THE EFFECT OF PERENNIAL RYEGRASS-ENDOPHYTE SYMBIOTA ON MORTALITY AND FECUNDITY OF MEALY GRASS ROOT APHID

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Participating organisations:

Agriculture Services Victoria
RAGT (Formerly Seed Force)

Executive Summary

An in vitro bioassay was used to evaluate perennial ryegrass-endophyte symbiota for effectiveness in mealy grass root aphid *(Aploneura lentisci)* control.

Significant differences in aphid mortality and fecundity were observed when aphids were fed a root-sucrose diet from perennial ryegrass (prg)-endophyte symbiota compared to prg-WE (without endophyte). Multiple statistical tests were employed to analyse and validate the data.

When ranking symbiota for effectiveness in root aphid control 1137-RGT18, 1137-RGT15 and prg-NEA2 provided better whole-of-life cycle control than prg-SE, prg-AR37 and prg-WE.

The alkaloids (peramine, ergovaline, lolitrem B and epoxy-janthitrem I) are present in roots of 12-day old seedlings and occur at concentrations lower than that observed in shoots of the same seedlings.

Methods

The effect of perennial ryegrass endophyte on the mortality and fecundity of the mealy grass root aphid, Aploneura lentisci Passerini (Homoptera, Aphididae), was examined using an in vitro bioassay.

Aphid diet preparation

Roots from a pooled sample of c.100 12-day old seedlings were ground to a fine powder in liquid nitrogen and suspended in a 20% sucrose/ultrapure water solution. A pooled sample reduces symbiotum-symbiotum variation and provides an indication of the population average.

Sample preparation for alkaloid analysis

Alkaloid concentrations were measured in the roots and shoots of a pooled sample of c.100 12-day old seedlings. Plant material (10mg ±0.1mg) was extracted twice with 1 mL of methanol:water (80:20, v:v) (Merck LiChrosolv ≥99.9%; MilliQ water). Supernatants were combined and dried under a stream of nitrogen gas for two hours, and reconstituted in 100 µL of methanol:water (80:20, v:v). For alkaloid quantitation, peramine nitrate (BDG Synthesis, Wellington, NZ), ergotamine (Sigma-Aldrich, St Louis, USA) and lolitrem B (isolated in-house) were used to construct concentration curves from 1 to 2000 ng/mL (peramine and ergotamine) and 6 to 2400 ng/ml (lolitrem B) in matrix (perennial ryegrass-WE).

LCMS analysis

Extracts were analysed using a 100 mm x 2.1 mm Themo Hypersil Gold 1.9 µm HPLC column fitted to a Thermo Fischer Scientific Vanquish liquid chromatograph (Thermo Fischer Scientific, Bremen). Metabolites were eluted from the column using a gradient mobile phase, A (0.1% formic acid in water, Thermo Fischer Scientific) and B (0.1% formic acid in acetonitrile, Thermo Scientific) at 0.3 mL/min. Initial conditions were 98% A before initiating a linear gradient to 0% A over 11 minutes, and this was maintained for 4 minutes before returning to the initial gradient conditions. The compounds were detected with a Thermo Fisher QExactive Plus mass spectrometer (Waltham, MA, USA; Thermo, Bremen, Germany), operating in the ESI mode with a HESI probe for positive data acquisition. The sample extract (3 µL) was injected onto the LCMS system and analysed using a Thermo Fisher QExactive Plus mass spectrometer

13 Future Court, Shepparton VIC 3630, Australia
T +61 3 5832 3800 **E** info@ragt.au
RAGT Australia | ABN 11 118 991 272
ragt.au

(Waltham, MA, USA; Thermo, Bremen, Germany) in FT positive mode over a mass range of 80-1200 amu with resolution set at 35,000. Typical mass accuracy for the alkaloids was 3-5 ppm. Elution times for peramine, ergovaline and lolitrem B were 3.77±0.02 min, 5.56±0.01 min and 11.07±0.02 min, respectively. Quantitative results with matrix-matched standards were calculated for ergovaline, peramine and lolitrem B. Relative quantitation (expressed as peak area) were determined for epoxy-janthitrems I at 11.08±0.02 min.

