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Mass Screening of Temperate Fruit Germplasm against Major Viruses for developing a 'Clean Stock' Programme in Himachal Pradesh, India

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A B S T R A C T

The importance of viruses and related pathogens on the quality of planting material is largely recognised as a major component in a certification protocol aimed at developing 'Clean Stock' programme. Since majority of the viruses associated with temperate fruits are of latent nature, the significance of a quick and reliable method for early detection of these viruses is of paramount importance. This paper discusses the use of DAS-ELISA as an efficient tool for the detection of major viruses of temperate fruit crops and also highlights the value of DAS-ELISA in mass screening and development of 'Clean Stock' programme. Early detection of viruses in the propagative material is a pre-requisite for checking their effective spread and to guarantee a sustainable 'Clean Stock' programme. This programme will also help in the production of planting material of known variety and plant health status for local growers by controlling the propagation of pathogen-tested mother plants.

Introduction

Maintaining 'Clean Stock' of propagating material of temperate fruits is often considered a high risk pathway for the movement of plant pathogens particularly the graft transmissible pathogens (GTPs) including viruses, viroids and phytoplasma as these pathogens are likely to be transmitted through infected scion wood and other means of plant propagation from mother blocks and nuclear stock to the commercial growing units and individual fruit growers. Viruses and related pathogens are often associated with the fruit tree production system all over the world. While some viruses are known to have a minor impact on infected plants, other viruses can lead to long term impact on the entire propagation and fruit production system and can lead to major crop losses over a period of time. Since GTPs are integrated into the entire production chain, plants once infected with GTPs, cannot be cured. The only way to remove GTPs from a progeny orchard or nuclear stock is by destroying the infected plants and by replacing these with indexed clean plants. The present paper discusses an approach which is being used for developing a 'Clean Stock' programme for mass screening of major temperate fruit germplasm against viruses in Himachal Pradesh, India. The current system employs DAS-ELISA for screening of

germplasm as it has been found to be a quick and reliable diagnostic tool.

The 'Clean Stock' programme described in this paper uses a systematic approach in order to produce virus indexed fruit tree nursery stock. The approach essentially involves various independent components such as virus-testing employing DAS-ELISA, regular field inspection, maintaining proper isolation distances and vector control wherein all these components are synchronised to work in tandem to minimize the presence and the spread of viruses and related pathogens. It is however very important that the 'Clean Stock' programme thus devised for the state of Himachal Pradesh should harmonize with similar programmes in the country including nursery certification scheme so that the programme can provide the nurseries involved in commercial marketing of temperate fruit plants with guidelines for a single cohesive streamlined procedure.

Materials and Methods

Collection of samples

Random samples from different cultivars of apple, pear, plum, cherry, rootstocks of pear and plum collected by the field functionaries of the Department of Horticulture, Government of Himachal Pradesh were supplied through the respective incharges of the progeny-cum-demonstration orchards (PCDO) from Shimla, Chamba, Kullu and Solan districts of Himachal Pradesh. The samples were brought to the laboratory for serological indexing against major viruses of temperate fruits. All samples were subjected to serological indexing using DAS-ELISA with antibodies against apple mosaic virus (ApMV), apple chlorotic leafspot virus (ACLSV), apple stem pitting virus (ASPV), apple stem grooving virus (ASGV) and prunus necrotic ring spot virus (PNRSV) procured from BIOREBA, Switzerland in Microscan MS 5608A ELISA plate reader from ECIL, Hyderabad, India. Additionally, samples were also collected from the Model Farm of Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan. In all, 47 samples from different cultivars and rootstocks of temperate fruits were indexed serologically using DAS-ELISA to ascertain the sanitary status of temperate fruit planting material maintained at different PCDOs and other farms.

ELISA detection

The protocol given by Clark and Adams (1977) was followed for conducting DAS-ELISA tests. Wells of the microtitre plate (NUNC maxisorp certified microplates) except those of the top and bottom rows and rows on the extreme left and right, were filled with 100 µl aliquots of coating antibodies diluted in 1X coating buffer (1:500 ratio v/v). The plate was incubated in humid box for 4 hours at 37˚C. The coating antibody suspension was removed by shaking out the plate over the wash basin. The wells were filled with 1X PBS-Tween and kept for 2 minutes with gentle shaking. The plate was emptied and filled again with PBS-Tween. The washing was repeated three times. The test samples were ground in 1X extraction buffer (1:10 ratio w/v). All coated wells were filled with 100 µl aliquots of test sample (each sample in duplicate) besides positive and negative control wells. The plates were incubated in humid box overnight at 4±1˚C. The washing steps were repeated as mentioned above. The alkaline phosphatase (ALP) conjugate antibodies were filled in each well with 100 µl aliquots after diluting it in 1X conjugate buffer at a ratio of 1:500 (v/v). The plate was incubated in humid box for 2 hours at 37˚C. The washing was done as mentioned above. The p-nitrophenyl phosphate (pNPP) substrate was dissolved in 1X substrate buffer.

