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Transgenic glyphosate-resistant canola (*Brassica napus*) can persist outside agricultural fields in Australia

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ABSTRACT

In the last two decades the cultivation of transgenic crops has steadily increased worldwide. In Western Australia transgenic glyphosate-resistant canola (GR) has been cultivated since 2009. This study was conducted to examine the potential for transgene persistence outside agricultural fields after commercialization of GR crops. Propagule pressure, population fluctuations and reproductive output of GR canola plants have been assessed in semi-natural (roadside) and natural environments over consecutive years. The estimation of demographic parameters (plant survival and fecundity) suggest that GR canola has low likelihood to become invasive, as plants are subjected to biological and abiotic stressors likely limiting the fitness. This was particularly evident in a natural environment in which a propagule of 300 GR canola plants accidentally introduced by a wind storm could persist for three years before extinction. Thus, in natural areas GR canola populations did not show a positive population turnover and declined overtime. Conversely, on roadsides the significant correlation ($r = 0.975$) between mean plant fecundity (seed rain) and the soil seedbank density in the following year suggests that local recruitment contributed to canola persistence for at least three years. As, no individual GR plants were found with stacked genes for multiple herbicide resistance we suggest that GR volunteer canola plants can be controlled by simple mixture of herbicide modes of action different to glyphosate although an integrated management including mechanical control operations would be the optimal strategy.

1. Introduction

The cultivation of transgenic glyphosate herbicide-resistant (GR) crops has been the most rapidly adopted crop technology in modern agriculture. Introduced in 1996 in USA, GR crops have generated benefits to farmers (e.g. simplified and effective weed control, profit, etc.) (Duke, 2015). At present, transgenic crops are grown on an area in excess of 180 million ha (James, 2014) comprising 80% of GR crops as the dominant trait (Green, 2014).

GR canola (*Brassica napus* L.) is widely planted in Canada (Beckie et al., 2006) and more recently and to a lesser extent in Australia. All varieties of cultivated canola retain vestiges of wild traits such as a degree of seed shattering and secondary seed dormancy which allowed plants to disperse and seed to persist within the soil seedbank prior to domestication (Hall et al., 2005). Thus, it is important to understand how transgenic traits can affect plant fitness, whether they can increase crop plant weediness and what are the mechanisms allowing movement of transgenic traits into nearby natural ecosystems (reviewed by Chèvre et al., 1997; Chapman and Burke, 2006; Warwick et al., 2009).

In Europe studies showed little seed movement from canola crops to adjacent fields, but roadside canola populations were evident from repeated spillages from trucks transporting seed to delivery points (Crawley and Brown, 1995; Devos et al., 2009). Similarly, in Japan and Canada spillages of imported herbicide-resistant canola varieties have occurred along roadsides connecting harbors to oil factories (Yoshimura et al., 2006; Kawata et al., 2009). Other studies in the U.S. and Europe confirm that transgenic herbicide-resistant canola can establish on roadsides (Schafer et al., 2011; Munier et al., 2012) and persist for several years (Pessel et al., 2001).

In agricultural fields, volunteer canola plants can infest subsequent crops and the problem is exacerbated if volunteer plants are able to stack traits for resistance to multiple herbicides through sequential crossing (Hall et al., 2000). Secondary dormancy contributes to the persistence of *B. napus* seeds in the soil seed-bank (Gruber et al., 2004) with an estimated persistence of seeds in the soil seed-bank up to four years or longer depending on the level of disturbance (Lutman et al., 2003). Australian studies confirmed a more rapid decline of the seed-bank in a minimum

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versus no-till system and indicated a complete decline of the soil seedbank over 3.5 years with no germination recorded in either tillage system (Baker and Preston, 2008). The risk of gene escape from cropped fields through intra-specific crop-to-crop cross-pollination of canola between fields can occur at considerable distance at low frequencies (Rieger et al., 2002). In Australia the risk of inter-generic crop-to-weed gene flow with major cruciferous weeds such as *Raphanus raphanistrum* L. is also very unlikely (reviewed by Rieger et al., 1999, 2001). Canola is a poor competitor and therefore its presence or persistence on roadsides is often related to recurrent seed spillages (Yoshimura et al., 2006). However, where glyphosate is applied to roadsides for weed control, GR canola could survive and persist on roadsides or corridors between farms and grain delivery sites (Knispel and McLachlan, 2010). In Western Australia (WA) spillages of GR canola have been reported (McCauley et al., 2012; D. Bowran, unpublished data), but the persistence of GR canola outside agricultural fields in disturbed areas such as roadsides or natural environments has not been investigated (i.e. GR segregation analysis and progeny tests).

