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Patterns of mixed virus infections: a 3-year study of symptomatic cereal and grass hosts in Australia

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ABSTRACT

Context. Yellow dwarf viruses (YDVs) form a complex of economically important pathogens that can significantly reduce grain yield in cereals. Mixed infections, or infection with two or more YDV species, can be particularly damaging. **Aims.** We aimed to examine the proportion of single and multiple virus infections present in symptomatic cereal and grass plants in Victoria, south-eastern Australia. **Methods.** Over 3 years (2020–2022), symptomatic cereal and grass plants from within and around cereal fields in Victoria, Australia were individually tested using tissue-blot immunoassay (TBIA) for barley yellow dwarf virus PAV, barley yellow dwarf virus MAV, cereal yellow dwarf virus RPV, wheat streak mosaic virus, and with a generic TBIA test that can detect multiple luteovirus and/or polerovirus species. **Key results.** Across 2020–2021, 34% of virus-positive plants were infected with multiple YDV species. The proportion of mixed infections was similar in each individual year. However, higher proportions of wheat (*Triticum aestivum*, 47%) and wild oat (*Avena fatua*, 36%) plants were infected with multiple YDV species compared to barley (*Hordeum vulgare*, 8%) and brome grass (*Bromus* spp.,17%). **Conclusions.** The proportion of virus-positive plants infected with multiple YDV species found was almost four times higher than previously reported in a similar study in Victoria, Australia in 1985. The proportion of plants infected with multiple YDV species varied more with host type than between individual years. **Implications.** These findings demonstrate the complex epidemiology of these damaging viruses, and the challenges associated with developing virus-resistant cereal cultivars, while also highlighting the importance of regular surveillance over multiple years.

Keywords: aphid-transmitted viruses, barley (*Hordeum vulgare*), cereals, epidemiology, oats (*Avena sativa*), surveillance, viruses of plants, wheat (*Triticum aestivum*).

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Introduction

Cereals such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oat (*Avena sativa*), and triticale (*Triticum* sp × *Secale cereale*) are a critical human and animal food source worldwide. In Australia, cereals are the largest grain commodity by volume, with an average annual production of 52 million tonnes during 2020–2022, compared to 6.6 million tonnes of canola and 3.9 million tonnes of pulses (ABARES 2023). Yellow dwarf viruses (YDVs) are damaging pathogens of cereals that cause significant yield and quality losses worldwide. Yield losses of up to 84% in wheat and 64% in barley have been caused by artificial inoculation with a barley yellow dwarf virus (BYDV) isolate in south-eastern Australia (Nancarrow *et al.* 2021), where YDV incidences of up to 58% have been observed in individual cereal fields (Nancarrow *et al.* 2018). Grain yield is reduced with every 1% increase in YDV infection (Gill 1980; Smith and Sward 1982; McKirdy *et al.* 2002; Nancarrow *et al.* 2021). Virus infection can be particularly damaging when more than one virus species is present within a plant; however, the relationships and interactions between different virus species, their aphid vectors and their plant hosts can be both complex and specific (Baltenberger *et al.* 1987; Wen *et al.* 1991; Haber 1995; Leclercq-Le Quillec *et al.* 2000; Hall and Little 2013; Lacroix *et al.* 2014; Malmstrom *et al.* 2017).

YDVs have a wide host range and can infect more than 150 species belonging to the Poaceae family (D'Arcy 1995), including cultivated cereal hosts such as wheat, barley, oat, maize (*Zea mays*), rice (*Oryza sativa*) and many wild grass species (Oswald and Houston 1951; Watson and Mulligan 1960; Guy *et al.* 1987). YDVs are transmitted from plant to plant by aphids (Hemiptera: Aphididae) (Oswald and Houston 1951). The most common and important vectors of YDVs in south-eastern Australia are *Rhopalosiphum padi* (bird cherry-oat aphid), *Rhopalosiphum maidis* (corn aphid), and *Metopolophium dirhodum* (rose grain aphid) (Waterhouse and Helms 1985; Guy *et al.* 1987; Sward and Lister 1988; McKirdy and Jones 1996; Thackray *et al.* 2005). Typical symptoms of YDV infection include stunted plant growth and yellow and/or red leaf discolouration (Oswald and Houston 1951).

Currently, at least 12 YDV related species that can infect cereals or grasses are formally recognised by the International Committee on Taxonomy of Viruses (ICTV) (Sõmera *et al.* 2021*a*; Walker *et al.* 2021). This includes luteoviruses (family *Tombusiviridae*), such as barley yellow dwarf virus PAV (BYDV PAV, *Luteovirus pavhordei*), barley yellow dwarf virus PAS (BYDV PAS, *Luteovirus pashordei*) and barley yellow dwarf virus MAV (BYDV MAV, *Luteovirus mavhordei*), and poleroviruses (family *Solemoviridae*) such as cereal yellow dwarf virus RPV (CYDV RPV, *Polerovirus CYDVRPV*), cereal yellow dwarf virus RPS (CYDV RPS, *Polerovirus CYDVRPS*), maize yellow dwarf virus RMV (MYDV RMV, *Polerovirus MYDVRMV*) and barley virus G (BVG, *Polerovirus BVG*).

