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An overview of Potyviruses infecting daffodil and their disease management

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REVIEW ARTICLE

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ABSTRACT

Daffodil (*Narcissus tazetta* L.) is a bulbous ornamental plant which belongs to the family *Amaryllidaceae*. It is popular for sweet-smelling beautiful flowers and is used for the fragrant oil and perfumes. It is also a source of alkaloids used in traditional medicines. Daffodil is commercially important ornamental plant, therefore, it is important to maintain its quality and production. Various type of viruses affect daffodil cultivation and among them, potyviruses are of great concern as they cause more economic losses to its commercial cultivation in term of reduction in bulb size, number of bulbs and the quality of daffodil flowers. In this review, a general overview of daffodils, genus *Potyvirus*, disease symptoms in daffodils linked to potyvirus infection, potyviruses described in daffodils, *Potyvirus* identification methods and possible management strategies of potyviruses in daffodil cultivation are described in detail. The study will be helpful to daffodil growers for improvement of the production/yield and quality of daffodil crop.

KEYWORDS

Daffodil (*Narcissus tazetta*), *Potyvirus* genus, disease symptoms in daffodils, *Potyviruses* described in daffodils, *Potyvirus* identification methods

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1. GENERAL OVERVIEW OF DAFFODILS

Daffodil (*Narcissus tazetta* L.) is propagated vegetatively through its bulbs. It is worldwide accepted as an ornamental plant for cut-flower in the floriculture industry (Hanks, 2002). Most of the daffodil genus blooms in spring while some in late autumn or early winter (Graham and Barrett, 2004). It is reported to be the native of the Mediterranean region and Central-Europe (Spain, Portugal and the Iberian Peninsula) (Hanks, 2002; Thakur, 2017) whereas it grows naturally in meadows, woodlands, along watercourses, and in rocky outcroppings up to sub-alpine altitudes. Daffodil crop has been cultivated from the earliest sixteenth century in The Netherlands (Hanks, 2002). Some daffodil species are considered to be extending from Southern France, Italy, Balkans, and North Africa etc (Thakur, 2017). They are also found in Central Asia, Nepal, India, China, and Japan with increasing its international trade (Harvey and Selby, 1997; Hanks, 2002; Mathew, 2002). Most probably daffodils were brought in India by the Mughals and were cultivated in Kashmir valley for hundred years ago as the most scented flowers (Willis, 2012).

Different species of daffodil are present all over the world and are produced for commercial uses. Daffodil species are perennial geophytes, naturally occurring in a wide range of habitats and bloom in the late spring or early autumn (Thakur, 2017). Daffodil species are classified into thirteen horticultural divisions based on different flower forms or by botanical names (Kington, 1998; www.rhs.org.uk/plants/pdfs/plant-registration-forms/daffhortclass.pdf). They are listed as: Trumpet, Large-cupped, Small-cupped, Double, Triandrus, Cyclamineus, Jonquilla, Tazetta (Poetaz or Bunch-flowered), Poeticus (Poet's), Bulbocodium, Split-corona, Miscellaneous, Wild Variants, and Wild Hybrid daffodils. Daffodils are also the source of alkaloids like lycorine, homolycorine, and galantamine used as traditional medicines for a variety of disorders like cancer (Duke and Duke, 1983). The Bible also provides many references about Mediterranean daffodil, which have been used against cancer as it contains anticancer isocarbostyril constituents such as narciclasine, pancratistatin (Alexander and Antonio, 2008). The galanthamine alkaloid has been used for management of Alzheimer's disease (Bastida et al., 2006; Adam and Fred, 2013).

Daffodil is a genus of herbaceous perennial bulbous plant which dies back after flowering to an underground storage bulb. Daffodil bulb consists of a disc-shaped basal plate and the roots are formed at the edge of the basal plate. Depending on the bulb size, species and cultivar, a bulb produces 1 to 4 stems with 1–12 or more flowers per scape (David, 2017). Generally, daffodil flowers are white or yellow (orange or pink are also found) in color, having solid or different coloured tepals and corona. The fragrance of a daffodil flower remains for 7–10 day after blooming (Hanks, 2002).

2. GENERAL OVERVIEW OF THE GENUS *POTYVIRUS*

The potyviruses belong to the family Potyviridae which has been divided into six genera: *Potyviruses, Bymoviruse, Rymoviruse, Maculavirus, Tritimovirus* and *Ipomovirus* (Berger et al., 2005). According to new report of the International Committee on Taxonomy of Viruses (ICTV), the Potyviridae family has been divided into eight genera including six previous genera and two new genera *Brambyvirus* and *Poacevirus* (Wylie et al., 2017). All genera contain

a monopartite genome having single RNA (except one genus, *Bymovirus*), they contain bipartite genome with two RNA molecules i.e. RNA-1 and RNA-2 (Shukla et al., 1998; Wylie et al., 2017). The viruses of Potyviridae family are transmitted in nature by various vectors such as aphids, mites, whiteflies, fungus and nematodes.

Potyvirus is the largest group of Potyviridae family that infects various plant species in nature (Shukla et al., 1994). Potyviruses have positive sense RNA of ~9.4–11 kb in size, characterized by a 5′ VPg covalently linked untranslated region (5′ UTR), and a 3′ UTR region that contain poly-A tail. *Potyvirus* genome contains a single long open reading frame (ORF) which is translated into a large polyprotein of 340–370 kDa. The polyprotein auto cleaves itself into ten functional peptides, the characteristic of *Potyvirus* genus (Urcuqui-Inchima et al., 2001; Adams et al., 2005). These polypeptides were identified as: major inner capsid protein (P1 protein), helper component proteinase (HC-Pro), P3, 6K1 CI protein, 6K2, viral protein genome-linked (VPg), nuclear inclusion (Nla-Pro), Nlb and coat protein (CP) proteins (Shukla et al., 1998; Sharma et al., 2014). P1 is the major inner capsid (core) protein which is responsible for genomic replication and transcription. The cysteine protease NIa-Pro processes the C-terminal part of the polyprotein by seven cleavage events, while P1 and HC-Pro are responsible for their own release by a cis cleavage at their respective C-termini (López-Moya et al., 2009). Many functions were also attributed to P1 protein, such as cell-to-cell movement, systemic spread, and viral genome replication enhancement; P1 was later shown to strengthen the RNA silencing suppressor activity of HCPro (Valli et al., 2006).

