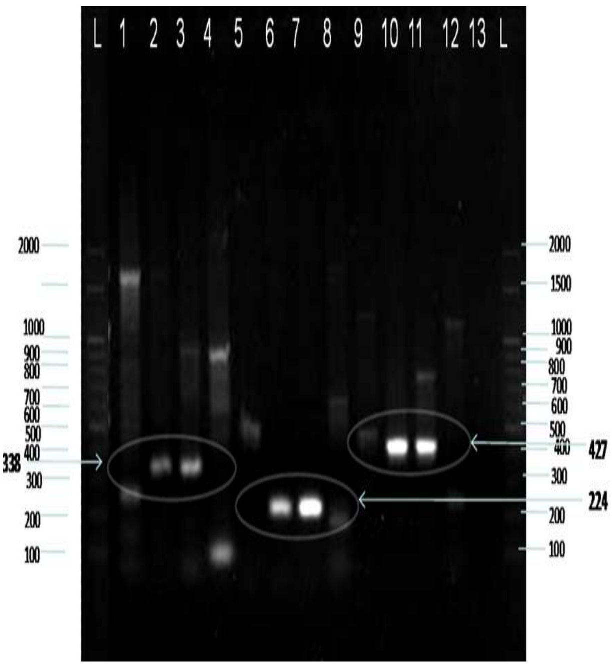

Fig. 2. *PCR based amplification of Rhizobium rhizogenes and Agrobacterium tumefaciens DNA templates with vir D2 and ipt primers.*
L: Ladder (100bp); 1,5,9: K84; 2,6,10: C58; 3,7,11: I_1; 4,8,12: UHFBA-212; 13: Control 1-4: *vir D2* primers A-E', 5 -8: *vir D2* primers A-C', 9-12: *ipt*primers CYT-CYT'

***In vitro* selection of antagonistic isolates:** Total 159 isolates of *Agrobacterium* were collected from rhizosphere soil from different locations of Himachal Pradesh. In addition to this, *R. rhizogenes* strain K84 as standard strain was also taken in present studies. *In vitro* evaluation of different isolatesagainst pathogenic *A. tumefaciens* with or without exposure to chloroform vapours revealed that they were able to inhibit the growth of *A. tumefaciens* (Table 4). Therefore, these isolates were considered as agrocinogenic. Maximum zone of inhibition (4.16 cm) without exposure was observed in UHFBA-212, whereas 3.90 cm maximum zone of inhibition was observed in Sam –21 (4) after exposure to chloroform followed by 3.57 cm in the UHFBA-212. The native isolate *R. rhizogenes* UHFBA -212 was superior to strain K-84 of Kerr (1972).Wang *et al.* (2003) reported that an antibacterial compound named Ar26 produced by non-pathogenic *Rhizobiumvitis* strain E26 inhibited the growth of some Ti strains of *Rhizobium* on culture plates. There are many reports where native strains are significantly controlling the crown gall of apple, stone fruits and grapewine (Kawaguchi, 2008;Gupta *et al*., 2010; Kawaguchi, 2013).

Evaluation of antagonistic isolate(s) for their antagonism against *A. tumefaciens* for gall development on potted tomato plants: In order to find out the effective biocontrol agent, different test antagonist (s) isolated from rhizosphere soil were tested against *A. tumefaciens*strain C58for their antagonistic activity by cross inoculation method on tomato plants. Non-pathogenic isolates when cross inoculated against pathogenic *A. tumefaciens*isolate is strain C58 on tomato plants showed varied reactions. Antagonisticisolates viz., UHFBA-13, UHFBA-14, UHFBA-15, UHFBA-16, UHFBA-17, UHFBA-18, UHFBA-19, UHFBA-20, UHFBA-21, UHFBA-40, UHFBA-41, UHFBA-42, UHFBA-43, UHFBA-44, UHFBA-45, UHFBA-46, UHFBA-47, UHFBA-48, K84 and UHFBA-212 completely inhibited the gall formation on indica-

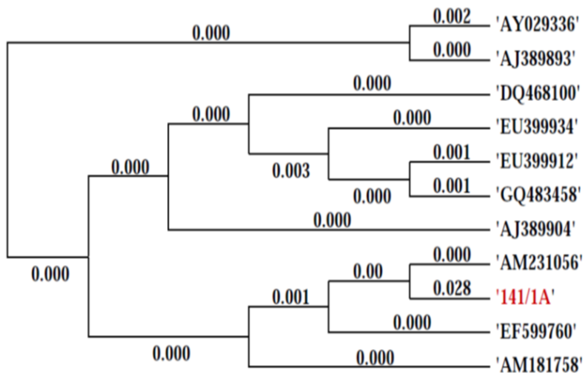

Fig. 3. *Neighbour-joining phylogenetic dendrogram based on 16S rDNA sequences showing relationships between different isolates.*

Table 1. Oligonucleotide primers and PCR cycling conditions used in the study.

