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Original Research Paper

Molecular and biological detection of impatiens necrotic spot virus (INSV) isolate from ornamental plants in Iran

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ABSTRACT

Impatiens necrotic spot virus (INSV), belong to genus Orthotospovirus, causes severe damage to greenhouse ornamental plants. INSV reported from almost every ornamental screen house in Iran. INSV general symptoms include necrotic leaf spot, chlorosis and stunting in infected ornamental host plants. A total number of 581 ornamental samples of 58 different plant species with symptoms similar to those of INSV infection were collected in 4 provinces of Iran. The results indicated an average of 20.13 per cent virus incidence. INSV infection was recorded to be 28% in Mahallat, 24.5% in Tehran, 22.1% in Mazandaran and 16.4% in Guilan provinces. No record of INSV infection on ornamental samples was obtained from Khouzistan province (Dezful). From the total of 58 ornamental species tested, 33 species recorded positive for INSV. ELISA positive samples were rechecked by RT-PCR using a set of specific primers directed to the N-gene region, which were designed to detect and characterize the virus species. The primers amplified a 777 bp product of the nucleoprotein as shown by agarose gel electrophoresis. The nucleotide sequence of amplicons was compared with related sequences, using Blast software available at NCBI GenBank, which showed highest similarity with impatiens necrotic spot virus (INSV) isolates. Accordingly, all genome components of the isolate shared 89-96.5 % nucleotide sequence identities with corresponding sequence of other Iranian and GenBank INSV isolates. The Iranian isolates were all placed in the same group and bore the most similarity to INSV isolates from the Netherlands, Italy and United States. In phylogenetic analysis based on the partial nucleotide and deduced amino acid sequences of N region, INSV from Iran appears to be closely related to the Japanese AB1099100 *Verbena* spp. isolate.

Keywords: DAS-ELISA, INSV, ornamental plants, RT-PCR

INTRODUCTION

The cultivated area of ornamental plants in Iran is approximately 4704 hectares, of which 83.7 hectares is under glass/greenhouses, 1076 hectares are wooden-plastic greenhouses, 1268 hectares are metal-plastic greenhouses, and 2276 hectares is cultivated in the open condition. 8588 producers are active in this sector and produce 1224 million cut flowers, 36 million flower pots, 137 million ornamental tree trunks and 842 million seasonal and nursery plants (Anonymous, 2018). One of the limiting factors for ornamental production is infection with various viral pathogens (Amruta et al., 2020; Daughtrey et al., 1997; Naidu et al., 2005). Impatiens necrotic spot virus-INSV- Orthotospovirus is among the most important Tospoviruses reported from ornamental plants in the world (German et al., 1992; Peters, 1998) and also in most ornamental plants growing greenhouses in central province of Tehran, Iran. Infection with viral diseases, not only reduced the quality, but also leads to leaf and flower blistering, dwarf plant, mosaic, marginal leaf chlorosis, leaf entanglement, deformity or lack of flower formation (Shahraeen & Ghotbi, 2003; Shahraeen et al., 2022). INSV is the most important harmful and predominant virus in ornamental plants in Iran and has been reported from various ornamental species in the country (Ghotbi et al., 2005; Shahraeen & Ghotbi, 2003; Pourrahim et al., 2012; Bayat & Nazerian, 2019).

Plants infected with INSV have no symptoms, in some cases, necrotic spots and necrotic rings are seen in young leaves. Symptoms vary from one plant to another and from one species to another, but in general there are symptoms such as: dwarfing, necrotic and yellow spots on the leaves, brown and black necrosis

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on the stem, circular spots, linear patterns, mosaic and vein necrosis is observed (EPPO, 1999; Peters, 1998; Ghotbi et al., 2003). This virus is transmitted by *Frankliniella occidentalis* in an unstable manner (Broadbent & Allen, 1995), which is considered as a quarantine pest in Iran, but in 2013 it was reported for the first time on ornamental plants in the Varamin region (Mehraban & Shahraeen, 2000; Shahraeen et al., 2002). Due to extensive import of ornamental plants from foreign countries, this investigation in Iran is brought in to focus. Therefore, in this study, collected ornamental samples were tested serologically and selectively by RT-PCR test.

MATERIALS AND METHODS

Serology

Enzyme linked immunosorbent assay (DAS-ELISA) test were applied on collected samples (Table 2) as described (Clark & Adames, 1977; German et al., 1992). Using INSV specific antibody antiserums provided by Dr. Winter (DSMZ/JKI-Germany). Leaf samples were extracted in the ratio of one gram of tissue with 5 mL of extraction buffer and 100 µL was added to each well (ELISA plate) at each stage. Replications of two control samples were also added. One hour after adding the substrate solution containing Paranitro-phenyl phosphate, the light absorption of the wells at 405 nm was measured using a microplate reader (Multiscan 334 Lab system, Finland).