3. SYMPTOMS IN DAFFODILS LINKED TO *POTYVIRUS* INFECTION

Potyviruses cause the different type of symptoms based on the species of host plant, cultivar, and the virus strain. Symptoms manifest by them are described as necrotic or chlorotic lesions, mosaic, stripe, vein banding, mottling, vein clearing (Sharma et al., 2014). A number of potyviruses viz. narcissus yellow strip virus (NYSV), narcissus latent virus (NLV), narcissus mosaic virus (NMV), narcissus late season yellows virus (NLSYV), cyrtanthus elatus virus-A (CyEVA), narcissus white streak virus (NWSV) and narcissus degeneration virus (NDV) are reported to cause various symptoms in daffodils. NYSV causes mosaic, chlorotic yellow stripes on leaves symptoms and size of bulbs reduces (Caldwell and Kissick, 1950; Brunt, 1966; Rees, 1966, 1995; Chen et al., 2006; Raj et al., 2019). NLV caused mosaic chlorotic spots on leaves and transient leaf tip chlorosis symptoms (Brunt, 1966; Wylie and Jones, 2012; Berniak et al., 2013). NMV caused yellow mosaic, leaf distortion and chlorosis, and flower colour breaking symptoms (Brunt, 1995; Miglino et al., 2005; Hunter et al., 2011). The infection of NLSYV in narcissus is referred to as late season yellows disease since the symptoms of chlorotic streaks on leaves and pale green to yellows of stem appears after flowering (hence called as 'late season') (Mowat et al., 1988). NLSYV also caused leaf streaking symptoms and reduced bulb size (Langeveld et al., 1997). CyEVA caused chlorotic stripes symptoms along with leaf distortion and stunting (Chen et al., 2003; Kumar et al., 2015; Ohshima et al., 2016). The complete genome sequence of an Indian strain of CyEVA investigated by Raj et al. (2018) caused mild to severe mosaic and yellow stripe symptoms on daffodil plants (Fig. 1). NWSV produced greenish-purple streaks symptoms on leaves that turn white to yellow reduced flower stalk, bulb size and yields (Hanks, 1993; Yan et al., 2008). NDV caused mosaic or chlorotic striping symptoms on leaves (Chen et al., 2006; Ward et al., 2009; Ohshima et al., 2016) (Table 1).

Fig. 1. Naturally virus infected daffodil plants growing under open field conditions exhibiting severe mosaic and yellow stripe (A) and mild symptoms (B) elicited by CyEVA

4. POTYVIRUSES DESCRIBED SO FAR INFECTING DAFFODILS

There are several RNA viruses reported to infect daffodils which include potyviruses: NYSV, NLV, narcissus tip necrosis virus (NTNV), NLSYV, NMV, CyEVA and bean yellow mosaic virus (BYMV) (https://doi.org/10.1007/978-81-322-3912-3608); Carlavirus: narcissus common latent virus (NCLV) and narcissus symptomless virus (NSV); Nepovirus: tobacco ring spot virus (TRSV) and arabis mosaic virus (ArMV); Carmovirus: NTNV and Cucumovirus: cucumber mosaic virus (CMV) (Brunt, 1977, 1980, 1995; Chen et al., 2003; Wylie and Jones, 2012) (Table 1). Amongst them, potyviruses are found most prevalent which cause severe mosaic, yellow stripe symptoms and reduced plant growth and production of bulbs (Brunt, 1995, 2008; Aminuddin et al., 1999; Yadav and Khan, 2008; Wylie and Jones, 2012).

The NDV, NLSYV, and NSV were reported on daffodil for the first time in New Zealand by Ward et al. (2009). The daffodil plants showing leaf mottling, flower distortion, and colour break were received from commercial nurseries in Taranaki (TK) in New Zealand's North Island and Canterbury (CB) in the South Island. Filamentous virus particles (700–900 nm long) were observed in crude sap of both plants under a transmission electron microscope. Further, total RNA was isolated from the leaves of both plants with an RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA), and cDNA was synthesized by Superscript III (Invitrogen, Carlsbad, CA). cDNA was used in PCR to test the potyviruses. Both plants tested were found positive for potyvirus using generic potyvirus primers (Marie-Jeanne et al., 2000). The amplicons obtained from both the plants were directly sequenced. The sequence from the CB plant showed 97% nucleotide identity with NLSYV (EU887015) and NDV (AM182028) sequences, respectively. The sequence of TK plant also showed 97% nucleotide identity with NLSYV. Both the plants were also tested for NLSYV, NLV, and NSV by indirect ELISA (Neogen, Lansing, MI). Results confirmed the presence of NLSYV in both plants. NLYSV has been reported to be a possible cause of leaf chlorosis and striping and NDV has been associated with chlorotic leaf striping in *N. tazetta* plants (Ward et al., 2009).

Various potyvirus have been identified based on full length genome sequence identities and phylogenetic relationships with respective sequences of other potyviral genome database available in GenBank. The full length genome sequences of NYSV, CyEVA and NLSYV potyvirus isolates reported on daffodil worldwide have been listed in Table 2.

Table 1. List of viruses of various groups/families reported on narcissus

Virus	Family	Symptom on narcissus	Reference
Narcissus yellow stripe virus	*Potyviridae*	Mosaic, chlorotic yellow stripes on leaves, size of the bulbs reduced. Flower wing stunted, stripes or mottle symptoms on the leaves.	Caldwell and Kissick (1950), Rees (1966), Brunt (1995), Chen et al. (2006), Raj et al. (2020).
Narcissus latent virus	*Potyviridae*	Mosaic, chlorotic spots on leaves and transient leaf tip chlorosis	Brunt (1966), Wylie and Jones (2012), Berniak et al. (2013).
Narcissus mosaic virus	*Potyviridae*	Yellow mosaic, leaf distortion and chlorosis, and flower colour breaking	Brunt (1995), Miglino et al. (2005), Hunter et al. (2011).
Narcissus late season yellows virus	*Potyviridae*	Leaf streaking, stunting and reduced bulb size	Langeveld et al. (1997), Wylie et al. (2014), Wylie and Jones (2012).
Cyrtanthus elatus virus-A	*Potyviridae*	Chlorotic stripes along with leaf distortion and stunting	Chen et al. (2003), Kumar et al. (2015), Ohshima et al. (2016), Raj et al. (2020).
Ornithogalum mosaic virus	*Potyviridae*	Mosaic or chlorotic striping on leaves.	Hong Ying et al., (2009).
Narcissus white streak virus	*Potyviridae*	Greenish-purple streaks on leaves that turn white to yellow reduced flower stalk.	Hanks (1993), Yan et al. (2008).
Narcissus degeneration virus	*Potyviridae*	Mosaic or chlorotic striping on leaves.	Chen et al. (2006), Ward et al. (2009), Ohshima et al. (2016), Raj et al. (2020).
Arabis mosaic virus	*Secoviridae*	Yellow stripe symptoms on leaves.	Mitsuro and Yasuo (1974).
Tomato black ring virus	*Secoviridae*	Ringspot symptoms on leaves like lettuce ringspot	Kamenetsky and Okubo (2012).
Narcissus common latent virus	*Flexiviridae*	Symptomless	Chen et al. (2003), Yan et al. (2008).
Narcissus symptomless virus	*Flexiviridae*	Mild mosaics symptoms on leaves	Chen et al. (2006), Ward et al. (2009).
Narcissus tip necrosis virus	*Tombusviridae*	Necrotic lesions on tip of the leaf	Mowat et al. (1988).
Cucumber mosaic virus	*Bromoviridae*	Leaf mosaic or mottling symptoms or leaf yellowing	Clark and Guy (2000).

