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***The cause of color break in reverse bicolor
daffodils***

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1 *Executive summary*

The cause(s) of color break in reverse bicolor daffodils is the subject of some debate. One possible explanation is that viruses are responsible. Crop and Food Research have investigated the cause of color break in reverse bicolor daffodils using a range of analytical tools and flowers collected from three different locations in New Zealand.

Color-broken daffodil flowers were found to have substantially higher quantities of plant viruses in their perianths than their non color-broken counterparts.

Transmission electron microscopy (TEM) revealed the presence of rod-type particles in the color-broken tepals predominantly of 550 nm in length. This is known to be the length of the potyvirus *Narcissus mosaic virus* (NMV). The viral particles in the color-broken daffodils were generally located in the perianth tissue, with lower levels observed in leaves and bulbs.

The enzyme-linked-immunoassay (ELISA) procedure with an antigen specific to the potyviral group showed that the presence of potyviruses was not strongly associated with color break.

Sap collected from the color-broken extracts of the ten flowers tested and rubbed onto leaves of the indicator plant *Gomphrena globosa* caused the leaves to display red necrotic local lesions characteristic of NMV infection.

The reverse transcriptase-polymerase chain reaction (RT-PCR) procedure confirmed that the color-broken flowers had much higher levels of virus than the non-broken flowers. Sequencing of the RT-PCR generated products and comparison with web-based databases revealed that the color-broken flowers were infected with NMV.

We were able to cause previously non-broken reverse bicolor flowers to color break by inoculating them with tepal sap from 'broken' flowers.

The mechanism by which NMV causes the flowers to 'break' is still not known, but it does not appear to be through interfering with the gene expression of the biosynthetic enzymes that make the yellow colour of the tepals (lutein).

We conclude that viruses are the cause of color break in the reverse bicolor daffodil flowers and that the virus(es) has the potential to be spread by growers of the flower. Growers should therefore remove the infected plants immediately being careful not to transfer sap to their other plants.

The virus that most likely causes the color break is NMV. It was found in all reverse bicolors that 'broke'. However, to unequivocally demonstrate this, we would need to be able to cause colour break by inoculating with a pure isolate of NMV. Until this is done we cannot say that NMV alone causes the break as it is still possible that other viruses in addition to NMV are needed.

Future research that would increase our understanding of color break could include:

- Isolating the NMV strain that is associated with color break in the reverse bicolor daffodils and testing whether it alone causes the flowers to color break.
- Isolating the sequence of the NMV strain that infects daffodil and placing a fluorescent green marker in it so that we can monitor in real time where the virus is located in the daffodil. This will enable us to:
 - test whether the virus is preferentially located in the white areas of the petal (those areas will fluoresce green under UV if this is so).
 - Monitor over time how the virus moves (via movement of green fluorescence) in the infected daffodils.
 - Assess whether there are particular conditions which reduce the vigor of the virus. (Does this explain the reported seasonal variation in the occurrence of color break?)
- Metabolite profiling of color breaking flowers to investigate which point in the color formation pathway is responsible for the color breaking effect.
- Using NMV to investigate further the basis of color changes in the flower. The knowledge generated from this research would lead to increased understanding on:
 - What colors the daffodil flowers have the potential to make.
 - New approaches to adding novel colors to the flower.

2 *Introduction*

Color break occurs in the petals of a range of flowers and results from either an increase or decrease in the normal amount of pigment. Reverse bicolor daffodils are prone to a certain type of 'break' where the solid yellow of the flower 'breaks' into patches of white. The cause of this 'break' has not been studied in the reverse bicolor daffodils and growers still debate its cause(s). Some believe that it is caused by viruses since viruses cause color break in other flowers. However, others have continued to question whether the 'break' is of viral origin since:

1. color breaking predominantly affects only reverse bicolor-type daffodils, and
2. the flowers that 'break' do so from plants that otherwise look healthy.

Viruses have been shown to cause color break in daffodil flowers. Rees, (1966) found that 74% of Minister Talma daffodil flowers (1-Y-Y) inoculated with a combination of *Narcissus yellow stripe virus* (NYSV) and *Tobacco rattle virus* (TRV) developed white blotches on their perianth segments. However, these viruses also dramatically affected general plant health,

inhibiting leaf growth and promoting early leaf senescence. Therefore, it is unlikely that these viruses would be the cause of color break in the reverse bicolor flowers. There is also little evidence for other viruses causing daffodils to display color break and, interestingly, some researchers showed that even when NYSV is present color break is not always observed (Clark & Guy 2000).

Given that viruses are commonly found in daffodils, and often in complexes of up to four different types (Brunt 1966; Clark & Guy 2000) it is perhaps surprising that the incidence of color break is so low if viruses are the cause. It is unlikely to be the inability of the virus(s) to infect the floral tissue, because complexes of up to at least three different viruses have been found in petals of non-broken flowers (DA Hunter, unpublished data) and researchers have reported that daffodil floral extracts, not vegetative extracts, serve as the best inocula for viral inoculation studies (Brunt 1966).

In this report, we detail our findings on whether viruses are the cause of the color break observed in the reverse bicolor daffodils.

3 *Methods*

3.1 *Flower sources*

Color-broken and non color-broken daffodils of the same variety were kindly supplied for this study by five growers in four different regions throughout New Zealand: Bob MacDonell (JRM, Ashhurst), Reg Cull (RC, Foxton), John Hunter (JAH, Nelson) Kevin Kerr (KK, Nelson) and Peter Ramsey (PR, Hamilton).

3.2 *Types of color break*

Daffodil flowers (reverse and non reverse bicolor daffodils) can show different types of color break. Figure 1 shows flowers displaying either increased (Figure 1A) or decreased (Figure 1C) production of the yellow pigment lutein. In some flowers there is a sector-like pattern to the color break (Figure 1A and C) whereas in others the break appears more mottled in appearance (Figure 1B, 2B, C, E and F).

We observed that when the flower of a parent bulb was 'broken' the attached daughter bulbs were also broken. However, we also discovered that the flower of an attached offset bulb could be 'broken' when its mother bulb was not.



Figure 1: Different patterns of color break in daffodils. Flowers showing increased accumulation of pigment (A), decreased accumulation of pigment (mottled appearance) (B) and decreased accumulation of pigment (sectored appearance) (C).



