Indexing Tools for Indian Citrus Ringspot Virus (ICRSV)

Sanjeev Sharma[*,1], Balwinder Singh[2], Avinash Nagpal[3], G. S. Virk[3] and A. A. Zaidi[4]

[1]*Lyallpur Khalsa College, Jalandhar-144001, India*

[2]*Khalsa College, Amritsar-143001, India*

[3]*Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar 143005, India*

[4]*Molecular Plant Virology Laboratory, Institute of Himalayan Bioresource Technology, Council of Scientific and Industrial Research, Palampur 176061 (H.P.), India*

Abstract: *Indian citrus ringspot virus* (ICRSV) is known to cause serious disease problem in Kinnow (*Citrus nobilis* Lour × *C. deliciosa* Tenora). This paper reports the various methods *viz.* Bioassay, ELISA and RT-PCR for indexing of ICRSV. Bioassay was performed on *Chenopodium amaranticolor, Cucumis sativus, Nicotiana glutinosa, N. tabacum, Petunia hybrida* and *Phaseolus vulgaris.* However necrotic local lesions were observed only in case of *Chenopodium amaranticolor* and *Phaseolus vulgaris*. Infected trees were also found positive by indirect ELISA. RT-PCR of the infected plants showed an amplification of 539 bp fragment corresponding to coat protein gene and gene for nucleic acid binding protein.

Keywords: Kinnow, ICRSV, Bioassay, ELISA, RT-PCR.

INTRODUCTION

Mandarins, loose skinned citrus fruits, constitute a commercially important group of fruit trees. Kinnow mandarin, a hybrid between King and Willow mandarins (*Citrus nobilis* Lour x *C. deliciosa* Tenora) is one of the most important fruit crops and its cultivation is considered to be a highly paying proposition in India. In recent years, tremendous loss in yield and quality of this fruit crop has been observed due to various fungal, viral and bacterial diseases. A number of viruses are known to infect Kinnow trees and their consequences range from latent infection with little apparent effect on the host to its death. Among different viruses known to infect Kinnow, *Indian citrus ringspot virus* (ICRSV) is widely distributed throughout the country limiting the production of quality fruits [1-5]. The disease was first described by Wallace and Drake (1968) from California and has since been reported from many areas worldwide [6]. The leaves of infected plants exhibit typical chlorotic rings of variable diameter and drop prematurely. Several affected trees show dieback and decline type of symptoms and thus become less productive. A decrease in fruit weight, number, size and juice content of infected trees has also been observed [2,4,7]. During a survey recently conducted by us in major Kinnow growing zones of Northern states of India, the percent incidence of the disease ranged from 66 to 91.33% [8].

*Address correspondence to this author at the Department of Botany, Lyallpur Khalsa College, Jalandhar-144001, India;
E-mail: sanbot@rediffmail.com

Accurate information on the tools for indexing of virus-infected trees is highly important for decisions to implement a virus eradication or suppression programme. The literature on ICRSV identification had been noticeably confusing over the years. The disease was earlier described as a "graft transmissible disorder" of citrus in California by Wallace and Drake in 1968 and they assigned it the name "citrus ringspot" [6]. The infected plants showed symptoms of chlorotic flecking of young leaves and yellow blotches and rings on mature leaves. In most cases, the infected trees showed bark scaling symptoms of psorosis disease. Timmer and Benatena (1977) suggested that *Citrus ringspot virus* (CRSV) was related to *Citrus psorosis virus* (CPsV) and caused bark scaling in the infected plants [9]. A number of studies have suggested that ringspot and psorosis are variants of the same disease [10-12]. Until the year 2000, the name *Citrus ringspot virus* had been used for two quite different viruses. One of them is *Citrus psorosis virus* belonging to genus *Ophiovirus* [13-15] and the other is *Indian citrus ringspot virus* (ICRSV) [16, 17]. However, Rustici *et al.* [17] while studying an isolate of ICRSV from kinnow mandarin in India demonstrated that purified virus yielded a major band of 34 kDa and a ssRNA of about 7.5 Kb, which was infectious [16]. Keeping in view very little and confusing information available about indexing of ICRSV, the present study was planned to standardize methods for its indexing *viz*. Bioassay, ELISA and RT-PCR.

MATERIALS AND METHODS

Bioassay

Extracts were prepared from young symptomatic leaves of kinnow plants. Infected leaves were detached from the tree, washed with tap water followed by double-distilled

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water to remove any extraneous matter. The leaves were dried and ground more or less homogenous in 0.1 M phosphate buffer at pH 7.0. The homogenate was squeezed through two layers of muslin cloth and the filtrate was used as a standard extract and applied to the leaves of test plants *viz. Chenopodium amaranticolor*, *Cucumis sativus*, *Nicotiana glutinosa*, *N. tabacum*, *Petunia hybrida* and *Phaseolus vulgaris* after abrasing the leaves with corborandum powder with the help of forefinger stroking from petiole to tip of the leaf whist supporting the leaf with other hand. Immediately after inoculations, the leaves were washed with distilled water to remove excess inoculum and corborandum powder and were labeled. Observations on appearance of visual symptoms on the test plants were recorded daily.