Table 2. Details of full length genome of narcissus yellow stripe virus (NYSV), narcissus late season yellows virus (NLSYV) and cyrtanthus elatus virus-A (CyEVA) potyvirus isolates reported on narcissus worldwide

Virus	Isolate	Host	Location	Genome size (nt)	GenBank Accession	Source
NYSV	NAR2	*Narcissus tazetta*	India	9650	KU516386	Raj et al. (2019)
NYSV	NY-OI1	*Narcissus tazzeta*	Japan	9626	LC314391	Ohshima et al. (2018)
NYSV	NY-KM1O	*Narcissus tazzeta*	Japan	9637	LC314392	Ohshima et al. (2018)
NYSV	NY-KM1P	*Narcissus tazzeta*	Japan	9639	LC314393	Ohshima et al. (2018)
NYSV	NY-CB5	*Narcissus tazzeta*	Japan	9630	LC314394	Ohshima et al. (2018)
NYSV	NY-EH173	*Narcissus tazzeta*	Japan	9630	LU314395	Ohshima et al. (2018)
NYSV	NY-HG19	*Narcissus tazzeta*	Japan	9629	LU314396	Ohshima et al. (2018)
NYSV	NY-HG27	*Narcissus tazzeta*	Japan	9629	LU314397	Ohshima et al. (2018)
NYSV	Zhangzhou-1	*Narcissus tazetta*	China	9650	AM158908	Chen et al. (2006)
NYSV	Zhangzhou-2	*Narcissus tazetta*	China	9650	NC_011541	Chen et al. (2006)
NYSV	ZZ2	*Narcissus tazetta*	China	9654	JQ911732	Unpublished
NYSV	Marijiniup3	*Narcissus* spp.	Australia	9647	JQ395042	Wylie and Jones (2012)
NLSYV	Zhangzhou	*Narcissus tazetta*	China	9651	JQ326210	Lin et al. (2012)
NLSYV	Marijiniup8	*Narcissus* spp.	Australia	9687	KC691259	Wylie et al. (2014)
NLSYV	Marijiniup9	*Narcissus* spp.	Australia	9577	JX156421	Wylie et al. (2014)
CyEVA	NBRI16	*Narcissus tazetta*	India	9942	KX575832	Raj et al. (2018)
CyEVA	Marijiniup7-1	*Cyrtanthus elatus*	Australia	9908	NC_017977	Wylie and Jones (2012)

Virus Acronyms: NYSV = narcissus yellow stripe virus; NLSYV = narcissus late season yellows virus; CyEVA = cyrtanthus elatus virus-A.

Chen et al. (2006) reported the complete nucleotide sequence of the genomic RNA of NYSV isolated from Chinese daffodil in Zhangzhou city, China. The complete nucleotide sequence was 9650 nt long, excluding the polyA tail, and had the typical genome organisation for a member of the genus *Potyvirus*. The first AUG codon at 128–130 nt appears to be the initiator for the long ORF, which terminates with a UAA codon at 9437–9439 nt, followed by a 3 -NCR of 211 nt. The predicted translation product of this ORF contained 3103 amino acids (aa) with a calculated Mr of 351.9 kDa (polyprotein). The polyprotein amino acid sequence was aligned with those of the other completely sequenced potyviruses, and the characteristic proteolytic cleavage sites of the ten mature potyvirus proteins were identified. Previous analysis demonstrated that many *Potyvirus* species have a consistent pattern of residues at position P2 amongst the seven sites cleaved by the NIa cysteine protease. NYSV resembled with many other potyviruses in having H at this position. The ten proteins all contained the expected, well-characterised motifs. The deduced CP was 274 aa long and had a predicted molecular weight of 31.1 kDa (Chen et al., 2006).

An isolate of NDV from Chinese daffodil (*N. tazetta* cv. Chinensis) was characterized by Chen et al. (2006) based on virus transmission, western blot assay and analysis of the complete genome sequence. The NDV isolate was successfully transmitted mechanically from Chinese daffodil to three species of daffodil and to *Lycoris radiata*. In western blot immuno-assay, the coat protein reacted strongly with antiserum of NDV (UK isolate). The complete nucleotide sequence (9816 nt) had the typical genome organisation for a member of the genus *Potyvirus*. Sequence comparisons and phylogenetic analysis showed that the NDV isolate was different from all previously sequenced potyviruses but distantly related to onion yellow dwarf and shallot yellow stripe viruses (Chen et al., 2006).

The complete genome sequences of three potyviruses: NDV, NYSV and Vallota speciosa virus (VSV) have been reported from daffodil plants in Australia (Wylie and Jones, 2012). The NDV genome was of 9,816 nt that shared 95% nt and 97% aa identities with the reference genome (9,816 nt) of NDV isolate Zhangzhou. It was named as NDV isolate Marijiniup2. The NYSV genome was of 9,647 nt that shared 72% nt and 79% aa identities with the reference genome sequence of NYSV-Zhongzhou (9,650 nt). It was named as NYSV isolate Marijiniup3. While VSV genome was of 9,908 nt which shared highest identities (92–99%) to the CP sequences of five isolates of the VSV from Australia, New Zealand and UK. The virus was named as VSV isolate Marijiniup7. The genome sequence of VSV Marijiniup7 encoded an ORF of 3,102 aa with a molecular weight of 355 kDa (Wylie and Jones, 2012).

The complete genome sequence of a Chinese daffodil isolate of NLSYV from Zhangzhou, China (NLSYV-ZZ) was determined by amplification and sequencing of viral RNA genome (Lin et al., 2012). The viral genome was of 9,651 nucleotides in length, excluding the 3′-terminal poly (A) tail. The viral genome contains a single long open reading frame of 9,615 nucleotides encoding a polyprotein of 3,105 amino acids. The polyprotein was predicted to be cleaved into ten mature proteins by three viral proteases. Complete genome sequence comparisons and phylogenetic analysis indicated that NLSYV-ZZ was most closely related to NYSV that was also isolated from a daffodil plant. These viruses shared 69.9% identity in their complete nucleotide sequences and 77.0% identity in their polyprotein amino acid sequences (Lin et al., 2012).

The complete genome sequences of two new NLSYV isolates of Australia were compared with the genome sequences of NLSYV isolate of China and with two complete genomes of NYSV isolates of Australia and China. On the basis of symptoms on natural and experimental

host species and genome sequence identities, the isolates could either be classified as closely related members of three different species or placed together in one taxon (Wylie et al., 2014).

The genetic diversity of daffodil viruses related to turnip mosaic virus (TuMV) was investigated to discriminate potyvirus species (Ohshima et al., 2018). In this study, the daffodil plants (*Narcissus tazetta* var. Chinensis) showing mosaic or striping leaves were collected from around Japan and tested for virus infections using potyvirus-specific primers. Many of them were found to be infected with a macluravirus and mixtures of different potyviruses, one third of them were NYSV-like viruses. The full length genomes of nine NYSV-like viruses were sequenced and, together with four already published sequences provided data for phylogenetic and pairwise identity analyses of their place in the TuMV phylogenetic group. Using existing ICTV criteria for defining potyvirus species, the narcissus viruses in TuMV group were found to be from five species: the previously described NLSYV and four new species: narcissus virus 1 (NV-1) and NYSV-1, NYSV-2 and NYSV-3. However, all are from a single host species and natural recombinants with NV-1 and NYSV-3 parents have been found in China and India. It was concluded that they could be considered to be members of a single mega species, narcissus virus; the criteria for defining such a *Potyvirus* species would be that their polyprotein sequences have greater than 69% identical nucleotides and greater than 75% identical amino acids (Ohshima et al., 2018).