Figure 2: Different patterns of color break in reverse bicolor daffodils. Photos of normal and 'broken' Twelve Gauge (A-C), and Lighthouse Reef (D-E) flowers. Both radial (B) and mottled (C, E and F) 'breaks' were observed. Flowers were supplied by RC and JRM, respectively.

4 Results

4.1 Presence of virus particles in color-broken flowers

Transmission electron microscopy (TEM) analysis revealed large quantities of virus particles in the tepals of color-broken flowers. Numerous rod-type virus particles were identified in the tepal extracts of a variety of color-broken reverse bicolor flowers and a color-broken non reverse bicolor Culfind (2Y-YOO) collected from different regions within New Zealand (Figure 3, Table 1). Only low numbers of virus were observed in the tepal extracts of the same types of flowers that were not color broken. Interestingly the virus associated with color break in Culfind (ca. 645 nm in length) was different to that associated with color break in the reverse bicolor daffodils (ca. 550 nm [the size of *Narcissus mosaic virus*, NMV]). Viral numbers were also much lower in the vegetative parts of the plant (leaves, bulb scales) than in the perianth tissue (Table 1).

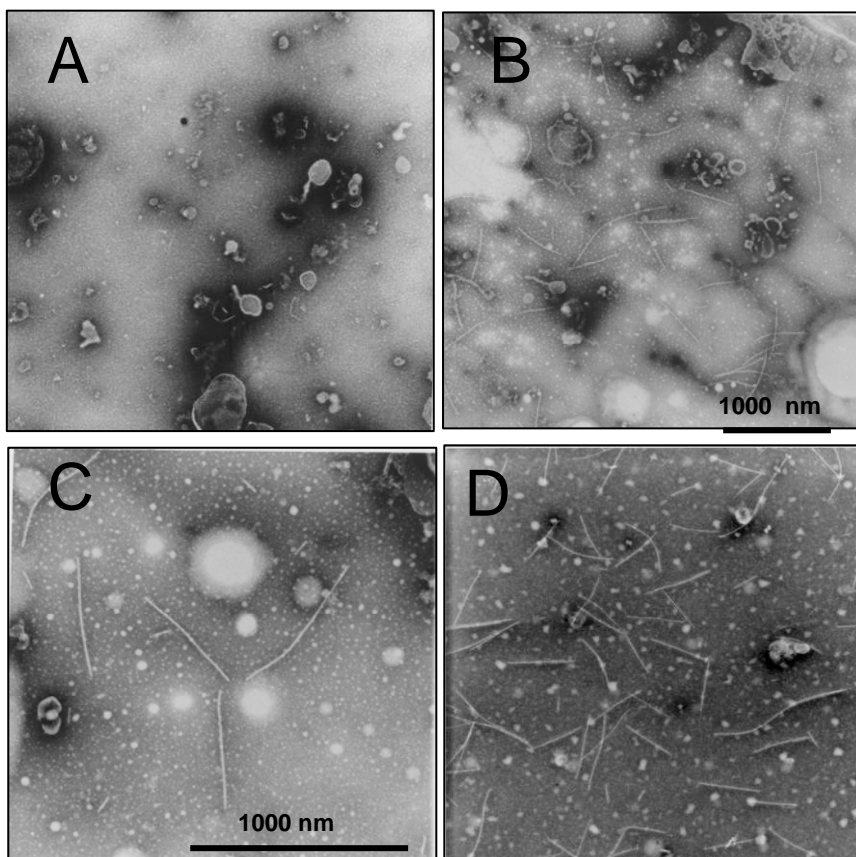


Figure 3: TEM photos of the virus particles observed in tepal extracts of non color-broken (A) and color-broken (B, C) Twilight Zone flowers (JRM, Ashhurst) and (D) Lighthouse Reef(JRM).

The correlation between presence of virus and color break was particularly well illustrated by the finding that the 'broken' flower of the attached offset

bulb of Culfind contained lots of virus whereas the non 'broken' flower of the mother bulb did not (Table 1).

Table 1: Number of viral particles found in the tepal extracts of color-broken and non-broken flowers using TEM.

Identity	Particle length ⁴ (nm)	Flower symptom	Source/ location	Viral count tepals	Viral count leaves	Viral count bulbs
Culfind (<u>offset</u> Bulb) (Non-reverse bicolor)	640	Color Broken	JAH/Nelson	15.0±0.6	-	-
Culfind (<u>parent</u> Bulb)		Not Broken	JAH/Nelson	1.7±0.3	-	-
21/92A seedling (Twilight Zone x R.Cull 2YP)	550	Color Broken	JAH/Nelson	51.3±14.1	3.5±0.5	1±0 ²
21/92A seedling		Not Broken.	JAH/Nelson	0 ¹	0 ¹	0 ¹
Twelve Gauge	550	Color Broken	RC/Foxton	12.3±2.8	-	-
Twelve Gauge		Not broken	RC/Foxton	1 ²	-	-
Twilight Zone	550	Color Broken	JRM/Ashhurst	29.4±4.2	-	-
Twilight Zone		Not Broken	JRM/Ashhurst	1 ³	-	-
Lighthouse Reef	550	Color Broken	JRM/Ashhurst	28.2±4.5		
Daydream	550	Color Broken	JAH/Nelson	20±2.3	-	-
20/87H (Royalist x [1W P Sdg] x Fintona) x Kabonova	550	Color Broken	JAH/Nelson	16.9±0.6	-	-

Viral counts were determined from TEM photos. The photos were taken from randomly chosen observations under magnification of 21200 X. ¹No viruses found anywhere in extract; ²very hard to find viruses and petal extract continuously scanned to find them; ³only one virus found in whole extract. – not measured. JAH = John Hunter, RC = Reg Cull; JRM = Bob MacDonell. ⁴approximate size of the majority of the particles measured in the extracts.

4.2 *Effect of color-broken tepal extracts on indicator plants*

Tepal extracts of color-broken flowers caused visual damage of *Gomphrena globosa* consistent with infection by NMV.