This study took advantage that in 2009 for the very first time GR transgenic canola was commercially grown in WA crop fields. From 2009 we monitored over four consecutive years a site near Quairading, WA, where transgenic GR canola was first grown on a 50 ha field in 2009. In 2012 we also surveyed roadsides near one grain delivery site for transgenic GR canola to assess the ability of transgenic canola plants to persist on WA roadsides. Here we report the potential for populations of GR canola to persist in bushland or road margins in the south-western Australian environment.

2. Materials and methods

2.1. Roadside survey of volunteer canola plants

In October 2012 a canola population that had established from truck-vectored spillages on a 3500 m roadside (RS) transect was surveyed near a grain storage point that accepts deliveries of GR (CBH, Perth Metro grain center, −31.9746, 115.9849, Western Australia). Along 38 roadside sub-transects (approximately 92 m in length starting exactly from the facility's main gate), flowering volunteer canola plants were counted on road margins and/or in the 6 m-wide unpaved median strip. The number of fertile canola siliquae produced by each individual was counted for subsequent estimation of seed production. GPS coordinates were taken at the beginning of each sub-transect. The total plant number for each sub-transect was divided by the corresponding area surveyed in order to calculate plant density (plants m^{-2}). The total number of plants found along a 3500 m transect, divided into 500 m distances (0–500 m, 501–1000 m, 1001–1500 m, 1501–2000 m, 2001–2500 m, 2501–3000 m, 3001–3500 m) is presented. Twenty seeds per siliquae was the calculated mean number of several fertile siliquae ($n > 50$) inspected during the surveys. We emphasized that the majority of surveyed plants were observed growing in isolation or at much lower mean densities (<1 plant m^{-2}) than in agricultural field conditions in southern Australia (50–100 plants m^{-2}). Thus, canola volunteer plants were not substantially affected by intra-specific competition and we assumed the seed number produced by each siliquae on mother plants contained 20 viable seeds (Buzza, 1979).

2.2. Survey of volunteer canola plants in natural areas

A 50 ha GR canola (variety: Hyola 502 RoundUp Ready) crop at Wamenusking, Quairading, WA (−32.0106120; 117.4024570) was windrowed prior to harvest in October 2009. A wind storm the next day moved GR canola plants into immediately adjacent areas of remnant natural bushland (two areas of natural land NL1 and NL2, each approx. 2 ha). Subsequent careful inspections of the two areas shortly after the wind storm revealed several siliquae had been already detached from mother canola plants and had dispersed seeds scattered on the ground as the plants moved into these bushland areas. Native vegetation mainly consisted of *Eucalyptus* spp. woodland as described by Keighery et al. (2001). Since 2010, in spring when canola plants are flowering (August–September), we have carefully monitored and counted any canola individuals present in these two bushland areas to assess the persistence of transgenic canola plants in remnant bushland. At natural land site NL1 an estimated 300 transgenic GR canola plants were wind-transported in late 2009 approximately 50–100 m from the closest field edge. Some seeds released from those canola plants resulted in established plants that in 2010 produced seed although in 2010 the growing season April–October rainfall (160 mm) was significantly lower than average (330 mm). Plants were counted and geo-referenced and leaf material collected from plants to test for the presence of the *CP4-EPSPS* transgene. Again in 2011 there were canola plants which established, flowered and produced seed in the bushland site. Plants were counted and geo-referenced and their morphological traits were measured (height, number of branches, number of fertile siliquae) to estimate plant fecundity as seed production. The survey of canola plants continued in the spring of 2012 and 2013, a total of four consecutive years after the unique movement of the 2009 wind-dispersed GR canola plants into the bushland site from the nearby windrowed GR canola. At the other natural land paddock NL2 site no canola plants were ever evident between 2010 and 2014. No canola was grown in the fields nearby NL1 and NL2 in the following years.