The first complete genome sequence of NLV from daffodil was determined by Gao et al. (2018). The complete sequence of a narcissus virus isolated from the Netherlands (Narv-NL) was determined to be 8172 nucleotides in length with an open reading frame encoding for 2624 amino acids. Sequence analyses indicated Narv-NL shared 67–69% nucleotide and 51–68% amino acid sequence identity with artichoke latent virus (ArLV) and Chinese yam necrotic mosaic virus (CYNMV) either in the complete ORF or the coat protein (CP) gene, suggesting that Narv-NL was a member of NLV from the genus *Macluravirus*. Macluraviruses resemble classifiable members of the genus *Potyvirus* in their transmission by aphids; however, their virions are slightly shorter. NLV is distributed widely throughout the major planting areas of Japan, New Zealand, and European countries. It is one of the most common viruses infecting daffodil plants that caused significant yield losses and quality deterioration in daffodil bulbs and flowers (Gao et al., 2018).

The full-length genome sequence of CyEVA associated with leaf chlorotic stripe disease of *N. tazetta* cv. Paperwhite was reported for first time in India (Raj et al., 2018). In this study, the full-length genomic sequence of CyEVA-NBRI16 (KX575832) had been determined that consisted of 9942 nucleotides, excluding the polyA tail, and encoded a single large polyprotein of 3226 amino acids with the genomic features typical of a *Potyvirus*. It shared highest 93% nucleotide sequence identity and closest phylogenetic relationship with sequences of CEVA-Marijiniup7-1 and CyEVA-Marijiniup7-2, both reported from Australia on *Cyrtanthus elatus* host. The genome of CyEVA-NBRI6 (KX575832) isolated from daffodil consisted of the linear, single-stranded positive-sense RNA of 9942 nucleotides long containing 5′UTR, a single large predicted ORF from nucleotide positions 460–9765 encoding large polyprotein of 3226 amino acid residues with a calculated molecular weight of 387.12 kDa and 3′UTR (Fig. 2). The polyprotein further yields the predicted ten mature proteins, characteristic of the potyvirus genus, identified as P1, HC-Pro, P3, 6K1, CI, 6K2 proteins, VPg, Nla-Pro, Nlb and CP having the amino acid/molecular weight of 316/37.9, 458/54.9, 382/45.8, 52/6.2, 635/76.2, 52/6.2, 185/22.2, 241/28.9, 520/62.4 and 259/31.0, respectively. The putative proteolytic cleavage sites in CEVA-NBRI16 isolate were Y/S, G/G, Q/H,

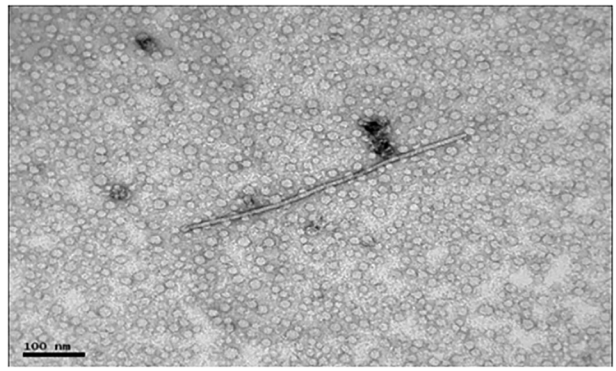

Fig. 2. Schematic representations of the full-length genome of CyEVA isolate NBRI16. Arrow represents the orientation of ORFs present in the virus genome. The numbers above the ORF indicate their starting site while the length (amino acids) and predicted molecular weight (in kDa) of ORF is shown below and above by arrows

Q/S, Q/S, Q/G, E/N, Q/S and Q/S (Fig. 2), identified from alignments with full length polyprotein sequences of other potyviruses, which have similarity with the Marijiniup7-2 isolate (JQ723475) of CyEVA previously known as VSV (Wylie and Jones, 2012). This was the first report from India on full-length genome sequence of CyEVA associated with leaf chlorotic stripe disease in Indian daffodil (*N. tazetta*) cv. Paperwhite (Raj et al., 2018).

Complete genome sequence of NYSV isolated from *N. tazetta* cv. Paperwhite exhibiting leaf chlorotic stripe symptoms was determined for the first time from India. The viral genome sequence contained 9650 nucleotides that encode a large polyprotein (372.36 kDa) of 3103 amino acids. The comparison of the NYSV genome sequences with corresponding sequences of other potyviruses revealed 90–97% identities and closest phylogenetic relationships with NYSV-Zhangzhou-1 and NYSV-ZZ-2 isolates infecting *N. tazetta* reported from China. Therefore, the NYSV isolate was identified as a new member of NYSV and designated as NYSV-NAR2 (Raj et al., 2019).

In a recent study of the detailed evolutionary analyses of wild and domesticated narcissus viruses from Japan was conducted and daffodil plant was considered as a melting pot for potyviruses (Probowati et al., 2022). They reported that NLSYV and NDV are the major viruses of daffodil causing serious disease outbreaks in Japan. In this study, they also showed that NLSYV is widely present in Japan whereas NDV is limited to its Southwest parts (Probowati et al., 2022).

5. DAFFODIL INFECTING POTYVIRUSES DESCRIBED IN INDIA

The potyviruses infecting various bulbous ornamental plants including daffodils have been characterized by various workers based on symptomatology, virus transmission by aphids, particle morphology, serological detection by ELISA, their serological relationships (Aminuddin et al., 1999). The association of an unidentified *Potyvirus* with severe mosaic disease of *N. tazetta* was investigated in India by Aminuddin et al. (1999) based mainly on biological, serological and

electron microscopic properties. The electron microscopy, SDS-polyacrylamide gel electrophoresis of coat protein, serological relationship and RT-PCR were carried out to identify the virus isolate. Electron microscopy of purified virus preparation revealed the presence of flexuous particles of 730 × 13 nm. One band with a molecular weight of 33 kDa was obtained by SDS-polyacryl amide gel electrophoresis. Serological relationship of the virus isolate was established with BYMV in electroblot immuno-assay. Further, RT-PCR was performed employing the potyvirus specific primers and a band of about 300 bp was obtained from the core region of the coat protein that confirmed the association of a potyvirus with the mosaic disease of *N. tazetta* (Aminuddin et al., 1999).