Gene name	Primer	Sequence 5'–3'	Anneal. temp. (◦C)	Reference
virD2	A	ATGCCCGATCGA GCT CAA GT	52	
	E'	CCTGACCCAAACATCTCGGCTGCCCA		Haas *et al.*,1995
	C'	TCGTCTGGCTGACTTTCGTCATAA		
ipt	CYT	GATCG(G/C)GTCCAATG(C/T)TGT	55	
	CYT'	GATATCCATCGATC(T/C)CTT		

Table 2. Initial population of bacterial culture used for seed and root dip treatments.

Bacterial culture	CFU × 10^6
K84	182
UHFBA-212	180
I_1	184
C58	182

tor tomato plants.*Agrobacterium tumefaciens*strain C58 and native isolate $I_2$from Bajaura resulted in 7.33 and 4.00 number of wounds showing galls respectively (Table 5). Similar results of gall development on tomato seedlings were observed by different workers (Kerr, 1969); New and Kerr, 1972; Jindal and Sharma, 1988; and Gupta *et al.*, 2005).

Detection of *virD2* and *ipt* genes in antagonistic strains: Total genomic DNA of *Rhizobium rhizogenes*UHFBA-212, strain K84and pathogenic isolates was amplified with virulence *vir D2* and *ipt* gene specific primers.There was no amplification in genomic DNA of *Rhizobium rhizogenes*UHFBA-212 and strain K84, suggesting absence of virulence genes. *Agrobacterium tumefaciens*(C58 and I_1) isolates resulted in 338, 224 and 427 bp product with vir D2 AE, AC and iptpimer (Fig. 2).

Colonization of inoculated seeds/roots by antibiotic resistant mutants: Colonization of root surface by *R. rhizogenes* isolates is one of the most important factor contributing to biocontrol. The antibiotic resistant mutants of *R. rhizogenes* and *A. tumefaciens* were used for enumeration of their population from the day of sowing till the uprooting of nursery plants after treating roots of cherry rootstock Colt and bitter almond seeds.

Colonization on root dip (RD) treated cherry rootstock Colt plants: Maximum population (269.67 × 10^6cfu/g of soil) was recovered in native *R. rhizogenes* isolate UHFBA-212 followed by 207.00 × 10^6cfu/g of soil in K84 applied as root dip on cherry rootstock Colt. UHFBA-212 co-inoculated with *A. tumefaciens* I_1 as root dip gave 145.00 × 10^6cfu/g and it was statistically at par of C58 population (143.31 × 10^6cfu/g) when *A. radiobacter* K84 was co inoculated with C58 after one month. After a period of three and six months population of both *R. rhizogenes* and *A. tumefaciens* were either nil or negligible. Maximum population (11.33× 10^6cfu/g) was observed for *A. tumefaciens*I_1 co inoculated with UHFBA-212 after three months. Population after six months (23.00× 10^6cfu/g) was observed for C58 as root dip treatment followed by (15.00× 10^6cfu/g) for *A. tumefaciens*I_1. No population could be observed for most of the treatments after six months (Table 6).

There was an increase in population at the time of uprooting of plants i.e. ninth month. Maximum population(329.33 × 10 ^{6}cfu/g) was observed for K84 followed by 285.33 × 10 ^{6}cfu/g for UHFBA-212 applied alone. Minimum population (9.33 × 10 ^{6}cfu/g) was observed for *A. tumefaciens*I_1 as root dip treatment on cherry rootstock Colt. Untreated cherry rootstock Colt plant showed no population up to the time of uprooting of plants. Mean values of three and six month periods were statistically at par with each other. The mean values showed that root dip treated K84 and UHFBA-212 are statistically at par in Duncan's multiple comparison tests ($p \leq 0.05$).

