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Seed longevity of glyphosate resistant transgenic creeping bentgrass (*Agrostis stolonifera* L.) lines

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Abstract
Studies to estimate seed longevity and dormancy of creeping bentgrass (*Agrostis stolonifera* L.) were conducted from 2000 to 2005 at Corvallis and Hermiston, Oregon. Seeds from three transgenic glyphosate resistant creeping bentgrass lines, 48-10, 48-13, and ASR368, and one non-transgenic glyphosate susceptible line, SR1020, were used. Creeping bentgrass seeds were buried at 3, 18 and 31 cm in 2000 and removed 6, 12, 18, 24, and 51 months later. Soil type and climatic conditions were different at the two locations. At Corvallis, the soil was a Malabon silty clay loam, and the winters wet and mild. The soil at Hermiston was an Adkins fine sandy loam, and winters drier and colder. Seeds of all creeping bentgrass lines deteriorated faster at Corvallis than at Hermiston. The estimated half-lives of creeping bentgrass lines buried at Corvallis were 8.4 to 20.2 months, while those buried at Hermiston were 8.4 to 37.7 months. At both sites, seeds of the glyphosate resistant lines, 48-10 and 48-13, deteriorated faster than the susceptible line, SR1020. However, seed deterioration in the resistant line, ASR368, was slower than all other creeping bentgrass lines. Based on the germination test, exhumed intact seeds at Corvallis were more dormant than those at Hermiston. If buried, it could be expected that viable creeping bentgrass seeds will persist more than 4 years after the seeds are introduced to a site, but environmental conditions can influence both seed longevity and dormancy.

Key words: *Agrostis stolonifera*, deterioration, germination, glyphosate, GMO, seed burial

INTRODUCTION

The introduction of genetically modified (GM) crops over the past two decades has led to new options for agriculture. Modifications include resistance to herbicides, diseases, and insects. Barton and Dracup (2000) divided the traits added to crops into two categories, an output trait (a value enhancing trait for the end user) and an input trait (agronomic traits which benefit the growers). The use of molecular biological techniques for the manipulation of DNA provides farmers with alternative management strategies for weeds, pests, and diseases (James 1998). Herbicide resistant crops provide multiple advantages to growers. Growers can design simpler weed management strategies based on fewer herbicides (Duke 1998). Herbicide resistant crops allow control of difficult weeds that could not be controlled in conventional crops. The herbicide resistant crop may increase the period of time that weeds can be controlled without injury to the crop.

Creeping bentgrass (*Agrostis stolonifera* L.) has been

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the most widely used turfgrass for golf course fairways and putting greens in North America for over 100 years (Duich 1985). This species is well adapted to the high maintenance requirements of golf courses, due to tolerance for close mowing and its ability to spread by stolons. The earliest creeping bentgrass used on golf courses was introduced from a South German bentgrass mix which consisted of a mixture of *Agrostis* species, including colonial bentgrass (*Agrostis tenuis* Sibth.), redtop (*Agrostis gigantea* L.), velvet bentgrass (*Agrostis canina* L.), and creeping bentgrass. The Scotts Company and Monsanto developed glyphosate-resistant Roundup Ready™ creeping bentgrass varieties in 1998. The glyphosate resistance would allow for the control of a broad spectrum of weeds, including difficult to control grasses. However, concerns have been raised over the release of an outcrossing, perennial plant, and the potential for seed and stolon escapes as well as pollen flow to related species. Transgenes of GM crops can be transferred to their wild relatives by pollen or seed flow, and cause a potentially serious problem when introduced into the natural environment (Snow and Palma 1997). Zapiola et al. (2008) reported the potential for transgene escape and gene flow of GM creeping bentgrass at a landscape level.

The introduction of herbicide resistant seeds that persist in the seed bank for more than a single year is a management concern for growers because seeds that remains viable for many years can increase the risk of plant escapes in the future. Carryover of viable seeds in the soil from previous years can buffer the effects of weed control, and hence maintain the weed problem (Barralis and Chadoeuf 1987). Herbicide resistant seed remaining in the seed bank after the removal of the herbicide resistant crops present a similar problem. Information about seed dormancy, viability, and longevity in the soil is important in developing strategies for herbicide resistant crop volunteer management (Mennan 2003).