The characterization and identification of a of lycoris potyvirus associated with severe mosaic and light green to yellow leaf stripes disease of daffodil sp. in India was done by Yadav and Khan (2008). RT-PCR was performed with potyviridy specific primers: PotI (CACG-GATCCCGGG(T)17VGC)/PotII (ACCACAGGATCCGGBAAYAAYAGY where B = T or G or G, Y = C or T and D = T or G or A) (Gibbs and Mackenzie, 1997) to amplify part of the NIb gene, the coat protein (CP) gene and the 3′ untranslated region (UTR). The amplified DNA fragment of c. 1·5 kb was cloned and sequenced (GenBank accession DQ991145). The CP gene nucleotide sequence showed 97–98% identity with NCLV and 95% with lycorsis potyvirus. This was the first specific identification of a *Potyvirus* from daffodil in India (Yadav and Khan, 2008).

Yadav and Khan (2015) reported molecular characterization of a potyvirus associated with severe mosaic disease of daffodil in India. The nucleotide sequences of its 3′-terminal genome (~2900 bp) comprising Nla-Pro (nuclear inclusion protein a protease), Nlb, CP and 3′NTR showed 93% and 85% identity whereas; CP gene showed 97% and 87% identities with NYSV at amino acid and nucleotide level, respectively. Phylogenetic analyses with neighbour-joining algorithm in MEGA software version 4.0 (Tamura et al., 2007) using bootstrap value of 1000 replicates of present viral genome showed its close relatedness to NYSV at nucleotide and amino acid level using CP and NIb sequences. Thus, it was identified as a new strain of NYSV from India (Yadav and Khan, 2015).

The genome characterization of two potyviruses in daffodil was investigated by Kumar et al. (2015) and Raj et al. (2019) based on sequence identities and phylogenetic relationships of ~1.5 kb amplicons obtained by RT-PCR using potyvirus specific Pot1 and Pot2 primers (Gibbs and Mackenzie, 1997). The positive clones were sequenced and obtained sequence data of were assembled and analyzed to eliminate any sequence ambiguity. The obtained sequence data were analyzed using published sequences available in GenBank through BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and *Genomatix DiAlign* tools (https://www.genomatix.de/cgi-bin/dialign/dialign.pl) for obtaining the global and specific sequence identity. The virus isolates under study were identified as two potyvirus isolates: CyEVA (KF430816) (Kumar et al., 2015) and NYSV (KU516386) in daffodil samples (Raj et al., 2019). The CyEVA (KF430816) isolate shared 98–80% sequence identities with CyEVA isolates (JQ723475, GU812282, GU812284, GU812283, DQ417604 and FJ032248) reported worldwide. While NYSV (KU516386) isolate shared 97–75% identity with other NYSV isolates (AM158908, LC31498, LC3149, JQ911732, LC31493, and LC31495) reported all over the word (Raj et al., 2019).

Raj et al. (2020) reported the identification of three potyvirus species: CyEVA, NYSV and NDV in *N. tazetta* cv. Paperwhite based on sequence analyses of ~1.5 kb genomic fragments obtained from seven RT-PCR amplifications of infected daffodil samples. *Potyvirus* species associated with yellow leaf stripe disease of Indian daffodil (*N. tazetta* L.) cv. Paperwhite has

been studied by sequence analyses of ~1.5 kb genomic fragments obtained from seven RT-PCR amplifications of infected samples. Sequence analysis revealed the occurrence of three *Potyvirus* species: CyEVA (KF430815, KF430816, KM066973, KM066974); NYSV (KM066972, JQ686724) and NDV (MK572806). The existence of three *Potyvirus* species: CyEVA, NYSV and NDV have been reported in Indian daffodil. To know the probable reasons for occurrence of three species potyvirus daffodils grown at National Botanical Research Institute, seven infected samples were analyzed by RT-PCR amplification using potyvirus degenerate primers. The study suggested prevalence of diverse *Potyvirus* species present in the vicinity and probability of emergence of new recombinant species is high (Raj et al., 2020).

6. *POTYVIRUS* IDENTIFICATION METHODS IN DAFFODILS DESCRIBED BY DIFFERENT AUTHORS

It is very critical to diagnose and identify the disease caused by potyviruses in daffodils. Various strategies are described by different workers for diagnosis and identification of potyviruses that are based on their electron microscopy, biological, biochemical and genomic (nucleic acid) properties.

6.1. Electron microscopic study

Electron microscopy is a valuable tool in plant virology. Particularly, transmission electron microscopy (TEM) is a very useful tool for virus detection and for basic and applied research of plant viruses. TEM is being used to observe and diagnose the potyvirus particles in leaf dip and purified preparations, and also in situ location in ultrathin sections of plant tissue. TEM may be used to 1) detect the presence of virions, 2) provide information of morphology and size and 3) monitor the purity and relative amount of virus during purification (Baker et al., 1985). During a TEM study of the virus isolated from infected daffodil leaf, the typical flexuous filamentous particles of about 680 nm in length and 11 nm width (in diameter) were observed (Fig. 3) at 80,000 magnification that indicated the presence of *Potyvirus* (Raj et al., 2019).

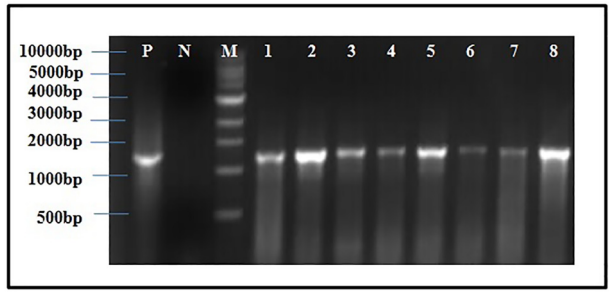

Fig. 3. Transmission electron micrograph of the virus isolated from infected daffodil leaf showing flexuous filamentous particles of size 680 × 11 nm (LxW) similar to the potyvirus. Bar = 100 nm (Raj et al., 2019)

Mowat et al. (1988) reported the properties and serological detection of NLSYV in infected daffodils plants showing symptoms characteristic of late season yellows. They observed NLSYV particles measuring of c. 750 ± 12 nm by Immuno-electron microscopy (ISEM) in a purified preparation from fresh leaf extract of the infected daffodil plants. ISEM tests confirmed the occurrence of a distinct potyvirus (NLSYV) in daffodil cv. Grand Soleil plants affected by white streak disease. Detection of the virus by ISEM was found as sensitive as by ELISA (Mowat et al., 1988).

6.2. Enzyme-linked immunosorbent assay (ELISA)

Mowat et al. (1988) also detected NLSYV by ELISA in 14 of the 18 daffodil cultivars examined using antibodies from antisera prepared to purified virus particles of NLSYV. The virus was detected by ELISA only in samples collected after flowering and capsid antigen concentration was greatest in the distal region of leaves. The reliability of detection depended on the cultivar (Mowat et al., 1988).

A distinct *Potyvirus* having close resemblance with NLSYV and NYSV has been reported on daffodil from India based ELISA detection (Chandel et al., 2010). The daffodil samples showing chlorosis were collected from Shimla, Solan, Kangra, and Kullu districts of Himachal Pradesh (in India). Positive results for the presence of a *Potyvirus* were shown by samples collected from Kangra district with ELISA using potyvirus group specific antibodies. Further, they isolated the RNA from theses samples and performed RT-PCR using potyvirus group specific primers that resulted in positive amplification of ~800 bp fragment. The sequence analysis of amplified fragment confirmed the identity of the virus infecting daffodil as a potyvirus. The phylogenetic analysis demonstrated that the virus infecting daffodil had close resemblance with NLSYV and NYSV reported from China but was distinct from both of them (Chandel et al., 2010).