Colonization on seed treated (ST) bitter almond plants: Maximum population 212.89 x10^6/g was observed for seed treated UHFBA-212 was co inoculated with C58 followed by 198.67 x10^6/g for K84 when co inoculated with *A. tumefaciens*I_1 after one month on bitter almond. Minimum population of UHFBA-212 (20.28 x10^6/g)was observed in seed treated bitter almond plants. After three and six months, the population decreased below the range of detectable limits. Maximum viable counts (6.33 x10^6/g) were observed for UHFBA-212 seed treated bitter almond plants after three months followed by 4.67 x10^6/g in K84. After six months no viable counts were observed for K84 and UHFBA-212. Maximum population 17 x10^6/g was observed for C58 and it was statistically at par with (16 x10^6/g) in *A. tumefaciens*I_1 as seed treatment. At the time of uprooting maximum viable counts (311.33 x10^6/g) was observed for K84 followed by 193.67 x10^6/g in UHFBA-212 seed treated bitter almond plants. Minimum population 87 x10^6/g was observed for UHFBA-212 when co inoculated with C58. Mean values for treatments showed maximum population

Table 3. Primers used for 16SrDNA sequencing.

Gene name	Primer	Sequence 5'–3'	Anneal.temp. (°C)	Reference
16S rDNA	F667-pA-res	AGAGTTTGATCCTGGTGAG	57	Bruce *et al.*, 1992
	F668-pH-res	AAGGAGGTGATCCAGCCGCA		

Table 4. *In vitro* evaluation of *Agrobacterium radiobacter* isolate (s) for antagonism and detection of agrocin production after exposure to chloroform.

Isolate (S)	Zone of inhibition (cm)	
	Without exposure to chloroform vapours	**After exposure to chloroform vapours**
UHFBA-14	2.60	3.07
UHFBA-15	3.26	1.89
UHFBA-16	2.90	2.07
UHFBA-17	1.54	1.63
UHFBA-18	1.50	2.16
UHFBA-21	1.23	2.09
UHFBA-42	2.45	2.76
UHFBA-43	1.54	2.33
UHFBA-44	3.40	1.98
UHFBA-45	3.00	2.78
UHFBA-46	1.54	2.47
UHFBA-47	2.76	2.99
UHFBA-48	1.74	3.04
Sam 21(4)	2.78	3.90
UHFBA-212	4.16	3.57
K84	2.92	1.21

(111.58 x10^6/g) in K84 followed by 89.92 x10^6/g in K84 co-inoculated with *A. tumefaciens*I_1. Mean values for months showed maximum viable counts i.e. 137.37 x10^6/g after nine months followed by 109.17 x10^6/g after one month while three and six months mean were statistically at par in Duncan's multiple comparison test (Table 7).

Population densities of agrobacteria were minimum or almost nil during the growth period which suggest that these bacteria might have dispersed from the treated sites to nearby sites, thereby these were not within the detectable limits of routinely used dilution plate method. The same observations were also observed by Raio *et al*., (1997). As most of studies have been done on strain K84 no information available for other isolates. The population sizes of native *R. rhizogenes* strain UHFBA-212 and K84 were similar to those reported for other rhizospheric bacteria (Bull *et al.*, 1991; Gross, 1988 and Loper *et al.*, 1985). Penalver and Lopez (1999) suggested that the ability of strain K84 to colonize and persist on roots is important in the biological control process. Maximum viable counts were observed for strain K84 at the time of uprooting in cherry rootstock colt and bitter almond plants followed by native strain UHFBA-212. The population of *R. rhizogenes* isolate UHFBA-212 with that of *A. tumefaciens* I_1 when it was co inoculated and applied as root dip and soil drench, indicated that both *R. rhizogenes* and *A. tumefaciens* isolates coexist in the rhizosphere soil and if the population of antagonist become low, it give chance to pathogen to cause disease. Similar re-

Table 5. *In vitro* evaluation of antagonistic isolates for inhibition of gall development on potted tomato plants