There is currently little information available on the persistence of creeping bentgrass in the soil seed bank. The Rampton and Ching study (1970) used an older colonial bentgrass cultivar called 'Highland'. However, many of the current creeping bentgrass lines have been bred to increase germination and decrease dormancy in order to achieve a quick and even establishment on playing surfaces. Therefore, current creeping bentgrass varieties may differ from Highland colonial bentgrass.

The objective of this study was to determine the seed longevity and dormancy of transgenic and non-transgenic creeping bentgrass lines at two different locations.

MATERIALS AND METHODS

Seed burial experiments were initiated in 2000 at the Hermiston Agriculture Experiment Station, Hermiston, Oregon and at Lewis Brown Horticulture Research Farm, Corvallis, Oregon. The soil was a Malabon silty clay loam at Corvallis and an Adkins fine sandy loam at Hermiston. The winters are wetter and milder at Corvallis than at Hermiston.

Seeds of three transgenic creeping bentgrass lines (48-10, 48-13, and ASR368) and one non-transgenic line (SR1020) were provided by the Scotts Seed Company. The three transgenic lines of creeping bentgrass, resistant to glyphosate were harvested from nursery plots in western Oregon (ASR368) and in eastern Washington (Lines 48-10 and 48-13). The seeds from western Oregon were hand harvested and cleaned, while seeds from eastern Washington were hand harvested and cleaned using a hammer mill. The non-transgenic bentgrass line, SR1020, was from western Oregon, and was machine harvested and cleaned.

Seed burial preparations

One hundred seeds of each creeping bentgrass line were placed into a 5-cm square, 60-micron nylon mesh packet, with a plastic bead color coded for each bentgrass line and secured to a cord. The openings in the mesh were large enough to allow the movement of air, water and microorganisms. The cord was secured perpendicular to a wooden stake, with packets suspended at 3, 18 and 31 cm from the underside of the stake. Five removal dates were replicated three times at each location. Control samples were maintained at 20 ± 5 °C in the dark in the laboratory.

Holes at both locations were spaced 60 cm apart and dug to a depth of approximately 40 cm. Stakes were placed perpendicular to the hole, allowing the cord to hang freely. The holes were then filled until level with the underside of the stake.

Seed removal and germination

Creeping bentgrass seeds were buried in November and December 2000 at the Corvallis and Hermiston, respectively. Then, seeds were exhumed 6, 12, 18, 24, and 51 months after seed burial at both locations. A hole was dug 10 to16 cm to the side of the original hole to a depth of 50 cm. Soil was removed by hand from around the cord. The cord with packets attached was removed from the stake, placed into a tub of water, and rubbed gently to remove

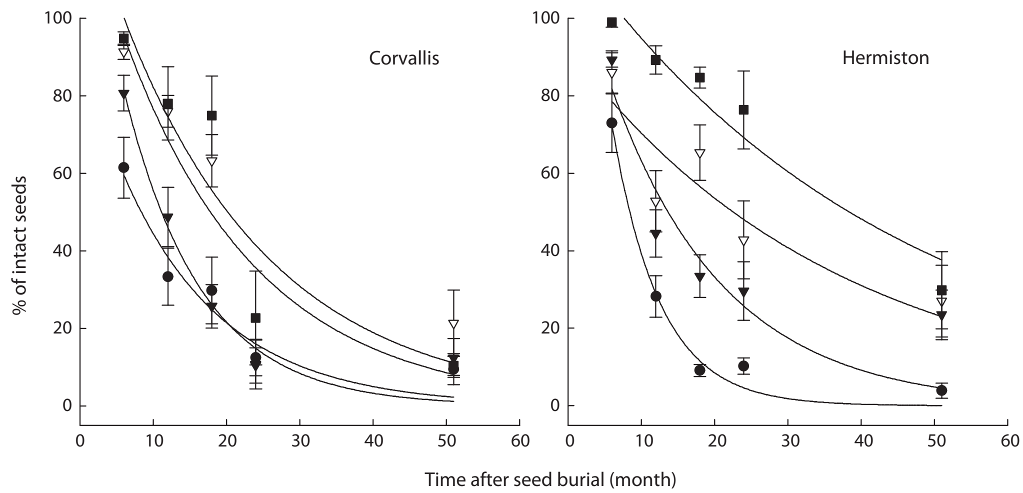

Fig. 1. Deterioration and an exponential decay curves of glyphosate-resistant (▼ 48-10, ● 48-13, ■ ASR269) and –susceptible (▽ SR1020) creeping bentgrass seeds buried at Corvallis and Hermiston Oregon.

excess soil and debris. The cords and packets were then allowed to air-dry for 1 hr prior to counting and planting. The seeds were separated from remaining soil and debris within the packet. Intact seeds, which were counted and planted into water soaked 18 × 42 mm Jiffy Forestry Pellets®(Jiffy, Lorain, OH, USA). The stored sample in the lab (around 25 °C on the shelf) was used to compare the effects of seed age on germination, without the environmental and burial factors. Seeds were germinated in a growth chamber at 25 °C day (8 h) and 15 °C night (16 h) for 21 days. Germination was defined as the emergence of a coleoptile from the seed.