6.3. Molecular identification methods of potyviruses in daffodils

6.3.1. Nucleic acid spot hybridization tests using potyviruses specific probes. The detection of CyEVA in naturally infected daffodil plant samples was done by nucleic acid spot hybridization (NASH) in 18 randomly selected daffodil plant exhibiting yellow leaf stripe symptoms (Raj et al., 2018). For detection of *Potyvirus*, approximately 2.0 µg total genomic RNA was blotted in a replica nitrocellulose membrane. The 2.0 µg RNA from a healthy daffodil plant (as negative control) along with 200 ng cloned DNA of CP gene of CyEVA (KF430816), as positive control was also blotted on nitrocellulose membrane. The blotted membrane was hybridized with probe prepared by random primer labelling method (Fienberg and Vogelstein, 1983); and pre-hybridization, hybridization, and washing steps were performed according to the standard method (Sambrook et al., 1989), and the hybridization signals were recorded by phosphorimaging using a phosphorimager. The NASH with CyEVA probe showed strong positive signals in 16 samples out of 18 samples tested. The intensity of signals was strong similar to as a positive control (cloned DNA of CyEVA) while two plants (5 and 10) do not show signals (Fig. 4). The intensity of the hybridization signals from samples varied and was in proportion to the viral load in tested samples. These results indicated that the probe-based detection method successfully detected the virus (CyEVA). It is suggested that the method may be utilized for mass indexing of the bulk daffodil propagative materials (Raj et al., 2018).

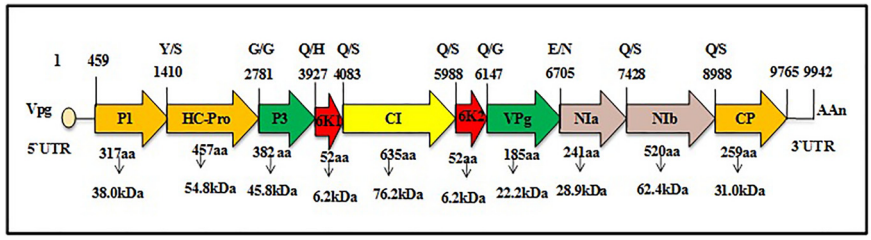

Fig. 4. Detection of CyEVA in daffodil leaf samples by NASH method using probe developed from cloned coat protein gene of CyEVA isolated from daffodil (Raj et al., 2018)

6.3.2. Reverse Transcription-Polymerase chain reaction for* Potyvirus *identification in daffodil. Reverse Transcription-Polymerase chain reaction (RT-PCR) is a molecular-based most effective technique for the diagnosis and identification of RNA viruses. In this technique virus gene-specific primers are used during PCR for amplification of viral genomic fragment. For potyvirus detection different primers of conserved region were used in the past. Since CP is the most conserved region of *Potyvirus*, hence conserved sequence like WCIEN box or QMIKAA motif universal primers were designed to detect and identify potyviruses (Langeveld et al., 1991; Colinet and Kummert, 1993; Pappu et al., 1993; Bateson and Dale, 1995). The most conserved motif (GNNSGQPSTVVDN) of the NIb region (Gibbs et al., 2003) and forward primer of this conserved motif are used for detection of specific numerous members of *Potyviridae* family (Gibbs and Mackenzie, 1997; Chen and Adams, 2001). Then after HC-Pro, C-terminal region of NIb, C-terminal of CP were also used for detection of *Potyvirus* (James et al., 2003; Kajic et al., 2008; Gadiou et al., 2009; Raj et al., 2018).

For molecular detection of potyviruses, the leaf samples of daffodil plants showing mosaic and yellow stripe symptoms were collected. The total RNA was isolated from 100 mg leaf samples of infected and healthy daffodil plants using the RNA isolation kit (Sigma-Aldrich, USA), and isolated RNA from each was suspended in 30 µl nuclease-free water. The concentration of RNA was quantified through spectrometer and their OD at the ratio of absorbance at 260/280 nm was used and the ratio was ~160–190 µg ml^{-1} suggesting the quality of RNA was appropriate. The RT-PCR was performed using two sets of degenerate primer pairs first is Pot-9502 (5′-GCGGATCCTTTTTTTTTTTTTTTT-3′)/CPUP(5′-TGAGGATCCTGGTGYATH-GARAA YGG-3′ where R = A or G, Y = C or T, W = A or T, K = T or G, and H = A or T or C) that amplify complete CP region (Van der Vlugt et al., 1999) and second PotI (CACGGATCCCGGG$(T)_{17}$VGC)/PotII (ACCACAGGATCCGGBAAYAAYAGY) spanning 3′-UTR and partial NIb region of *Potyvirus* (Gibbs and Mackenzie, 1997). The total RNA of eight infected daffodil leaf samples was used as a template and oligo-dt primer was used for cDNA synthesis. PCR reaction was performed in a 25 µl reaction volume which contained 1x Taq buffer, 2.5 mM $MgCl_2$, 200 mM each of dATP, dCTP, dGTP, dTTP, 1.0-unit Taq DNA polymerase, 10 nM primers (Pot9502/CPUP), nuclease-free water (to make up the final reaction volume) and 5 µl cDNA as template. Amplified products were electrophoresed in 1% agarose gel with 1 kb DNA ladder used as size marker. Results revealed the expected size ~1.5 kb amplification with CP gene specific primers similar to as the positive control, indicated the presence of *Potyvirus* infection in daffodil plants (Fig. 5).

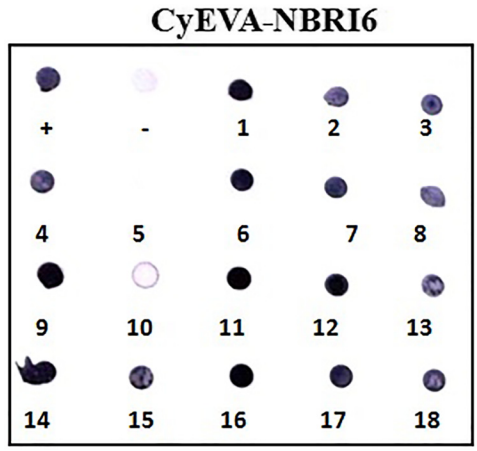

Fig. 5. Agarose gel electrophoresis of RT-PCR products obtained by potyvirus specific degenerate primers showing ~1.5 kb amplicon in eight symptomatic diseased leaf samples (lanes 1–8) as compared to a positive control (P, CyEVA, Accession number KF430816) (Kumar et al., 2015) while no such amplicon in an asymptomatic healthy leaf sample that confirmed the potyvirus infection in all diseased plants

