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Ryegrass resistance to glyphosate and amitrole is becoming common in New Zealand vineyards

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Abstract The prevalence of herbicide resistance in ryegrass (*Lolium* spp.) in the wine-growing regions in New Zealand is poorly understood. Cases of glyphosate, glufosinate and amitrole-resistant ryegrass were documented in a few vineyards in New Zealand in 2013, but there have been no regional surveys for resistance. To address this knowledge gap, 106 vineyards were visited across the important New Zealand wine-growing regions of Marlborough and Waipara in late February 2021, and Hawke's Bay and Gisborne in late February 2022, and seed samples from individual plants at each site surviving weed-control measures were collected. Ryegrass was found in more South Island (68%) than North Island (20%) vineyards. These seeds, and those from a susceptible ryegrass population were sown in marked rows into trays (10-20 seeds per herbicide) and grown in a glasshouse. When seedlings reached the 3-4 leaf stage, trays were sprayed at the highest recommended label rate of glyphosate. Samples with enough seed were also screened against additional herbicides, amitrole, glufosinate or clethodim. The results indicated 39% of the surveyed vineyards contained glyphosate-resistant ryegrass, with cases detected across all regions, including 58% of vineyards in Marlborough. Eleven of the 27 Marlborough vineyards screened contained amitrole-resistant ryegrass; six samples were also resistant to glyphosate. However, glufosinate and clethodim were still effective against ryegrass at the sites tested. Considering the levels of herbicide resistance to ryegrass observed in this study, growers should explore alternative weed-suppression measures, including tilling, cover-crops, grazing, mowing and the use of herbicides with different modes of action.

Keywords weeds, herbicide resistance, wine, pesticide, *Lolium perenne, Lolium multiflorum*

INTRODUCTION

The Group-9 herbicide glyphosate is the most widely used in vineyards in New Zealand (Dastgheib & Frampton 2000). Its use has led to the repeated selection of resistant ryegrass (*Lolium* spp.) biotypes in vineyards in Australia, France, Greece, Italy, Portugal, South Africa and the United States (Heap 2022). It is also known that frequent spraying with glyphosate in New Zealand vineyards has favoured resistant ryegrass biotypes and naturally glyphosate-tolerant broadleaved weeds like *Malva* and *Epilobium* species (Dastgheib & Frampton 2000, Ghanizadeh et al. 2013). Vine growers have access to alternative herbicides with six other modes of action for weed control in vineyards but choosing between herbicides creates a number of trade-offs, including cost, specificity, restrictive pre-harvest withholding periods, rules regarding timing of application, number of applications per season, notification and record-keeping (New Zealand Winegrowers unpublished[a]).

Populations of glyphosate-resistant ryegrass (*Lolium multiflorum* Lam. and *Lolium perenne* L.) were documented from a few vineyards in the Nelson and Marlborough regions of New Zealand in early in the 2010s, although detection was via ad-hoc reporting (Ghanizadeh et al. 2013). These populations had apparently also evolved resistance to herbicides with different modes of action, amitrole (Group 34) and glufosinate (Group 10) (Ghanizadeh et al. 2015a).

[a] New Zealand Winegrowers Members-only Report. *Vineyard Spray Schedule 2020/2021*. https://www.nzwine.com

Ghanizadeh et al. (2015c, 2016) considered that a mix of target site and non-target site mechanisms were causing the glyphosate resistance. Some glyphosate-resistant populations that had a non-target site resistance mechanism became susceptible when sprayed under cooler winter conditions (Ghanizadeh et al. 2015a).