Resistance test

After 21 days in the growth chamber, creeping bentgrass seedlings from resistant lines were moved to the greenhouse. The seedlings were grown for two weeks and sprayed with glyphosate at a rate of 1.12 kg/ha. The number of dead and alive plants was counted 28 d after application. The living plant counts were divided by dead plant counts to obtain a ratio of resistance to susceptible plants. The ratio test was performed because at the time of seed burial the glyphosate resistant lines seed were heterozygous at a 1:1 resistant to susceptible ratio. Prior to burial, glyphosate resistant creeping bentgrass lines were tested to confirm the resistance to susceptible ratio.

Statistical analysis

An analysis of variance (ANOVA) was performed and Fisher's Protected LSD was used for mean separation using SAS/STAT (SAS Institute Inc 1987). An exponential decay curve was fitted to the intact seed fraction to estimate the deterioration of seeds. Estimated seed half-life of buried creeping bentgrass lines at both locations was determined using the following equation:

$$y = ae^{bt}$$

where a is the initial number of seeds buried, b is the decay rate, y is the proportion of seeds remaining in the soil and t is time in months.

RESULTS

Based on the ANOVA, differences in deterioration or germination were not detected among burial depths at either location. Therefore, the germination or deterioration data were combined over burial depths by location.

An exponential decay curve was fitted to the intact seed fraction for all creeping bentgrass lines at both locations (Fig. 1). Seeds of creeping bentgrass lines deteriorated faster at Corvallis than at Hermiston. Except line 48-13, the estimated rate of seed decay for each creeping bentgrass line at Corvallis was shorter than at Hermiston (Table 1). At Corvallis, differences in seed decay rates were small among creeping bentgrass lines (0.049-0.095) when compared to at Hermiston (0.023-0.153). The differences are likely due to the wetter climate at Corvallis and the soil type. At Corvallis, seeds of 48-10 and 48-13 deteriorated faster than seeds of ASR368 and SR1020. The estimated half-lives of ASR368 and SR1020 were approximately twice that of 48-10 and 48-13 (Table 1). At Hermiston, seeds of the glyphosate-resistant lines, 48-10 and 48-13, deteriorated faster than the susceptible line, SR1020. However, seed deterioration in the resistant line, ASR368, was slower than the susceptible line, SR1020. The estimated half-life of ASR368 seeds was about 40 month, which is approximately 1.5 times that of SR1020 (Table 1).

Shelf-stored seeds of ASR368 and SR1020 had the highest germination rate (about 90%) throughout the study. However, the germination rate of shelf-stored 48-10 and

Table 1. Seed decay parameters of creeping bentgrass lines buried at Corvallis and Hermiston, Oregon

Location	Line*	Predicted decay rate ± s.e.	R^2	Estimated half-life** (month ± s.e.)
Corvallis	SR1020	0.055 ± 0.009	0.61	17.7 ± 3.0
	48-10	0.095 ± 0.013	0.71	11.1 ± 1.5
	48-13	0.073 ± 0.017	0.45	8.4 ± 2.1
	ASR368	0.049 ± 0.010	0.58	20.2 ± 4.1
Hermiston	SR1020	0.027 ± 0.007	0.35	22.8 ± 6.3
	48-10	0.064 ± 0.011	0.53	13.7 ± 2.4
	48-13	0.153 ± 0.020	0.79	8.4 ± 1.1
	ASR368	0.023 ± 0.004	0.59	37.7 ± 6.8

Data for estimated half-life are mean of nine replications.
*SR1020, non-transgenic line; 48-10, 48-13 and ASR368, transgenic line.
**$y = ae^{bt}$, where a is the initial number of seeds buried, b is the decay rate, y is the proportion of seeds remaining in the soil and t is time in months.