2.3. Glyphosate assays to confirm presence of GR roadside canola

Approximately 220 individual seed samples (one individual plant per sample) were randomly collected from canola individuals growing along the 3500 m roadside transect surveyed, labeled with a unique number, and kept in separate paper bags. The seeds of each sample were subsequently scattered in flat trays (one 20×30 cm tray per sample) containing standard potting mix (50% river sand, 25% peat, 25% pine bark), watered as required (>80% field capacity) and fertilized weekly with KNO_3 (50 mg kg^{-1} potting mix). Emerging seedlings (two-leaf stage) were treated with a lethal dose of glyphosate (600 g glyphosate ha^{-1}). The treatment was applied as RoundUp PowerMax® (Nufarm, Melbourne, VIC, Australia) (540 g a.e. L^{-1}) with a cabinet track sprayer mounted with twin flat-fan nozzles and delivering a water volume of 120 L ha^{-1} per pass at a pressure of 200 kPa. Surviving canola plants were counted 28 days after spraying and a second glyphosate treatment, identical to the first, was then applied (plants were at the 6-leaf stage, prior to bud formation). A final count of survivors was conducted 28 days after the second glyphosate treatment. A commonly grown non-transgenic, commercial canola variety (TT Thunder) was also sprayed with glyphosate as a susceptible control. In addition, a transgenic GR canola variety (RR Hyola) was included as a positive GR control.

As triazine-resistant and imidazolinone-resistant canola varieties are grown in WA, sub-samples of plants surviving both glyphosate treatments, now at the rosette stage, were evaluated for resistance to either of two different herbicide modes of action applied at the recommended rates: atrazine (a photosystem II inhibitor) at 2000 g ha^{-1} and imazamox + imazapyr (acetolactate synthase inhibitors) at 17 + 8 g ha^{-1}. A total of 823 and 698 plants surviving glyphosate were treated with atrazine or imazamox + imazapyr, respectively.

2.4. Roadside survey of volunteer canola soil seed bank

In April 2013 the soil seedbank was surveyed on the same roadside 3500 m transect. Ten soil samples approximately 350 m apart were collected in areas where standing volunteer 2012 canola stems were evident. Each soil sample consisted of 20 bulked sub-samples collected over an area of approximately 100 m^2 and was collected at 0–5 cm depth with a cylindrical sampler (0.02 m radius). Soil samples were weighed and then placed in flat trays (two replicated 20×30 cm trays per soil seedbank sample), regularly watered as required (80% field capacity) and kept outdoors for a period of six months, during which time the sequential germination of canola seedlings was assessed. Emerged seedlings (two-leaf stage) were counted and treated twice with a glyphosate dose (600 g ha^{-1}) known to be lethal to non-transgenic control canola plants. Surviving plants were fertilized weekly with KNO_3 (50 mg kg^{-1} potting mix), assessed and re-counted 28 days after each glyphosate treatment to confirm the presence of GR canola seed in the soil seedbank and then removed to allow staggered germination of new canola seedlings to emerge.

The soil seedbank density SSBD (seeds m^{-2}) was estimated using the following formula:

$$\text{SSBD}(\text{seeds m}^{-2}) = \frac{x}{\Pi((0.02 \times 0.02) \times 20)};$$

with x being the number of emerged canola seedlings from plastic trays with a 2 cm radius, $\Pi = 3.14$ and 20 the number of sub-samples collected for each sample.

