i. Molecular analysis of agrocin producing Agrobacterium radiobacter for biological control of crown gall in stone fruits

Summary

Full proof protocol for isolation, identification and characterization of *A. radiobacter* and *A. tumefaciens* isolates has been developed. As per this protocol; isolation of Agrobacteria from rhizosphere soil of stone fruit plants, is done on D1 medium, biovar characterization on 1A and 2E media (Plate 1). The results were authenticated by biochemical tests, scanning electron microscopy and 16S rRNA sequence analysis. Universal primers VirD2 and ipt- of *A. tumefaciens* were also used to identify and characterize trans-conjugant of *A. tumefaciens* and *A. radiobacter*. Among three hundred forty seven *A. radiobacter* isolates from stone fruit nurseries, thirty two isolate were agrocin producing. Isolate UHFBA-212 (141-1A-1), UHFBA-215 (Cherry 1A-7-2), UHFBA-217 (Cherry 1A-10-2) and UHFBA-218 (Cherry 2E-2-2) showed 2.43, 2.13, 2.29 and 1.89 cm zone of inhibition, respectively after exposure to chloroform as compared to 1.21 cm zone of inhibition produced by *A. radiobacter* strain K-84 . All the *A. tumefaciens* isolates were sensitive to agrocin K-84 produced by *A. radiobacter* strain K-84 which indicate their nopaline utilizing behaviour.

Mean values of 2010-11 and 2011-12 indicate that isolate UHFBA-218 (Cherry 2E-2-2) controlled crown gall by 89.1 per cent as compared to 78.0 per cent by strain K-84 as seed treatment on peach. The disease incidence in untreated plants was 72.6 per cent. Isolate UHFBA-218 also controlled crown gall by 66.0 per cent on cherry rootstock-Colt as compared to 64.1 (average of 2010-11 and 2011-12) per cent disease control by strain K-84 as root dip. The disease incidence in untreated plants was 54.6 per cent. On peach this isolate provided 69.8 per cent disease control as compared to 30.2 per cent control in K-84 treated plants applied as root dip. The disease incidence in untreated peach plants was 50.1 per cent.

Oligonucleotides FGPS6 (5’GGAGAGTTAGATCTTGGCT-CAG3’) and FGPL132 (5’CCGGGTTT-CCCCATTCGG3’) were used to amplify a ribosomal region containing 1,479 bp (99.5%) of the 16S rRNA, the intergenic spacer region between 16S and 23S rRNA genes, and 132bp of the 23S rRNA genes of the seventeen isolates belonging to native *A. radiobacter* and *A. tumefaciens* along with standards- *A. radiobacter* strain K-84 and *A. tumefaciens* C-58 to further identify the relatedness of strains/ isolates of native *A. radiobacter* and *A. tumefaciens*
with standard isolates. Amplicons were digested with TaqI, RsaI and AvaI. The studies revealed that native *A. radiobacter* isolates viz., 139-1A-2, 139-1A-5-1, 1401A-7, Cherry 2E-3-2 and Cherry 2E-2-2 and strain K-84 are of the same group, whereas *A. tumefaciens* strain C-58, Peach 2E-1, Peach 2E-3 and Peach 2E-10 have similarity with each other (fig. 1).

A unique phenomenon of complete loss of Ti plasmid (loss of ability to produce galls) with the simultaneous acquisition of pAg plasmid (agrocin producing ability) was confirmed in *A. tumefaciens* (identified on the basis of 16S rRNA) isolate - UHFBA-215 (Cherry 1A-7-2) by using VirD2 and ipt universal primers- of *A. tumefaciens* This isolate also provided 93.4 per cent control of crown gall (average of 2010-11 and 2011-12) as seed treatment on peach and 68.1 per cent control as root dip on cherry rootstock-Colt under field conditions.

To confirm whether this isolate UHFBA-215 (Cherry 1A-7-2) can further acquire Ti plasmid, *A. tumefaciens* (Peach 2E-10 @ 0.001 ml having 10^{12} cfu/ml) isolate was inoculated after a period of 30 minutes, 1h and overnight on the same wounds where isolate UHFBA-215 (Cherry 1A-7-2 @ 0.001 ml having 10^{6} cfu/ml) was inoculated on the stem of 4 weeks old potted tomato plants. Among 48 agrocin producing isolates recovered from galls developed on inoculated tomato plants after 4 weeks, amplification with Vir D2 and ipt primers were observed only in one isolate. There was no formation of galls when wounds were inoculated with the same number of viable bacterial cells (0.001 ml containing 10^{12} cfu/ml) of *A. radiobacter* UHFBA-215 (Cherry 1A-7-2) or UHFBA 218 (Cherry 2E-2-2) prior to inoculation of same wounds with *A. tumefaciens* (0.001 ml containing 10^{12} cfu/ml) after a incubation period of 30 minutes, 1h and overnight on stem of artificially inoculated 4 weeks old potted tomato plants. This confirmed that for a successful biological control of crown gall, the population of antagonist-*A. radiobacter* either should be the same or higher than that of *A. tumefaciens*.

All the recommended pesticides used in stone fruit nurseries viz., chloropyriphos (0.1%), carbendazim (0.05%), glyphosate (0.1%), atrazine (0.5%), oxyfluorfen (0.05%), phorate (0.05%), carbofuran (0.05%) and mancozeb (0.25%) marginally reduced the growth by 6 to 22 per cent of *A. radiobacter* isolate UHFBA-218 whereas copper oxychloride (0.3%) completely inhibited its growth. The population of UHFBA-218 applied as seed treatment persisted in the rhizosphere soil of bitter almond plants and it was 294x10^{6} cfu/g of rhizosphere soil as against of 187x10^{6} cfu/g of strain K-84 at the time of uprooting of plants after nine months of seed sowing.