6.3.3. Multiplex RT-PCR and RT-qPCR assay for identification of potyviruses in daffodil. A protocol was developed by Jin et al. (2017) for detection of NYSV and NMV was in daffodil via multiplex TaqMan-based reverse transcription-PCR assay. The development of a multiplex TaqMan RT-qPCR assay was done for simultaneous detection of NYSV and NMV which frequently caused mixed infection in daffodil. The primers and probes were designed based on the conserved CP gene regions of NYSV or NMV and their suitability for singleplex and multiplex TaqMan RT-qPCR assays as well as for conventional RT-PCR. Conventional RT-PCR, singleplex and multiplex TaqMan RT-qPCR assays proved to be NYSV and NMV specific. The *P*-values and coefficients of variation of TaqMan RT-qPCR assays indicated high reproducibility. They achieved significantly increased sensitivity as compared to conventional RT-PCR. The detection limit of both viruses was found 103 copies with superior correlation coefficients and linear standard curve responses between plasmid concentrations and Ct values. The infections of both NYSV and NMV viruses were successfully detected in leaves, tepals, and bulbs of daffodil via the multiplex RT-qPCR method in 1–25 mg leaf tissue. This assay provided rapid, specific, sensitive and reliable testing for simultaneous detection of NYSV and NMV using primers for NYSV (TF-TGGATGGAGAAGAACAAGTTGAATT/TR-GCCATTATCTGCCT-GAGTGTAGGT) and NMV (F-ACTCAGTCGCACCCGCTATG/R-GTGCTTCAATGGCG-TACATGG) that was useful in routine monitoring for different daffodil samples (Jin et al., 2017).

7. DISEASE MANAGEMENT STRATEGIES OF POTYVIRUSES IN DAFFODIL CULTIVATION

Potyviruses cause significant economic losses in daffodil crops due to various diseases they cause in daffodils; therefore, their disease management seems to be essential for the

improvement of its crop production. The genus *Potyvirus* is one of the most devastating group of plant pathogens in the world and yield losses due to infection of this virus is commonly ranges from 8% to 35%, although losses are as high as 94% have been recorded. Daffodil is propagated by bulbs and if mother stocks are infected with virus then they can act as a source of virus spread from one generation to other and continue to many generations. Therefore, their management is essential by various management strategies (Verma et al., 2004).

7.1. Sanitation of cropping field and control of vectors

For the disease management of potyviruses, sanitation of field and planting practices are used. Vectors could be also avoided by early sowing of seeds, growing plants in isolated or remote areas, and rotation of crops to minimize specifically soil-borne virus diseases. The use of non-host plants in and around the cultivated area may be a successful means of reducing the virus infection has been suggested by Matsuura et al. (2006). Vectors are the main source for virus spreading from infected to healthy plants. Mehrotra in 1991 suggested for use of some barriers like cages towards the onset of insect vectors. Khan et al. (2006) used Malathion insecticide for the control of aphids and whiteflies in chilli crop. Shukla et al. (2016) used insecticidal fern protein in cotton for protection against whiteflies. Therefore, the potyvirus disease management from mother stock (bulbs) of infected daffodil plants is essential. In natural conditions, potyviruses are mainly transmitted through aphid, whiteflies vectors and nematodes (Poutaraud et al., 2004; Adams et al., 2005) from infected plants to healthy plants growing nearby. Potyviruses can also be transmitted through seeds and vegetatively propagated tubers (Aminuddin and Singh, 1993; Johansen et al., 1994; Shukla et al., 1994; Sastry, 2013).

7.2. Control of virus transmitting vectors through insecticides

Aphids are a well-known vector of potyviruses. Vectors can affect various crops, including daffodil, by sucking the sap of the virus-infected plant and transferring the virus infection from one plant to other healthier plants. Therefore, the control of the aphid vector is necessary. In a study, three sprayings of 0.2% Malathion insecticide (w/v) solution in water at 21 day intervals have attempted in daffodil field which resulted in the significant decrease in aphid population and potyvirus like disease symptoms (Raj, 2019).

7.3. Developing virus-free daffodil plants

In general, virus accumulates in different plant parts including bulbs and survives in the growing tissues of mother bulbs of daffodil and therefore is a major reason for virus dissemination to their subsequent generation and from one place to another cultivation area (Aminuddin and Singh, 1993; Johansen et al., 1994), hence, management of potyviruses was considered as highly required for quality and for quantity improvement of daffodil bulblets.

Different therapies were used for the management of potyvirus disease in daffodil plants. The success rate was varied from different protocols due to the relationship between host, virus, and treatment (Panattoni and Triolo, 2010). Recently, the three different therapies: electrotherapy, chemotherapy, and combination of electrotherapy with chemotherapy have been used for elimination of potyvirus infection in daffodils (Raj et al., 2022).

7.4. By *in vitro* chemotherapy

In this therapy ribavirin is used as an antiviral chemical, this has a capability for virus elimination from infected plants by restricting their replication (DeFazio et al., 1978; Verma et al., 2004). Antiviral effect of ribavirin forced RNA viruses into error catastrophe (Crotty et al., 2001). Ribavirin (1-D-ribofuranosyl-1, 2, 4-triazole3-carboxamide) is a base analog of adenine or guanine. The different concentration of ribavirin in MS medium and duration of treatment should be optimized for different hosts and viruses (Hu et al., 2012). Due to the phytotoxicity of ribavirin, it can affect the growth of *in vitro* plants (Hansen and Lane, 1985; Paunovic et al., 2007). In a study, Panattoni et al. (2013) showed that several other antiviral drugs such as: inosine monophosphate dehydrogenase (IMPDH) inhibitor, S-adenosyl homocysteine hydrolase (SAH) inhibitor and neuraminidase (NA) have been also used. Ribavirin induces broad-spectrum activity against several RNA and DNA viruses due to its guanosine analog base (Leyssen et al., 2005). In the process of transcription of triphosphate ribonucleotides, IMPDH inhibitors are directly involved and therefore assist to inhibition of viral nucleic acid replication (Crotty et al., 2001). The main target of antiviral activity of these molecules is the inosine monophosphate dehydrogenase, which is an enzyme that catalyzes the conversion of inosine 5′-monophosphate (IMP) in xanthosine 5′-monophosphate and it is able to change the pathway for the production of guanosine mono-di-and triphosphate. SAH hydrolases is other inhibitors, which are involved in virus replication (Panattoni et al., 2013). In trans-methylation reactions, S-adenosylmethionine (SAM) is used and donates methyl groups to a large range of acceptors together with nucleic acids, viral proteins, and phospholipids, and then converted them to S-adenosyl homocysteine. The methylation process is regulated them negatively by an increase in SAH and a decrease of SAM or SAM/SAH ratio. Removal of SAH plays an important role and it is initiated by SAH hydrolase, which has an ability to convert this molecule into homocysteine and adenosine. Thus, the collection of SAH makes their conversion and resulting blockage of the maturation of viral RNA impossible, especially without stopping the formation of "cap" (DeClercq, 2005).

