

Presence of *Epichloë* Fungus in the Endosperm-Side of the Seed Predicts the Symbiotic Status of the Seedling

P.E. Gundel*, A.C. Ueno, M. Panteix and L.J. Iannone

ABSTRACT

Some important forage grasses of the genera *Festuca* and *Lolium* establish persistent symbiotic associations with vertically-transmitted fungal endophytes (genus *Epichloë*). In certain cases, the fungus causes livestock diseases due to fungal toxins that accumulate in the plant biomass. Killing the fungus in the seed is a possibility for getting rid of the problem. However, since the symbiosis is mutualistic, the inoculation of elite cultivars with non-toxic but still beneficial endophytes is a current breeding strategy. Additionally, the symbiosis has become a model system to study in ecology and evolution, where the manipulation of the symbiotic status of plants is critical for the experiments. In this study, we confirmed that testing for the endophyte's presence or absence in the endosperm-side of the seed was a reliable predictor of the symbiotic status of the seedling. We built on this previously proposed concept by (i) estimating the high correspondence between the infection status in one side of the seed (either + or -) and the infection status of the other side, and (ii) demonstrating that cutting the seed in two halves did not affect seed germination, normal seedling growth, nor the endophyte transmission to the seedling. We also showed that cutting the seed reduced seedling size, an impact that increased with endophyte presence provided the fungus was alive. The strengths and weaknesses of the technique, as well as its potential use in other species, are discussed.

INTRODUCTION

Persistence and multiplication of certain plant symbionts rely on the successful transmission through the seed. This is the case of certain *Epichloë* species (Clavicipitaceae), the fungal endophytes of grasses (subfamily Poöideae) (26, 33). *Epichloë* fungal endophytes became critically important when researchers discovered that they were the responsible agents behind cattle and sheep intoxications when grazing on tall fescue (*Festuca arundinacea* Schreb.) and perennial ryegrass (*Lolium perenne* L.) (2, 11), probably the two most important forage grasses of the temperate regions of the world (18, 21, 34). In order to eliminate such a threat, the first strategy consisted of eliminating the fungi from seeds of commercial cultivars (5). However, due to the abundant evidence indicating that the symbiosis improved plant persistence and productivity, the strategy turned to selection of nontoxic endophytes, the so-called

P.E. Gundel and A.C. Ueno, IFEVA, Universidad de Buenos Aires, CONICET, Facultad de Agronomía, Buenos Aires, Argentina; M. Panteix, Undergraduate student, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina; L.J. Iannone, Instituto de Micología y Botánica (INMIBO), Universidad de Buenos Aires-CONICET, Ciudad Autónoma de Buenos Aires, Argentina. *Corresponding author (gundel@agro.uba.ar). Accepted 16 Nov. 2018.

“safe endophytes,” which are inoculated into elite cultivars (4, 13, 20). From the 1970’s to today, the grass-endophyte symbiosis has become a model system not only in agronomy and plant physiology (mainly in forage grasses), but also in ecology and evolution (23, 30).

Manipulation of the plant symbiotic status aims to control the “endophyte factor” and experimentally estimate its effects on different aspects of host physiology and ecology. Depending on the goals, studies could address problems at the population or individual level. At the population level, each experimental unit would consist of a large number of individuals either highly infected (E+) or uninfected (E-) with fungal endophytes. One simple possibility is to start with a batch of endophyte-infected seeds, and to subject half of them to a treatment for the removal of the fungi. The treatments can be *i*) application of a systemic fungicide (17), *ii*) soaking seeds in hot water (24), *iii*) heat-treatment of seeds in an oven (22, 31), or *iv*) aging the seeds. Since fungi longevity is shorter than that of the seeds, aged seeds can produce endophyte-free seedlings (14, 35). Apart from the fact that each technique has to be optimized for each species and cultivar, it is not recommended to conduct experiments with fungicide-treated and untreated seeds, as there would be confounding effects. Fungicide-treated and untreated seeds should be sown in a common field plot and plants allowed to exchange pollen during flowering (those grass species are usually self-incompatible and wind cross-pollinated), then work done using F1 seeds as, for example, reported by Gundel et al. (15, 16). When the interest is the interaction of plant genotype and endophyte symbiosis, it is possible to split the plant into ramets (clones) and to treat some with fungicide in hydroponic conditions (10). Before being used experimentally, those ramets (fungicide-treated and untreated) should be grown for a while to clean any potential phytotoxic effects or any fungicide-induced imprinting on the genome (epigenetic). Moreover, the ramets can be transplanted in a common field plot to produce seeds that will be available to work with in future experiments, after confirming that the fungicide succeeded in removing endophytes from plants.