Indicator plants are used by virologists to aid in the identification of certain viruses. These plants show characteristic lesions when infected with particular viruses. For example, the indicator plant *Gomphrena globosa* shows ringspot lesions when infected with NMV (Brunt 1966; Asjes 1972). We have studied the response of *G. globosa*, *Chenopodium amaranticolor*, *Phaseolus vulgaris* and *Nicotiana tabacum* to infection with tepal extracts from color-broken and non-broken reverse bicolor flowers. Our results suggest that the tepal extracts from each one of the 10 color-broken flowers examined contained enough virus to cause viral damage in *G. globosa*, whereas extracts from the non-broken flowers did not (Table 2). The damage observed was characteristic for infection with NMV (Figure 4).



*Figure 4: Viral damage caused by inoculating *G. globosa* with a tepal extract from 1 of 10 color-broken flowers. The viral damage observed is typical of NMV infection. It was observed after every inoculation with color-broken tepal extracts.*

Table 2: Identification of virus in color-broken tepals using indicator plants and ELISA. Color- broken and non-broken *Narcissus* tepal tissue extracts were used to mechanically inoculate the indicator plants *C. amaranticolor* (Ca), *G. globosa* (Gg), *P. vulgaris* (Pv) and *N. tabacum* (Nt) and the host reaction determined. NS = no symptoms; LL = local lesion. DIBA ELISA was also used to test for the presence of viruses in the Potyviridae viral family.

Cultivar name	Flower symptom	Host reaction	Potyvirus group assay
82/90 [JAH] (Rich Reward X [Daydream X Empress of Ireland])	Color broken	LL on Gg turning systemic	-
	Not broken	NS	-
Daydream [JAH]	Color broken	LL on Gg turning systemic	-
	Not broken	NS	-
Twilight Zone [JRM]	Color broken	LL on Gg turning systemic	-
	Not broken	NS	(+)
Twilight Zone [JRM]	Color broken	LL on Gg turning systemic LL on Nt	(+)
	Not broken	NS	-
Twelve gauge [RC]	Color broken	LL on Gg turning systemic LL Ca	-
	Not broken	NS	-
Startracker [JRM]	Color broken	LL on Gg turning systemic	-
	Not broken	NS	-
Honeybird [RC]	Color broken	LL on Gg turning systemic	-
	Not broken	NS	(+)
20/87 [JAH] (Royalist X [1W P Sdg] X Fintona) X Kabonova	Color broken	LL on Gg turning systemic	-
Daydream Mitsch [JAH]	Color broken	LL on Gg turning systemic	(+)
Lighthouse Reef [JRM]	Color broken	LL on Gg turning systemic	
Trumpet Warrior [KK]	Not broken	NS	
Gold Convention [JRM] (non reverse bicolor)	Not broken	NS	+
Control PVY	-	-	+
Control sap	-	-	-
LL C am. Twelve Gauge	-	-	-
LL G g Daydream	-	-	-

ELISA identified members in the potyviridae that were present in both color-broken and non-broken flowers.

The technique known as ELISA (enzyme linked immunosorbent assay) can be used to detect and partially identify viruses in tissue extracts. Rather than detecting the RNA of a virus, ELISA works by detecting the type of protein that coats the RNA. Each virus family has a particular type of protein which can be specifically recognised in the assay. The ELISA we used was able to detect the presence of viruses in the potyviridae family. Like the RT-PCR approach, ELISA is a very sensitive procedure, capable of detecting very low levels of virus. We found evidence for viruses in this family both in color-broken and non-broken flowers (Table 2). The absence of a correlation between the presence of potyviruses and presence of color break suggests that color break is not caused by potyviruses.

4.3 *Identification of the types of viruses present in the color-broken tepal extracts by RT-PCR and gene sequencing*

Viruses are very simple organisms. Many of them are just protein-coated RNA. Their occurrence in flower tepals can be revealed by testing for the presence of either their RNA or protein. Reverse transcriptase polymerase chain reaction or RT-PCR is a very powerful and sensitive technique that can detect viruses in tissue based on the presence of viral RNA. If the viral RNA is present, the procedure makes huge numbers of copies of this RNA (cDNA). After the copies are made, their sequence can be determined by passing them through a DNA sequencing machine. Once the researchers have the sequence, they can very quickly identify the organism it belongs to by searching a sequence database publicly available on the internet. For example, a sequence that was made by the RT-PCR procedure from color broken tepals of Twelve Gauge (RC, Foxtan) was:

```
TTAAACGGAATTTTTGGCTGCCAAATCAAGTCTCGTCGTGAGGATCT
GTGTCATCTCACCCACGAGAATCCTTACACCACCGATCCTAAGGGA
GTAGTCGTGGCCACGAACAAGAAGTCATCAACTTGCTGTTGCAAC
ACGGTTGCCCGTGACTCCCACTCAACATCTTTGGGGCCTGACCAT
TCCAGTTGTGAGCGTCTACCTTACCTCCATCACCTCCTTGCCACTG
TTGACCGCGCCAATCTCTTCTGAGTCTCACTAGACATTCAAAGCG
CTCCACATTTTCGAATTCGATGCCTGGTTTCACGCCACCTGTAACT
ACGAGCAGGTGTACAAAGTTTTGGCTATTGGATTCTGCTCTGCGCT
TCTATCTATTGTCTGAGGTCTAA
```

When this sequence was placed into the BlastN search site at the web address www.ncbi.nlm.nih.gov/BLAST/ the sequence showed highest similarity to a sequence present in *Narcissus mosaic virus* (NMV). A portion of the readout is presented in Figure 5.

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>gi|37905677|gb|AY225449.1 Narcissus mosaic virus strain New Zealand,
complete sequence Length=6956 Score = 690 bits (348), Expect = 0.0
Identities = 384/396 (96%), Gaps = 0/396 (0%) Strand=Plus/Plus

seq entered TTAAACGGAATTTTGGCTGCCAAATCAAGTCTCGTCGTGAGGATCTGTGTCATCTCACC 60
|||||
NMV          TTAAACGGAATTTTGGCTGCCAAATCAAGTCTCGTCGTGAGGATCTGTGTCATCTCACC 5451

seq entered CACGAGAATCCTTACACCACCGATCCTAAGGGAGTAGTCGTGGCCCACGAACAAGAAGTC 120
|||||
NMV          CACGAGAATCCTTACACCACCGATCCTAAGGGAGTAGTCGTGGCCCACGAACAAGAAGTC 5511

seq entered ATCAACTTGCTGTTGCAACACGGTTGCCCGTGACTCCCACTCAACATCTTTGGGGCCTG 180
|||||
NMV          ATCAACTTGCTGTTGCAACACGGTTGCCCGTGACTCCCACTCAACACCTTTGGGGCCTG 5571

seq entered ACCATTCCAGTTGTGAGCGTCTACCTTACCTCCATCACCTCCTTGTCCACTGTTGACCGC 240
|||||
NMV          ACCATTCCAGTTGTGAGCGTCTACATTACCTCCATCGCCTCTTTGTCTACTGTCGACCGA 5631

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Figure 5: The sequence of the RT-PCR products showed similarity to the sequence of NMV in the web-based databases.