Recent surveys in the arable sector have revealed higher than expected rates of herbicide resistance, with 48% of farms having resistant weeds, though weeds resistant to glyphosate were *not* recorded in the randomly selected arable farms tested (Buddenhagen et al. 2021). New Zealand vineyards were identified as a priority sector for herbicide resistance surveys (Buddenhagen et al. 2019) due to the limited knowledge of herbicide resistance there. Therefore, the main goal of this study was to detect glyphosate-resistant ryegrass in vineyards and, for the first time, estimate the proportion of vineyards with resistant ryegrass (prevalence) in the most important wine-growing areas in New Zealand. Resistance to herbicides with other modes of action amitrole (Group 34), glufosinate (Group 10) or clethodim (Group 1) was also tested for sites where ryegrass seeds were available in sufficient quantities. Documenting the prevalence of herbicide resistance should facilitate increased grower awareness about this issue and can be used as a starting point for the adoption of mitigation measures, as it has for the arable sector (Espig et al. 2022).

MATERIALS AND METHODS

Vineyard selection

A list of 692 New Zealand vineyards maintained by the Bragato Research Institute (BRI) was used as a basis for the survey. This list was randomised, and growers were called in random order to obtain permission to carry out weed and herbicide-resistance surveys. On the South Island, 12 randomly selected vineyards were located in Waipara and 38 randomly selected sites were located in the Marlborough region. On the North Island, another 30 vineyards were randomly selected in Gisborne and Hawke's Bay and the owners agreed to participate. BRI also sent a notice to growers asking for volunteer survey participants in the Marlborough region and owners of 26 vineyards volunteered (self-selected).

In total, the owners of 106 vineyards around the country allowed us to collect weed seeds from their property for this study (Fig 1). This range of vineyards meant that the sampling area was representative of the largest wine-growing areas in New Zealand (i.e., those containing 90% of the vineyard hectares in the country (New Zealand Winegrowers 2022).

Ryegrass seed collection

The goals of this study were to: (a) detect any resistance in each vineyard; and (b) estimate prevalence across vineyards within a region (proportion with resistance). Therefore, growers were asked to point out any weedy areas or areas suspected of having resistance. Search areas on each vineyard were approximately 100 m long. Four adjacent inter-rows under vines at each site were sprayed by the owner with one of the industry permitted herbicides: glyphosate; amitrole; glufosinate; or clethodim, presumably at the recommended rates (New Zealand Winegrowers unpublished[a]). Seeds from up to seven mature ryegrass plants that had survived herbicide treatment were collected before grape harvest in late February 2021 on the South Island and late February 2022 on the North Island. The interval between the last spray and seed collection varied and was not known to the authors. The number of mother plants sampled was determined by abundance; not all vineyards had ryegrass survivors in sprayed areas. Seed from individual mother plants were bagged separately, not bulked. The collected seed was stored at 5 °C until it was planted for resistance testing. Most seeds were *Lolium perenne* but *Lolium multiflorum* or probable hybrid seeds were also collected at some sites.

Herbicide-resistance screening

The 'tray' method of Buddenhagen et al. (2021) was used to detect cases of resistance. Seed samples (10-20 seeds per sample) from each herbicide-resistant mother plant were sown into one of six tagged rows or lanes in a (22 cm x 35 cm x 5 cm) tray filled with commercial potting mix. Seedlings were grown in temperature-controlled glasshouse (18-24 °C). The susceptible control is described in Ghanizadeh et al. (2016) and known to be susceptible to all the herbicides used in this study. This population originates from a commercial seed source and has been used as a susceptible standard in several studies. Herbicide-resistant control populations were also planted into a row on each tray if a previously studied source population was available (see Buddenhagen et al. 2021). Thus, a tray could have four or five field samples and one (susceptible) or two (one susceptible and one resistant) control populations.

South Island seeds were planted in mid July 2021 and North Island seed in mid May 2022. When plants were at the 3-4 leaf stage (one tiller), they were counted and then sprayed using a moving-belt sprayer fitted with a single TeeJet TT11002 fan nozzle at 200 kPa, positioned 440 mm above the top of the trays and calibrated to apply 200 L ha^{-1} of glyphosate 1440 g.ai.ha^{-1} (trade name Crucial and the surfactant Pulse at 0.1%, Nufarm). This rate is regarded as a "discriminating" dose (sensu Beckie et al. 2000), i.e., one that can be used to separate resistant from susceptible biotypes. The highest recommended "label" rates for *L. perenne* are often higher than rates recommended for *L. multiflorum* so all seed was considered to be *L. perenne* for the purposes of this study.