Recently, the *in vitro* chemotherapy was done for elimination of CyEVA from infected daffodil bulbs using virazole (chemical name ribavirin). For the standardization of *in vitro* chemotherapy, they used three different concentration of ribavirin 30 mg L^{-1}, 40 mg L^{-1} and 50 mg L^{-1} in MSg3 media. Result showed that at high concentration 50 mg L^{-1} ribavirin germination rate was poor (16%) because they are toxic to plant and at 40 mg L^{-1} ribavirin germination rate was moderate (36%) but at low concentration 30 mg L^{-1} ribavirin germination rate was high (56%) because these levels were less toxic. The virus-free daffodil plants obtained were 13%, 26%, and 46% in ribavirin at 30 mg L^{-1}, 40 mg L^{-1} and 50 mg L^{-1} concentrations respectively (Raj et al., 2022).

7.5. By *in vitro* electrotherapy

In this technique, plant tissue is treated with electric current for the elimination of virulence activity of viruses by means of disrupting the viral nucleoproteins. It is suggested as a presumption that when plants tissue is treated with the electric current then inside the tissue, nucleoproteins of viruses may be denatured because of increased temperature (Black et al., 1971; Lozoya-Saldaña et al., 1996). In a research work it was shown that when nucleoproteins are present within the cells then, thermal inactivation may not be worked because of cells are

thermally isolated by the cell wall. In 1974 Blanchard obtained virus-free plants using direct current (Blanchard, 1974). This study established a basis for electric current mediated for management of plant RNA virus.

Gonzalez et al. (2006) proposed that viral particles may be denatured in apoplastic space during transportation through the plasmodesmata. Hormozi-Nejad et al. (2010) proposed that due to denaturation of a nucleoprotein, virus cannot move from cell to cell, therefore, prevention of virus movement into healthy cells. These studies reveal a basis for electric current induced removal of plant RNA viruses. Since the 1990s it has appeared that the electric current is used in the number of the crop for the production of virus-free plants. This technique was first utilized on potato plants for the elimination of potato virus X (PVX) on a large scale. In this technique, an electric current of 15 mA for 5 min leads to 60–100% elimination of PVX in various plants cultivars (Lozoya-Saldaña et al., 1996).

In a recent study, three different electric current were used during *in vitro* electrotherapy for elimination of *Potyvirus* in infected daffodil bulb explants (Raj et al., 2022). In this study, the virus infected bulbs were sterilized and kept for therapy of electric current in electrophoresis tank in 1× TAE buffer and placed medium for germination under aseptic condition. Electrotherapy was used with different electric current (10, 20 and 30 mA) for 20 min. After 30 days germination rate was recorded, the rate of germination was higher 63% in case of 10 mA for 20 min electric current and the percent of virus-free plants 40% were obtained from 20 mA/20 min electric current. The combinations of electrotherapy with chemotherapy was also used for elimination of *Potyvirus* from daffodil bulb explants using a different combination of ribavirin (30–50 mg L^{-1}) and electric current (10–30 mA/20min). The percentage of virus-free plants were found highest in the combination of chemotherapy and electrotherapy (30 mg L^{-1} ribavirin +20mA/20min electric current) 50% as compared with other therapies; hence the therapy efficiency rate was also the highest (Raj et al., 2022).

7.6. By combination of *in vitro* chemotherapy with electrotherapy

The previous studies showed that the use of a combination of chemotherapy with electrotherapy was found best results in case of PVY elimination from potato (Mahmoud et al., 2009) and BYMV from gladiolus (Kaur et al., 2019). In a recent study at NBRI, Lucknow (India), the combination of electrotherapy and chemotherapy was attempted for elimination of CyEVA potyvirus from infected daffodil bulbs (Raj et al., 2022). The three combinations: ribavirin (30.0 mg L^{-1}) + electric current (10 mA/20 min), ribavirin (30 mg/lt) + electric current (20 mA/20 min), and ribavirin (30 mg/lt) + electric current (30 mA/20 min) were used. After 30 days regeneration responses were recorded. It was found that 60% explants were germinated in 30.0 mg L^{-1} ribavirin with 10 mA/20 min electric current, 53% explants germinated in 30.0 mg L^{-1} ribavirin with 20 mA/20 min electric current while only 33% explants germinated in 30.0 mg L^{-1} ribavirin with 30 mA/20 min electric current. The total regenerated explants obtained from three therapies were hardened and potted in small pots and maintained in glass house. Various growth stages of the development of virus-free daffodil plants are shown in (Fig. 6). The regenerated plants were screened by RT-PCR using CyEVA potyvirus specific primers to confirm the presence/absence of CyEVA virus. The highest number of virus-free (50%) daffodil plants were obtained from the combination of chemo with electrotherapy (30 mg L^{-1} ribavirin +20 mA/20min). The virus-free daffodil plants were maintained in glasshouse conditions to observe their growth parameters and performance. The virus-free

Fig. 6. Various growth stages during the development of virus-free daffodil plants

daffodil plants showed better growth and produced more bulbs as compared to the controls plants. The developed protocol may further be utilized for virus disease management in daffodil (Raj et al., 2022).

8. DISCUSSION (CONCLUSION)

Different plant viruses including: potyviruses (NYSV, NLV, NTNV, NLSYV, NMV, CyEVA and BYMV), *Carlavirus* (NCLV and NSV), *Nepovirus* (TRSV and ArMV), *Carmovirus* (NTNV) and *Cucumovirus* (CMV) are reported to infect daffodils. Amongst them, potyviruses are found most prevalent which cause severe mosaic, yellow stripe symptoms and reduced plant growth, and production of Paperwhite and Chinensis cultivars of daffodils in India and abroad. In a recent study, Probowati et al. (2022) collected daffodil plants showing mosaic or striped leaves along with asymptomatic plants in Japan for evolutionary analyses and showed that NLSYV is widely present in Japan whereas NDV is limited to its southwest parts. They also found three novel recombination type patterns in NLSYV comprising of at least five distinct phylogenetic groups whereas NDV had only two groups. The infection of viruses in daffodils often occurs as co-infection with different viruses, isolates of the same virus, and in the presence of quasispecies (mutant clouds) of the same virus in nature. Hence, the wild and domesticated daffodil plants

are considered like a melting pot of potyviruses (Probowati et al., 2022). Daffodil growers may manage these viruses in the field by clean cultivation practises and avoiding plantation of bulbs from infected plants. The most widely or more commonly used potyvirus detection and identification techniques are electron microscopy, ELISA, RT-PCR and multiplex TaqMan-based RT-PCR assays in daffodils. In a recent study, elimination of CyEV-A potyvirus from infected narcissus bulblets has been achieved by chemotherapy, electrotherapy alone and in combination of chemotherapy with electrotherapy. They suggested that virus-free narcissus bulbs may be multiplied for mass propagation that would enhance the quality and production of flowers, and ultimately enriching the floriculture industry and daffodil growers (Raj et al., 2022). This study would accelerate extensions of virus-free plants to practical agricultural production for improvements of yield and quality of crops. Therefore, daffodil growers are advised to use the certified virus-free planting material and proper sanitation practices.

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