Root aphid bioassays

Colonies of root aphids were reared on mature endophyte-free perennial ryegrass plants, in a controlled environment room (CER) maintained at 20 ± 2 °C and 62 ± 5% RH, with a photoperiod of 14 h light and 10 h dark.

Single adult aphids were placed in 35 mm petri dishes. A 200 µl aliquot of the diet was sandwiched between two layers of parafilm, creating a feeding membrane. Feeding chambers were then inverted so that the aphids sat directly on top of their food source. Feeding chambers were enclosed inside an additional large petri dish with a layer of moistened filter paper to maintain a humid environment. A total of 14 aphids were used for each treatment. Adult mortality, nymph production and nymph survival were monitored for eight days.

Separate statistical analyses were performed for the response variables (adult mortality, nymph production and nymph survival) and the six endophyte treatments (WE, SE, AR37, NEA2, RGT15 and RTG18) over the eight-day period. Adult survival was analysed using one-way Analysis of Variance (ANOVA) on the factor of endophyte strain for each period (day 1-8). Pairwise t-test assuming unequal variance tested significance ($P<0.1$) between the control (WE) and individual endophyte strains.

To meet the statistical assumption of homogeneity of variance between endophyte treatments, nymph production and nymph survival were square root transformed before performing one-way ANOVA. Pairwise t-tests assuming unequal variance were then utilised to test for significance ($P<0.1$) between the WE control and individual endophyte strains. For all analyses, multiple comparison Bonferroni correction was employed to assess any significance between means at $a = 0.05$.

Shapiro-Wilk test for normality and Levene's test for equal variance were also performed, and where deviance from normality was found, non-parametric Kruskal-Wallis tests were conducted to check for difference between the means. This was followed by post-hoc Dunn's test to check pairwise differences.

Additionally, binary logistic regression models (for adult survivorship) and multiple linear regression models (for the number of nymphs produced and survival) were developed to test significance between the different treatments. The independent variables were day; assay plate number; endophyte treatment, and the dependent variables were survivorship or number of nymphs born.

Results

The number of adults and nymphs surviving on perennial ryegrass-endophyte root-sucrose diet were determined over eight days. The symbiota tested were perennial ryegrass (prg)-SE, prg-AR37, prg-NEA2, 1137-RGT15 and 1137-RGT18. Prg-WE (without endophyte) was used as an endophyte-free control (see Table 1).

Table 1. The mortality and fecundity of pasture root aphids exposed to a root-sucrose diet derived from perennial ryegrass-endophyte symbiota. The total number and percentage of adults surviving (A), nymphs born (NB) and nymphs surviving (NA) was assessed over eight days.

	prg-WE[1]			prg-SE			prg-AR37			prg-NEA2			RGAS1137 1.11.15			RGAS1137 1.4.18		
DAY	A	NB	NA	A	NB	NA	A	NB	NA	A	NB	NA	A	NB	NA	A	NB	NA
1	12	17	17	14	12	12	14	16	16	13	7	7	13	5	5	13	6	6
	100%		100%	100%		100%	100%		100%	93%		100%	93%		100%	93%		100%
2	12	30	30	14	22	22	12	28	28	12	14	13	11	13	13	13	20	20
	100%		100%	100%		100%	86%		100%	86%		93%	79%		100%	93%		100%
3	12	40	39	11	28	18	8	38	37	11	20	17	11	31	28	13	24	20
	100%	-	98%	79%		64%	57%		97%	79%		85%	79%		90%	93%		83%
4	12	48	40	8	38	20	6	44	36	10	25	20	5	32	23	5	28	16
	100%		83%	57%		53%	43%		82%	71%		80%	36%		72%	36%		57%
5	8	55	30	6	39	21	5	46	29	7	27	19	4	31	17	5	28	17
	64%		55%	43%		54%	36%		63%	50%		70%	29%		55%	36%		61%
6	5	55	20	4	39	11	5	46	11	4	27	9	3	31	10	5	28	15
	43%		36%	29%		28%	36%		24%	29%		33%	21%		32%	36%		54%
7	1	55	4	3	39	1	0	46	3	1	27	4	2	31	4	3	28	10
	7%		7%	21%		3%	0%		7%	7%		15%	14%		13%	21%		36%
8	1	55	0	0	39	0	0	46	0	0	27	0	0	31	4	1	28	2
	7%		0%	0%		0%	0%		0%	0%		0%	0%		13%	7%		7%