While BYDV was reported in Australia for the first time in 1957 (Smith 1957), the YDV species BYDV PAV, BYDV MAV, CYDV RPV, and MYDV RMV were distinguished from each other serologically and reported in Australia during the 1980s (Waterhouse and Helms 1985; Guy *et al.* 1986; Guy *et al.* 1987; Sward and Lister 1987, 1988) and are regularly found in Australia (McKirdy and Jones 1993; Hawkes and Jones 2005; Milgate *et al.* 2016; Trębicki *et al.* 2017; Nancarrow *et al.* 2018). BVG, CYDV RPS, and BYDV PAS have recently been reported in Australia based on molecular identification (Nancarrow *et al.* 2019, 2023, 2024); however, their incidence and geographic distribution in Australia is yet to be determined.

Wheat streak mosaic virus (WSMV) (*Tritimovirus tritici*, family *Potyviridae*) also occurs with high incidence, and has resulted in substantial yield losses, in wheat fields in some regions of Australia (Jones and Burges 2006; Coutts *et al.* 2008*b*). WSMV is transmitted by the wheat curl mite (WCM, *Aceria tosichella* Keifer) (Slykhuis 1955; Singh *et al.* 2018), which is present throughout the major grain growing regions of Australia (Halliday and Knihinicki 2004; Carew *et al.* 2009). WSMV was found in Australia for the first time in 2002 in the Australian Capital Territory (ACT) (Ellis *et al.* 2003*a*); and subsequently detected in other Australian states, including Victoria (Ellis *et al.* 2003*b*; Jones *et al.* 2005; Coutts *et al.* 2008*a*). More recently, WSMV was found with BVG in volunteer cereals in Victoria between growing seasons (Nancarrow *et al.* 2019); however, little information is available about its occurrence more broadly in commercial cereal fields in the region.

The primary aim of this study was to examine the proportion of single and multiple virus species in individual symptomatic cereal and grass plants within and around commercial cereal fields. This information is critical for the development of more effective virus-resistant cereal cultivars and enhances our understanding of cereal virus epidemiology. Data on the occurrence and distribution of BYDV PAV, BYDV MAV, CYDV RPV, and WSMV in south-eastern Australia is also reported.

Materials and methods

Field locations

Plant samples were collected from around and within the town of Horsham, Victoria, Australia (36°43′0.0012″S, 142°11′59.9892″E) (Fig. 1). The long-term (1998–2022) annual mean maximum temperature and rainfall, measured at the Horsham aerodrome weather station (79100), are 22.1°C and 378 mm, respectively. The annual mean maximum temperature was below average during each year of this study, while annual rainfall was close to average during 2020 and 2021 but well above average during 2022 (Table 1) (www.bom.gov.au).

Collection of plant material

During October–November in 2020, 2021, and 2022, samples were collected from 1313 symptomatic plants from 42 locations in the major cereal production region of south-eastern Australia. Samples were collected from symptomatic cereal plants from 40 cereal fields (wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.)) and from symptomatic grass and/or volunteer cereal plants from 31 of the 40 cereal fields, as well as a roadside and a riverside location (Table 2; see Supplementary Table S1). Targeted symptoms included yellow and/or red-purple leaf discolouration and stunted plant growth, which are common indicators of virus infection in cereals and grasses (Fig. 2). While wheat and barley fields were primarily targeted for sample collection, symptomatic wild oat (*Avena fatua* L.) and brome grass (*Bromus* spp.) plants were regularly observed either within or around (i.e. near to the fence line) the targeted fields. Samples were collected from these four hosts in each year of the study, along with volunteer wheat plants from one barley field in 2020, and volunteer barley plants from two wheat fields in 2021. Samples were also collected from symptomatic plants from oat fields in 2021 and 2022, and symptomatic phalaris (*Phalaris* spp.) and rye grass (*Lolium perenne* L.) plants from along the fence lines of three wheat fields during 2022. Additionally, samples were collected from symptomatic brome grass plants from alongside the Wimmera River in the Horsham

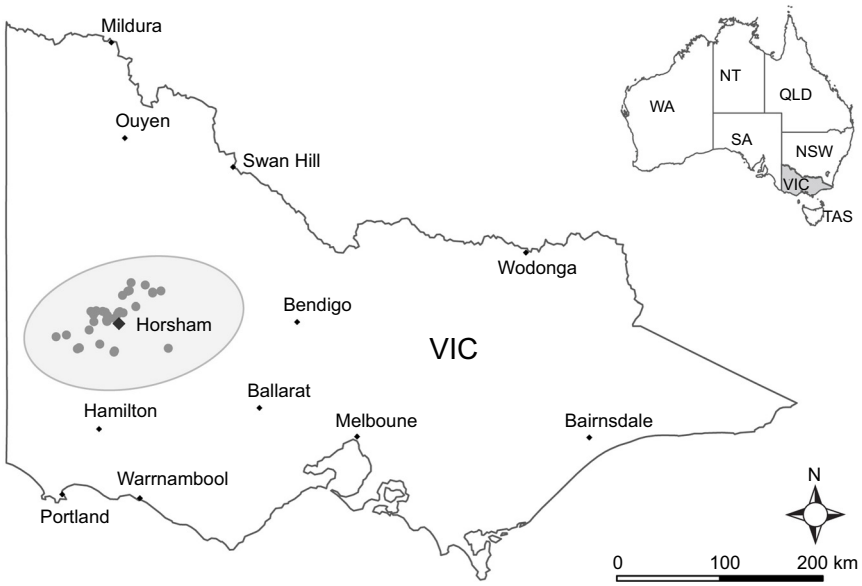

Fig. 1. Map of the 42 locations (grey points) in Victoria (Vic), Australia, where symptomatic plants were sampled during 2020, 2021, and 2022. The inset map of Australia shows the location of the following Australian states and territories: Western Australia (WA), Northern Territory (NT), South Australia (SA), Queensland (Qld), New South Wales (NSW), Victoria (Vic), and Tasmania (Tas).