2.5. CP4-EPSPS *gene identification in transgenic volunteer canola in natural areas*

Leaf material (two 5 mm leaf disks) was collected from the youngest leaf of each of 16 individual plants collected from NS1 to perform PCR test for the presence of the *CP4-EPSPS* glyphosate resistance transgene. Genomic DNA was extracted from leaf tissues as described by Yu et al. (2008). To detect the presence of the *CP4-EPSPS* gene, 10–20 ng of extracted genomic DNA was used as a PCR template in a reaction containing 20 mM Tris–HCl pH 8.4, 50 mM KCl, 50 mM $MgSO_4$, 20 mM dNTPs, 1 U high fidelity Taq DNA polymerase (Invitrogen, Australia) and 10 μM of each primer in a total volume of 25 μL. Primers GT73Fwd [5′-CCA TAT TGA CCA TCA TAC TCA TTG CT-3′] and GT73Rev [5′-GCT TAT ACG AAG GCA AGA AAA GGA-3′] were designed to amplify a 108 bp fragment of the recombination region between the GT73 insert and the plant genome (Monsanto, 2002). DNA integrity was confirmed using primers BnGAPDHF 5′ACTCGAGAAAGCTGCGACCT′3 and BnGAPDHR 5′ACCCATTCGTTGTCGTACC′3, designed to amplify a 200 bp fragment of the *B. napus* housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (accession number DQ097338). PCR conditions were set as follows: 2 min denaturing at 94 °C; 35 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at 60 °C for both primers, and 25 s elongation at 68 °C; with a final extension of 7 min at 68 °C. PCR products were visualised on 1.5% (w/v) agarose gels stained with 1x SYBR® safe DNA gel stain (Invitrogen, Australia), electrophoresed in 1x UltraPure™ TAE buffer at 100 V and photographed under UV light (302 nm).

2.6. Statistical analysis

To evaluate the ability of transgenic canola to persist on roadsides, correlation analysis was performed to identify the degree of relationship between plant fecundity (seed rain) and soil seed bank on a 3500 m transect surveyed in two consecutive years. We used GraphPad Prism (GraphPad Software, Inc., La Jolla, CA 92037 USA) to calculate Pearson's correlation coefficient (r), 95% confidence intervals and two-tailed P-values for pair wise combination of estimated seed rain and soil seedbank, assessed at equal distances from the grain storage site.

Transgenic glyphosate-resistant canola plants are hemizygous F_1 hybrids for the bacterial *CP4-EPSPS* and *GOX* genes, which are inserted at a single locus in the canola 'A' genome (Monsanto, 2002). This feature allowed easy phenotypic characterisation of glyphosate resistance and segregation analysis in canola plants carrying the *CP4-EPSPS* trait. Segregation of glyphosate resistance endowed by the *CP4-EPSPS* transgene was assessed to monitor and infer the persistence of volunteer canola on roadsides or in natural areas. Phenotypic survival data or genotypic frequency values were subjected to chi-square (χ^2) analysis. Canola is predominantly self-pollinating and all transgenic GR canola plants possess the *CP4-EPSPS* gene in the hemizygous state. Thus, the null hypothesis H0 was that the expected mortality to a lethal dose of glyphosate would have decreased by 50% at each generation from an initial 25% of glyphosate-susceptible individuals in the first seed progeny from plants grown in the field. Survival/mortality proportions of glyphosate-treated canola plants were assessed by a goodness of fit chi-square (χ^2) test to compare the observed to the expected calculated values according to a one-resistance gene segregation model.

3. Results

3.1. GR canola growing on WA roadsides

In October 2012 a roadside survey close to the major Perth Metro grain storage facility established that volunteer canola plants were growing on a 3500 m roadside transect that connected to this grain receival site. The number of established roadside canola plants was highest in the 500 m closest to the grain receival site (Fig. 1A). The majority of these canola plants had developed mature seed siliquae, indicating the ability of roadside canola plants to produce viable seed (Fig. 1B). The total canola seed output in the surveyed 3500 m transect from 2578 counted plants was estimated to be 510,620 seeds (Table S1). Some 73% of plants produced seed. The 27% of the plants that were late emerging and only at the cotyledons or two-leaf stage did not produce seed (Fig. 1B). Thus, calculated proportion of seeds germinating without leaving progeny is 0.11% of the total seed rain as ratio of total number of emerged plants found with no fertile siliquae and total seed rain (data not shown). As a greater number of volunteer GR canola plants was found in the 500 m closest to the grain receivalthere was a greater total seed production near the grain receival facility (Fig. 2). The estimated seed output decreased with distance from the storage site and a similar trend was observed with the quantification of the canola soil seed bank (Fig. 2). There was a positive and significant correlation ($P < 0.001$) between mean seed rain and soil seed bank values at distance from the grain receival facility with a calculated Pearson coefficient of $r = 0.975$.