*[1] at the beginning of the experiment adult aphids were n=12 for prg-WE, all other symbiota n=14

Adult aphids exposed to prg-WE diets survived longer than those exposed to perennial ryegrass-endophyte symbiota (Figure 1). For example, 37% of adult aphids exposed to 1137-RGT15 and 1137-RGT18 survived to four days, compared to 100% for prg-WE. Differences in adult survival at four days, for the symbiota tested, were statistically significant (ANOVA, P = 0.0039). Significant differences (P<0.05) were observed between symbiota and prg-WE (adult aphid survival average and variance 1,0) at four days: prg-SE (0.571,0.264; P=0.008), prg-AR37 (0.438,0.264; P=0.0011), prg-NEA2 (0.714,0.220; P=0.040), 1137-RGT15 (0.357,0.247; P=0.0003) and 1137-RGT17 (0.357,0.247; P=0.0003). No significant differences (P<0.1) were determined between other pairwise combinations of prg-endophyte symbiota. Highly significant results were determined for endophyte treatment (P<0.001) and analysis day (P<0.0001) according to binary logistic regression, but the effect of plate number was not significant (P=0.562). This was also true for regression models conducted for day four, whereby endophyte treatment was statistically significant (P=0.001), but plate number had no significant effect (P=0.632). All endophytes reduced adult survival. When considering ranking of symbiota for effectiveness in reducing adult survival 1137-RGT18 = (1137-RGT15 > prg-AR37) > prg-SE > prg-NEA2 > prg-WE.

Aphid fecundity, measured as nymph production, ceased at five days (Figure 2). Compared to prg-WE (average and variance for nymph production at five days 4.583,13.356), less nymphs (P=0.153) were born when exposed to root-sucrose diet of perennial ryegrass-endophyte symbiota prg-NEA2 (1.929,5.918; P=0.045), 1137-RGT15 (2.214,5.72; P=0.071) and 1137-RGT18 (2,5.692; P=0.051) but not prg-AR37 (3.286,10.066; P=0.348) or prg-SE 2.786,7.412; P=0.176). No significant differences (P<0.1) were determined between other pairwise combinations of prg-endophyte symbiota. Similar results were found via non-parametric testing and after data normalisation. Multiple linear regression determined the effect of endophyte treatment was significant throughout (P<0.0001), and specifically, at five days (P=0.084). The symbiota 1137-RGT15, 1137-RGT18 and prg-NEA2 effectively reduced aphid fecundity. When considering ranking of symbiota for effectiveness in reducing fecundity (1137-RGT18 = 1137-RGT15 = prg-NEA2) > (prg-SE = prg-AR37 = prg-WE).

Maximum nymph survival was observed at four days (Figure 3). Significant differences (P<0.1) were observed between symbiota (ANOVA, P=0.088). Compared to prg-WE (average and variance for nymph survival 3.33,8.42), significantly (P<0.1) less nymphs survived when exposed to prg-SE (1.429,2.72; P=0.06), prg-NEA2 (1.429,3.495; P=0.067), 1137-RGT15 (1.643,3.324; P=0.098) and 1137-RGT18 (1.143,2.132; P=0.031) but not prg-AR37 (2.571,8.11; P=0.507). No significant differences (P<0.1) were determined between other pairwise combinations of prg-endophyte symbiota. Further analysis via non-parametric testing and data normalisation supports the original findings. Multiple linear regression models determined the effect of endophyte treatment across all time points (P= 0.007) and at four days (P=0.128). The symbiota 1137-RGT15, 1137-RGT18, prg-SE and prg-NEA2 effectively reduced nymph survival. When considering ranking of symbiota for effectiveness in reducing nymph survival 1137-RGT18 > (prg-SE = 1137RGT15 = prg-NEA2) > (prg-AR37 = prg-WE).