Table 1. Summary of the 3-monthly and annual rainfall (mm) and mean maximum temperature (°C) during 2019–2022, and the long-term (1998–2022) mean rainfall (mm) and temperature (°C), for Horsham, Victoria, Australia.

	Year	Jan–Mar	Apr–Jun	Jul–Sep	Oct–Dec	Annual
Rainfall (mm)	2019	15	125	93	40	273
	2020	77	122	106	94	399
	2021	58	94	134	81	366
	2022	111	119	230	209	670
Long-term mean rainfall (mm)	1998–2022	64	103	122	90	378
Temperature (°C)	2019	31.1	18.6	15.1	26.3	22.8
	2020	28.4	17.1	15.5	25.3	21.6
	2021	28.4	18.3	15.8	24.7	21.8
	2022	29.8	17.7	14.8	22.5	21.2
Long-term mean temperature (°C)	1998–2022	29.5	18.1	15.6	25.4	22.1

township and from a roadside near Horsham during 2022. Each sampling location, which will be referred to as a field from this point on, was recorded using GPS coordinates. A single tiller was collected from each symptomatic plant, placed into a plastic bag (one bag per field), and stored at 4°C until processed.

Virus testing by tissue blot immunoassay

Each individual tiller was cut with a sterile scalpel blade and sap from the tiller was immediately blotted onto a nitrocellulose membrane (Whatman Protran, 0.45 μm, ThermoFisher Scientific, USA). Sap from each tiller was blotted onto five individual membranes, then one membrane per virus test was used. Membranes were tested by tissue blot immunoassay (TBIA) using polyclonal antibodies for barley yellow dwarf virus PAV (BYDV PAV, Agdia 27500), barley yellow dwarf virus MAV (BYDV MAV, DSMZ AS-0540), cereal yellow dwarf virus RPV (CYDV RPV, DSMZ AS-0539) and wheat streak mosaic virus (WSMV, DSMZ AS-0544), and a monoclonal antibody

Table 2. The number of wheat, barley, and oat fields that were sampled, and the number of samples collected from symptomatic plants of each host type in individual years (2020, 2021, and 2022) and overall (2020–2022).

Host	2020		2021		2022		Overall	
	Number of fields	Number of samples	Number of fields	Number of samples	Number of fields	Number of samples	Number of fields	Number of samples
Wheat (*Triticum aestivum*)	7	261	10	130	11	239	28	620
Barley (*Hordeum vulgare*)	5	179	4	63	1	18	10	260
Oat (*Avena sativa*)	0	0	1	17	1	30	2	47
Brome grass (*Bromus* spp.)	7	21	12	43	6	85	25	149
Wild oat (*Avena fatua*)	8	103	7	54	7	52	22	209
Phalaris (*Phalaris* spp.)	0	0	0	0	3	11	3	11
Rye grass (*Lolium perenne*)	0	0	0	0	3	7	3	7

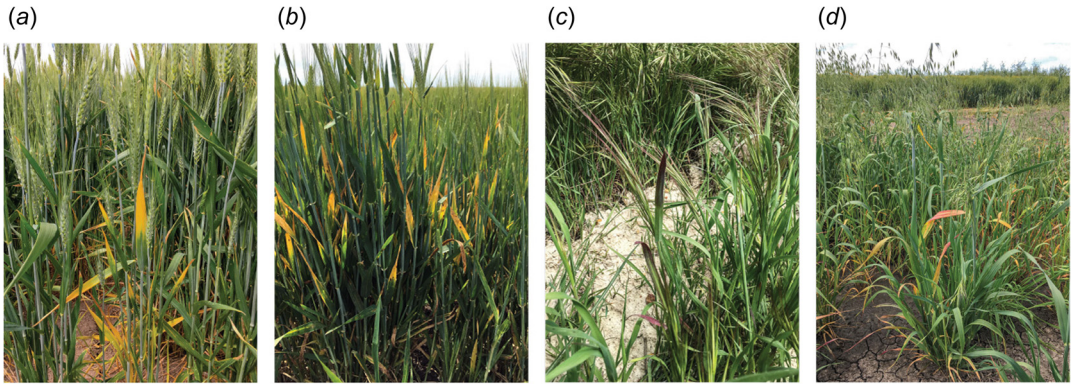

Fig. 2. Virus symptoms in (*a*) wheat, (*b*) barley, (*c*) brome grass, and (*d*) wild oat that were targeted for sample collection during 2020, 2021, and 2022.

(DSMZ AS-0227/1) that can detect a range of luteovirus and/or polerovirus species, as described previously (Trębicki *et al.* 2017; Nancarrow *et al.* 2019). After testing was completed in 2021, the BYDV MAV antibody was no longer available; therefore testing for BYDV MAV was not included in 2022. After processing, membranes were examined using a dissecting microscope, where a plant sample was considered positive if dark purple staining was observed in the phloem, or negative if dark purple staining was not present.