Indirect ELISA

Symptomatic leaves from infected kinnow plants were tested for the presence/absence of ICRSV employing indirect ELISA. Leaf samples were triturated in extraction buffer (1:10 w/v). Antibodies, positive and negative controls were procured form Dr. Y.S Ahlawat, Indian Agriculture research institute (IARI), New Delhi (India). Enzyme conjugate and substrate were procured from Bangalore Genei Pvt. Ltd. (India). The procedure adopted for ELISA was as described By Clark and Adams [18]. The absorbance at 405 nm was measured with flow ELISA microplate reader. The reaction was considered positive only if the mean absorbance value was more than three times of negative control.

RT-PCR

Symptomatic leaves (100mg) of kinnow plants were used for total RNA extraction by Tri-reagent (Sigma, USA) according to manufacturer's instruction. Specific primers for ICRSV (Upstream (U): 5′ CCAACTGGATGAAAT 3′, Downstream (D): 5′ GAGCCAAGCGTTCAGA 3′) were designed by aligning the sequences available in the EMBL Database to amplify partial coat protein gene and gene for nucleic acid binding protein and were got synthesized from Bangalore Genei Pvt. Ltd. (India).

Reverse transcription was performed in 0.2ml thin walled tubes using the total RNA as the template. The reaction was carried out in a total reaction volume of 50 µl at 37^{o}C for 1.15h with 5x reverse transcriptase buffer (Promega, USA), 10 mM each dNTP, 200 ng down stream (D) primer, 25 units of RNase inhibitor and 200 U of M-MLV reverse transcriptase (Promega, USA). This was followed by incubation at 70^{o}C for 5 min and the reaction mixture was transferred to ice immediately.

PCR was carried out in thermocycler (Gene Amp PCR system 9700, Applied Biosystems, Singapore) with 50 µl of total reaction mixture containing 3 µl cDNA, 1 µl (0.2 µg) of upstream primer (U), 1µl (0.2µg) of downstream primer (D), 5µl of 10xTaq Polymerase buffer (Genei, Bangalore, India). The reaction mixture was heated to 94^{o}C for 1 min, annealing at 52 ^{o}C for 1 min and elongation at 72 ^{o}C for 5 min were performed. PCR product was run on 1 % agarose gel at 80 V, stained in ethidium bromide (0.5 mg/ml) and visualized with UV transilluminator.

RESULTS AND DISCUSSION

Bioassay

Local necrotic lesions were observed only in leaves of *Chenopodium amaranticolor* and *Phaseolus vulgaris*. These lesions developed in about 10-12 days of inoculation. However, such symptoms were not observed in other host species tested *viz. Cucumis sativus*, *Nicotiana glutinosa*, *Nicotiana tabacum* and *Petunia hybrida*.

Bioassay is probably the most widely used approach for virus indexing because specialized skills are not required to perform the test. The technique involves the use of indicator plants whose leaves react by developing highly visible necrotic spots, when treated with an extract of a virus-infected plant [19]. In the present investigation, bioassay was performed using *Chenopodium amaranticolor*, *Cucumis sativus*, *Nicotiana glutinosa*, *N. tabacum*, *Petunia hybrida* and *Phaseolus vulgaris* as indicator host species for detection of

(a) (b)

Fig. (1). Necrotic local lesions observed in *Chenopodium amaraticolor* when inoculated with extracts of symptomatic leaves of Kinnow plants (a) Control (b) Infected leaf.

(a) (b)

Fig. (2). Necrotic local lesions observed in *Phaseolus vulgaris* when inoculated with extracts of symptomatic leaves of Kinnow plants (**a**) Control (**b**) Infected leaf.

ICRSV. Local necrotic lesions were observed only in leaves of *C. amaranticolor* and *P. vulgaris* (Figs. **1** and **2**). However earlier studies reported that ICRSV could not be transmitted by mechanical inoculations on herbaceous hosts and by some citrus cultivars [2, 20].

Indirect ELISA

The absorbance at 405 nm was measured with flow ELISA microplate reader. The reaction was considered positive only if the mean absorbance value was more than three times of negative control. The mean absorbance values at 405 nm for negative and positive controls were 0.021 and 0.401, respectively. The absorbance values for all the plants used for this analysis ranged between 0.346-0.433, indicating ICRSV infection of these plants (Data not shown).