Mortality of seedlings was assessed after 4 weeks when susceptible controls had died. However, a previous study (Ghanizadeh et al. 2015b) had revealed that plants with a non-target site mechanism of glyphosate resistance became susceptible under cold conditions. Therefore, in the current study, plants from South Island seed were kept out-doors for more than a week and resprayed using the moving-belt sprayer when they had 1-3 tillers (4-12 leaves). Spraying occurred on a cool morning after a night of sub-10 °C temperatures (4 August 2021). Sprayed plants were returned to the cool outdoor location and mortality reassessed at an appropriate time 3-4 weeks later. A sample or vineyard was defined as resistant when one or more seedling plants survived spraying at the label rates and defined as susceptible when there were zero survivors. Any samples where less than 4 seedlings germinated were omitted – regardless of survival rates. For example, one Gisborne sample had only three seedlings germinate and, although they survived, we designated this as "untested". However, other samples from the same vineyard were resistant so we still counted the vineyard as resistant. If any of the susceptible controls in a particular tray survived spraying then that tray was omitted from the results.

Sufficient seed was available from some South Island sites (but no North Island ones) to test additional herbicides in the following order of priority (using the same calibrated sprayer described above for glyphosate): amitrole 4000 g.ai.ha^{-1} (Weedazol, Nufarm); glufosinate-ammonium 1500 g.ai.ha^{-1} (Buster + 0.5% Actiwett, BASF); or clethodim 120 g.ai.ha^{-1} (Sequence + Bonza 0.5%, Nufarm). Clethodim was included because it is a Group-1 herbicide registered for vineyards and commonly recommended as a control measure for glyphosate-resistant plants.