Data analysis

The proportion of virus-positive plants within the population being evaluated was calculated using the formula [number of infected plants/number of plants tested] × 100. The co-occurrence of virus species was estimated using the Jaccard index of similarity. Sum totals of virus positive plant samples were calculated using GenStat 22nd Edition (VSN International Ltd, Hemel Hempstead, UK). R ver. 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria) was used to calculate Jaccard similarity index coefficients and to generate maps, graphs and area proportional Euler diagrams.

Results

Only virus-positive plant samples were used to examine the proportions of symptomatic plants infected with single or multiple viruses. A relatively large proportion (46%) of the 639 virus-positive samples across 2020–2021 was positive with more than one of the five virus tests, while a small proportion (3%) was positive with all four of the BYDV PAV, BYDV MAV, CYDV RPV, and generic tests (Fig. 3, Table 3*a*). Of the 639 virus-positive samples, all of which included testing for BYDV MAV, approximately half (52%) contained single infections of BYDV PAV; a third (33%) were positive with the generic test; 22% were positive for CYDV RPV, and a quarter (26%) were positive with both the BYDV PAV and generic tests (Fig. 3). The proportion of plant samples infected

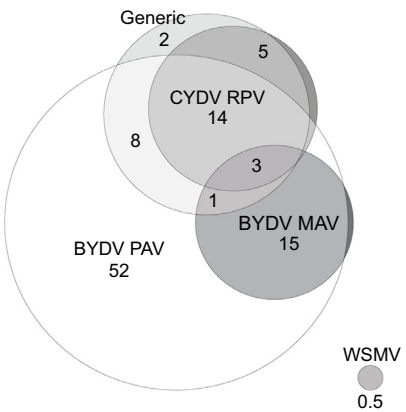

Fig. 3. A Euler diagram showing the proportion (%) of virus-positive plants infected with single and multiple cereal viruses overall across 2020 and 2021 (n = −639). Proportions of less than 1% are not labelled (excluding WSMV). 'Generic' refers to the generic test that can detect multiple luteovirus and/or polerovirus species.

with both BYDV PAV and BYDV MAV (19%) was similar to the proportion infected with both BYDV PAV and CYDV RPV (17%) (Fig. 3, Table 4). Additionally, 34% of the plant samples that were positive with at least one of the three YDV specific tests (BYDV PAV, BYDV MAV, and CYDV RPV) across 2020 and 2021 were infected with multiple YDV species (Table 3*b*). Interestingly, only one of the 117 BYDV MAV-positive plant samples detected during this study occurred as a single infection.

The proportion of symptomatic plants infected with multiple cereal viruses was similar between years, with a similar proportion of plant samples positive with the CYDV RPV and/or generic tests in 2020 (35%), 2021 (30%), and 2022 (37%) (Fig. 4). However, a somewhat higher proportion of those samples was infected with BYDV PAV as well in 2020 (30%) and 2022 (26%) than 2021 (15%). Additionally, fewer of the total 1313 plant samples were positive with at least one virus test in 2021 (53%) than 2020 (84%) and 2022 (79%), although WSMV was only detected in 2021 (Table 5).

Table 3. The proportion (%) of virus-positive symptomatic plants from 2020 and 2021 that were positive with single or multiple virus tests (*a*) when all five virus tests (BYDV PAV, BYDV MAV, CYDV RPV, WSMV, and the generic test) were examined, (*b*) when only the three specific YDV tests (BYDV PAV, BYDV MAV, and CYDV RPV) were examined, and (*c*) in individual host types.

		Percentage (%) of positive samples		
		2020	2021	Overall (2020–2021)
(*a*)	Number of viruses			
	1	53	58	54
	2	27	30	28
	3	17	9	15
	4	3	2	3
	5	0	0	0
	Number of samples	476	163	639
	Number of fields	12	14	26
(*b*)	Number of viruses			
	1	64	73	66
	2	33	25	31
	3	3	3	3
	Number of samples	468	155	623
	Number of fields	12	14	26

		Barley	Brome grass	Wheat	Wild oat	Overall (2020–2021)
(*c*)	Number of viruses					
	1	92	83	53	65	66
	2	8	17	42	34	31
	3	0	0	5	2	3
	Number of samples	159	24	311	113	607
	Number of fields	7	13	16	12	25

Furthermore, fewer symptoms of virus infection were observed and therefore, fewer samples were collected, during 2021 than 2020 and 2022 (Table 5). In each year, a higher proportion of plant samples was infected with BYDV PAV than any other virus (Table 5), with 51% and 53% of samples infected with only BYDV PAV during 2020 and 2021, respectively (Fig. 4).

