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Infection of perennial ryegrass (*Lolium perenne*) by an endophyte fungus (*Neotyphodium lolii*) decreases the abundance and diversity of predators and parasitoids

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ABSTRACT

Perennial ryegrass is one of the most important food sources in animal production. However, several pests affect this crop, and one of the primary control strategies is the symbiotic relationships between ryegrass endophyte fungi. This fungus produces alkaloids that exhibit toxic activity against arthropods. Furthermore, the effect of fungi may extend to higher trophic levels, including predators (spiders and/or insects), decreasing their abundance and diversity. Given the importance of spiders and insects as predators, whether the symbiotic interaction between perennial ryegrass and endophyte fungus reduces the abundance and diversity of predators pose an important question. To address this question, natural enemies in perennial ryegrass were collected and analyzed over a year, and the percentage of endophyte fungus was evaluated by the presence of hyphae from two ryegrass cultivars, Jumbo (E-) and Alto AR1 (E+). We observed an 80% endophyte infection rate for (E+) and 0% for (E-). Moreover, 222 individual spiders corresponding to 10 families were identified in both perennial ryegrasses, including 209 individuals for (E-) and 13 for (E+). The most abundant spider family was Lycosidae, representing 71.17% of the total spiders. In addition, 65 insects were collected, corresponding to 6 families, with Carabidae being the most abundant. Furthermore, the Simpson index indicated the dominance of the family Lycosidae. Overall, spider and insect abundance and diversity were reduced in (E+), suggesting a negative effect of the endophyte on predator populations.

Introduction

Crops can be colonized by several organisms (Compant et al., 2019), which can lead to damage, disease or death. Other organisms, including fungal organisms, can act to promote growth and protection from biotic or abiotic stresses (Ademe, 2020). These fungal organisms, termed endophytes, can inhabit their host without causing damage. In general, endophyte fungi (EF) have been reported to increase yield production and improve the uptake of nutrients from the soil as well as the ability to cope with insect pests in perennial ryegrass (PR) (*Lolium perenne*) (Clay, 1996; Ahmad et al., 2020). This plant–fungus symbiosis can generate resistance in the PR-EF complex to insect pests. For instance, *Listronotus bonariensis* (Coleoptera: Curculionidae) has been reported to cause damage to pastures in New Zealand, Australia, and South America, as the adult weevil feed on the leaves of PR (Lanuza et al.,

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2003; Leuchtmann et al., 2014; Shields et al., 2022). One of the strategies implemented to achieve control over *L. bonariensis*, the most serious pest in PR, has been the symbiotic association with EF of the genus *Neotyphodium.* Mutualistic symbionts are widespread and may have bottom-up influences on the structure of communities (Finkes et al., 2006). For example, the symbiosis between this endophyte fungi and PR leads to lower larval survival and oviposition in *L. bonariensis* (Popay et al., 2003). This toxic effect on insects is attributed primarily to nitrogenous compounds produced by endophyte fungi alkaloids, including indole diterpenes, ergot alkaloids, pyrrolopyrazine, and pyrrolizidine (PA). For instance, Moore et al. (2015) reported that in *L. multiflorum,* PA N-formylloline was detected at concentrations from 496 to 1,031 mg/L when EF was present. In addition, Trigo (2011) reported that PAs can flow to different trophic levels, mediating several ecological interactions between herbivores and predators.

Furthermore, the PA peramine in PR has been described as a deterrent for *L. bonariensis,* reducing their feeding score from 4.2 to 2.9 per plant and oviposition from 10.7 to 1.4 eggs/plant in lines infected with endophyte fungi (Jensen et al., 2009; Keathley and Potter, 2011). Thus, the symbiosis with EFs has a positive impact on the control of agricultural pests but can also affect trophic levels, including herbivore insects (HI) and predators such as spiders and/or insects (Wise, 2006). These alkaloids generate toxic and antifeedant effects in HI, but the influence of EFs on these herbivore insects could cascade to other trophic levels (Rudgers and Clay, 2005). For instance, Fuchs et al. (2013) indicated that EFs can harm predator or parasitoid insects by cascading the toxicity generated by fungi through trophic levels (food chain). These authors provided the first evidence that peramine was present in the aphid *Rhopalosiphum padi* (Hemiptera: Aphididae) and in the predatory insects of this aphid when they were raised in ryegrass infected with endophytes, leading to a decrease in the performance of these predators (*Harmonia axyridis*, *Adalia bipunctata* (Coleoptera: Coccinellidae) and *Chrysoperla carnea* (Neuroptera: Chrysopidae)). Similarly, these authors concluded that peramine is responsible for longer pupal stages in the ladybird *H. axyridis*. However, predators and parasitoid insects are not the only beneficial organisms in the field. For example, spiders are considered generalist predators and help to keep pest insect populations below the economic threshold (Mammola et al., 2016). These traits make spiders one of the most important terrestrial predators, and they play a critical role in the biological control of phytophagous insects in grasslands (Cardoso et al., 2011). Unfortunately, information about the impact of endophyte fungi on spider populations present in PR is scarce. The structure of spider communities may be affected by endophyte fungi, decreasing diversity and morphospecies richness and leading to a shift in the relative abundance of predators. For example, in tall fescue subjected to an endophyte infection, a strong negative impact on the native spider population was observed relative to endophyte-free fescue. In addition, a negative effect on the abundance of Linyphiidae and Thomisidae spider families, which were reduced by 47 and 57%, respectively, was observed in PR infected by endophyte fungi (Finkes et al., 2006).