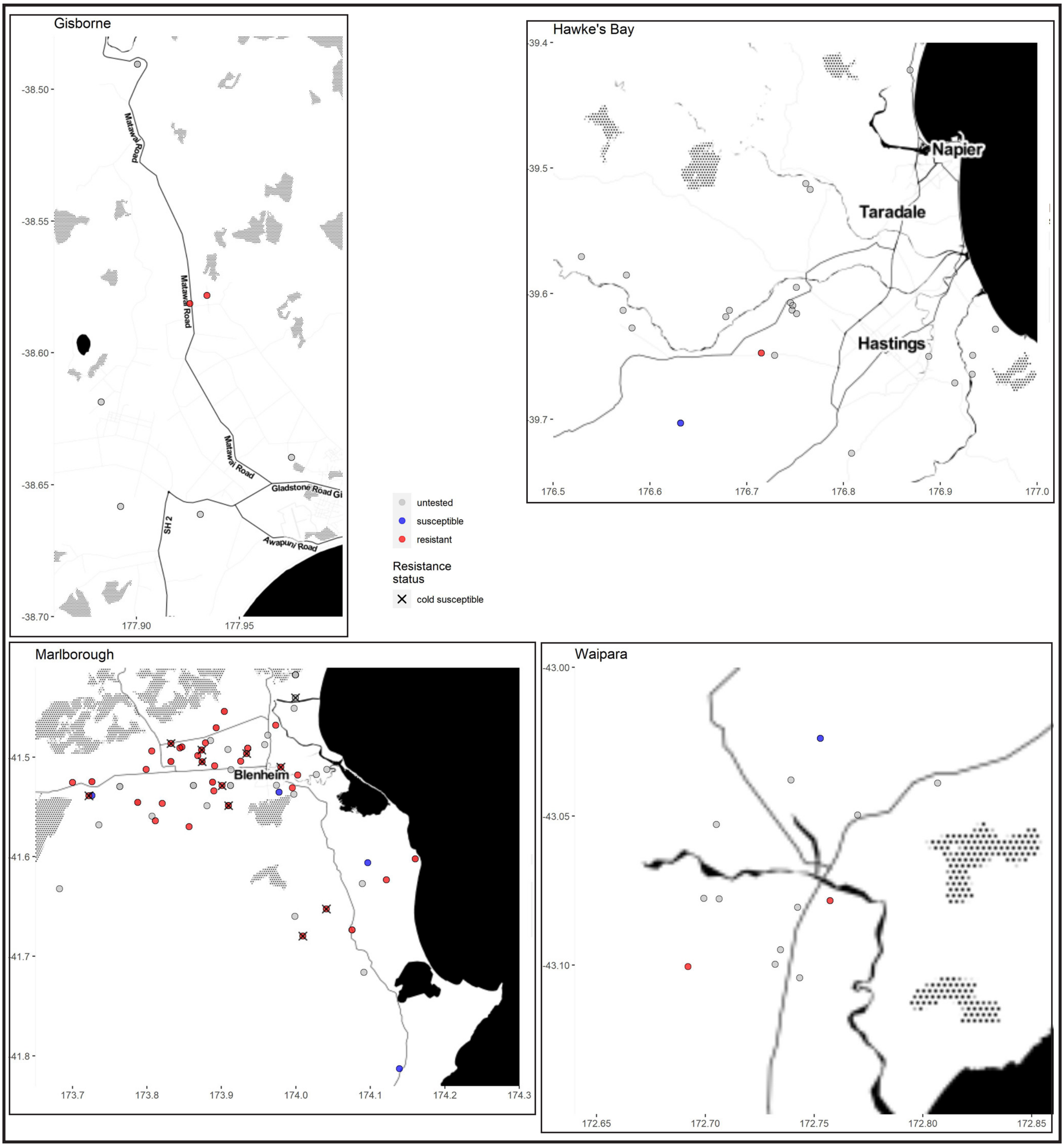

Figure 1 Vineyards with red dots contained seeding ryegrass (*Lolium* spp.) plants, the seeds of which produced seedlings that survived being sprayed with glyphosate. Blue dots represent farms with susceptible populations, red dots represent where resistant populations (see key). Crosses indicate cases where glyphosate resistance was lost under cold conditions. Gray circles indicate locations where ryegrass plants were not found or did not germinate and so were untested. Latitude and longitude are in decimal degrees.

Table 1 Counts of vineyards where ryegrass samples produced seedlings with different resistance statuses to glyphosate, as determined from glasshouse-based glyphosate trials for vineyards that had ryegrass plants with mature seed prior to grape harvest. Note for vineyards counts: In some vineyards ryegrass was not present (No ryegrass) or had poor or no germination – these are designated "Untested". Glyphosate application rates are specified in the Methods section. The number of samples relates to the number of samples found in the vineyards with this status. All seedlings from the susceptible control population in each tray died.

Region	Spray	No. of vineyards			No. Seed Samples		No. Seedlings Treated		No. Seedlings Surviving	
		(No ryegrass) Untested	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
Gisborne	Glyphosate	(4) 1	0	2	2	5	11	39	0	12
Hawke's Bay	Glyphosate	(21) 0	1	1	4	2	28	11	0	3
Marlborough	Glyphosate	(16) 7	4	37	20	75	135	539	0	219
Waipara	Glyphosate	(9) 0	1	2	3	3	17	17	0	3

[1] A sample was designated "resistant" when four or more seedlings from a single sample were treated and one or more survived.

Table 2 For South Island samples, additional herbicides were tested if enough ryegrass seed was present at a particular site. Counts of vineyards, samples, and seedlings with different resistance statuses are shown, as determined from glasshouse-based trials for vineyards that had ryegrass plants with mature seed prior to grape harvest. Herbicide application rates are specified in the Methods section. The number of samples relates to the number of samples found in the vineyards with this status. All seedlings from the susceptible control population in each tray died.