Approximately 220 separate seed samples were collected from roadside canola individuals and 93% of the treated plants were established to be glyphosate-resistant (Table 1). Chi-square analysis confirmed that the calculated frequency of phenotypic glyphosate resistance was not significantly different from the expected segregation of the hemizygous *CP4-EPSPS* transgene in F_4 seed progeny (i.e. second seed progeny obtained from a cultivated hybrid F_1 plant) (Table 1). Conversely, the expected segregation frequencies of F_3 (2nd seed progeny) or F_5 (4th seed progeny) families were significantly different from the observed canola plant mortality to glyphosate (Table 1). A greater number of canola plants growing in close proximity to the delivery site could indicate

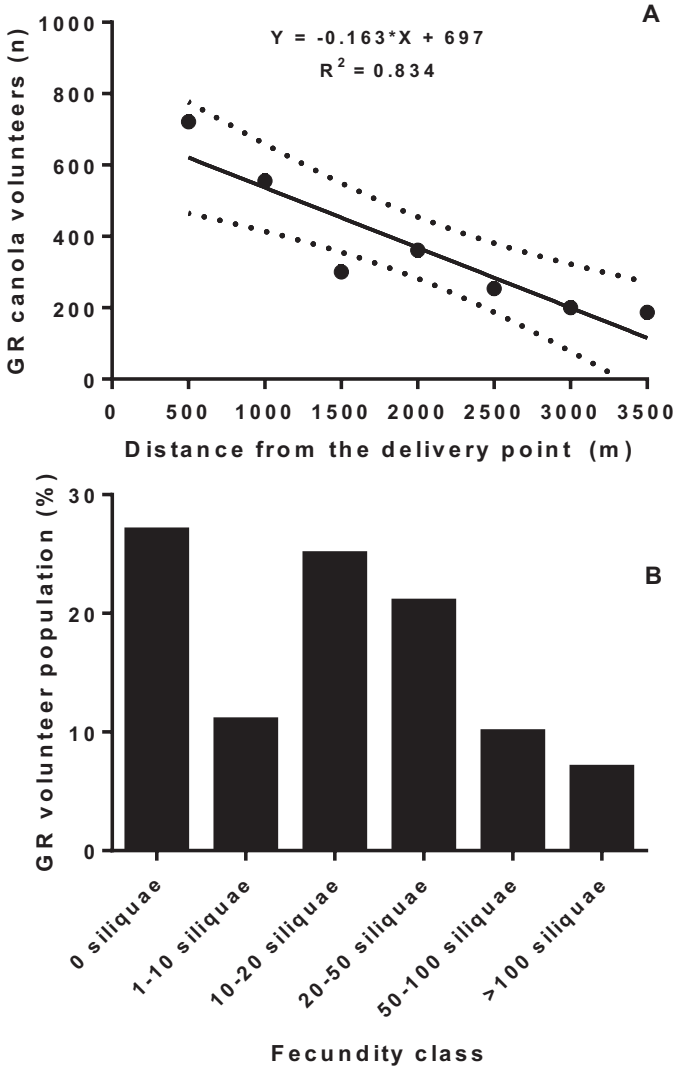

Fig. 1. (A) Total number of volunteer canola plants surveyed in 2011 along a 3500 m main transect at regular intervals of 500 m from a major grain receival facility (crop bulk handlers storage site, Perth Metro, Western Australia). Solid circles are the total number of plants as sum for each of the seven categories of distance (0–500, 501–1000, 1001–1500, 1501–2000, 2001–2500, 2501–3000, 3001–3500). Linear regression is represented with 95% confidence intervals (B) distribution (%) of plant fecundity (number of fertile siliquae per plant) in the canola plant population surveyed along the same transect.

more frequent delivery truck seed spillages occurring each year. However, the segregation analysis of the canola samples collected from within 500 m from the delivery point was not significantly different ($P = 0.48$) from the expected segregation frequency of a F_3 population (data not shown). A total of 823 and 698 plants surviving glyphosate were treated with atrazine or imazamox + imazapyr, respectively. However, no individual plants survived the sequential herbicide application thus suggesting that no individuals carried traits for multiple resistance to glyphosate and atrazine or glyphosate and imazamox + imazapyr (data not shown).

Glyphosate treatments were also applied to the emerging canola seedlings in the soil seed bank study and nearly all seedlings survived glyphosate whereas all glyphosate susceptible controls were killed. The mortality recorded after glyphosate treatment was not significantly different from the expected segregation ratio of a F_3 or F_4 population (data not shown).