We initially used the RT-PCR technique to test for the presence of different virus types (potexvirus, carlavirus and potyvirus) in the color-broken and non-broken tepals of Twelve Gauge flowers. Figure 6 shows the initial results obtained using primers (listed in Table 3) that test for the presence of potexviruses. Potexvirus RNA was detected only in the tepals of color-broken flowers. When the RNA sequence was compared with sequences in the internet database it was identified as NMV (Figure 5, Table 3). The results we obtained using primers for the carlavirus and potyvirus groups are also shown in Table 3. Interestingly, although the primers were expected to identify carlaviruses and potyviruses, they also identified NMV, indicating a strong correlation between color break and the presence of NMV.

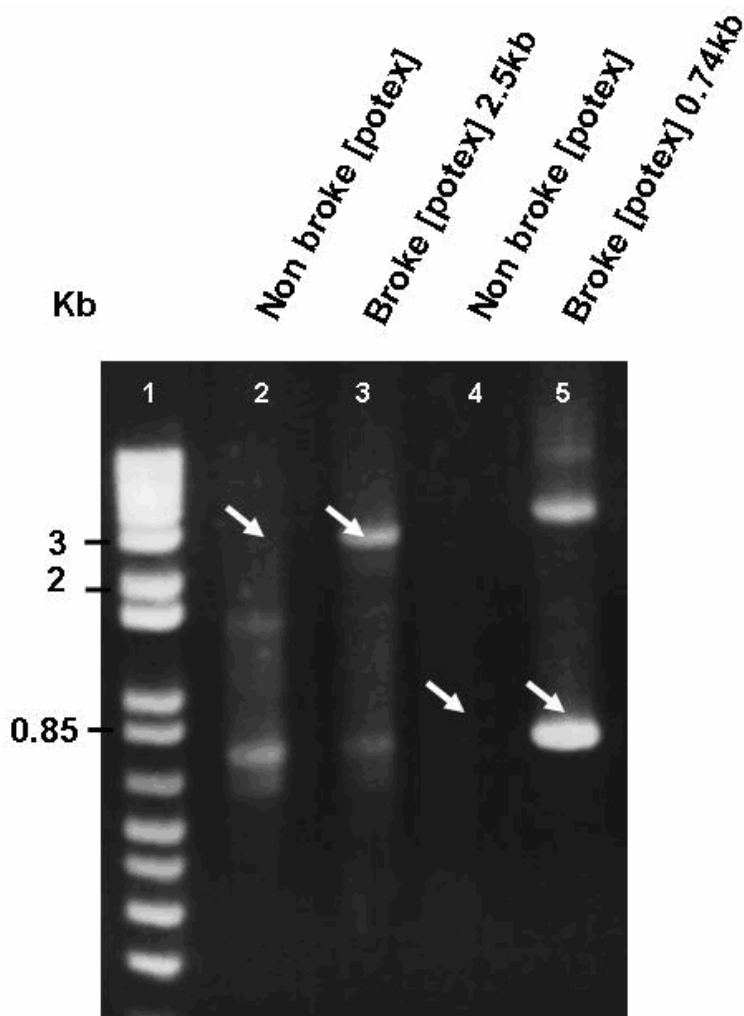


Figure 6: RT-PCR results indicating the presence of viral RNA in the tepals of the color-broken but not the non-broken Twelve Gauge flowers. The presence of a white band of the correct size indicates that viral RNA has been detected. The white arrows indicate where the cDNA copy of the viral RNA of the correct size was expected to be. Note that cDNA of the correct size was only found in tepals of the color-broken flowers.

We repeated the RT-PCR on six new varieties from two additional regions in New Zealand (Nelson and Ashhurst) to confirm that the broken flowers contained greater quantities of virus and to confirm that NMV was the virus predominantly associated with the color-break phenomenon. Figure 7 clearly shows that viral RNA sequences are much more prevalent in the tepals of the color-broken flowers, independent of the regions from which they were collected. It should also be noted that although the potyvirus and carlavirus primer sets identified viral bands sequencing subsequently showed them to be identifying the potyvirus NMV (Table 3). In fact, of the 19 RT-PCR products we sent away for sequencing, 17 of them were NMV sequences and the other two were *Pea streak virus* (PSV) and *Narcissus late seasons yellow virus* (NLSYV) (Table 3).

We then performed a more specific RT-PCR procedure that would only work if NMV or NLSYV was present in the extracts of the flowers. The results are shown in Figure 8. and confirm that the presence of NMV was strongly associated with the occurrence of color break and that the potyvirus NLSYV was not.