Table 2. A comparison of shelf seed germination among removal dates

Lines*	Month after seed buried				
	6	12	18	24	51
			- % germination ** -		
SR1020	90.3 ± 3.2 a	90.2 ± 10.7 a	87.8 ± 3.4 a	94.0 ± 0.9 a	87.3 ± 3.1 a
48-10	75.3 ± 4.7 c	73.5 ± 2.1 b	74.0 ± 1.5 b	66.3 ± 3.1 b	38.7 ± 2.7 b
48-13	82.0 ± 1.7 b	55.3 ± 10.7 c	48.5 ± 1.2 c	46.3 ± 3.7 c	40.7 ± 2.1 b
ASR368	87.7 ± 5.5 a	87.8 ± 10.6 a	90.0 ± 4.1 a	90.0 ± 5.0 a	88.3 ± 5.8 a

Means (N = 6) within column followed by the same letters were not significantly different based on a Fisher's Protected LSD at 5% level.
*SR1020, non-transgenic line; 48-10, 48-13 and ASR368, transgenic line.
**Seeds were stored on the shelf in a lab (around 25°C).

Table 3. A comparison of seed germination among removal dates at Corvallis, Oregon

Lines*	Month after seed buried				
	6	12	18	24	51
			- % germination** -		
SR1020	77.6 ± 19.7 a	24.8 ± 30.3 a	36.4 ± 32.2 a	8.1 ± 16.0 a	7.1 ± 17.5 a
48-10	39.9 ± 21.9 b	22.7 ± 17.6 a	15.6 ± 13.4 ab	3.4 ± 6.7 a	6.6 ± 12.0 a
48-13	31.0 ± 17.4 b	14.0 ± 18.3 a	7.1 ± 11.0 a	1.6 ± 2.6 a	4.1 ± 8.1 a
ASR368	75.6 ± 21.7 a	36.2 ± 39.3 a	35.6 ± 40.1 a	1.8 ± 2.4 a	2.0 ± 1.6 a

Means (N = 9) within column followed by the same letters were not significantly different based on a Fisher's Protected LSD at 5% level.
*SR1020, non-transgenic line; 48-10, 48-13 and ASR368, transgenic line.
**Seeds were stored on the shelf in a lab (around 25°C).

48-13 seeds decreased rapidly and was less than 50% at 51 months (Table 2). The germination of creeping bentgrass seeds decreased quickly over time and was less than 10% at 24 month after seed burial at Corvallis (Table 3). Germination rates for SR1020, 48-10, and 48-13 seeds at Hermiston were similar. However, ASR368 had the greatest germination rate at all seed removal dates except at 51 months (Table 4).

DISCUSSION

Seed deterioration often depends on the seed burial depth. Because of microorganisms, moisture, and air near the soil surface, seeds close to the soil surface deteriorate faster than seeds far from the soil surface (Taylor et al. 2005). In this study, however, there were no differences in seed deterioration and germination among seed burial depths.

The greater germination and longer half-lives observed at Hermiston may in part be attributed to climate. When weather data are compared at the two locations, Corvallis had higher precipitation and cooler summer and warmer winter air temperatures than Hermiston (Fig. 2).

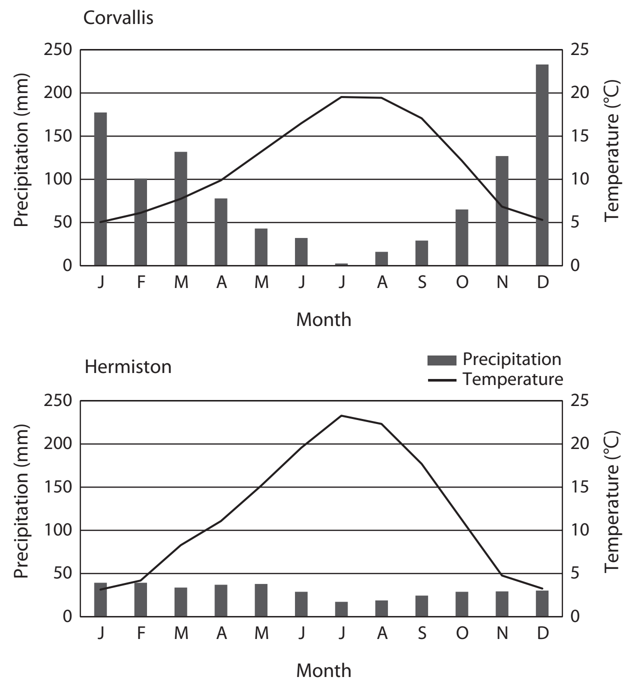

Fig. 2. Average monthly temperature and precipitation from 2000 to 2004 in two study sites, Corvallis and Hermiston (Source: National Oceanic and Atmospheric Administration).