The possibilities are more restrictive in annual species such as the annual ryegrasses (e.g., *Lolium multiflorum* Lam., *L. rigidum* Gaudin), as there is no time to grow plants or ramets for long periods. For these species, determining the infection status of a plant requires establishment of the seed infection status before sowing. However, the standard seed squash technique, the most widely used method for evaluating endophyte infection frequency of seed lots, is destructive. Briefly, the squash technique consists of seed imbibition in 5% NaOH for at least 8 h, the seed is squashed over a slide, stained, and covered with a coverslip, and then examined for endophyte hyphae under a light microscope (1, 6, 25). In 2015, Moore et al. (28) proposed a methodology to segregate endophyte-infected and endophyte-free seeds from a common seed lot by searching for the fungal hyphae in the endosperm-side of the seed. In our study, we further built on their technique by exploring some needed aspects to ensure its reliability. The successful implementation of this methodology would depend on the confirmation that (*i*) there was a high correspondence between infection status in endosperm-side and the embryo-side, (*ii*)

cutting the seed in two halves did not affect seed germination and the ability to produce a normal seedling, and (iii) it did not affect the ability of the endophyte to colonize the seedling. We conducted a suite of experiments in order to address all these aspects. Some limitations of the presented technique are also discussed.

MATERIALS AND METHODS

Seeds of *Lolium multiflorum* were collected from a successional grassland in Inland Pampa, Argentina (Carlos Casares County, 34°06'S, 60°25'W). The seeds showed a high endophyte infection frequency (95%), determined by evaluating 100 seeds under a microscope [seed squash technique; Bacon and White (1) and Card et al. (6)]. Part of the seed lot was treated with a systemic fungicide to kill the fungus (Baytan® 150 FS, 150 g a.i. kg⁻¹; Bayer AG). Treated and untreated seeds were sown in 1 m² contiguous plots at the experimental field, College of Agronomy, University of Buenos Aires (34°35'S, 58°35'W). Seeds produced by those plants in a common garden were checked for endophyte infection resulting in two seed lots: one with high (E+: 95%, n = 100) and one with low (E-: 5%, n = 100) endophyte infection. These two seed lots were annually cultivated for maintenance and multiplication.