The proportion of symptomatic plants infected with multiple cereal viruses varied with host (Fig. 5, Table 4). In each year, virus symptoms were observed in wheat, barley, brome grass, and wild oat. Therefore, the majority of plant samples were collected from these hosts. A higher proportion of single infections of BYDV PAV was observed in barley (75%) and brome grass (69%) than wheat (45%) and wild oat (36%), while a higher proportion of wheat plants (34%) were infected with both BYDV PAV and BYDV MAV than wild oat (6%), barley (1%), and brome grass (0%) (Table 4*c*, Fig. 5). Additionally, a higher proportion of wild oat (29%) plants were positive with both BYDV PAV and CYDV RPV than wheat (17%), brome grass (15%), and barley (7%), and a higher proportion of virus-infected barley (79%) and brome grass (77%) plants were positive with only one of the five virus tests compared to wheat (47%) and wild oat (39%) (Table 4*c*, Fig. 5). Furthermore, the proportion of plants that tested positive with the generic test that were also positive for CYDV RPV was lower in barley (32%) than wheat (80%), brome grass (74%), and wild oat (68%) (Table 4*c*, Fig. 5). When analysing only the plant samples that were positive with at least one of the BYDV PAV, BYDV MAV, and CYDV RPV tests across 2020 and 2021, a higher proportion of wheat (47%) and wild oat (36%) than barley (8%) and brome grass (17%) plants were infected with more than one of the three YDV species (Table 3*c*). While only two oat fields were sampled across the 3 years, a higher proportion of oat plants were positive with the CYDV RPV (45%) and generic (60%) tests than any other host (Table 5). Although BYDV PAV and CYDV RPV were detected in wheat, barley, wild oat, brome

Table 4. Co-occurrence of virus species (calculated using the Jaccard index of similarity) in virus-positive plants, (*a*) overall (2020–2021), (*b*) in individual years (2020 and 2021), and (*c*) in individual host types (barley, brome grass, wheat, and wild oat).

		Number of samples	Number of fields	Co-infection (virus species per test)					
				BYDV PAV and BYDV MAV	BYDV PAV and CYDV RPV	BYDV PAV and generic test	BYDV MAV and CYDV RPV	BYDV MAV and generic test	CYDV RPV and generic test
(*a*)	Overall (2020–2021)	620	26	0.20	0.17	0.26	0.08	0.08	0.63
(*b*)	2020	476	12	0.19	0.20	0.29	0.08	0.08	0.61
	2021	144	14	0.21	0.10	0.13	0.08	0.07	0.73
(*c*)	Barley	165	8	0	0.08	0.20	0	0	0.33
	Brome grass	26	14	0	0.17	0.15	0	0	0.75
	Wheat	312	16	0.35	0.18	0.23	0.10	0.11	0.73
	Wild oat	117	12	0.07	0.31	0.44	0.04	0.04	0.68

Co-occurrence coefficients greater than 0.30 are shaded in grey.

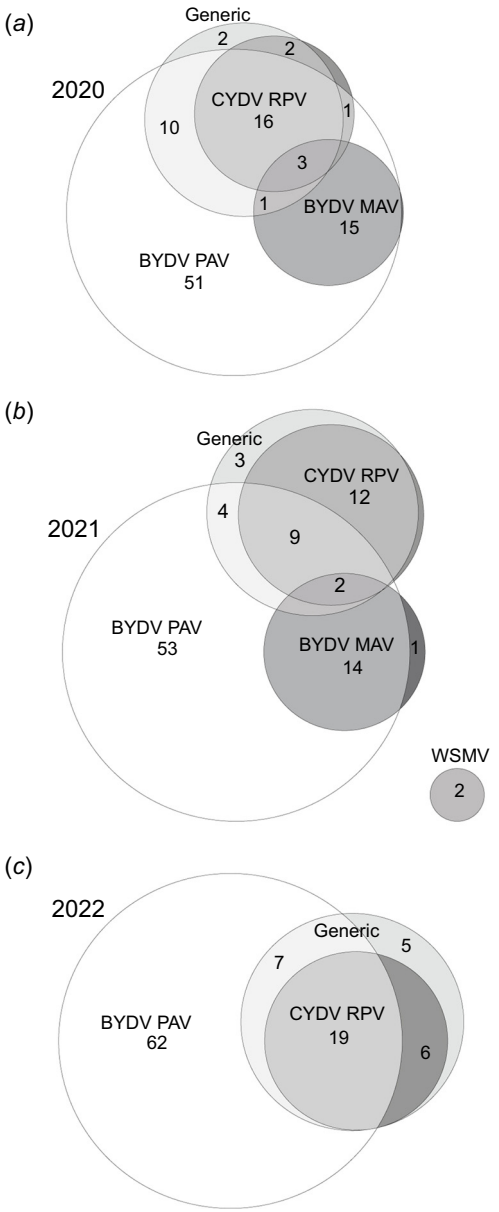

Fig. 4. Euler diagrams showing the proportion (%) of virus-positive plants infected with single and multiple cereal viruses during (*a*) 2020 (n = 476), (*b*) 2021 (n = 163), and (*c*) 2022 (n = 348). Proportions of less than 1% are not labelled. Plant samples collected during 2022 were not tested for BYDV MAV. 'Generic' refers to the generic test that can detect multiple luteovirus and/or polerovirus species.

grass, and oat, BYDV MAV was most often detected in wheat (29%), but was only found at low levels, or was not found at all, in the other four hosts (Table 5). Additionally, while some of the 11 samples collected from symptomatic phalaris plants were positive for CYDV RPV (36%) and with the generic test (45%), none were positive for BYDV PAV (Table 5). In contrast, none of the seven symptomatic rye grass plants were positive with the CYDV RPV or generic tests, although 29% were positive for BYDV PAV (Table 5).