Fig. 2. Estimated seed rain from volunteer canola plants and soil seedbank surveyed in October 2012 on a 3500 m transect on roadsides near the Perth Metro CBH receival facility, Western Australia. Solid circles and squares are mean values and bars standard errors for seed rain and seed bank, respectively ($n \geq 2$). Significant correlation ($P < 0.001$) seed rain and soil seed bank density was observed $r = 0.975$.

3.2. GR canola growing in natural bushland areas

Before harvest in October 2009, approximately 300 windrowed GR canola plants were wind-dispersed up to 200 m into two immediately adjacent remnant natural bushland areas (NL1 and NL2 of two ha each). As prior to 2009 no GR canola had ever been commercially grown in the very large WA grain-belt, then any GR canola in the bushland in 2010 must have resulted from wind-blown 2009 GR canola plants. No GR canola has been grown in this area before and none since 2009, so no further GR canola seed could have reached this site. In the 2 ha bushland area of NL2 no emerging canola was ever observed despite the presence of 2009 transported seed-carrying canola stems. This, was most likely due to a large colony of vertebrate herbivory (i.e. rabbits) infesting the NL2 area. Conversely, in the 2 ha bushland site NL1, GR canola plants were evident, flowered, produced seed and populations were monitored over the following four years. In 2010 (<50% of normal April–October rain) only 19 individuals were observed. Mature seed siliquae were counted ($n = 145$) with an estimated seed rain of 2900 seeds in 2010 (data not shown). In 2011, a total of 96 individuals were identified, geo-referenced and fecundity traits (fertile siliquae) measured, showing that >50% of individual plants plants produced at least 50 fertile siliquae (Fig. 3B). A total of 1229 mature siliquae were counted corresponding to an estimated seed rain of 24,580 seeds (data not shown). Some 25 plants did not produce siliquae. Thus, the observed fatal germination (i.e. late emerging plants with no siliquae) was only <0.01% of the total seed rain in 2010 and 2011 (data not shown).

Despite the abundant 2011 seed production only five canola plants emerged and flowered in this area in 2012 (Fig. 3A), most likely due to another low rainfall year (60% of normal April-October rain). In 2012 we also recorded very limited emergence of other *Brassica* weed species in the same bushland natural area (data not shown), whereas *Lolium rigudum* and *Bromus* grass species were abundant. In 2013 and 2014 no canola plants were found at this location. Nuclear DNA was successfully recovered from leaf tissue of 16 plants from those 19 emerged plants growing in the natural bushland area surveyed in 2010. PCR analysis revealed that 12 plants (75%) possessed the *CP4-EPSPS* gene and 4 (25%) did not. Chi-square analysis of the observed GR and -susceptible genotype frequencies, although calculated on a limited number of plants surveyed in 2010 ($n = 16$), corresponded to the expected segregation values of an F_2 family, i.e. the first seed progeny obtained from

Table 1
Natural area—Segregation analysis of the *Agrobacterium tumefaciens* strain *CP4-EPSPS* transgene obtained from leaf material harvested of volunteer canola plants found in a natural area (i.e. emerged plants from the 1st seed F_2 progeny accidentally wind-transported into remnant bushland) in 2010 in Quairading, Western Australia. Roadside—phenotypic segregation as plant survival to 600 g glyphosate ha^{-1} in seedlings obtained from a seed population collected along a 3500 m roadside transect near a grain receival facility in 2011 (Perth Metro CBH). GS = glyphosate-susceptible (GS) canola individuals to a lethal dose of 600 g glyphosate ha^{-1} due to genotypic or phenotypic segregation of the single hemizygous transgenic *CP4-EPSPS* trait. GR = glyphosate-resistant (GR) canola individuals surviving 600 g glyphosate ha^{-1} due to the presence of the *CP4-EPSPS* transgenic trait.

Seed progeny	Segregation frequency Expected GS	Natural area Observed GR	Natural area Observed GS	χ^2	*P*	Roadside Observed GR	Roadside Observed GS	χ^2	*P*
Crop = F_1	0.00	–	–			–	–		
1st = F_2	0.250	12	4	0	1				
2nd = F_3	0.125					1521	116	26.2	<0.001
3rd = F_4	0.063					1521	116	0.79	0.37
4th = F_5	0.031					1521	116	25.8	<0.001

hybrid F_1 hemi-zygous GR transgenic canola crop in the 2009 neighboring agricultural field (Table 1).