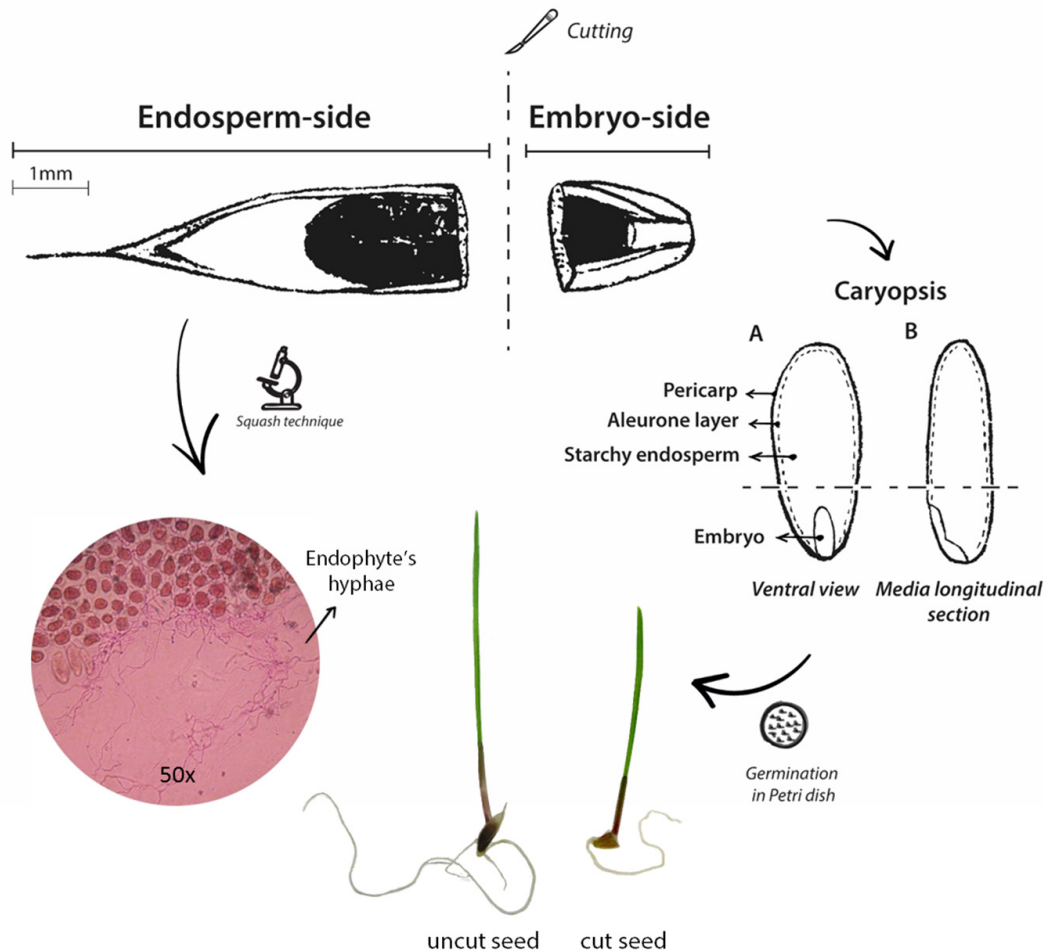
Besides the endophyte infection status, we tested seed lots stored for different time periods. The experiments presented in this article were completed in 2016 on what was designated as 'new seeds' produced in 2015 and 'old seeds' produced in 2013. At the time of the experiments, new seeds had been stored for 6 months while old seeds had more than two years of storage. Seeds were stored under cold temperatures (5 °C) and dry conditions (paper bags containing seeds were placed inside a plastic box with silica gel).

Experiments

Correspondence essay. To determine to what extent observation of an infection status in one side of a seed was a reliable evidence of the infection status of the other side, a correspondence essay was performed. The essay consisted of evaluating endophyte presence in both sides of the seed, the endosperm-side and the embryo-side. New and old E- and E+ seeds (about 100 seeds each) were cut into two parts, separating the endosperm-side from embryo-side (Fig. 1). Each part was placed in one of two corresponding 96-well microplates, using multiple microplates per population. Each well was filled with NaOH (3%) to fully cover the seed part. After an incubation period of at least 8 h in NaOH, both the endosperm- and matching embryo-side of the seed were subjected to the squash technique: each seed part was squashed on a slide, stained with rose bengal, and observed under a light microscope (×100), and infection status determined.

Effects of removing the endosperm-side of the seed on germination, seedling performance and endophyte transmission. The effect of cutting the seed (cut vs. uncut) on the ability of the embryo side to germinate, produce a normal seedling, and transmit the endophyte fungus, in new and old E- and E+ seeds, was evaluated. Thirty whole-seeds and 30 embryo-side (or endosperm-removed) seeds from each population (endophyte symbiotic status and seed

FIGURE 1. Scheme depicting steps used to determine the infection status of a mature plant through searching for fungal mycelia in the endosperm-side of the seed and germinating the embryo-side. The circular magnified image shows mycelia of the fungal endophyte and aleurone round-type cells following the seed squash technique (6, 32). Bottom center photos show normal seedling development from cut and uncut seeds.