The alkaloids (peramine, ergovaline, lolitrem B and epoxy-janthitrem I) are present in roots of 12-day old seedlings and occur at concentrations lower than that observed in shoots of the same seedlings (see Table 2).

Table 2: Peramine, ergovaline, lolitrem and epoxy-janthitrem I in shoot and root tissue from a pooled sample of c.100 12-day old seedlings of perennial ryegrass-endophyte symbiota.

Association	Tissue	Peramine ppm (mg\kg)	Ergovaline ppm (mg\kg)	Lolitrem B ppm (mg\kg)	Epoxy-janthitrem I Response
prg-WE	Shoot	NF	NF	NF	NF
	Root	NF	NF	NF	NF
prg-SE	Shoot	29.85	1.96	2.19	NF
	Root	1.28	0.47	0.26	NF
prg-NEA2	Shoot	n.d	n.d	n.d	n.d
	Root	0.28	0.46	0.04	NF
1137-RGT15	Shoot	20.80	1.77	NF	NF
	Root	0.77	1.05	NF	NF
prg-AR37	Shoot	NF	NF	NF	66491029
	Root	NF	NF	NF	16266897
1137-RGT18	Shoot	NF	NF	NF	63715307
	Root	NF	NF	NF	17880423

NF = not found. nd = not determined. Epoxy-janthitrem I is measured as an arbitrary response

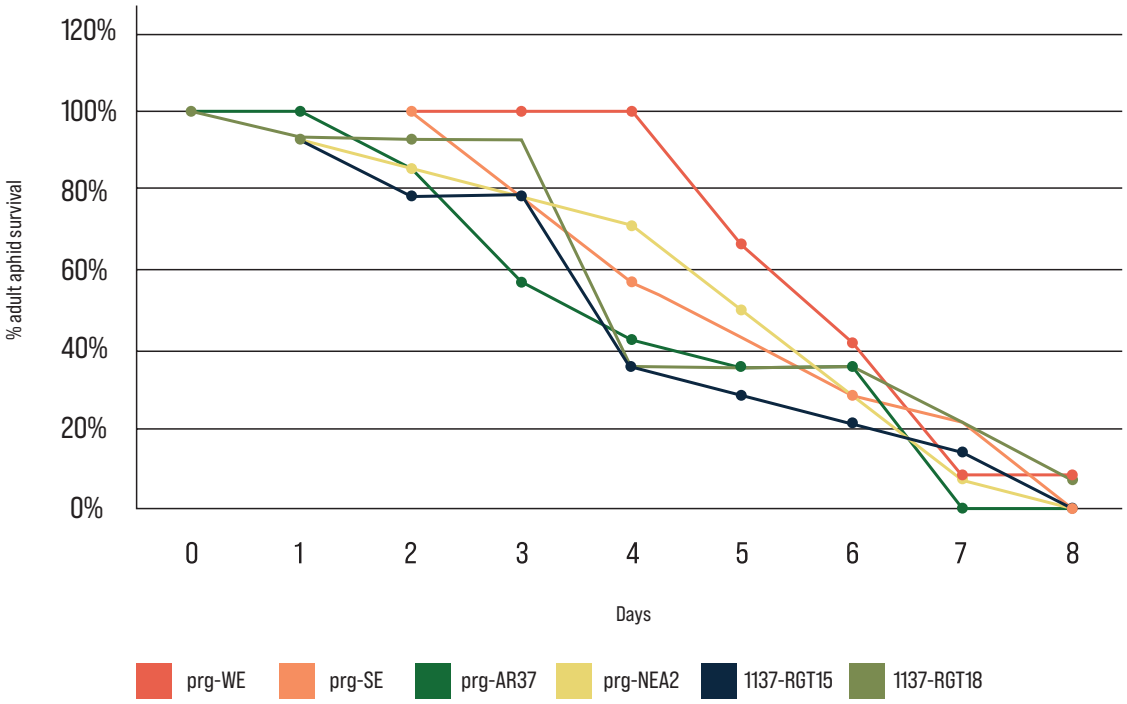


Fig.1. Percentage of adult aphids surviving on a diet supplemented with finely ground root tissue from perennial ryegrass-endophyte symbiota.

