Received: 14 December 2023 | Accepted: 8 January 2025

DOI: 10.1002/csc2.70002

Crop Science

ORIGINAL ARTICLE

Crop Breeding & Genetics

Higher seed yield through selection for reduced seed shattering in Italian ryegrass

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Assigned to Associate Editor Patrick Conaghan.

[Correction added on 8 August 2025 after first online publication: The copyright line was changed and legal statement added.]

Funding information
Agroscope; Delley Seeds and Plants Ltd.

Abstract

Seed shattering, that is, the loss of seeds at ripening stage shortly before or during seed harvest, is strongly reducing seed yield in Italian ryegrass (*Lolium multiflorum* Lam.). The aim of this study was to evaluate the possibility to reduce seed shattering within breeding germplasm via recurrent phenotypic selection on spaced plants. Starting from a founder population of 300 plants not previously selected for seed shattering, two cycles of phenotypic selection for high and low seed shattering were performed based on spaced plants. The resulting five populations with different levels of selection for seed shattering (selected once or twice for low seed shattering, selected once or twice for high seed shattering, and no [random] selection) were phenotyped in plot trials with two harvesting dates (early and late). Seed shattering was highest in the population selected twice for increased seed shattering (7.92% for early and 28.44% for late harvesting), followed by the population selected once for increased seed shattering (7.34% early, 21.56% late), the non-selected population (6.12% early, 20.22% late), the population selected once for decreased seed shattering (4.13% early, 16.73% late), and the population selected twice for decreased seed shattering (2.41% early, 9.31% late). Selecting twice for low seed shattering resulted in a reduction of seed shattering by 10.91 percentage points at late harvest when compared to the non-selected population. Thus, recurrent phenotypic selection on spaced plants is appropriate to efficiently reduce seed shattering and to increase seed yield in sown plots, which is highly relevant for seed production.

Plain Language Summary

Seed shattering, where seeds fall off before or during harvest, lowers seed yields in Italian ryegrass. This study aimed to reduce seed shattering through selective breeding. We started with 300 plants and conducted two rounds of selection for high and low seed shattering, resulting in five groups: no selection, selected once and twice for high shattering, and selected once and twice for low shattering. Field tests with early and late harvests showed that the group selected twice for high seed shattering lost the most seeds (7.92% early, 28.44% late), while the group selected twice for low shattering lost the least (2.41% early, 9.31% late). Selecting twice for low shattering reduced seed loss by 10.91 percentage points at late harvest compared to the non-selected group. The proposed method proved to effectively reduce seed shattering and increase seed yield, benefiting seed production in Italian ryegrass.

1 INTRODUCTION

Italian ryegrass (*Lolium multiflorum* Lam.) is one of the most abundant grass species in temperate grassland used for forage production. It is an annual species, valued for its high biomass yield and fast ground cover in intensive, short-term grassland management systems (Humphreys et al., 2010). Italian ryegrass flowers multiple times a year and is an obligate outcrossing species, resulting in a high degree of heterozygosity and a large genetic variation within natural and breeding populations. Breeding of Italian ryegrass is mainly based on recurrent phenotypic selection on spaced plants, followed by cultivar development via open pollination in a polycross and testing of cultivar candidates in plot trials (Conaghan & Casler, 2011). One limitation of this procedure is that phenotypic observations for complex traits on spaced plants cannot always be directly transferred to sward conditions as observed in a plot (Casler et al., 1996). For example, traits related to seed yield, such as number of fertile tillers or spike length, phenotyped on spaced plants only poorly correlated with the results from plot trials (Elgersma, 1990b). In general, prerequisites for successful phenotypic selection are a high heritability of the trait of interest, adequate phenotyping methods, and a large phenotypic and genotypic variation within the population. The main breeding targets in Italian ryegrass are biomass yield, digestibility and palatability, disease resistance, and persistence (Lüscher et al., 2019). Since low seed yield makes seed production more expensive, seed yield is also a crucial trait for a cultivar to be successful on the market (Boelt & Studer, 2010).

The potential seed yield is defined as the total number of ovules per area present at flowering time (Falcinelli, 1999). Many factors, such as unsuccessful pollination and fertilization, abortion of seeds during development, as well as seed shattering (i.e., the loss of seeds before harvest), can lead to realized seed yields that are substantially lower than the potential seed yield (Boelt & Studer, 2010; C. Marshall, 1985). Higher seed-yielding varieties may be achieved by breeding for an improved potential seed yield, for example, via increasing numbers of spikes per area, spikelets per spike, or flowers per spikelet. Alternatively, factors leading to reduced realized seed yield, as discussed above, may be addressed. In Italian ryegrass, seed losses of up to 54% due to shattering have been observed (Maity et al., 2021), and even higher rates of up to 78% were observed for perennial ryegrass (*L. perenne* L.; Tubbs & Chastain, 2022). Therefore, seed shattering seems to be a relevant breeding target to develop Italian ryegrass cultivars with improved seed yield.