age) were sown in 9-cm Petri dishes lined with Whatman-1 filter paper moistened with 5 mL of distilled water, with three replicates per treatment. The dishes were incubated under optimal conditions for germinating *L. multiflorum* seeds, namely 15/25 °C alternating temperature and 12/12 h light/dark cycles (19). One week after sowing, seed germination (radicle protrusion as evidence of root emergence from the caryopsis) was evaluated, and two weeks after sowing, the number of normal seedlings determined.

Concurrently, we evaluated the effect of treatments (endophyte infection status, age of the seed lot, and cutting) on seedling biomass and length. Seedling length was measured from the caryopsis-shoot juncture to the tip of the first leaf, on 15 randomly selected normal seedlings per Petri dish. Biomass of seedlings was determined by weighing 10 seedlings per Petri dish after drying in an oven at 60 °C for 72 h.

To evaluate the effect of seed lot age and cutting treatment on transmission of the endophyte to the seedlings, 100 normal seedlings were transplanted into soil in seedling trays and kept outdoors. When seedling had 3–4 leaves, they were harvested and evaluated by microscopic examination of the base of the pseudostems stained with rose bengal (1, 3). This was only performed on the E+ populations.

Statistical analyses

With the correspondence essay, we evaluated the likelihood that any endophyte infection status, whether positive or negative, in one side of the seed (either endosperm-side or embryo-side), would have exactly the same status in the other side. We modelled the individual probability of correspondence for each population based on a binary response [correspondence (+,+ or -,-) = 1, and non-correspondence (+,- or +,-) = 0], as affected by endophyte infection status (E– and E+), seed age (old and new), and cutting (cut and uncut). The model was a generalized linear model (GLM) using the binomial error distribution and logit link function (9, 37). There was no over-dispersion.

Similar models were used to evaluate the effects of the same factors on seed germination and proportion of normal seedlings. Germination was modelled (GLM) using a quasi-binomial distribution and logit link to account for over-dispersion. The “normal seedling/germinated seed” was also modelled (GLM) using the binomial distribution and logit link function (9, 37). No over-dispersion was observed for this latter case.

The proportion of endophyte-infected seedlings (infected = 1; uninfected = 0) as affected by seed age (new and old) and cutting treatment (cut and uncut), was estimated by GLM using binomial distribution and logit link (9, 37). No over-dispersion was observed. Analyses of deviance were performed to estimate the effect of each factor (Chi-square test).

Seedling biomass (DW) and seedling height (cm), as affected by endophyte infection status, seed age, and cutting, were estimated through standard ANOVA using GLM. The assumptions of ANOVA were achieved without data transformation.

All statistical analysis were performed in R software (R Core Team, 2011; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Correspondence in symbiotic status between endosperm-side and embryo-side. The average correspondence of the presence/absence of fungal hyphae in the endosperm-side and the symbiotic status of the embryo-side across all populations was 98% (Table 1). This was neither affected by endophyte symbiotic status ($\chi^2_{1,403} = 0.959$, $p = 0.327$) nor by the age of the seed lot ($\chi^2_{1,404} = 2.954$, $p = 0.086$).

Effects of removing part of the endosperm-side on capacity to germinate and produce a normal seedling. Germination was not affected by the symbiotic status of the seeds ($\chi^2_{1,21} = 0.404$, $p = 0.534$) but affected by a two-way interaction between the seed lot age and cutting treatment ($\chi^2_{1,18} = 9.443$,

TABLE 1. Average level of correspondence (proportion and estimated limits of the Wald-type confidence interval for binomial distributions) of endophyte infection status between the endosperm-side and embryo-side of each seed, for new and old *Lolium multiflorum* seed lots with low (E–) and high (E+) incidence of the fungal endophyte *Epichloë occulta*.