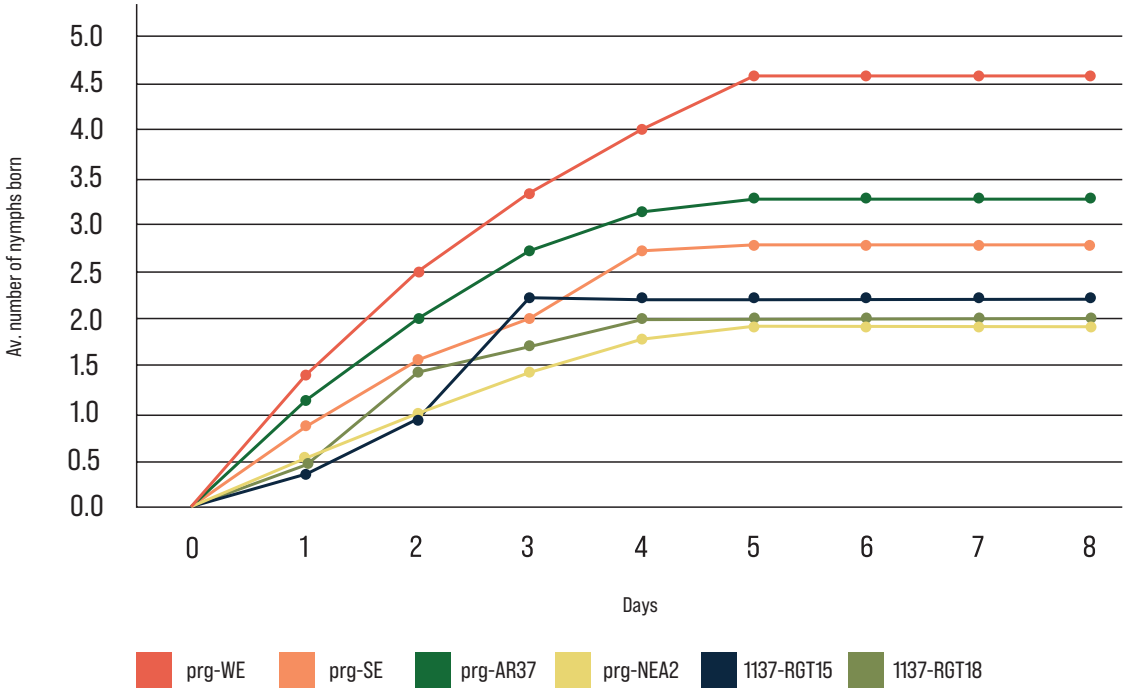


Fig. 2. The average number of nymphs born on a diet supplemented with finely ground root tissue from perennial ryegrass-endophyte symbiota