Biological tests

Infected INSV samples were selected and leaf extracted (1:5) by cold phosphate buffer of 0.01 M containing 0.15% antioxidant 2-mercaptoethanol pH=7, were mechanically inoculated to specific indicator plants of *Chenopodium amaranticolor*, *Datura metel, Nicotiana tabacum* cv. Samson, *D. stramoniom* and *Vigna unguiculata* (Table 1). Inoculated plants were kept in greenhouse free of insect vectors, and infectivity and symptoms appearance were studied and recorded by Elisa test (Zavareh et al., 2013; Ghotbi & Shahraeen, 2022).

Table 1 : Results of biological tests on indicator plants

Indicator plant	INSV	
	local	systemic
N. tabacum cv. samson	-	CL, Mo
V. unguniculata	MNL	-
D. metel	CL, RS	LD, Mo
D. stramonium	CL, RS	CL, Mo
Ch. amaranticolor	NL	NL, RS

CL: chlorotic lesion, De: death, LD: leaf deformation, MNL: mild necrotic lesion, Mo: mosaic, NL: necrotic lesion, NT: not tested, RS: ring spot, SNL: sever necrotic lesion, VCL: vein clearing

In biological tests, *N. tabacum* cv. Samson reacted with systemic mosaic, *V. Unguiculata*, *D. Metel*, *D. stramonium* and *Ch. amaranticolor* reacted with local and systemic infection (Fig. 1a-d).

Fig. 1a : INSV infected Chrysanthemum spp. with brown necrotic and leaf malformation

Fig. 1b : Scindapsus spp. with leaf chlorosis, narrowing infected by INSV

Fig. 1c : Philodendron spp. with leaf narrowing and stunting infected by INSV

Fig. 1d : *Dianthus* spp. with yellowing and necrosis infected by INSV

Molecular diagnostic, RT-PCR

INSV coat protein gene carried out using specific primers (GeneBank, Acc. No. AB1099100) (Ghotbi & Nazerian, 2010; Ghotbi & Shahraeen, 2012). Positive INSV samples in ELISA tests was selected and total RNA extracted by commercial solution RNXTM-plus (SinaGen Company, Iran) according to the method recommended by the manufacturer. RNA extracted using INSV (Gene Bank Access No. AB1099100) specific primers (F: 5'-GTAGCATTAACATGCTGTAAATG-3'; R: 5'-GTCAAGCTTTTTG ACTCAATCTGAT-3') with two steps RT-PCR. The primers amplified a 777 bp product of the nucleoprotein as shown by agarose gel electrophoresis (Chung et al., 2006).

Amplification and sequencing

In order to determine the nucleotide sequence of the fragment for isolation of INSV from *anthurium*, 20 microliters of PCR product with specified primers (with a concentration of 50 picomol) was sent to the representative of Kiagen Biotech Korea in Iran. The fragment was sequenced and the sequences obtained from PCR products were compared with the information and sequences in the Gene Bank (NCBI) by BlastN software at the nucleotide level. The nucleotide data sequenced for the INSV virus were then multiple-aligned with virus isolates already available in the NCBI database using MEGA5 and ClustalX software, the progeny analysis was based on nucleic acid using the Neighbor-Joining method. MEGA5 software. The offspring tree was drawn in 1000 boot strap using MEGA5 bioinformatics software (Tamara et al., 2013; Nei and Kumar 2000). All branches merged with a bootstrap value of less than 70%. In this analysis, *Peanut stunt virus* (PSV) ER isolate (U15730) was used as an out-group with *cucumber mosaic virus* sequences (Poelwijk et al., 1997, Altschul et al., 1997).

Results obtained in the ELISA test (DAS-ELISA) for total of 581 ornamental samples from 58 different species with symptoms of leaf necrosis, chlorosis and dwarfism in 5 provinces of the country (Gilan 128 samples, Mazandaran 159 samples, Tehran 122 samples, Central 108 sample, Khuzestan 64 samples) indicates the contamination of 117 different samples from 5 provinces. Therefore, 20.13% of all the collected samples were infected with INSV. The highest contamination was related to central provinces. Tehran with 30 samples (24.5%), Mazandaran (22.01%) and Gilan 16.40%. Khuzestan samples were not found to be infected with INSV virus. According to the studies conducted in this research, 33 species (56.89%) of 58 ornamental species were infected with INSV virus (Table 2).