Seed lot age	Infection status	N	Level of correspondence (95% CI)
New	E–	106	0.9528 (0.9487–0.9568)
	E+	98	0.9796 (0.9767–0.9824)
Old	E–	94	0.9894 (0.9872–0.9915)
	E+	108	0.9907 (0.9889–0.9924)

TABLE 2. Average percentage (\pm SE, $n = 3$) germination and normal seedlings of seeds from new and old *Lolium multiflorum* seed lots, with high and low infection incidence of the fungal endophyte *Epichloë occulta* (E– and E+), subjected to either removal of part of the endosperm (cut) or not (uncut).

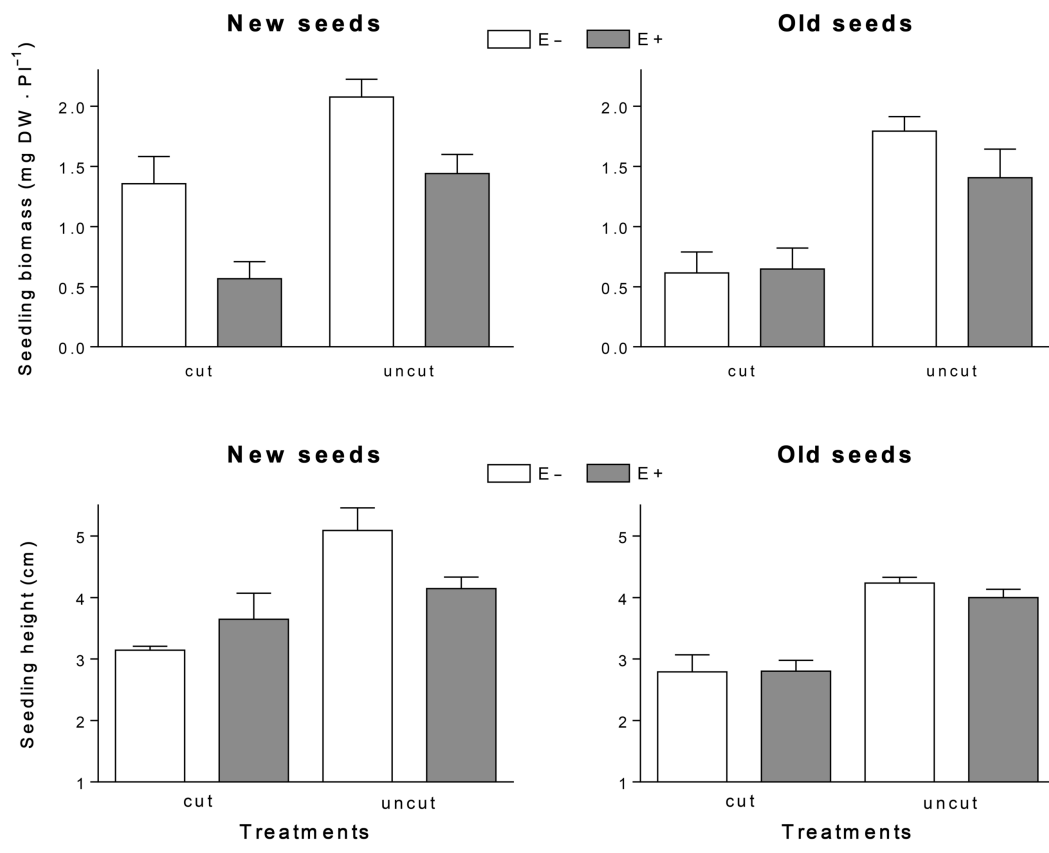
Seed lot age	Treatment	Endophyte incidence	Germination	Normal seedlings
			(%)	(%)
New	Cut	E–	100.00 (0.00)	98.88 (1.11)
		E+	98.88 (1.11)	100.00 (0.00)
	Uncut	E–	94.44 (2.94)	96.58 (1.92)
		E+	96.66 (1.92)	100.00 (0.00)
Old	Cut	E–	98.66 (1.33)	100.00 (0.00)
		E+	97.77 (2.22)	98.85 (1.15)
	Uncut	E–	96.62 (1.92)	98.81 (1.19)
		E+	93.22 (2.02)	98.81 (1.19)

$p = 0.003$) (Table 2). Removing the endosperm-side led to a small increase in proportion of germinated seeds ($\approx 4\%$).