Only three of the 1313 samples (less than 1%) were positive for WSMV; these three samples were all collected from the same wheat field on the same day in 2021 and were not positive with any of the other four virus tests. However, viruses other than WSMV were also detected at this field (Field 19, Supplementary Fig. S1). Although 75% of the 1313 symptomatic plants were positive with at least one virus test, the remaining 25% were not positive with any test, despite displaying symptoms of virus infection at the time of collection (Table 5*a*). At least one virus was detected at each of the 42 fields examined, with BYDV PAV detected at all fields except for one (98%), CYDV RPV detected at 88% of fields, WSMV detected at 2% of fields, while samples from 90% of fields were positive with the generic test; BYDV MAV was detected at 63% of the 27 fields that were examined (Supplementary Fig. S1). Almost 10% of the 339 plant samples that were positive using the generic test did not test positive with any of the other four virus tests. While almost all (98%) of the 228 CYDV RPV-positive plant samples were also positive with the generic test, only 29% of the 902 BYDV PAV-positive samples, and 19% of the 117 BYDV MAV-positive samples, were also positive with the generic test.

BYDV PAV was the most commonly detected species in the 1313 plant samples that were tested; 69% were positive for BYDV PAV, 17% were positive for CYDV RPV, 26% were positive using the generic test while 13% of the 871 plant samples that were tested for BYDV MAV were positive (Table 4). Furthermore, of the 987 virus-positive plant samples collected across the 3 years, 91% were positive for BYDV PAV, 23% for CYDV RPV, <1% for WSMV and 34% were positive using the generic TBIA test. BYDV MAV was detected in 18% of the 639 virus-positive plant samples that were tested for that virus species.

Discussion

The proportion of single and multiple virus species present in 1313 symptomatic cereal and grass plants from south-eastern Australia during 2020–2022 was examined and quantified. Overall, 34% of the virus-positive plants from 2020–2021 were infected with more than one of the three YDV species (BYDV PAV, BYDV MAV, and CYDV RPV) included in the study. This proportion is almost four times higher than the 9% previously reported by Sward and Lister (1988) when they tested 200 individual cereal and grass plants in one of the few similar published surveys conducted in the region (i.e. Victoria, south-eastern Australia) during 1985. Furthermore, Sward and Lister (1988) reported that mixed infections of BYDV PAV and CYDV RPV were the most common, while two mixed infection combinations were similarly common in our study: (1) BYDV PAV and CYDV RPV; and (2) BYDV PAV and BYDV MAV. Infection with two or more YDV species can have a particularly damaging effect on grain yield (Baltenberger *et al.* 1987) and can also challenge the effectiveness and

Table 5. The proportion (%) of the 1313 symptomatic plants that were positive with each virus test, (*a*) overall (2020–2021), (*b*) in individual years (2020 and 2021), and (*c*) in individual host types.

		Number of samples	Number of fields	Number of samples tested for BYDV MAV	Proportion (%) of virus-positive plants							
					BYDV PAV	CYDV RPV	Generic	Average	BYDV MAV	WSMV	Positive with at least one virus test	No virus detected
(*a*)	Overall (2020–2022)	1313	42	871	69	17	26	37	13	<1	75	25
(*b*)	2020	564	12	564	81	18	28	42	16	0	84	16
	2021	307	15	307	44	13	16	24	9	1	53	47
	2022	442	15	0	70	20	30	40	N/A	0	79	21
(*c*)	Wheat	620	29	381	80	18	24	41	29	<1	83	17
	Barley	270	12	252	63	6	17	29	0	0	66	34
	Brome grass	149	25	64	59	9	14	27	0	0	64	36
	Wild oat	209	22	157	61	29	43	44	4	0	75	25
	Oat	47	2	17	38	45	60	48	0	0	77	23
	Phalaris	11	3	0	0	36	45	27	N/A	0	45	55
	Rye grass	7	3	0	29	0	0	10	N/A	0	29	71

The average includes BYDV PAV, CYDV RPV and the generic test and excludes BYDV MAV and WSMV. Plant samples collected during 2022 were not tested for BYDV MAV. 'Generic' refers to the generic test that can detect multiple luteovirus and/or polerovirus species.