4. Discussion

4.1. GR canola growing on WA roadsides

This study, three years after the commercial introduction of GR transgenic canola in Western Australia, reports for the first time that GR canola plants can grow on roadsides near a canola grain receival facility. As herbicide resistance is a strong marker trait to detect gene flow (reviewed by Mallory-Smith et al., 2015) we analysed the phenotypic segregation of the hemi-zygous *CP4-EPSPS* transgene conferring resistance to glyphosate in cropped canola plants to infer seed-mediated gene flow and population persistence on roadsides. We hypothesized that due to self-pollination and genetic segregation the frequency of glyphosate-susceptible seeds would have declined and halved at each generation from an initial 25% in the first seed generation produced by hemi-zygous GR canola crop plants from the field.

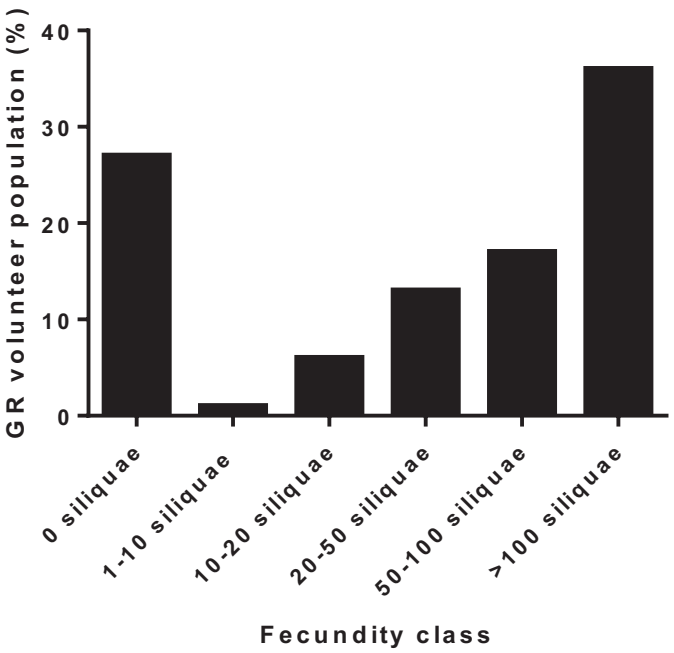

Fig. 3. Distribution (%) of plant fecundity (number of fertile siliquae per plant) in the volunteer population ($n = 96$) of transgenic GR canola surveyed in a natural area (remnant bushland) in 2011 in Quairading, WA.

Thus, truck seed spillage probably occurred in 2010, resulting in the establishment of a transgenic canola population growing along the 3500 m transect surveyed. Similar phenomena have been reported in Europe (Crawley and Brown, 1995; Pessel et al., 2001; Devos et al., 2012), U.S. (Schafer et al., 2011; Munier et al., 2012), Canada (Hall et al., 2005; Yoshimura et al., 2006; Beckie and Warwick, 2010) and Japan (Kawata et al., 2009). The observed infestation pattern, i.e. a larger population size near the delivery site, suggests that seed spillages are likely to be greater and more frequent near the delivery point. Yet, segregation data of the 2012 population growing within 500 m of the delivery site indicated that the seed spillages had occurred two years before. Thus, the high correlation between the fecundity of volunteer canola plants (seed rain) and the soil seedbank in the following year suggests that on the surveyed roadsides, local seed recruitment can contribute to GR canola persistence (Harker et al., 2006). In this study a large proportion of established GR canola plants produced seed. Thus, late germination, allowing the establishment of plants not able to produce leave seed progeny, was was a minimal proportion of the total seed rain (Pivard et al., 2008; Knispel and McLachlan, 2010). Thus, our work suggests that seed spillages are frequent near grain delivery/receival facilities and persistence on roadsides for GR canola can occur at least for three years in this environment. Inspections in April 2015 confirmed the presence of GR canola mother plants and emerging seedlings at the basis of those mother plants in an area surveyed at a 3500 m radius from the delivery site.