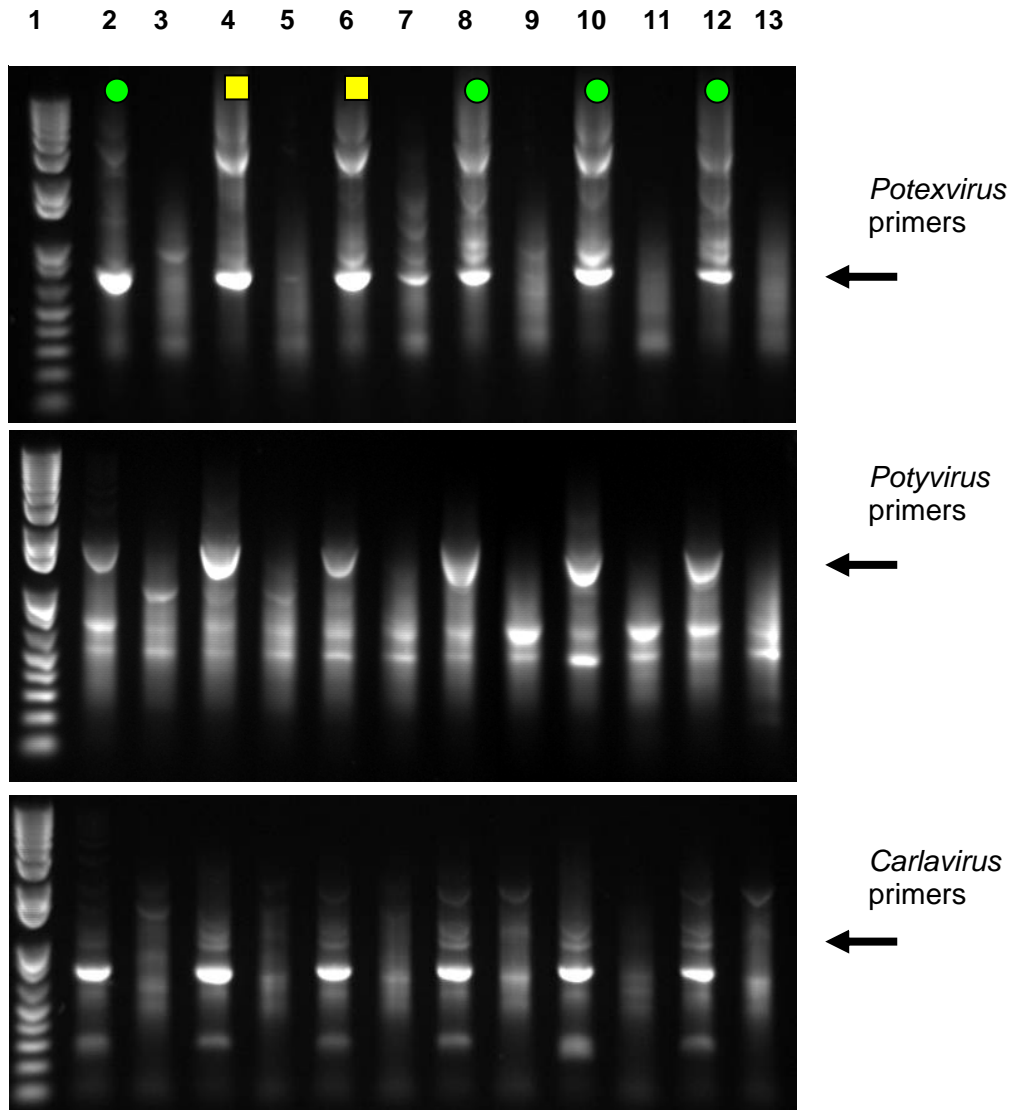


Figure 7: Correlation between presence of color break and presence of virus for flowers in different regions of New Zealand (■Nelson ●Ashhurst) Lanes 2, 4, 6, 8, 10, 12 are from petal extracts of broken flowers. Lanes 3, 5, 7, 9, 11, 13 are from petal extracts of non broken flowers. Flowers examined were Lighthouse Reef lanes 2, 3; Daydream, 4, 5; 20/87 seedling [JAH] 6, 7; Twilight Zone[JRM], 8, 9; Flying Cloud, 10, 11 and Twilight Zone[JAH] 12, 13, 40 cycles of PCR was performed using primers to identify potyviruses, potexviruses and carlaviruses. Arrows indicate the region in the photo where the viral sequences are expected to be. These viral sequence-containing white bands were purified and their sequence was determined and compared with known viral sequences in web-based sequence databases.