Test antagonist (s)	Number of wounds inoculated	Number of wounds showing galls	Number of galls per wounds	Gall size (cm)
Non pathogenic				
UHFBA-13	9.00	0.00	0.00	0.00
UHFBA-14	9.00	0.00	0.00	0.00
UHFBA-15	9.00	0.00	0.00	0.00
UHFBA-16	9.00	0.00	0.00	0.00
UHFBA-17	9.00	0.00	0.00	0.00
UHFBA-18	9.00	0.00	0.00	0.00
UHFBA-19	9.00	1.00	0.12	0.73
UHFBA-20	9.00	0.00	0.00	0.00
UHFBA-21	9.00	0.00	0.00	0.00
UHFBA-40	9.00	0.00	0.00	0.00
UHFBA-41	9.00	0.00	0.00	0.00
UHFBA-42	9.00	0.00	0.00	0.00
UHFBA-43	9.00	0.00	0.00	0.00
UHFBA-44	9.00	0.00	0.00	0.00
UHFBA-45	9.00	0.00	0.00	0.00
UHFBA-46	9.00	0.00	0.00	0.00
UHFBA-47	9.00	0.00	0.00	0.00
UHFBA-48	9.00	0.00	0.00	0.00
UHFBA-212	9.00	0.00	0.00	0.00
K84	9.00	0.00	0.00	0.00
Pathogenic				
*Agrobacterium tumefaciens*C58	9.00	7.33	1.81	3.76
Agrobacterium tumefaciensI_1	9.00	7.67	2.63	4.43
Agrobacterium tumefaciensI_2	9.00	4.00	1.04	1.93
Control	9.00	0.00	0.00	0.00
Mean	9.00	0.83	0.23	0.45

Table 6. Colonization of *Rhizobium radiobacter and Agrobacterium tumefaciens* isolate(s) on root dip (RD) treated cherry rootstock Colt plants.

Treatments	Population {CFU (x10^6/g) of rhizospheric soil after period of}				
	One Month	**Three month**	**Six month**	**Nine months**	**Mean (treatments)**
K84/RD with K84	207.00	7.00	0.00	329.33	135.83[a]
UHFBA-212/RD with UHFBA-212	269.67	5.33	0.00	285.33	140.08[a]
C58/RD with C58	127.33	4.00	23.00	37.33	47.92[bc]
Agrobacterium tumefaciens I_1/RD with *Agrobacterium tumefaciens* I_1	82.78	4.67	15.00	9.33	27.94[c]
K84/RD with K84 and C58	98.67	0.00	6.00	53.67	39.58[bc]
C58/RD with K84 and C58	143.31	6.33	0.00	64.00	53.41[b]
UHFBA-22/RD with UHFBA-212 and *Agrobacterium tumefaciens* I_1	145.00	5.00	0.00	83.33	58.33[b]
Agrobacterium tumefaciens I_1/RD with UHFBA-212 and *Agrobacterium tumefaciens* I_1	127.00	11.33	0.00	103.00	60.33[b]
Control	0.00	0.00	0.00	0.00	0.00
Mean	133.42[a]	4.85[c]	4.89[c]	107.26[b]	

Table 7. Colonization of *Rhizobium radiobacter* and *Agrobacterium tumefaciens* isolate(s) applied as seed treatment (ST) on bitter almond plants.

Treatments	Population {CFU (x10^6/g) of rhizospheric soil after period of}				
	One month	**Three month**	**Six month**	**Nine Month**	**Mean (treatments)**
K84/ST with K84	130.33	4.67	0.00	311.33	111.58[a]
UHFBA-212/ST with UHFBA-212	20.28	6.33	0.00	193.67	55.07[c]
C58/ST with C58	76.67	3.67	17.00	92.67	47.50[c]
Agrobacterium tumefaciensI_1/ST with *Agrobacterium tumefaciens*I_1	121.33	3.00	16.00	159.00	74.83[bc]
UHFBA-212/ST with UHFBA-212and C58	212.89	3.33	0.00	87.00	75.81[bc]
C58/ST with UHFBA-212and C58	72.67	1.67	0.00	130.00	51.08[c]
K84/ST with K 84 and *Agrobacterium tumefaciens*I_1	198.67	3.00	0.00	158.00	89.92[b]
Agrobacterium tumefaciensI_1/ST with K 84 and *Agrobacterium tumefaciens*I_1	149.67	2.00	1.00	104.67	64.33[bc]
Control	0.00	0.00	0.00	0.00	0.00
Mean (months)	109.17[b]	3.07[c]	3.78[c]	137.37[a]	

sults were also found by Kerr and Htay (1974), Raio *et al.* (1997) and Gupta *et al*. (2010) who reported that for a successful biological control, the population *R. rhizogenes* either must be of the same proportion or higher in number to that of *A. tumefaciens*.