Region	Spray	No. Vineyards		No. Samples		No. Seedlings Treated		No. Seedlings Surviving	
		Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
Marlborough	Amitrole	17	11	53	12	385	82	0	26
Marlborough	Clethodim	31	0	64	0	458	0	0	0
Marlborough	Glufosinate	32	0	67	0	443	0	0	0
Waipara	Amitrole	2	0	6	0	40	0	0	0
Waipara	Clethodim	2	0	7	0	46	0	0	0
Waipara	Glufosinate	2	0	7	0	47	0	0	0

[1] A sample is designated "resistant" when four or more seedlings from a single sample are treated and one or more survive.

Data were analysed in R by creating pivot tables and tallies of the numbers of vineyards, samples, seedlings treated, and seedlings surviving using the tidyverse package (Tables 1 and 2), and maps were made using the ggmap package (Kahle & Wickham 2013, Wickham et al. 2019, R Core Team 2021). The prop.test function in base R was used to estimate the binomial confidence interval for the proportion of randomly selected vineyards with resistant plants. The prop.test function in base R was also used to compare the proportion of vineyards with detected resistance in randomly-selected versus grower-selected vineyards in Marlborough. Data are available from BRI or the corresponding author.

RESULTS and DISCUSSION

Site sampling

Overall, 106 vineyards were sampled and 56 (52%) of these contained ryegrass with seeds on the dates sampled (Fig. 1; Table 1). The lack of ryegrass at some vineyards may be because previous control measures were effective while others may never have had ryegrass growing there. Ryegrass with mature seed was found, collected and tested in more South Island 51 (68%) than North Island 4 (13%) vineyards (Table 1). Ryegrass samples from some vineyards had poor or no-germination so are designated "untested" in Table 1 (e.g. 7% of North Island vineyards had inadequate germination).

There was no significant difference between the proportion of vineyards with glyphosate resistance between randomly-selected and grower-selected sites in Marlborough (53% versus 65% respectively, chi-squared = 0.57295, df = 1, p-value = 0.4491) so the data from all Marlborough vineyards are presented together. Instances of resistance were documented by vineyard and the proportion of vineyards with resistance were reported along with the number of plants treated and surviving (Table 1).

Resistance to glyphosate

Rather than step through the results in the main text tables we highlight some cases, to help interpret them. In Hawke's Bay, eleven ryegrass seedlings germinated from two resistant samples at one vineyard and were treated. Three of these seedlings survived, so this vineyard was added to the resistant tally (Table 1, Fig 1). In Marlborough, 37 vineyards had 75 resistant samples and produced 539 seedlings, of which 219 survived glyphosate (a 40% survival rate, Table 1). Of the 56 vineyards that contained ryegrass, 42 (75%) contained plants with glyphosate resistance (Table 1). On the North Island, glyphosate resistance was detected at two of the three Gisborne vineyards that contained ryegrass (66%). A further four vineyards contained no ryegrass. In the Hawke's Bay, glyphosate resistance was detected at one of the two vineyards containing ryegrass (50%) while 21 sites contained no ryegrass (Fig 1, Table 1).

On the South Island, glyphosate resistance was detected at one of the three Waipara vineyards that contained ryegrass (33%). In Marlborough, glyphosate resistance was detected at 37 out of the 48 vineyards containing ryegrass (77%). For samples that produced resistant seedlings, the proportion of propagated seedlings that survived glyphosate treatment in the glasshouse ranged between 6% and 77% with a mean of 35% (SD=18%). Resistant seed from only ten vineyards had >50% seedling survival, and only four of these had > 66% survival. It is possible that these results are skewed by both false negative and false positive results. We consider that false negatives (i.e., resistant plants were present but not collected by us) are more likely than false positives. We contend that sprayed plants would be unlikely to survive a herbicide unless they are resistant (given that all the plants from a known susceptible population died). As such, our estimate of the proportion of vineyards with glyphosate resistance is conservative. The 95% binomial confidence interval for resistance to glyphosate detected in the 80 randomly-selected vineyards across all regions was 31% ± 11%. These results provide an estimate of the proportion of vineyards that contain glyphosate-resistant ryegrass in vineyards across two North Island and two South Island regions (prevalence across, not within, vineyards).