Table.1 Details of varieties and rootstocks of temperate fruits

Sample No.	Location	District	Crop	Cultivar/rootstock
1	PCDO, Chamba	Chamba	Apple	Jeromine
2				Jeromine
3				Jeromine
4				Gale Gala
5				Gale Gala
6				Gale Gala
7				Auvial Early Fuji
8				Auvial Early Fuji
9				Auvial Early Fuji
10	PCDO, Duttnagar	Shimla	Apple	Jeromine/M-9
11				Redcap Valtod/MM-106
12				Super Chief/MM-106
13				Redlum Gala/M-9
14				Sun Fuji/M-9
15				Scarlet Spur II/MM-106
16				Granny Smith/M-9
17				Clonal rootstock M-9
18				Clonal rootstock EMLA-9
19			Cherry	Glory/Gisella-5
20				Regina/Gisella-5
21	PCDO, Bathara	Shimla	Apple	Redlum Gala/M-9
22				Scarlet Spur II/MM-106
23	PCDO, Patta Mahlog	Solan	Pear	Rootstock BA-29
24	PCDO, Darlaghat		Plum	Myrobalan
25				Pixy
26	University Model Farm, YSPUHF, Nauni		Apple	Red Velox
27				Modi
28				Buckeye Gala
29				Scarlet Spur III
30				Jeromine
31	Fruit Development Project, Bajaura	Kullu	Apple	Gale Gala/M-9
32				Redlum Gala/M-9
33				Granny Smith/M-9
34	PCDO, Chowai	Kullu	Apple	Redcap Valtod/MM-106
35				Sun Fuji/M-9
36	PCDO Bagthan	Sirmour	Apple	Redlum Gala
37				Sun Fuji
38				Gale Gala
39				Red Fuji
40				Red Velox
41				Granny Smith
42				Auvial Early Fuji
43				Jeromine
44				Super Chief
45				Recap Valtod
46			Pear	Carmen
47				Concorde