Among the various possible ways to detect plant viruses, serological techniques are frequently favoured because of their specificity, speed and the scope they provide for standardization. The technique utilizes the ability of antibodies raised in animals to recognize proteins, usually the coat protein, of the virus of interest [21]. Among the various forms of ELISA, available for the detection of plant viruses *viz*. double antibody sandwich ELISA (DAS-ELISA), triple antibody sandwich ELISA (TAS-ELISA) and indirect ELISA, indirect ELISA is one of the reliable and cost effective methods used for detection of a large number of plant viruses like *Andean potato latent virus* (APLV) [22], *Apple stem grooving virus* (ASGV) [23], *Florida hibiscus virus* (FHV) [24], *Pepper yellow mosaic virus* (PYMV) [25], *Plum pox virus* (PPV) [26]. In the present study also, indirect ELISA was used successfully as a tool for ICRSV indexing.

RT-PCR

RNA Isolation

The isolated RNAs from infected samples were run on 1% agarose gel to confirm presence/absence of viral RNA (Fig. **3**). A band corresponding to 7.5 Kb fragment typical of ICRSV RNA was observed in lane 1, in which RNA from an infected plant was loaded. Such band was not observed in lanes 2 and 3 where RNAs from healthy plants were loaded (Fig. **3**). In an earlier study, Rustici *et al*. [16], reported ICRSV to possess a ssRNA genome of 7500 nucleotides and a capsid protein (CP) of 34 kDa.

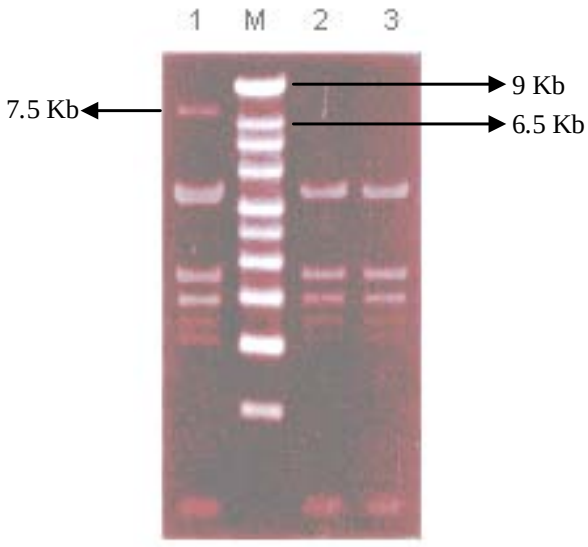

Fig. (3). Agarose gel electrophoresis of RNAs isolated from healthy and virus infected plants. (Lane M-Mellinium marker; Lane 1-RNA from ICRSV infected plant showing 7.5 Kb fragment of viral RNA; Lanes 2 and 3-RNAs from healthy plants).

RT-PCR

RT-PCR analysis of kinnow plants tested positive for ICRSV by bioassay and indirect ELISA has shown an amplification of 539 bp fragment corresponding to partial coat protein gene and gene for nucleic acid binding protein (Fig.

4). As ELISA lacks the sensitivity required for the detection of plant viruses, which occur in very low concentrations in infected tissues, many workers have used RT-PCR for indexing of different viruses to overcome this problem [27-34]. The procedure is extremely sensitive, fairly inexpensive and requires minimal skills to perform [35].

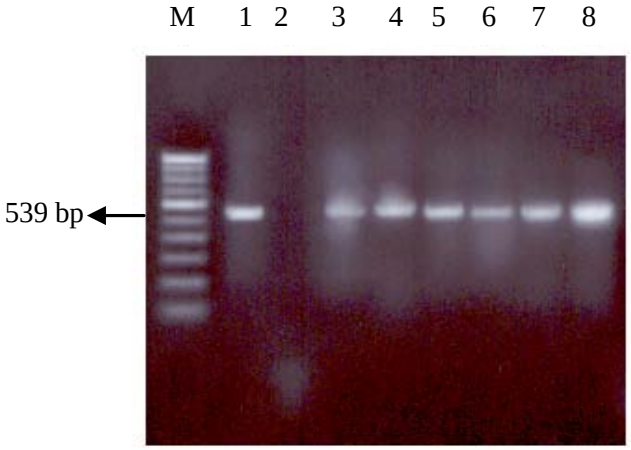

Fig. (4). Agarose gel electrophoresis of RT-PCR products of ICRSV infected kinnow plants. Lane M - 100 bp DNA ladder, Lanes 1 and 2 - Positive and Negative controls respectively, Lane 3 - 8 infected kinnow plants.

CONCLUSION

Indian citrus ringspot virus (ICRSV) is known to cause serious disease problem in Kinnow. As kinnow is propagated vegetatively, the use of infected budwood results in widespread occurrence of ICRSV. Accurate information on the tools for indexing of virus-infected trees is highly important for decisions to implement a virus eradication or suppression programme. In the present study, methods have been standardized for indexing of ICRSV *viz.* bioassay, ELISA and RT-PCR. The protocols developed for ICRSV indexing in the present investigation seems to be much effective as large number of the plants can be indexed in relatively lower costs and less time.

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