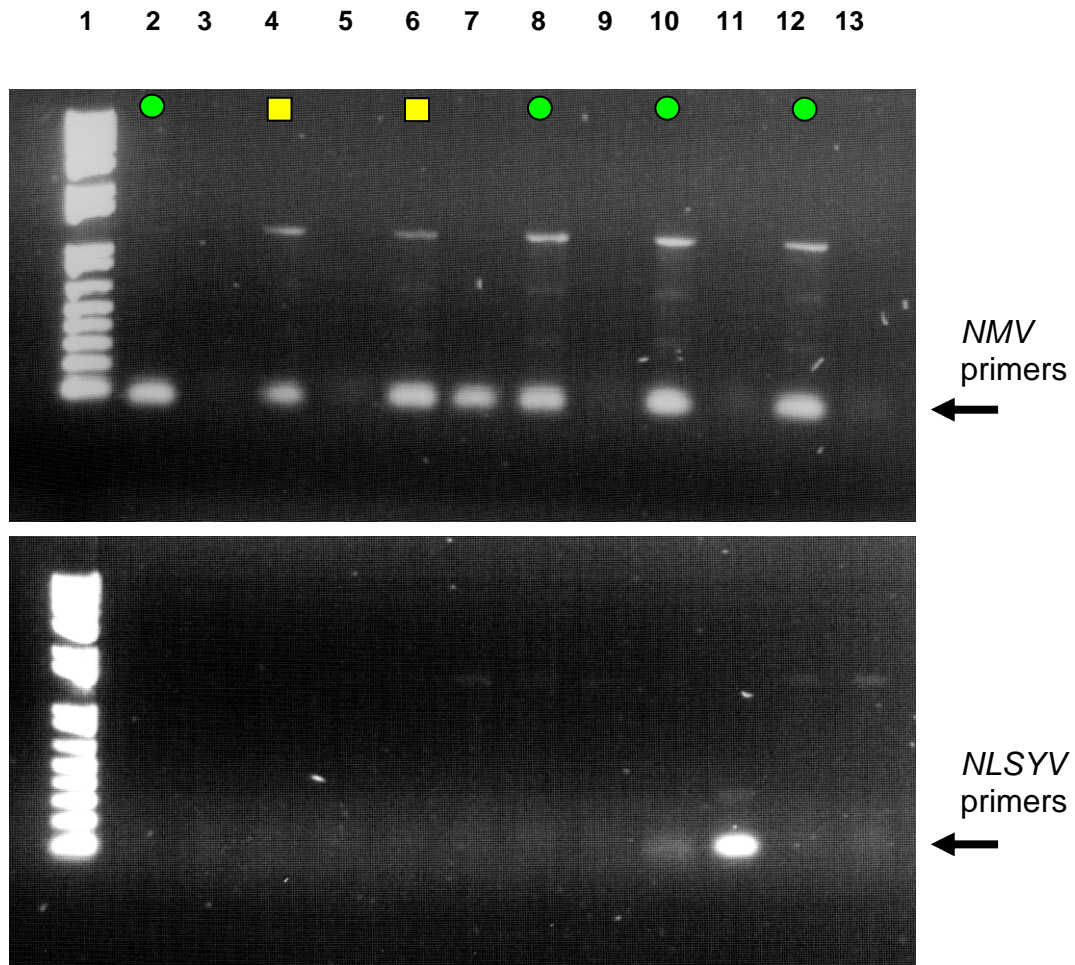


Figure 8: Correlation between presence of color break and presence of NMV but not NLSYV virus for flowers in different regions of New Zealand (■ Nelson ● Ashhurst) Lanes 2, 4, 6, 8, 10, 12 are from petal extracts of broken flowers. Lanes 3, 5, 7, 9, 11, 13 are from petal extracts of non broken flowers. Flowers examined were Lighthouse Reef lanes 2, 3; Daydream, 4, 5; 20/87 seedling [JAH] 6, 7; Twilight Zone[JRM], 8, 9; Flying Cloud, 10, 11 and Twilight Zone[JAH], 40 cycles of PCR was performed using primers to specific to NMV and NLSYV. Arrows indicate the bands that correspond to where NMV and NLSYV sequences were expected to be. These viral sequence-containing white bands were purified, and their sequence determined and compared with known viral sequences present in web-based sequence databases.

Table 3: Viruses identified in color-broken tepal extracts of flowers collected from different regions within New Zealand.

Primer names	Primer sequences	Genus expected	Product size expected/obtained	Sequence results	Genus obtained
Sprimer(S) KS ³ (AS)	GGNAAYAAYAGYGGNCARCC ¹ CGGTACCGATAAGCTTGA	Potyvirus	1.75/1.75	NLSYV (1) NMV (3)	Potyvirus Potexvirus
pCar-1(S) KS ³ (AS)	ATGCCNCTNANNCCNCC ¹ CGGTACCGATAAGCTTGA	Carlavirus	1.8/0.8	NMV (9)	Potexvirus
Potex1RC(S) Potex5(AS)	TCAGTRTTDGCRTCRAARGT ² CAYCARCARGCMAARGAYGA ²	Potexvirus	0.74/0.74	NMV (5) PSV (1)	Potexvirus Carlavirus
pVX(S) KS ³ (AS)	GGNGARGGNCCNACNTT ¹ CGGTACCGATAAGCTTGA	Potexvirus	2.5/2.5	DNS	
NMV(S) NMV(AS)	TGCGCTTCTATCTATTGTCTGAGG GTGGGGAAGGGAATGAATGTTATC	Potexvirus	71	NMV ⁴	
NLSYV(S) NLSYV(AS)	TACGACAGAAGAGAACACGGAGAG ACACAATACTACACGCCCTTACG	Potyvirus	92	NLSYV ⁴	

¹Chen et al. 2002; ²van der Vlugt & Berendsen 2002; ³3'-RACE primer (was attached to oligodT in RT). Underlined primers were used successfully in sequencing reactions to identify viruses. For the degenerate primers mentioned: N = A, G, C or T; Y = T or C; R = A or G. NMV = *Narcissus mosaic virus*, NLSYV = *Narcissus late season yellow virus*, PSV = *Pea streak virus*. DNS = did not sequence. The number of times the particular virus was positively identified by sequencing is indicated ().⁴ These are primers specific for the potexvirus NMV and potyvirus NLSYV.

4.4 *Effect of sap from a color-broken flower on a non-broken flower*

We tested whether we could induce ‘non-broken’ reverse bicolor flowers to ‘break’ by rubbing the sap from a ‘broken’ flower on to the newly emerging daffodil buds. Of the eight buds we inoculated with color-broken sap, six of them subsequently ‘broke’ when they flowered. None of the mock inoculated flowers ‘broke’ (Table 4). A representative photo of the ‘break’ induced by the inoculation is shown in Figure. 9. Interestingly, the flowers of the two seedlings that did not ‘break’ also did not show the reverse bicolor phenotype, i.e. their coronas did not whiten as they aged. We also inoculated the leaves of one bulb of Trumpet Warrior with the tepal extract from a color-broken flower at the daffodil patch of John Hunter. The next year all three flowers from this infected plant color broke whereas the other 26 flowers of this cultivar (except one) in that row did not ‘break’.

We also tested whether a pure isolate of NMV that had been genetically engineered to glow green in the plant (under ultraviolet [UV] light) would cause the daffodils to color break. The virus was able to infect tobacco, as indicated by the appearance of the green color in Figure 10. In the absence of the virus the plant fluoresces red under UV due to the chlorophyll it contains (which in normal light makes the plant look green). We inoculated over 20 daffodil flowers, but none showed green fluorescence under UV or color break. Different isolates of the same strain of virus can show differing abilities to infect and move in plants. We believe that our isolate of NMV is unfortunately unable to infect daffodils.

Table 4: Sap from color-broken flowers cause non-broken flowers to color break. Color broken flowers of Watermusic Honeybird Lighthouse reef and Daydream were crushed in a plastic bag with water and carborundum and the resulting extract rubbed on to the newly emerging floral buds (spathe was slit open to reveal the bud) of previously unbroken reverse bicolor daffodils. The flowers were assessed 23 days later for whether they color broke or not.

Inoculated	Color broke	Mock inoculated	Color broke
8	6	7	0



Figure 9: The color break seen after inoculation of a young floral bud with tepal sap from color-broken flowers.