Currently, there are commercial PRs that possess the endophyte fungus Alto AR1 (E+), a line from New Zealand utilized in the control of *L. bonaeriensis*, as well as cultivars that do not have endophyte fungi, such as Jumbo (E-) (Torres et al., 2003). We hypothesized that endophyte fungi in perennial ryegrass from Alto AR1 (E+) would cause a reduction in the abundance and diversity of spider and insect predators. Therefore, to understand the effect of EFs, we analyzed two commercial cultivars of PR, Jumbo (E-) and Alto AR1 (E+) – to elucidate the underlying impacts of endophyte fungi on the abundance and diversity of predators.

Materials and methods

Establishment of commercial cultivars

PR Alto AR1 (E+) and Jumbo (E-) commercial cultivars were used. These cultivars were established in experimental plots of 11.2 m^2 (7 Rows 0.2 m x 8 m long) in a system of 3 blocks in April 2019. These experimental plots were established at the Instituto de Investigaciones Agropecuarias (INIA) Carillanca, Vilcún, Region of La Araucanía, Chile (38° 41'45.8 "S 72° 25'04.6" W). Perennial ryegrass was fertilized according to soil analysis. Fertilization included 60 units of nitrogen (Can 27), 60 units of phosphorus, 1 unit of sulfur and 26 units of calcium (Triple Superphosphate) and 150 units of potassium, 44 units of sulfur, 44 units of magnesium and 15 units of calcium (Vitramag). Finally, the PR was grown for two years in the experimental plots until use in experiments and samplings (2021).

Determination of the presence of endophyte fungus

Tillers from Alto AR1 (E+) and Jumbo (E-) were cut, and the inner epidermis of the leaf sheath was peeled off and placed on a glass slide. Two or three drops of Rose Bengal stain were applied to each sample. After 1-2 min, the samples were covered with a cover slip. Slides were examined under the microscope at 40X (optical Model B-353PLi), and samples exhibiting typical fungal mycelia were considered positive. Sixty replicates were analyzed per PR cultivar (twenty by plot), and the percentage of endophyte fungi-associated PR cultivars was calculated according to the number of plants infected with hyphae according to the report by Parra et al. (2013).

Spider and insect collections

For the collection of spiders, a manual 1,500 W vacuum cleaner (model VCBD8521-CL) was used using a fuel generator as the energy source. Each plot was manually aspirated for approximately 1 minute to reduce noise and the disturbance caused by aspiration and allow quick recoveries for the following sampling. The aspiration methodology was an adaptation of the suction network used by Donald (2012) for capturing psyllids, which used a DC dustbuster, fulfilling the same function as our conventional vacuum cleaner. The aspirate was performed from the forage stem toward the aerial part, facilitating the capture of spiders and insects. After collect, the samples were stored in vacuum-sealed plastic bags that were subsequently labeled. The samples were transferred to the Laboratorio de Química Ecológica at the Universidad de La Frontera (Temuco, Chile) and stored in 50-mL plastic tubes with Kahle's solution, which was composed of 57% distilled water, 28% ethanol, 4% glacial acetic acid and 11% formaldehyde (Peña, 2001). Spider and insect samplings were conducted in January, February, March, May, July, September, and November.