Table 2 : Result of ELISA tests for INSV infecting ornamentals samples in 5 provinces of Iran

Ornamentals	Totals from each province	INSV infected samples
Ardisia crenata (Myrsinaceae)	5 Ma*	-
Alestreomeria spp. (Alestreomeriaceae)	4 Ma	3Ma
Erica spp. (Ericacea)	6 G+7M	1G+4M
Orchis pharanopsis (Orchidaceae)	4M	-
Azali spp. (Ericaceae)	3M	-
Spathiphylum spp. (Araceae)	5G+3M	3G+1M
Sterlitzia reginae (Strelitziaceae)	5G+2M+4T+1Ma	-
Zinia elegans (Compositae)	2G+3T+1Ma	1T
Aglonema schott (Araceae)	4G+6M	-
Anthurium spp. (Araceae)	6G+4M+8T+5Ma	1G+2M
Bambusa spp. (Graminaceae)	3M	-

Begunia semperflorus (Beguniaceae)	2M+2T	-
Ficus benjamina (Moraceae)	3M+4Ma+2T	2M+1Ma
Viola spp. (Violaceae)	5T+4Ma	2T
Saintpaulia ioantha (Gesneraceae)	3T+5Ma	2T+3Ma
Pandanus veitchii (Pandaceae)	4G+4M	-
Scindapsus aureus (Araceae)	7G+3M+3T+2Ma	2G
Pilea cadieri (Urticaceae)	3Ma	2Ma
Bignonia capreolata (Bignoniaceae)	4G+3T	-
Zingiber spp. (Zingiberaceae)	2G+5M	3M
Impatiens spp. (Balsaminaceae)	2G+4M+4T+5Ma	3M+3T
Althea spp. (Malvaceae)	5G+6M+3T+3Ma+7Kh	3G+2M+1Ma
Chrysanthemum spp. (Compositae)	5G+5M+5T+10Ma	3G+3M
Dracaena Fragrans (Liliaceae)	7G+4M+3T+3Ma	4G
Diffenbachia amoena (Araceae)	4G+2M+3T	1M
Rosa spp. (Rosaceae)	9G+5M+5T+4Ma+42Kh	2G+1M+3T+2Ma
Petris cretrica (Polypodiaceae)	5M	-
Asplenium scolopendrium (Aspleniaceae)	5G+5M	-
Cupresus sempervirens (Cupressaceae)	5G	-
Salvia splendens (Labiatae)	2M+5T+4Ma	1M+2Ma
Lilium spp. (Liliaceae)	4M+4T+3Ma	-
Cycas spp. (Cycadaceae)	6M	3M
Cissus spp. (Vitaceae)	4M	2M
Syngonium podophyllum (Araceae)	3M+3T+2Ma	2M
Cheiranthus cheiri (Cruciferae)	4T+3Ma	1Ma
Sheflera arboricola (Araliaceae)	3G+2M	-
Pelargonium hortorum (Geraniaceae)	3G+3M+5T+3Ma	2Ma+1T
Pelargonium peltatum (Geraniaceae)	5G+4M+2T+3Ma	-
Aspidistra elatior (Liliaceae)	3G+4T	-
Ficus elastica (Moraceae)	2G+5M+4T+5Ma	2M
Philodendron spp. (Araceae)	2G+4M+3T+3Ma	3M+2Ma
Pinus spp. (Pinaceae)	2G	-
Bougainvillea spp. (Nyctaginaceae)	2G+3T+3Ma	2T
Camellia sinensis (Theaceae)	3M	-
Codiaeum variegatum (Euphorbiaceae)	6G+2M+3T+4Ma	3T+2G
Cordyline spp. (Liliaceae)	5M	-
Dahlia spp. (Compositae)	4G+3M+5T+8Ma	4T+2Ma
Gazania spp. (Compositae)	4G+2T+3Ma	3T+2Ma
Gladiolus spp. (Iridaceae)	5T+4Kh	2T
Lilium longiflorum (Liliaceae)	3M+4T	-
Beucarnea recurvata (Liliaceae)	5M	-
Zinia elegans (Compositae)	5T+4Ma	2Ma
Polianthes spp. (Amaryllidaceae)	11Kh	-
Dianthus spp. (Caryophyllaceae)	3T+3Ma	2T+2Ma
Phoenix Canariensis (Palmaceae)	2M	-
Chamaedorea elegans (Palmaceae)	4M	-
Calandula spp. (Compositae)	3G+5T+5Ma	2T+4Ma
Euphorbia pulcherrima (Euphorbiaceae)	4M	-
Total in number	581 128G+159M+122T+108Ma+64Kh	117 21G (%16/40)+35M (%22/0)+ 30T (%24/5)+31Ma (%28/70)

*Indicating provinces initials, M = Mazandaran, Ma = Mahallat, T = Tehran, G = Gilan, Kh = Khozestan, - = No infection found, + = Total no of infected samples