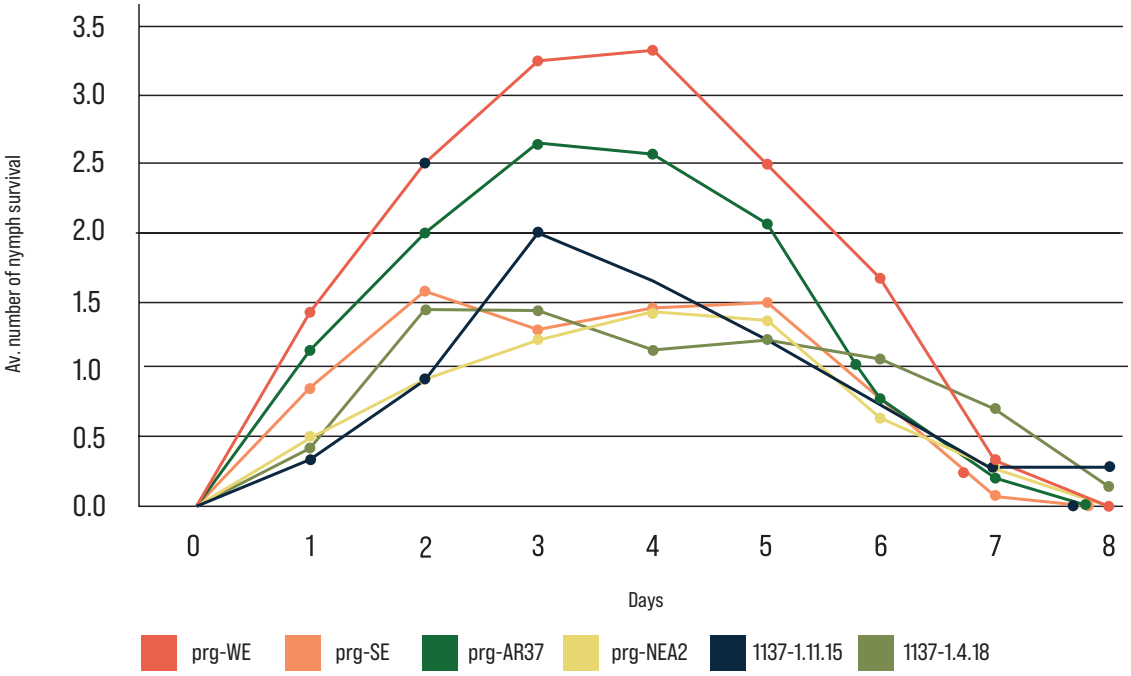


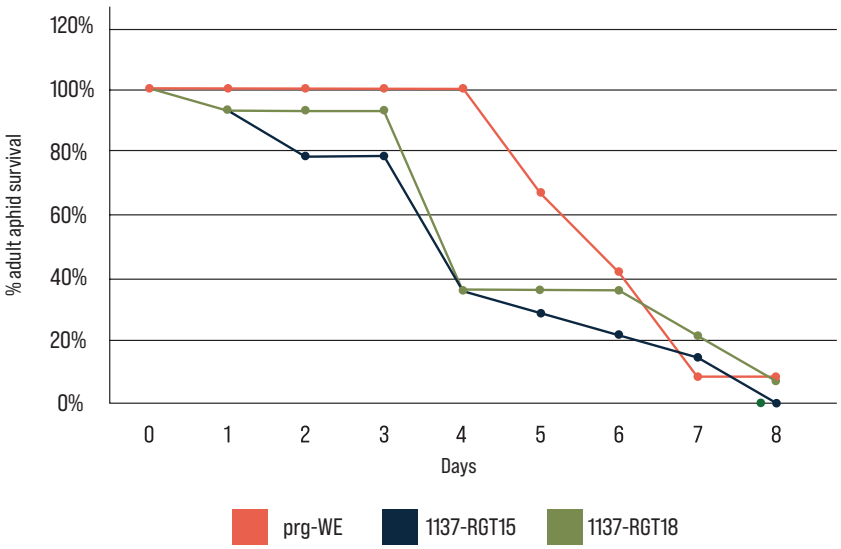
Fig.3. The average number of nymphs surviving on a diet supplemented with finely ground root tissue from perennial ryegrass-endophyte symbiota.