Spider and insect identification

The family level identification for each spider and insect species was performed under a Euromex laboratory magnifying glass (Stereo Blue model) integrated with a digital camera (Euromex HDII model), assisted by the software Image Focus 4 Photography for visualization through a desktop monitor. Each spider family found in PR was identified according to the taxonomic keys proposed by Aguilera and Casanueva (2005), Taucare-Ríos and Sielfeld (2013), and Wheeler et al. (2017). In addition, to identify the insect families belonging to the Hymenoptera order, Elgueta and Rojas (2000) taxonomic keys were used. In complement, the taxonomic keys provided by Artigas (1994) were used to determine the families belonging to the Coleoptera and Hemiptera orders.

Statistical analysis

The EF percentage in Alto AR1 (E+) and Jumbo (E-) PR were compared using Chi-square test with Statistix 10 software (Tallahassee, Florida, USA). Abundance, richness, and alpha diversity indices – Shannon, Simpson, Margalef, and Berger-Parker – were determined using PAST 2.17c (Hammer et al., 2001). The diversity indices were calculated as follows: Margalef index: $D_{mg} = S-1/\ln(N)$, where S = number of species in a sample and N = total number of organisms in the sample; the Shannon index: $H' = -\Sigma pi \operatorname{Log}_2 (pi)$; and the Simpson index: $D = \Sigma (pi)_{2}$, where pi = ni/N, ni = species abundance, and N = total number of organisms in the sample. In addition, for the measure of true diversity, we used order $q = 2$ (D^2), the inverse of the Simpson index, which can be interpreted as the number of dominant species according to reports by Alencar et al. (2015). For the calculation of significance, the lower and upper limits of confidence intervals were subjected to a bootstrapping process with 1000 iterations at 95% confidence for each diversity index. Chi-square test was performed to analyze the influence of the ryegrass cultivar (with and without endophytes) on the total number of spider and insect families. Data were considered significant at $p < 0.05$.

Results

The percentage of endophyte fungi in commercial PR was significantly higher ($p < 0.05$) in Alto AR1, with 80% EF infection, compared with Jumbo, where no infection was detected (0%) (Table 1). For to the total number of predators collected from Jumbo (E-) and Alto AR1 (E+), a significant 88.29% reduction in spider abundance was observed for Alto AR1 relative to Jumbo (E-). Similarly, a decrease of 13.25% in insect abundance was observed for Alto AR1 (E+) (Fig. 1). Spider sampling identified 222 individual adults of the order Araneae, all belonging to the suborder Araneomorphae. Members of the Lycosidae family were abundant, at 71.17%, followed by Dyctinidae, at 9.45%. The PR Jumbo (E-) exhibited 209 individuals, representing 94% of the total, whereas the PR Alto AR1 (E+) exhibited 6% of all spiders collected (Table 2). In addition, there was a significant reduction in the number of Lycosidae (154-4), Linyphiidae (10–2), Corinnidae (7–2), Dyctinidae (16–5) and Oonopidae (10–0) families for Alto AR1 (E+) in comparison with Jumbo (E-). A general reduction was also observed in the total number of predator insects. The PR Jumbo (E-) exhibited an insect abundance of 56.92%, but the PR Alto AR1 (E+) exhibit a reduction of 43.67% in predator abundance (Table 2). Concerning spider alpha diversity, the richness, and Shannon H and Margalef diversity indices of were not significant, with *p*-values of 0.752, 0.495, and 0.758, respectively. However, Simpson's diversity index differed significantly between Jumbo (E-) and Alto AR1 (E+), indicating a dominance of the family Lycosidae, consistent with the Berber-Parker index ($p = 0.003$) (Table 3). In addition, values for true diversity did not significantly differ between Jumbo and Alto AR1. To the insect predator/parasitoid alpha diversity, the richness and Shannon H, Simpson, and Margalef diversity indices did not differ significantly, with *p*-values 0.874, 0.294, 0.089, and 0.551, respectively. Finally, values for true diversity did not significantly differ between Jumbo and Alto AR1 (Table 3).

Table 1
Infection percentage of infection in Jumbo (E-) endophyte-free and Alto AR1 (E+) endophyte-infected ryegrass. * indicates a significant difference according to the chi-square test ($p < 0.05$).