RT-PCR

RT-PCR using specific INSV primer confirmed ELISA test results. Selected ornamental samples from different provinces yielded a desired amplicon of 777bp with INSV primer. No amplification was observed from healthy plants extracts (negative control). The nucleotide sequence of amplicons was compared with related sequences, using Blast software available at NCBI GenBank (Altschul *et al*., 1997), showed highest similarity with impatiens necrotic spot virus (INSV) isolate: Imp-Neth (X66972), Sol-Italy (DQ425096-1), Imp-USA (D00914-1), Ver-Jap (AB109100-1), Beg-serb (HQ724289), Glo-USA (DQ523598), unknown (L20886). Accordingly, all genome components of the isolate shared 89-96.5% nucleotide sequence identities with corresponding sequence of other Iranian and GenBank isolates. The Iranian isolates were all placed in the same group and bore the most similarity to INSV isolates from the Netherlands, Italy and United States. In phylogenetic analysis based on the partial nucleotide and deduced amino acid sequences of the N region, the INSV from Iran appears to be closely related to the Japanese AB1099100 *Verbena* spp. isolate (Fig. 2).

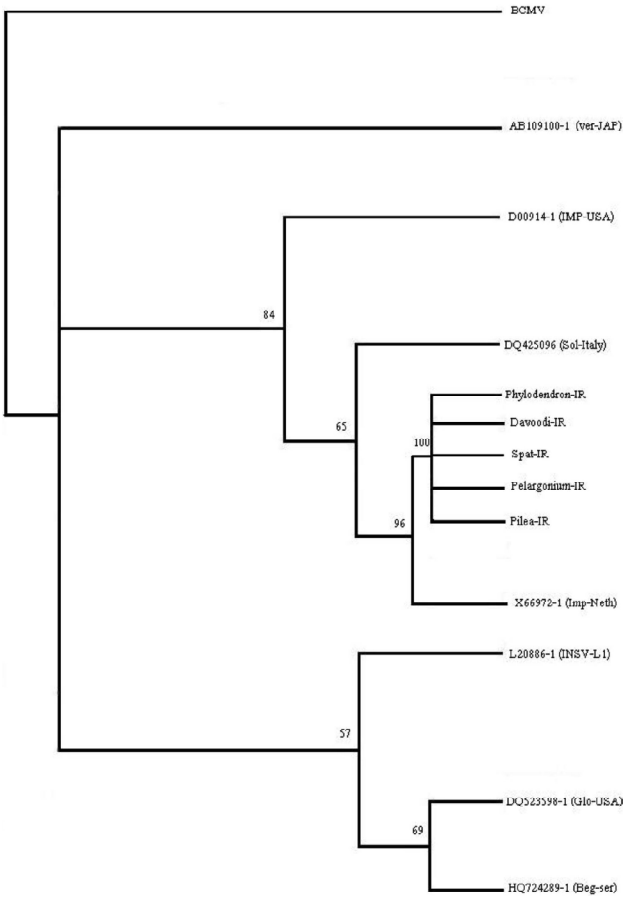

Fig. 2 : Phylogenetic tree using nucleic acid sequence of coat protein gene of INSV isolates from Anthurium using CLUSTAL X with seven other Genbank isolates. Coat protein gene sequence of BCMV was used as outgroup

Viral pathogens are one of the most economically damaging agents in the ornamental plants. The first report of the occurrence of Tospoviruses (TSWV species) in Iran was in 1996 (Bananej et al., 1996; Shahraeen & Bananej, 1995) from the hosts of Atlantic and tobacco and nightingale, button flower and cucumber from Varamin region (Tehran province). Then after, there were various reports of the occurrence INSV virus on different hosts from Iran (Pourrahim et al., 2012; Bayat & Nazerian, 2019; Ghotbi et al., 2005; Golnaraghi et al., 2001; Ghotbi & Shahraeen, 2012; Mehraban & Shahraeen, 2000). *Frankliniella occidentalis*, the potential vector of INSV reported for the first time in Iran in 2003 (Ghotbi et al., 2003) on the ornamental plants from Varamin region, thus, there remain an expectation of wider distribution of this virus in the ornamental production areas in the country (Ghotbi & Shahraeen, 2022).

INSV has a wide host range in ornamental plants of Iran (Ghotbi et al., 2005; 2003). This virus has a direct effect on the marketability by affecting the flowering and yield of ornamental plants, and is important to pay attention from an economic point of view. Cultivation of ornamental plant in Iran is both outdoor and indoor in screen-houses, unfortunately, the lack of awareness of growers about some common viral diseases of ornamental and other field crops may lead to spread and mechanical transmission of the viral agents. According to the above results, it is suggested to use healthy and virus-free imported samples as much as possible, and to use reliable methods like tissue culture for propagating ornamental plants in the country.

CONCLUSION

Regular examination of cultivation areas for infection by plant viruses, isolation and introduction of viruses identified in each area, removal of suspected weed and infected plant materials, stored in greenhouses or surrounding areas. Proper and timely use of insecticides is one of the useful strategies in controlling spread of INSV in ornamental plants.

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