Table 4. A comparison of seed germination among removal dates at Hermiston, Oregon

Lines*	Month after seed buried				
	6	12	18	24	51
			- % germination** -		
SR1020	57.6 ± 28.5 b	30.7 ± 20.0 b	32.2 ± 24.7 b	30.2 ± 28.6 b	11.9 ± 15.3 ab
48-10	49.8 ± 10.5 b	25.7 ± 15.0 bc	17.1 ± 15.3 bc	15.8 ± 14.0 bc	8.0 ± 11.3 ab
48-13	25.1 ± 16.0 c	10.4 ± 6.6 c	5.8 ± 5.0 c	6.4 ± 7.2 c	1.3 ± 2.2 b
ASR368	82.0 ± 13.5 a	53.2 ± 28.9 a	62.1 ± 22.7 a	59.9 ± 29.6 a	13.3 ± 15.3 a

Means (N = 9) within column followed by the same letters were not significantly different based on a Fisher's Protected LSD at 5% level.
*SR1020, non-transgenic line; 48-10, 48-13 and ASR368, transgenic line.
**Seeds were stored on the shelf in a lab (around 25°C).

Table 5. The ratio tests for resistant versus susceptible seeds pre-burial (November 2000) and after 24 month removal (November 2002)

Lines*	Corvallis		Hermiston	
	Pre-burial	After 24 month removal	Pre-burial	After 24 month removal
		- ratio** -		
48-10	1.03	0.96	1.07	1.01
48-13	1.10	0.93	1.12	0.76
ASR368	1.01	1.13	1.09	1.01
LSD (P = 0.05)	NS	NS	NS	NS

*48-10, 48-13 and ASR368, transgenic line.
**The number of alive plants (glyphosate resistant) divided by dead plants (glyphosate susceptible) 14 days after the glyphosate application.
LSD, least significant difference; NS, not significant.

The higher precipitation led to wet soils for much of the winter months at Corvallis. The high precipitation and above freezing air temperatures provided the potential for an environment conducive to soil microbe and seed pathogen activity. The data indicated that under the moist conditions found in Corvallis, the deterioration for creeping bentgrass lines increased rapidly and did not differ among the creeping bentgrass lines after 24 month removal. However, under the conditions found at Hermiston, the resistant line ASR368 had longer half-life and higher germination rate than any other lines throughout the study.

Differences in seed deterioration among line ASR368 and other transgenic lines may be attributed to poor seed quality of 48-10 and 48-13. Seeds from line 48-10 and 48-13 from the shelf had a lower germination than all other lines of bentgrass throughout the studies (Table 2). Lines 48-10 and 48-13 were subjected to an alternative seed cleaning process. The alternative seed cleaning process may have damaged the seed making them more susceptible to deterioration. The differences between the non-transgenic line, SR1020 and the transgenic resistant line ASR368 appear to be attributed to a line by environmental interaction and not the glyphosate resistance gene. The ratio between the heterozygous resistant and susceptible seed within the glyphosate resistant lines had not changed from the pre-burial seed ratio test to the 24 month test (Table 5). The ratio remained a 1 to 1 resistant to susceptible seed; thus, a greater portion of seeds that were viable were not resistant as would be expected if the glyphosate resistance gene was a factor.

Based on this study, it would be expected that seeds of the glyphosate-resistant lines, 48-10 and 48-13, would persist for a shorter time than the susceptible line, SR1020. However, seeds of the glyphosate-resistant line, ASR368, could persist longer than seeds of the susceptible line, SR1020, depending on environmental conditions. Exhumed intact seeds at Corvallis were more dormant than those at Hermiston. As the seeds were buried longer, deteriorated seeds were more and dormant seeds were fewer.

If buried, it could be expected that viable creeping bentgrass seeds will persist more than 4 years once introduced to a site, but environmental conditions can influence both seed longevity and dormancy. The persistence of a glyphosate resistance seed needs to be taken into account with future management of creeping bentgrass and other rotational crops used in bentgrass production.

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