Twelve South Island vineyards with glyphosate-resistant plants were susceptible when sprayed with glyphosate under winter conditions, suggesting they have a non-target mechanism of resistance as documented by Ghanizadeh et al (2015b). More work is needed to understand the mechanisms involved in the resistance, including whether any target site resistance is also involved.

Ryegrasses (*L. multiflorum*, *L. perenne*) are wind-pollinated obligate out-crossers (Matzrafi et al. 2021); resistance alleles are known to move via pollen between plants that are 35 and possibly as far as 3000 metres apart in the right conditions (Busi et al. 2008, Loureiro et al. 2016, Yanniccari et al. 2018). Some ryegrass seed in the inter-rows must be pollinated by resistant plants and produce resistant seed but it is unclear how many plants with the resistant phenotype may occur in these areas, nor how many may be present in the seedbank. Thankfully buried ryegrass seeds last no longer than a year and half in the soil (Cechin et al. 2021).

Resistance to other herbicides (South Island only)

Resistance of ryegrass to either amitrole, glufosinate or clethodim was not tested on the North Island populations due to insufficient seed supplies. Amitrole-susceptible ryegrass was found at two Waipara and 17 Marlborough vineyards. Neither of the Waipara sites successfully tested contained amitrole-resistant ryegrass while eleven Marlborough vineyards did (Table 2). Ryegrass from six samples sourced from these five vineyards were also resistant to glyphosate. In this case it bears noting that the tested seed samples were collected from individual mother plants, any given vineyard could have 1-7 samples. No clethodim-resistant plants were found at any site we tested. Previous results from another study detected resistance to glufosinate in a Marlborough ryegrass population (Ghanizadeh et al. 2015a), but glufosinate was found to be an effective herbicide when applied at the field rate on all the populations tested in this study. The earlier study used a range of application rates below the label rate in order to detect differences in glufosinate resistance compared to the susceptible control, while we used the label rate only.

Spray schedules for vineyards (New Zealand Winegrowers unpublished[a]) show that grower options for treating glyphosate- (Group 9) or amitrole- (Group 34) resistant ryegrass involve using herbicides with five other modes of action (defined by the Herbicide Resistance Action Committee 2022) including clethodim (Group 1), fluazifop-P-butyl (Group 1), glufosinate (Group 10), indaziflam (Group 29), pendimethalin (Group 3), and terbuthylazine (Group 5). Growers should be aware that some populations could have resistance to multiple herbicides. Glufosinate seems to be effective, as none of the vineyards surveyed in the current study had glufosinate resistance when tested at the full label rate. However, (Ghanizadeh et al. 2015a) reported glufosinate-resistant ryegrass in at least one New Zealand vineyard.

CONCLUSIONS

Glyphosate-resistant ryegrass populations are present in vineyards in all the regions sampled in this study. Amitrole resistance was detected only in Marlborough and either glufosinate or clethodim remained effective against ryegrass populations resistant to glyphosate in the locations tested. Some glyphosate-resistant plants become susceptible if sprayed during cool winter days, suggesting that glyphosate resistance in the tested samples is likely due to a non-target site mechanism of resistance documented previously (Ghanizadeh et al. 2015b). North Island populations were tested only for resistance to glyphosate as ryegrass and resistance was relatively rare. When ryegrass survives glyphosate treatment, growers should assume that it is resistant and remove plants using strategies discussed by Harrington et al. (2016). Simultaneous efforts to prevent ryegrass establishment using non-chemical methods such as tilling, grazing, mowing, and ground covers would also be helpful (Shields et al. 2016). The viability of alternative weed management measures needs to be better understood, especially if more herbicides are banned from use in vineyards (Buddenhagen et al. 2022).

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