No seed sample, collected separately from single canola volunteer plants, was fully susceptible to glyphosate. Susceptible canola plants were only found in small (e.g. 7%) proportions due to the Mendelian segregation of the *CP4-EPSPS* transgene. This suggests that roadside glyphosate treatment has likely occurred repeatedly in the surveyed roadside area. On roadsides, glyphosate is the herbicide of choice to control weeds infesting roadsides in Australia and many other parts of the world (Munier et al., 2012; Preston, 2015). Thus, the removal of inter-specific roadside weed competition through glyphosate use has allowed the transgenic GR canola plants to grown with minimal competition. The majority of canola grown in WA are maternally-inherited triazine-resistant (approximately 80%), followed by GR (approx. 15%) and imidazolinone-resistant (3%) varieties (Bucat and Seymour, 2016). There was no evidence of resistance to triazines or imidazolinone herbicides stacked with glyphosate resistance in any seed samples tested. Glyphosate used on roadsides would have controlled potential volunteer canola plants with resistance to triazine or imidazolinone herbicides grown from accidental truck spillages (see Beckie et al., 2004) and thus prevented any cross-pollination on roadsides and whereas gene flow between canola fields occurs at very low frequencies in Australia (Rieger et al., 2002).

4.2. Transgenic canola growing in natural areas

The accidental wind-transported movement of windrowed GR canola plants into adjacent remnant bushland area in 2009 allowed us to study GR canola persistence in a natural ecosystem. Persistence for a herbicide-resistant canola variety over a seven-year period was documented in Canada (Beckie and Warwick, 2010). However, other studies document the small probability of feral canola to persist as permanent ruderal populations (Dietz-Pfeilstetter et al., 2006).

It is well established that a greater propagule pressure can increase the probability of successful establishment and promote persistence by reducing demographic fluctuations and environmental stochasticity (reviewed by Simberloff, 2009). An estimated propagule pressure of 300 individuals allowed a total production of 35,000 seeds across three generations. However, over four consecutive years the population turnover of the transgenic canola was severely compromised and we observed population extinction at this site by a progressive decrease in the number of emerged plants. Fatal germination, as similarly observed on roadsides, was minimal if compared to the estimated seed rain from emerged plants. Thus, biotic stressors such as plant herbivory (reviewed by Gu et al., 2007) and fungal disease (West et al., 2001) may have contributed to a significant reduction of the population propagule over time. Shattered seeds probably remained on the soil surface and thus exposed to insect predation. It has been shown that post-dispersal seed predation and removal by ants in these environments is generally variable but can be up to 100% (reviewed by Andersen and Ashton, 1985; Jacob et al., 2006). Bird predation of green canola siliquae before seed maturation can significantly limit the production of viable seed (D. Bowran, personal communication). A numerous colony of rabbits most likely contributed to the early population extinction in one of the two natural areas. It is also possible that the canola plants suffered strong competition from other grass weeds invading this undisturbed area, further reducing their fitness and the likelihood of persistence. Secondary seed dormancy allows seed persistence of canola seeds in the soil seedbank, particularly in dry climates (Lutman et al., 2005). We suggest that canola seed burial in such an undisturbed environment was likely very limited and this might have limited the potential of the canola population to persist through secondary dormancy. It is emphasized that all these numerous abiotic and biotic stressors reducing fitness could have equally affected natural and roadside GR canola plants. However, the roadside GR canola population grew in a location with much higher rainfall (+100–400%), was not affected by inter-specific weed competition and likely suffered much less predation from vertebrate herbivory.

5. Conclusions

This study indicates that in a natural area the initial propagule of a transgenic GR canola population declined overtime and could persist for up to three years after being accidentally transported outside agricultural fields. In this natural undisturbed bushland area in southern Australia, the transgene conferring glyphosate resistance to canola plants did not confer a fitness advantage allowing a positive population's growth rate over time. Conversely, a more numerous transgenic GR roadside canola population produced a much greater number of seed progeny, established a soil seed-bank and persisted for at least three years in such a disturbed environment. However, the control of herbicide-resistant volunteer canola and other weeds infesting roadsides can be effectively achieved by mixtures of different herbicide modes of action (e.g. glyphosate + simazine) applied at the most sensitive plant stage in combination with other mechanical control operations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.agee.2015.12.028.

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