Conclusions

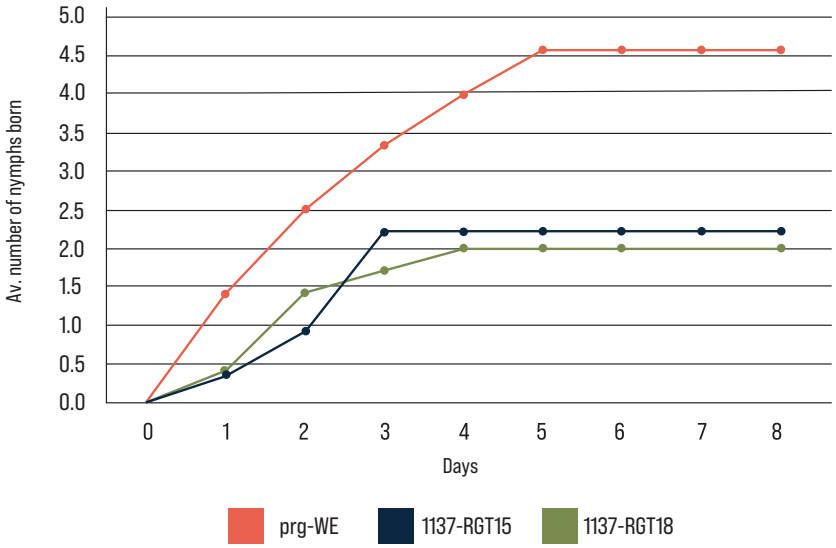
An in vitro bioassay was used to evaluate perennial ryegrass-endophyte symbiota for effectiveness in mealy grass root aphid *(Aploneura lentisci)* control. Aphids were fed a diet comprising a pooled sample of c.100 symbiota root samples suspended in sucrose. Use of a pooled sample provides an indication of the symbiota average.

Significant differences in aphid mortality and fecundity were observed when aphids were fed the root-sucrose diet. These findings were validated via parametric and non-parametric testing, data normalisation and regression models. The presence of an endophyte generally reduced aphid fitness compared to prg-WE. When considering ranking of symbiota for effectiveness in root aphid control 1137-RGT18, 1137-RGT15 and prg-NEA2 provided better whole-of-life cycle control than prg-SE and prg-AR37.

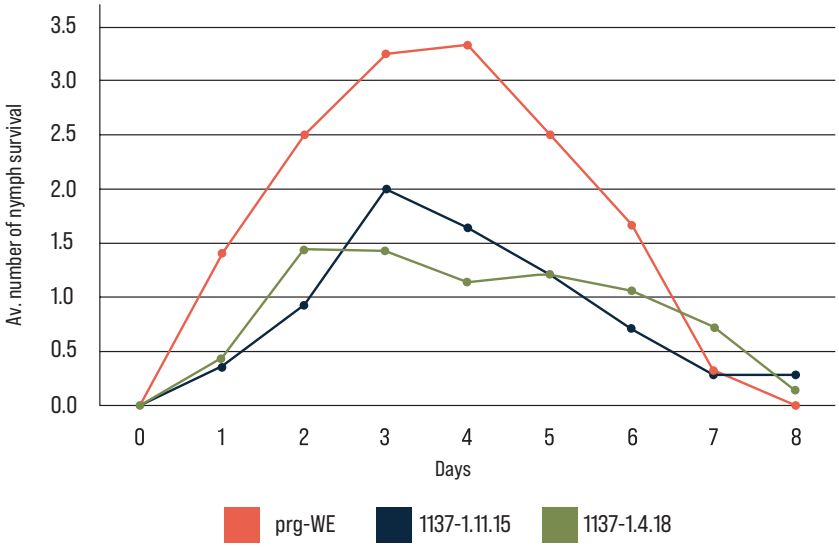
The alkaloids (peramine, ergovaline, lolitrem B and epoxy-janthitrem I) are present in roots of 12-day old seedlings and occur at concentrations lower than that observed in shoots of the same seedlings.



Supplementary Fig.1. Percentage of adult aphids surviving on a diet with finely ground root tissue from perennial ryegrass-endophyte symbiota.



Supplementary Fig. 2. The average number of nymphs born on a diet supplemented with finely ground root tissue from perennial ryegrass-endophyte symbiota.



Supplementary Fig. 3. The average number of nymphs surviving on a diet supplemented with finely ground root tissue from perennial ryegrass-endophyte symbiota.