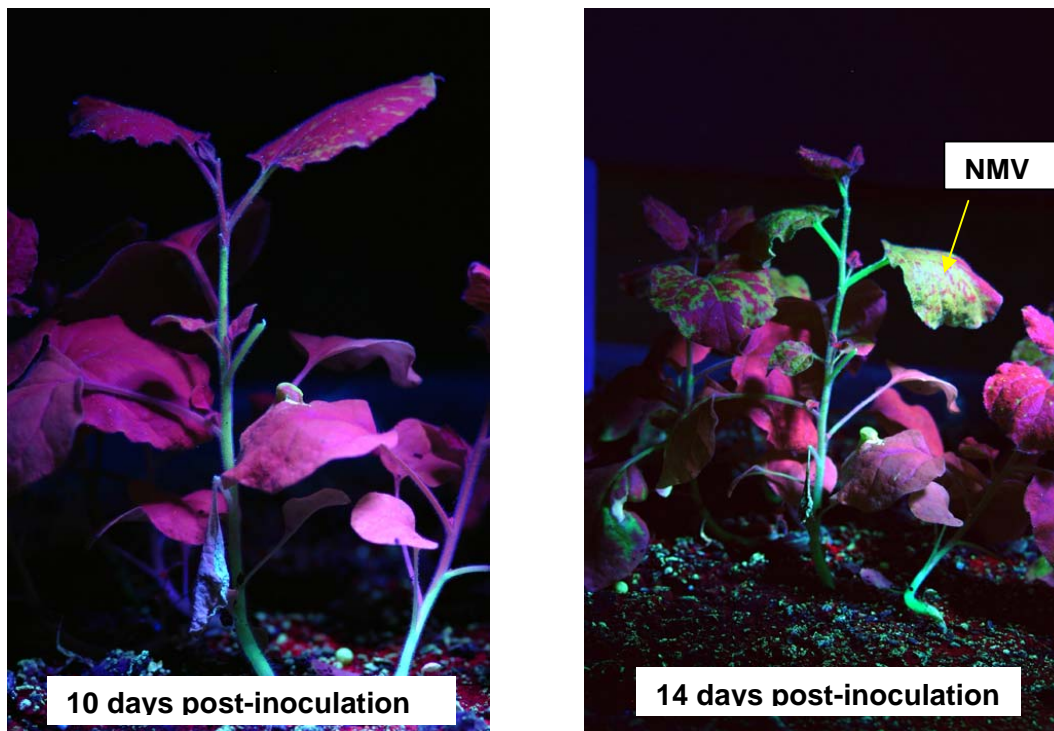


Figure 10: Movement of NMV in tobacco plants. The NMV was made to contain a protein (GFP) that fluoresces green under ultraviolet (UV) light. The green plants normally look red under UV light, which means we can monitor where the virus is in the plant by observing where the green fluorescence is observed (indicated by arrow in photo).

4.5 *Effect of virus on pigment synthesis pathways*

One possibility is that the loss of yellow pigment in reverse bicolor daffodils that occurs in response to viral infection may be due to the tissues' reduced ability to make the mRNA for phytoene synthase.

The enzymes that are required to make lutein (the pigment responsible for yellow color) are shown in blue in Figure 11. We examined whether the loss of lutein in the white tepals was due to a block in a particular step in the pathway that leads to the formation of lutein. We did this using Real Time PCR (Figure 12) to compare the mRNA levels for each of the enzymes in the white and yellow tissue from the color-broken flower of Trumpet Warrior. We also examined whether the white sectors contained higher levels of the NMV virus.

Our results suggest that the loss of yellow color is not due to the loss in mRNA for a particular enzyme needed for lutein formation. We did not find higher levels of virus in the white than the yellow tissue.

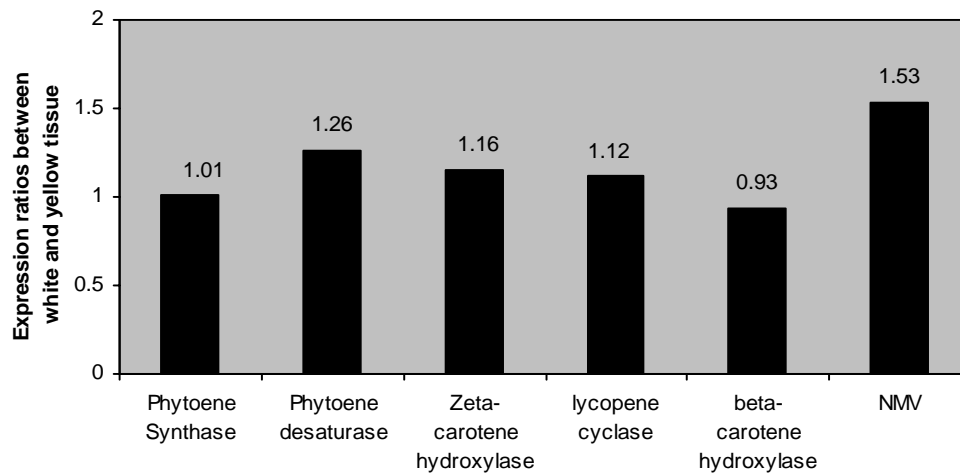


Figure 12: Comparative abundance of the transcripts that encode the colour forming enzymes in white and yellow tissue of colour-broken Trumpet Warrior flowers. White and yellow sectors of a 'broken' Trumpet Warrior' flower were dissected, and the amount of mRNA for each of the enzymes examined by Real Time PCR. The levels of mRNA were normalised to actin. An expression ratio of 1 means there was no difference in the levels of mRNA between the yellow and white patches in the flower. Data are the mean of two individual reverse transcriptase reactions done in duplicate.

5 Discussion

Our findings clearly indicate that color-broken reverse bicolor daffodils are full of virus, despite their foliage looking healthy. Viruses typically fall into two different categories, elongate or spherical. The electron microscopy images showed that elongated (rod) viruses of predominantly 550 nm in length were associated with the occurrence of color break in the reverse bicolor flowers and viruses of ca. 640 nm were associated with color break in the non reverse bicolor Culfind. All the color-broken flowers examined from the three regions in New Zealand had large numbers of these rod-type viruses in their tepals whereas their non broken counterparts did not. Often it was very difficult to see a single virus in the tepals of the non-broken flowers. More viruses were observed in the tepals of the plant than in the vegetative structures such as leaves and bulbs, suggesting that viruses multiply more rapidly once reaching the perianth tissue. Their apparent poor ability to multiply in the vegetative tissues may explain why it can take such a long time between inoculation of the daffodil leaves and the appearance of viral symptoms of infection. It has been reported to take 17 months for the inoculated leaves of King Alfred and Sulphur plants to show the inconspicuous mosaic pattern of NMV infection (Brunt 1966).