Effect of native *Rhizobium radiobacter* isolate and *Agrobacterium tumefaciens* isolate on the incidence of crown gall: Maximum incidence (100%) was observed on cherry rootstock Colt treated with *A. tumefaciens*I_1 followed by C58 (94.67%) on cherry rootstock Colt. Both the *A. tumefaciens* isolates viz., *A. tumefaciens* I_1 and C58 showed incidence of 82.67% on plants raised from bitter almond seed, respectively. Minimum incidence (2%) of disease was observed on bitter almond seed treated with UHFBA-212 and C58. Bitter almond seeds treated with *Rhizobium rhizogenes* strain K84 showed only 5 per cent incidence of crown gall compared to untreated (control) bitter almond plants raised from seed (23.67%) and cherry rootstock Colt (13.33%) (Table 8). When mutants of *R. rhizogenes* and *A. tumefaciens* were used in combination the incidence of disease remained quite low compared to the treatments where only pathogenic mutants of *A. tumefaciens* were applied both as seed and root dip treatment. The study conducted on strain K84 by Canfield and Moore (1991), Moore *et al.* (1988) and Trembley *et al.* (1987) indicate that even in galled tissue large population sizes of both pathogenic agrobacteria and biocontrol agent K84 are maintained for several months, indicating that the potential of plasmid transfer between these bacteria exists under field conditions, this is why that incidence of crown gall was observed in each treatment and none of these treatments provide hundred per cent control of crown gall.

Phylogenetic analysis of *R. rhizogenes* strain UHFBA-212 (141/1A) by 16S rDNA sequence analysis: *Rhizobium rhizogene* strain UHFBA-212 (141/1A) was showing maximum rhizosphere competence and found to be the most effective biocontrol treatment against*A. tumefaciens* under field conditions, therefore strain UHFBA-212 was identified to the species level by 16S rDNA sequence analysis. The 16S rDNA fragment was

Table 8. Effect of *Rhizobium radiobacter*co inoculated with *Agrobacterium tumefaciens*isolate (s) on the incidence of crown gall on bitter almond and cherry rootstock –Colt.

Treatments	Incidence of crown gall (%)
ST with K84 on bitter almond seeds	5.00(12.78) *
ST with UHFBA-212 on bitter almond seeds	2.22(4.99)
RD with K84 on Colt	9.33(17.71)
RD with UHFBA-212 on Colt	2.67(7.69)
ST with C58on bitter almond seeds	82.67(65.43)
ST with *Agrobacterium tumefaciens*I_1 bitter almond seeds	82.67(65.43)
RD with C58on Colt	94.67(74.28)
RD with *Agrobacterium tumefaciens*I_1 on Colt	100.00(90.00)
RD with K84 and C58 on Colt	9.33(17.71)
RD with UHFBA-212 and *Agrobacterium tumefaciens*I_1on Colt	6.67(14.80)
ST with K 84 and *Agrobacterium tumefaciens*I_1onbitter almond seeds	5.00(12.78)
ST with UHFBA-212 and C58on bitter almond seeds	2.00(4.73)
Control (bitter almond seeds)	23.67(27.71)
Control (Colt)	13.33(21.20)
Mean	31.37(26.56)

$CD_{0.05}$ (Treatments) =9.56 ST =Seed treatment, RD =Root dip, *Figures in the parentheses are arc- sine transformed values.

successfully amplified using designed primers and sequenced. Both the forward and reverse sequences soobtained were corrected, and unreadable and ambiguous sequences were deleted. Based on nucleotides homology and phylogenetic analysis, the strain UHFBA-212 (141/1A) was detected to be *Rhizobium* sp. Nearest homolog was found to be *Agrobacterium radiobacter* (Fig. 3). Phenotypic comparisons of *Agrobacterium spp*. and *Rhizobium spp*. have subsequently been supported by phylogenetic inferences based on 16S rDNA sequence analyses (Farrand *et al.*, 2003; Velazquez *et al*., 2010 and Mousavi *et al*., 2014).

Conclusion

Biological control is one of the indispensable approaches of integrated disease management. This study has shown the potential of *Rhizobium rhizogenes* strain UHFBA-212, as biocontrolagent against *A. tumefaciens* biovar 1 on bitter almond and cherry rootstock Colt. As far as we know, no comparative data concerning root colonization by the pathogen and the biocontrol agent have been obtained *in situ* in crown gall biocontrol experiments. Strain K84 has shown good rhizosphere colonization efficiency compared to the native strain UHFBA-212. In spite of the efficient biocontrol observed by strain K84 and UHFBA-212, average populations consisting of 10^6 agrobacteria per g of soil were found nine months after planting.Thus, these strains may be used as promising antagonists for the successful management of crown gall. However, the applicability of strain UHFBA-218 to other kinds of plants in the field should be investigated further.

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