The proportion of normal seedlings was dependent on the endophyte symbiotic status in interaction with the age of the seed lot ($\chi^2_{1,17} = 0.591$, $p = 0.002$) but not affected by the cutting treatment ($\chi^2_{1,22} = 0.159$, $p = 0.613$) (Table 2). Despite the significance of these factors' effects, the percentage of normal seedlings was very high across all treatments.

Effects of removing the endosperm-side on seedling size. Two weeks after seed sowing in Petri dishes, the accumulated biomass of the seedlings depended on the two-way interaction between the endophyte infection status and the seed lot age ($F_{1,16} = 4.589$, $p = 0.040$), and on the main effect of cutting treatment ($F_{1,16} = 49.736$, $p < 0.001$). In new seeds, endophyte presence caused a reduction in biomass of about 42%, from 1.72 mg to 1.00 mg, but the reduction was less pronounced in old seeds, from 1.20 mg to 1.02 mg, or 15% (Fig. 2). Conversely, cutting reduced the biomass of seedlings by about 53%, from 1.68 mg to 0.80 mg, independently of the symbiotic status or seed age (Fig. 2).

FIGURE 2. Biomass and height of seedlings from new and old *Lolium multiflorum* seed lots with low (E-) and high (E+) incidence of *Epichloë occulta* fungal endophyte, subjected to either removal of part of the endosperm (cut) or not (uncut). Values are averages \pm SE (n = 3).



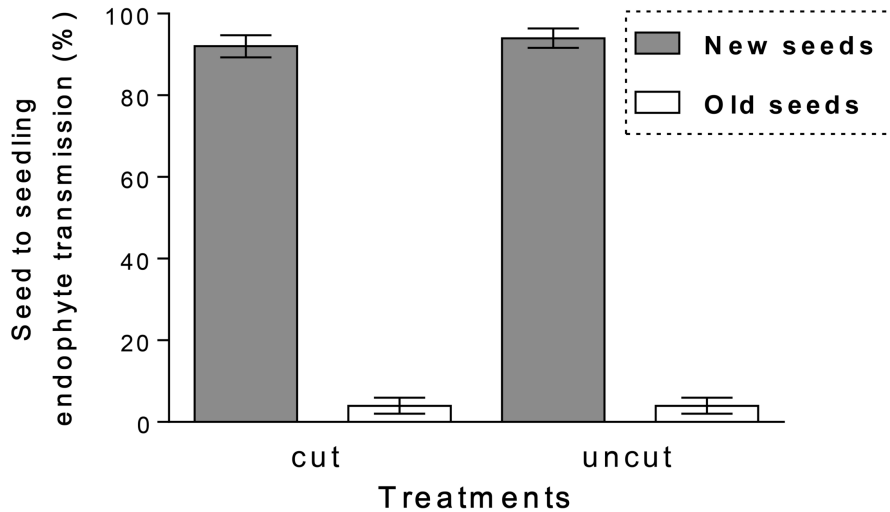
Seedling length was affected by endophyte infection status in interaction with cutting treatment ($F_{1,16} = 5.910$, $p = 0.027$) and by the main effect of seed lot age ($F_{1,16} = 9.898$, $p = 0.010$). Seedlings developing from E- uncut seeds were longer than E+ uncut seeds (4.66 and 4.07 cm, respectively), while this difference was not observed in cut seeds (Fig. 2). On average, seedling length of old seeds (around 3.45 cm) was 14% shorter than for seedlings of new seeds (around 4.00 cm) (Fig. 2).