Table.2 DAS- ELISA detection of viruses in test samples

Sample No.	O.D value (A_{405} nm)/reaction				
	ApMV	ACLSV	ASGV	ASPV	PNRSV
1	0.165 (-)	0.182 (-)	0.154 (-)	0.185 (-)	0.117 (-)
2	0.122 (-)	0.138 (-)	0.091(-)	0.137 (-)	0.125 (-)
3	0.139 (-)	0.146 (-)	0.134 (-)	0.144 (-)	0.114 (-)
4	0.106 (-)	0.142 (-)	0.135 (-)	0.145 (-)	0.131(-)
5	0.099 (-)	0.124 (-)	0.118 (-)	0.125 (-)	0.148 (-)
6	0.108 (-)	0.136 (-)	0.157 (-)	0.163 (-)	0.127 (-)
7	0.123 (-)	0.179 (-)	0.259 (-)	0.152 (-)	0.146 (-)
8	0.170 (-)	0.116 (-)	0.252 (-)	0.123 (-)	0.113 (-)
9	0.124 (-)	0.121 (-)	0.254 (-)	0.129 (-)	0.125 (-)
10	0.088 (-)	0.241 (-)	0.260 (-)	0.279 (-)	0.151 (-)
11	0.158 (-)	0.157 (-)	0.196 (-)	0.158 (-)	0.145 (-)
12	0.112 (-)	0.163 (-)	0.125 (-)	0.191 (-)	0.118 (-)
13	0.136 (-)	0.130 (-)	0.111 (-)	0.146 (-)	0.122 (-)
14	0.092 (-)	0.197 (-)	0.090 (-)	0.179 (-)	0.157 (-)
15	0.130 (-)	0.193 (-)	0.141 (-)	0.212 (-)	0.114 (-)
16	**0.390 (+)**	**0.820 (+)**	**0.404(+)**	**0.438 (+)**	0.160 (-)
17	0.131 (-)	0.135 (-)	0.136 (-)	0.170 (-)	0.124 (-)
18	0.108 (-)	0.124 (-)	0.156 (-)	0.059 (-)	0.138 (-)
19	0.144 (-)	0.117 (-)	0.108 (-)	0.086 (-)	0.154 (-)
20	0.137 (-)	0.121 (-)	0.133 (-)	0.131 (-)	0.137 (-)
21	0.124 (-)	0.236 (-)	0.132 (-)	0.115 (-)	0.096 (-)
22	0.066 (-)	0.106 (-)	0.135 (-)	0.158 (-)	0.127 (-)
23	0.095 (-)	0.146 (-)	0.188 (-)	0.104 (-)	0.118 (-)
24	0.107 (-)	0.122 (-)	0.096 (-)	0.109 (-)	0.166 (-)
25	0.118 (-)	0.238 (-)	0.135 (-)	0.141 (-)	0.163 (-)
26	0.129 (-)	0.283 (-)	0.197 (-)	0.242 (-)	0.131 (-)
27	0.135 (-)	0.187 (-)	0.163 (-)	0.161 (-)	0.112 (-)
28	0.149 (-)	0.106 (-)	0.106 (-)	0.182 (-)	0.086 (-)
29	0.093 (-)	0.112 (-)	0.186 (-)	0.124 (-)	0.119 (-)
30	0.171 (-)	0.262 (-)	0.194 (-)	0.246 (-)	0.134 (-)
31	0.130 (-)	0.163 (-)	0.186 (-)	0.145 (-)	0.122 (-)
32	0.146 (-)	0.132 (-)	0.172 (-)	0.153 (-)	0.108 (-)
33	0.151 (-)	0.131 (-)	0.188 (-)	0.151 (-)	0.112 (-)
34	0.125 (-)	0.148 (-)	0.131 (-)	0.109 (-)	0.111 (-)
35	0.150 (-)	0.165 (-)	0.144 (-)	0.136 (-)	0.139 (-)
36	0.137 (-)	0.154 (-)	0.212 (-)	0.149 (-)	0.206 (-)
37	0.127 (-)	0.156 (-)	0.219 (-)	0.149 (-)	0.115 (-)
38	0.079 (-)	0.174 (-)	0.132 (-)	0.153 (-)	0.090 (-)
39	0.104 (-)	0.151 (-)	0.145 (-)	0.116 (-)	0.134 (-)
40	0.131 (-)	0.163 (-)	0.216 (-)	0.103 (-)	0.167 (-)
41	0.096 (-)	0.159 (-)	0.121 (-)	0.132 (-)	0.138 (-)
42	0.141 (-)	0.151 (-)	0.172 (-)	0.126 (-)	0.140 (-)
43	0.152 (-)	0.167 (-)	0.156 (-)	0.159 (-)	0.153 (-)
44	0.163 (-)	0.154 (-)	0.126 (-)	0.144 (-)	0.142 (-)
45	0.148 (-)	0.201 (-)	0.183 (-)	0.169 (-)	0.177 (-)
46	0.096 (-)	0.237 (-)	0.200 (-)	0.183 (-)	0.162 (-)
47	0.110 (-)	0.252 (-)	0.189 (-)	0.176 (-)	0.153 (-)

Each well was filled with 100 µl aliquots of substrate. The plates were kept in humid box in dark condition at room temperature until a yellow colour was clearly visible in the positive control (usually between 30 to 60 minutes). If desired, the reaction was stopped by adding 50 µl of 3M NaOH to each well.

Results and Discussion

A total of 47 samples were collected from different locations in five districts of Himachal Pradesh and serologically indexed for the possible association of major viruses of temperate fruits to identify the infected plants, if any. The details of the samples tested in respect of different crops and their varieties and rootstocks are listed in Table 1.

Results of DAS-ELISA tests conducted on the test samples presented in Table 2 clearly indicate that out of 47 test samples of apple, pear, plum, cherry and rootstocks only one sample (sample No. 16) of apple variety Granny Smith representing PCDO Duttnagar in Shimla district tested positive against antibodies of ApMV, ACLSV, APSV and ASGV with O.D. values of 0.390, 0.820, 0.404 and 0.438, respectively. All other samples were found to be free from infection of all five major viruses considered for the studies. Systematic studies conducted on the detection of viruses in temperate fruits in different parts of the world have revealed the presence of one or more of these viruses either alone or in combination in apple and other temperate fruits (Polak, 2008; Rovira and Aramburu, 1998; Ylmaz *et al.,* 2005; Birisik *et al.,* 2008; Svoboda and Polak, 2008; Menzel *et al.,* 2003; Boulila and Marrakchi, 2001; Chandel *et al.,* 2008; Kapoor and Handa; 2017; Sanchez-Perez *et al.,* 2017).

Use of DAS- ELISA as a diagnostic tool for quick and reliable detection of viruses in planting material of temperate fruits in these studies has been found to be a highly efficient and effective approach as a step forward for developing 'Clean Stock' programme in Himachal Pradesh. A programme thus developed will pave the way for producing healthy planting material in other temperate fruit growing states of India that will eventually benefit the farming community as a whole.

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