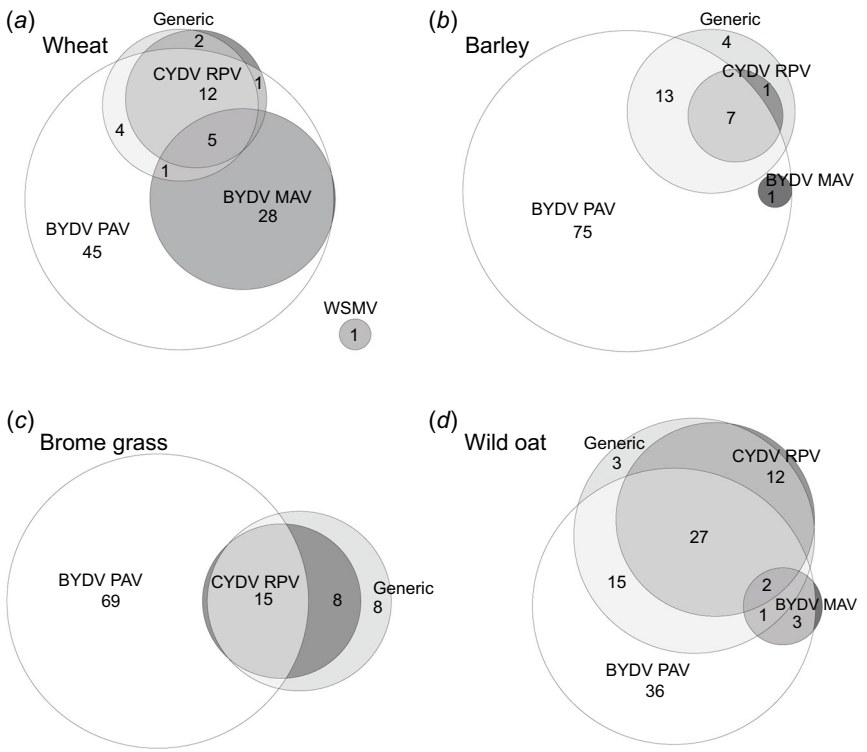

Fig. 5. Euler diagrams showing the proportions (%) of virus-positive (*a*) wheat ($n = 315$), (*b*) barley ($n = 165$), (*c*) brome grass ($n = 26$), and (*d*) wild oat ($n = 117$) plants infected with single and multiple cereal viruses across 2020 and 2021. Proportions of less than 1% are not labelled. 'Generic' refers to the generic test that can detect multiple luteovirus and/or polerovirus species.

development of virus-resistant cereal cultivars. These marked differences between the results of our study and those of Sward and Lister (1988) demonstrate the importance of regularly conducting epidemiological studies such as these. Relying on outdated or incomplete data can lead to misleading conclusions about the epidemiology and importance of these viruses.

Despite differences in environmental conditions during the 3 years, and the presence of more virus symptoms in some years compared to others, the relative proportions of almost all mixed infection combinations were similar in each year of the study. In contrast, host differences related to particular YDV species were much more pronounced. For example, a higher proportion of oat plants were positive with the CYDV

RPV and generic tests compared to wheat and barley. Host differences were also observed by Pereira (1993) in Portugal in 1988 where a higher proportion of mixed infections of BYDV PAV and CYDV RPV was observed in oats than in wheat, triticale or barley. Similar to this study, Pereira (1993) also found a relatively high proportion of wheat plants were infected with both BYDV PAV and BYDV MAV. Eagling *et al.* (1989) showed that BYDV PAV was prevalent in several cultivars of perennial ryegrass while BYDV MAV and CYDV RPV were found in some cultivars but not others. When Guy *et al.* (1987) tested poaceous plants from Tasmania, Australia, for BYDV PAV and CYDV RPV, they found that a higher proportion of plants from the subfamilies Arundinoideae and Panicoideae were infected with CYDV RPV than BYDV PAV, while a higher proportion of plants from the subfamily Pooideae were infected with BYDV PAV than CYDV RPV. Given these differences, the varying proportions of hosts collected in each year of this study may also help to explain why fewer plants were positive with all three of the BYDV PAV, CYDV RPV, and generic tests in 2021 than 2020 and 2022. The occurrence and distribution of YDV species is directly related to the populations and activity of the aphids that transmit them. Previously published transmission studies from Australia showed that *R. padi* was an efficient vector of BYDV PAV, BYDV MAV and CYDV RPV, *R. maidis* was an efficient vector of MYDV RMV, while *M. dirhodum* was a moderately efficient vector of BYDV PAV and BYDV MAV but did not transmit CYDV RPV or MYDV RMV (Waterhouse and Helms 1985; Sward and Lister 1988). The occurrence and prevalence of aphid species were not monitored throughout this study but will be the focus of future research of YDV disease in south-eastern Australia.

In contrast to the YDVs, and to the relatively high incidence of WSMV found in volunteer cereals at one particular field in Victoria outside of the growing season in 2018 (Nancarrow *et al.* 2019), WSMV was found in only three of the 1313 plants that were tested. Yield losses caused by WSMV infection can range from minimal up to almost 100% (Shahwan and Hill 1984; Edwards and McMullen 1987). In Australia, WSMV infection has caused particularly significant damage to irrigated wheat fields and early-sown wheat in New South Wales (Jones and Burges 2006; Dwyer *et al.* 2007; Coutts *et al.* 2008*b*) and has been detected with high incidence in commercial wheat fields in Western Australia (Coutts *et al.* 2008*a*). Although WSMV was reported as being present in Victoria for the first time in 2003 (Ellis *et al.* 2003*b*), little information has been published about its occurrence and distribution in commercial cereal fields in the region since it was first reported. The results of this study suggest that although WSMV is present, YDVs were the more important cereal viruses in the fields examined.

One quarter of the symptomatic plants did not test positive for any virus. It is possible that these were virus-infected but the virus concentration was too low to be detected by the TBIA tests, which are typically less sensitive than molecular methods. It is also possible that they were positive for YDV species or isolates that were not detected by the antibodies present in the TBIA assays used in this study. This possibility was illustrated by Malmstrom *et al.* (2017) when they observed that a CYDV RPS isolate collected from a non-crop perennial grass was not reliably detected by a serological test that targeted CYDV RPV, even though CYDV RPV and CYDV RPS are closely related. Given that CYDV RPS was recently reported for the first time in Australia (Nancarrow *et al.* 2023), it is possible that some of the plants that did not test positive for any virus were infected with a CYDV RPS isolate that was not detected by the CYDV RPV TBIA test. However, since it is difficult to diagnose plants based on symptoms alone, it is also possible that the plants that did not test positive for any virus were not infected with any virus. The symptoms observed throughout the study varied in severity. Some symptoms were obviously related to virus infection while others were less so. Moreover, more samples were collected from plants with 'less obvious' symptoms during 2021 when fewer symptomatic plants were available. Regardless, these 'virus-negative' plant samples require further investigation.