Influence of removing the endosperm-side of the seed on endophyte seed-to-seedling transmission. Seed-to-seedling transmission of the endophyte was not affected by cutting treatment ($\chi^2_1 = 0.190$, $p = 0.660$), but depended on the age of the seed lot ($\chi^2_1 = 385.68$, $p < 0.001$). In new seeds, the incidence of the fungal endophyte in seedlings was very high (93%), while it was very low (2%) in old seeds (Fig. 3).

DISCUSSION

By cutting the seed in two parts and checking for the endophyte symbiotic status in the endosperm-side, it was possible to predict the symbiotic status of the future seedling, as proposed by Moore et al. (28). For every observed presence or absence of fungal hyphae in the endosperm-side, there was a very

FIGURE 3. Proportion of seedlings infected with the fungal endophyte *Epichloë occultans* produced by new and old *Lolium multiflorum* seeds and subjected to removal of part of the endosperm (cut) or not (uncut). Values are averages \pm SE (n = 100).



high probability of obtaining the same result in the embryo-side (overall mean: 98%; n = 406). This high correspondence was not altered by the overall endophyte infection status or the age of the seed lot. In all cases, cutting the seeds did not have a negative effect on their ability to germinate or produce normal seedlings, but there was a consistent reduction in seedling growth in terms of accumulated biomass and height. Nevertheless, the endophyte transmission to seedlings was not affected by the cutting treatment in new seeds.

The method proposed by Moore et al. (28), and further explored in this study, offers a reliable alternative to segregating endophyte-infected and endophyte-free seeds before sowing, from seed lots with variable endophyte infection frequencies. It was demonstrated that irrespective of the overall endophyte infection status (high frequency of E+ or E- seeds) and age of seed lot (old or new), the probability of finding the same outcome in terms of endophyte infection (+ or -) in the endosperm-side and embryo-side of the same seed was very high (i.e., +/+ or -/-). Additionally, in support of this technique, we did not find any negative effects of removing part of the endosperm on the seed's ability to germinate and generate a normal healthy seedling.

What we did observe was a significant reduction in seedling size. Seedlings from seeds with partially removed endosperms were significantly shorter and lighter than seedlings from uncut seeds. However, the effect of cutting on seedling size was not evident in three-months-old plants, as referenced by Moore et al. (28), indicating that three months was enough time for seedlings to overcome the reduction in endosperm reserves. Interestingly, the presence of endophytes tended to consistently reduce the biomass of seedlings germinated from new seeds, but not from old seeds. The reduction in seedling size as a consequence of removing part of the endosperm was related to available reserves for growing during the heterotrophic phase of a plant. Interestingly,

seedlings also expressed the cost of harboring a fungal endophyte, a heterotrophic organism also deriving reserves from the seed's endosperm. Costs of endophytes are referred to as metabolic costs and have been found at different plant autotrophic stages (8). Those costs have been suggested to have a strong negative impact on host fitness when photosynthesis is limiting (7, 8). Costs at the seed stage should be more critical since stored reserves would be limited and never increasing. Strikingly, the cost we observed on seedlings produced by the new seeds was absent in seedlings derived from old seeds, which was surely related to the fact that endophytes were no longer alive (it should be noted that fungal endophytes were not present in seedlings from old seeds).

The investigated technique is appropriate for use on a small set of experimental plants, due to the difficulty of manipulating a large number of seeds. For an appropriately organized workflow, we used two separate 96-well microplates, one for endosperm parts and the other for embryos plus remaining endosperm portions, keeping track of individual seeds. This organization allowed for manipulation of a relatively large number of seeds in blocks of 96. More studies are needed to determine whether this technique is applicable to other grass-endophyte symbioses. There is a great variation in seed size among endophyte symbiotic grass species, ranging from big seeds such as *Bromus setifolius* J. Presl (29) to quite small ones such as *Festuca rubra* L. (12). In the latter case, for example, cutting very small seeds could increase the chances of damaging the embryos. Furthermore, the distribution of fungal mycelium in the seed can vary (27, 36), affecting the correspondence between the infection status of the endosperm-side and the embryo-side.

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