Commercial cultivar	Percentage of endophyte (%)	n
Jumbo (E-)	0	60
Alto AR1 (E+)	80*	60

Discussion

The incorporation of control strategies based on endophyte fungi symbiosis to protect against insect pests may reduce predator abundance and diversity at higher trophic levels due to the toxic effect of PAs (Rudgers and Clay, 2007). Harri et al. (2008) speculated that the alkaloids produced by endophyte fungi are responsible for fitness reduction in aphids and, therefore, the reduction in predator fitness when the aphids were fed with EF-infected plants, consistent with our results. The numbers of most predators and parasitoid insects were reduced in the presence of EFs, indicating a relationship between the decrease in the beneficial insect population and the presence of endophyte fungi. To provide an explanation for the points mentioned above, several investigations have put forth two potential causes: 1) Perennial crop systems (generally with endophytes in the case of forage) differ significantly from annual crop system in terms of their impact on natural enemy communities and 2) The production of alkaloids by endophytic fungi may have detrimental effects on the growth, development, and survival of parasites and predators that target herbivorous insects. There is evidence suggesting that these endophytic alkaloids can cascade through trophic levels, leading to alterations in the behavior of natural enemies. Surprisingly, in some cases, this alteration may results in higher herbivore loads rather than lower ones, as demonstrated in studies conducted by Faeth and Saari (2012) and Rusch et al. (2015). Furthermore, our results indicate that the spider family Lycosidae was the most abundant in PR, similar to the report by Finkes et al. (2006), who described Lycosidae as the primary spider family present in *L. arundinaceum* infected with

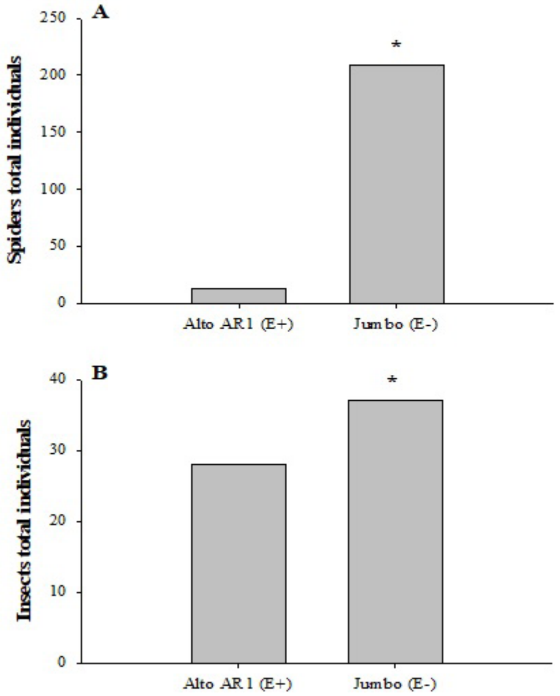

Fig. 1. Total number of predators collected from Jumbo (E-) and Alto AR1 (E+) perennial ryegrass. A) Number of spiders and B) the number of insects. * indicates significant differences according to the chi-square test.

Table 2
Number of total individuals per predator/parasitoid (spiders and insects) family collected for each experimental plot of perennial ryegrass. * indicates a significant difference according to the chi-square test.

Order	Family	Jumbo	Alto AR1	Total
Araneae	Lycosidae	154*	4	158
	Anyphaenidae	2	0	2
	Anapidae	4	0	4
	Linyphiidae	10*	2	12
	Corinnidae	7*	2	9
	Theridiidae	3	0	3
	Miturgidae	2	0	2
	Dyctinidae	16*	5	21
	Salticidae	1	0	1
	Oonopidae	10*	0	10
Coleoptera	Carabidae	7	14*	21
	Coccinellidae	11*	7	18
Hymenoptera	Braconidae	8*	3	11
	Ichneumonidae	1	0	1
	Figitidae	7*	3	10
Hemiptera	Nabidae	3	1	4
Total		**246***	**41**	**287**

Table 3
Alpha diversity estimations of predators/parasitoids for the two perennial ryegrass species analyzed: (Jumbo (E-) and Alto AR1 (E+)). The *p* value of the bootstrapping test evaluated the null hypothesis of no differences between the alpha-diversity estimations.