Color break in other flowers such as tulips is caused by a family of rod-type viruses known as potyviruses (Dekker et al. 1993). We used an antibody to test for the presence of potyviruses in our color-broken extracts and found that the occurrence of potyvirus did not correlate with the incidence of color break.

The size of the viral particles that were most prevalent in the color broken extracts of the reverse bicolor daffodils was 550 nm and this was most similar to that of a potyvirus known as NMV. The properties of this virus and its spread within daffodils have previously been documented (Brunt 1966).

NMV was found to be widespread in British crops of trumpet, large-cupped and double daffodils, but not in *Narcissus jonquilla* or *N. tazetta* and was able to be isolated from 27 of the 47 commercial daffodil stocks tested (Actaea, Aranjuez, Brunswick, Carlton, Cheerfulness, Fortune, Golden Harvest, Inglescombe, King Alfred, Magnificence, Minister Talma, Mount Hood, Royal Bride, and Zero). The plants where NMV was the only virus detected were either symptomless or showed inconspicuous mosaic symptoms at the base of their leaves. Notably, none of the plants exhibited color break but interestingly none were reverse bicolors (Brunt 1966).

Viruses can also tentatively be identified by the types of lesions they make on indicator plants. All 10 extracts from the color-broken reverse bicolor flowers from the three different regions in New Zealand produced red ringspots on the leaves of *Gomphrena globosa* whereas none of the non-broken flowers did. Red ringspots are characteristic of NMV infection (Brunt 1966), which, as mentioned above, was not previously reported to cause daffodils to color break.

The RT-PCR procedure enabled us to identify more specifically the types of viruses present in the color-broken extracts. This powerful technique clearly demonstrated that high quantities of viral RNA were present in the tepals that 'broke' and that the genetic sequence of the virus matched that of NMV. We appear therefore to have overwhelming evidence for an association of NMV with color break in the reverse bicolor daffodils.

The strong association of virus with the occurrence of color break is not proof that viruses are the cause of the 'break'. It could, for instance, be argued that a genetic component that causes the flower to 'break' also enables the viruses to multiply more readily in the perianth tissue. To prove causation we set up experiments to see whether we could make non-broken flowers 'break'. We did this by rubbing the sap of color-broken flowers on to the newly emerged floral bud of a previously 'non-broken' flower. We decided to inoculate directly on to the tepal tissue of the newly emerged bud rather than the leaves because according to the findings of Brunt (1966) it would take 17 months before we would be able to assess our inoculations. Our results suggest that we were successful in making previously unbroken flowers 'break' and that by directly inoculating on to the floral tissue we can reduce the time required for assessment from 17 months to 23 days.

We think it is likely that NMV is the cause of the color break in the reverse bicolor daffodils. We believe that the absence of color break observed by Brunt (1966) was because none of the flowers he examined were of the reverse bicolor type, and it is the interaction between NMV and the genetics

of this type of flower that is necessary for the 'break' to occur. However, we still, at this stage, cannot rule out the possibility that NMV requires interaction with other viruses to effect the 'break'.

Our initial attempts to test whether a pure NMV isolate could cause color break did not work. Crop & Food Research had previously obtained an NMV isolate that had been genetically engineered to contain a protein that made it fluoresce green under UV light (NMV:GFP). We were hoping that this would enable us to observe directly whether the virus was preferentially localised to the white 'broken' tissue by observing the white and yellow tissue under UV and seeing if the white tissue preferentially fluoresced green. Unfortunately our strain of NMV was not able to infect daffodils. Differences in infectivity of virus strains is commonly observed. In the future we hope to obtain a strain that will infect daffodils.

In the absence of a pure isolate of NMV:GFP that would infect daffodils we used Real Time PCR to see if we could detect greater levels of NMV in the white sectors versus the yellow sectors. Although a cruder analysis due to difficulty in dissecting the tissue, our Real Time PCR results suggest that the patchiness of the color break is not due to increased localised accumulation of the NMV in the white sectors of the tepals. This result does, however, have to be interpreted with some caution since it is possible that the virus could have initially been localised to the white tissue but moved into the yellow sectors prior to our harvest and analysis (a reason why the NMV:GFP would have been so informative).

Determining how NMV causes the tissue to 'break' into patches of white remains a fascinating area of research. Perhaps it could be argued that the break is not specific to NMV at all, but only requires the presence of large quantities of any type of virus. In this regard, it was interesting to find that the 'breaking' of the non reverse bicolor *Culfind* was associated with large quantities of a virus clearly different to NMV in the flower (Table 1). However, despite this, the virus interactions with the genetics of the reverse bicolor must be important, because otherwise color break would presumably be occurring more frequently in all types of daffodil since NMV is commonly found in floral tissue of commercial (non reverse bicolor) daffodils (Brunt 1966).

It is tempting to speculate that NMV causes color break by prematurely activating in reverse bicolors the normal developmental pathway that causes the corona to change from yellow to white as it ages. We hypothesised that the flower might do this by switching off the expression of one of the enzymes required for lutein (yellow) formation. Therefore we investigated whether the virus affected the levels of the mRNA that are needed to make the enzymes for lutein formation in the white color broken sectors. Our preliminary results (Figure 12) suggest that the virus is not causing the tepals to 'break' by interfering with the mRNA accumulation for any of the biosynthetic enzymes that we studied.

6 *Conclusions*

6.1 *We have discovered that:*

- Color-broken reverse bicolor flowers contained high levels of virus.
- NMV was the virus that was most common in the color-broken flowers.
- Color break could be passed onto non-broken reverse bicolor flowers by rubbing the floral sap of the color-broken flowers onto the leaves or newly emerged floral tissue of the 'non-broken' flowers.
- The virus does not cause the color-break by preventing production of the mRNA that encodes the enzymes necessary for yellow color of the tepals.

6.2 *This means that:*

- Color-broken reverse bicolor flowers should be removed to prevent viral spread to healthy flowers.
- When removing the plants try not to damage them as the released sap could potentially spread the virus.

6.3 *Future research challenges are:*

- To confirm that NMV alone can cause color break.
- To investigate how NMV causes color break.
- To determine what conditions enable NMV to spread within the daffodil.

7 *References*

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