Almost 10% of the samples that were positive with the generic test were not positive with any of the three YDV specific tests, indicating that other YDV species are circulating in the surveyed area. Additionally, only 33% of the virus-positive samples were detected with the generic test. Previously, this generic TBIA test detected BVG in cereal samples collected from south-eastern Australia (Nancarrow *et al.* 2019); therefore, it is likely that some plants that were positive with the generic test were infected with BVG. Interestingly, given that the proportion of plants that tested positive with the generic test that also tested positive for CYDV RPV was lower in barley than wheat, brome grass, and wild oat, it is also likely that a higher proportion of barley plants were infected with BVG than the other hosts. Furthermore, 98% of the CYDV RPV-positive plants were detected by the generic test. In contrast, excluding the CYDV RPV-positive plants, only a small proportion of the plants that were positive with the generic test were also positive for BYDV PAV and/or BYDV MAV, thus the generic test did not reliably detect these two YDV species. Therefore, although the generic test detects more than one YDV species, it is still not entirely clear which species it detects; this will only be clarified when molecular analysis is conducted on the samples collected during this study.

Across the 3 years, more plants were infected with BYDV PAV than any other virus species. This result is similar to many other published YDV surveys that have also shown that BYDV PAV is the most commonly detected YDV species in Australia (Guy *et al.* 1987; Sward and Lister 1987, 1988; Jones *et al.* 1990; Henry *et al.* 1992; McKirdy and Jones 1993; Trębicki *et al.* 2017; Nancarrow *et al.* 2018). In the survey conducted by Sward and Lister (1988), 79% of samples were positive for BYDV PAV, 11% were positive for BYDV MAV, 19% were positive for CYDV RPV, and 2% were positive for MYDV RMV. Excluding detections of MYDV RMV, which

was not included in our study, these findings were similar to those reported here. Additionally, at least one virus species was detected at all fields, and almost half of the virus-infected plants were positive with more than one virus test, further confirming that YDVs are widespread in south-eastern Australia. However, since symptomatic plants were targeted and plant samples were not randomly collected, this data cannot be considered representative of virus incidence more generally within a field or region.

Although serological tests such as TBIA and enzyme-linked immunosorbent assay (ELISA) are important tools for high-throughput virus testing and survey purposes, there are limitations with their use: these assays are unable to differentiate between some of the more closely related YDV species, they are less sensitive than molecular tests, are not available for the detection of all YDV species, antibodies from different sources can have different levels of cross-reactivity with other YDV species, and it can be difficult to continuously source some specific antibodies, which was demonstrated by the discontinued production of the BYDV MAV antibody that was used in the first 2 years of this study. However, in addition to the ease and efficiency with which serological tests can examine large volumes of samples, these methods are particularly effective initial screening tools because of their ability to detect a broad range of YDV isolates and their inability to distinguish between closely related YDV species. With the rapid increase in the description of new YDV species using molecular assays and viral genome sequencing, the required species resolution provided by the available serological assays is becoming a limiting factor, and these assays should be used in conjunction with more specific testing methods when identification of YDVs to species level is required.

With advances in molecular diagnostic technologies such as high-throughput sequencing (HTS), recent studies using these methods have shown that YDV diversity is greater than previously thought (Jo *et al.* 2018; Hodge *et al.* 2020; Welgemoed *et al.* 2020; Sõmera *et al.* 2021*b*). This is also the case in Australia, where polymerase chain reaction (PCR) and HTS were used to demonstrate for the first time that CYDV RPS and BYDV PAS are also present in Australia (Nancarrow *et al.* 2023, 2024). Therefore, with the previously mentioned limitations of virus testing using TBIA, it is likely that some of the plants identified with the CYDV RPV TBIA test were infected with CYDV RPS, and some of the plants identified with the BYDV PAV TBIA test were infected with BYDV PAS. Several studies have shown that when serological testing indicated that BYDV PAV was present, further molecular testing showed that BYDV PAS was often also present, and that it was usually more prevalent than BYDV PAV (Robertson and French 2007; Kundu 2008; Kundu *et al.* 2009; Sõmera *et al.* 2021*b*). Additionally, several studies have shown that BYDV PAS can cause more severe damage than BYDV PAV and that some sources of virus resistance that are effective against BYDV PAV can be overcome by BYDV PAS (Chay *et al.* 1996; Bencharki *et al.* 1999; Robertson and French 2007). However, Jarošová *et al.* (2013) found that BYDV PAV was more damaging than BYDV PAS in the Czech Republic, which further demonstrates the complex epidemiology of these viruses. Given these findings and the recent detection of BYDV PAS in Australia, further work is needed to determine the distribution, prevalence and impact of BYDV PAS in cereals in Australia. While BYDV PAV has been the main focus of screening for BYDV resistance in Australia (Zhou *et al.* 2015; Choudhury *et al.* 2018; Choudhury *et al.* 2019; Hu *et al.* 2019), the presence of these more recently reported YDV species, such as BYDV PAS, in Australia suggests that the epidemiology and management of these viruses in Australia is more complex than it is currently thought to be.

Supplementary material

Supplementary material is available online.

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Data availability. The data that support this study are available in the article and accompanying online supplementary material.

Conflicts of interest. The authors declare no conflicts of interest.

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