Class	Alpha diversity	Jumbo (E-)	Lower – Upper	Alto AR1 (E+)	Lower – Upper	*p*
Arachnida	Richness S	10	8 – 10	4	1 – 6	0.752
	Individuals	209	209 – 209	13	13 – 13	-
	Shannon H	1.006	0.860 – 1.225	1.306	0.981 – 1.517	0.495
	Simpson J	0.454	0.358 – 0.521	0.710	0.693 – 0.721	*0.036*
	Margalef	1.705	1.326 – 1.705	1.326	1.073 – 1.949	0.758
	Berger-Parker	0.765	0.673 – 0.801	0.384	0.461 – 0.923	*0.003*
	D^2	2.499	-	1.342	-	1.287
Hexapoda	Richness S	6	6 – 6	5	4 – 5	0.874
	Individuals	37	37 – 37	28	28 – 28	-
	Shannon H	1.623	1.433 – 1.707	1.291	0.905 – 1.470	0.294
	Simpson J	0.786	0.708 – 0.807	0.663	0.489 – 0.744	0.089
	Margalef	1.385	1.385 – 1.385	1.200	0.900 – 1.200	0.551
	Berger-Parker	0.297	0.243 – 0.459	0.500	0.357 – 0.678	*0.005*
	D^2	1.272	-	1.508	-	1.304

endophyte fungi (*N. lolii*). In addition, the same study indicated that the EF did not affect the total abundance of spiders but did impact the proportion of spider families, including Linyphiidae (47%) and Thomisidae (57%). Moreover, Keathley and Potter (2011) reported that the abundance of spiders in tall fescue (*L. arundinaceum*) plants was not affected by the presence of endophyte fungi. A similar situation occurs with the Carabidae family (Coleoptera), which was recorded mostly in ryegrass with endophyte (Table 2). Surveys of generalist arthropod predators in field plots suggest that this family may be insensitive to the presence of the endophyte, however, further studies are needed to corroborate this hypothesis (Rudgers and Clay, 2007). Otherwise, Rudgers and Clay (2008) reported that plots of tall fescue infected with EF exhibited 40% fewer arthropods than EF-free plots. Alderweireldt (1987) reported that the most abundant family in a pasture of Italian ryegrass (*L. multiflorum*) was Linyphiidae, which is consistent with the report by Pommeresche et al. (2013), who identified 37 species of the family Linyphiidae. Furthermore, these authors reported the identification of 13 members of the family Lycosidae, which represents the second most abundant families in barley crops after Linyphiidae. Together, these data support the theory that endophyte-infected ryegrass plants reduce the abundance and diversity of predators/parasitoids due to the cascading effect of toxic compounds and subsequent alteration of tri-trophic interactions. The prevailing belief among researchers has suggested that endophytic fungi, which inhabit certain plant species and produce toxic compounds, play a crucial role in shaping predator populations. These compounds deter herbivores from feeding on host plants, resulting in reduced herbivore populations and subsequently, a decline in predator abundance (Carbonell et al., 2022). However, we propose an alternative hypothesis that challenges this prevailing notion and highlights the importance of herbivore species reduction as a potential driver of predator population decline (Wootton et al., 2022). This hypothesis posits that predators follow the peaks of occurrence of herbivore species, relying on them as a primary food source. Consequently, a reduction in the number of herbivores within an ecosystem would inevitably lead to a decrease in the predator population. While the influence of endophytic fungi on herbivores cannot be entirely disregarded, it is crucial to consider the possibility that the decline in herbivores could have an equally significant impact on predator populations, if not more.

Conclusion

In general, our results suggest that the diversity of predators (spiders and insects) was lower in the presence of EFs in PR, particularly those of the Lycosidae family. Therefore, it is possible to assume that the bottom-up interaction could negatively affected by the presence of EFs, and that and endophyte fungi may reduce the predators´ population. Therefore, future work should aim to (1) determine the primary alkaloids present in the body of predators following consumption of their prey in Alto AR1 (E+) endophyte-infected PR, (2) quantify the alkaloids responsible for the decrease in the abundance and diversity of predators and (3) To investigate the relationship between herbivore species reduction and predator population decline. By presenting other possibilities that emphasize the potential significance of herbivores as drivers of predator dynamics and exploring these perspectives, it could be possible to contribute to the broader understanding of ecological systems. This endeavor will foster a more comprehensive and nuanced approach to ecosystem management and conservation. Finally, by presenting other possibilities that emphasize the potential significance of herbivores as drivers of predator dynamics and exploring these perspectives, it could be possible to contribute to the broader understanding of ecological systems. This endeavor will foster a more comprehensive and nuanced approach to ecosystem management and conservation.

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Conflicts of interest

The authors have declared that they have no competing interests.

Author contribution statement

MCF and LB conceived the study. MCF, ML, CR and DMC conducted experiments. LB and AQ contributed materials. MCF, WV and PF analyzed data and conducted statistical analyses. MCF and LB wrote the paper. LB secured funding. All authors read and approved the manuscript.

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