Several studies showed considerable genetic variation for seed shattering among different ryegrass accessions and cultivars, indicating the potential for improving the trait through recurrent phenotypic selection (Elgersma, 1990a; Harun & Bean, 1979; Hides et al., 1993; Tubbs & Chastain, 2022). Phenotyping of seed shattering, usually defined as the proportion of seeds lost from the total amount of seeds produced (lost and non-lost), has so far mainly relied on measurements on spaced plants. In perennial ryegrass, seed shattering was measured by rolling three spikes per plant over a steel bar and calculating the percentage of seeds shattered at a defined plant maturity stage (Tubbs & Chastain, 2022). Another approach consists of bagging inflorescences or parts of it after the end of flowering, and to determine the percentage of seeds fallen off until seed harvest (Kavka et al., 2023). Further, high-throughput phenotyping may be possible using an imaging pipeline for the description of spike architecture, which was found to be associated with seed shattering (Barreto Ortiz et al., 2020; Tubbs & Chastain, 2022).

Usually, grass breeding starts with the selection of single plants from large breeding populations in the spaced plant nursery. The selection could be made either directly based on the phenotypic data of the trait or by indirect selection, where the breeder improves the trait by selection for a secondary trait. A secondary trait could be beneficial if the phenotyping of the trait of interest is time consuming or not even possible. If an efficient and reliable phenotyping method is available, high selection intensities can be achieved at this stage for a trait like seed shattering. Further, to achieve a high selection efficiency, the genetic correlation between the secondary traits and the trait of interest as well as the heritability of the traits need to be high (Gallais, 1984).

On the other hand, seed production is usually done in swards and needs, therefore, to be tested on a plot level. Hence, a sufficient correlation of trait expression between spaced plant and sward level is a prerequisite for selection toward reduced shattering in spaced plants to be effective. Moreover, in an optimal selection system, undesired indirect selection affecting other traits like maturity should be avoided. To date, information on the efficiency of selection for reduced shattering in ryegrasses is still scarce. Employing a phenotyping system based on bagging inflorescences and standardized determination of harvest time, the objectives of this study were to (1) conduct a selection experiment to evaluate the response to recurrent phenotypic selection for this economically important trait, (2) test whether this selection on spaced plants also affects seed shattering and yield in swards, and (3) look for correlated traits that could be used as potential targets for indirect selection.

Core Ideas

- Seed shattering in Italian ryegrass can be assessed by bagging inflorescences after flowering.
- Phenotypic selection for low seed shattering in spaced plants also decreases seed shattering in swards.
- Reduced seed shattering enables higher seed yields under sward conditions.
- Reduced shattering is especially useful to ensure high seed yields under non-optimal harvest conditions.

2 | MATERIALS AND METHODS

Starting from a founder population (generation 0 [G_0]), this study is based on two cycles of divergent selection toward reduced and increased seed shattering on spaced plants. This resulted in five populations of generation 2 (G_2) differently selected for seed shattering: G_2 random (no selection for seed shattering), G_2+ (one cycle of positive selection [low seed shattering]), G_2++ (two cycles of positive selection), G_2- (one cycle of negative selection [high seed shattering]), and G_2-- (two cycles of negative selection; Figure 1; Table S1). In comparative trials on plot and spaced plant level, these five populations were phenotyped for different traits to assess the effect of selection.

2.1 | Determination of seed shattering

The following standard protocol was used for determination of seed shattering on spaced plants in the nursery. Plants were tied to bamboo sticks to prevent lodging and start of flowering was recorded. The complete inflorescence of a plant (i.e., all spike-bearing culms) was bagged after termination of flowering into a perforated plastic bag (Sealed Air, Cryovac, 330 mm × 750 mm) and tied to the bamboo stick. The time of harvest was determined using the temperature sum calculated on the average daily temperature sum 5 cm aboveground from a nearby weather station and a base temperature of 0°C. The day of harvest of the earliest flowering plant was determined manually according to experience, and the temperature sum accumulated from start of flowering up to harvest day was determined. All other plants were harvested on the day when the temperature sum accumulated since their start of flowering reached the same value as for the earliest flowering plant. At harvesting, the bagged inflorescences were removed from the plant and gently shaken three times by hand to ensure that seeds hanging loose but not yet shattered fall off the spikes into the bag. The bag was then opened, the inflorescence removed, and transferred to a second perforated plastic bag. Shattered seeds (still in the first bag) and non-shattered seeds (in the second bag, together with the inflorescence) were then dried in a drying cabinet under constant airflow at room temperature for 3 days. Non-shattered seeds were manually removed from the inflorescences. During cleaning of shattered and non-shattered seeds, empty seeds and debris were removed from fully developed seeds using an airflow-separator (Saugluft-Stufensichter T2, Baumann Saatzuchbedarf). The weight of cleaned shattered and cleaned non-shattered seed per plant (hereafter referred to as seed shattered and seed non-shattered, respectively) was determined using a digital scale (New Classic MS; Mettler Toledo; accuracy 0.01 g). Seed shattering (%) was then calculated following the formula:

$$\text{Seed shattering } (\%) = \frac{\text{Seed shattered } (\text{g})}{\text{Seed shattered } (\text{g}) + \text{ Seed non} - \text{shattered } (\text{g})} \times 100. \qquad (1)$$

2.2 | First cycle of selection

The founder population serving as starting point for selection (G_0) consisted of 10 synthetic populations (second-generation synthetic derived from multi-parental crosses (polycross) [Syn 2]) from the Agroscope breeding program of diploid Italian ryegrass (Table 1). Each Syn 2 originated from a polycross of variable size, where the seeds harvested from the polycross (first-generation synthetic derived from multi-parental crosses (polycross) [Syn 1]) were multiplied for another generation in the field and harvested in bulk. Two of the 10 Syn 2 are currently registered as cultivars (Xanthia and Bipes).

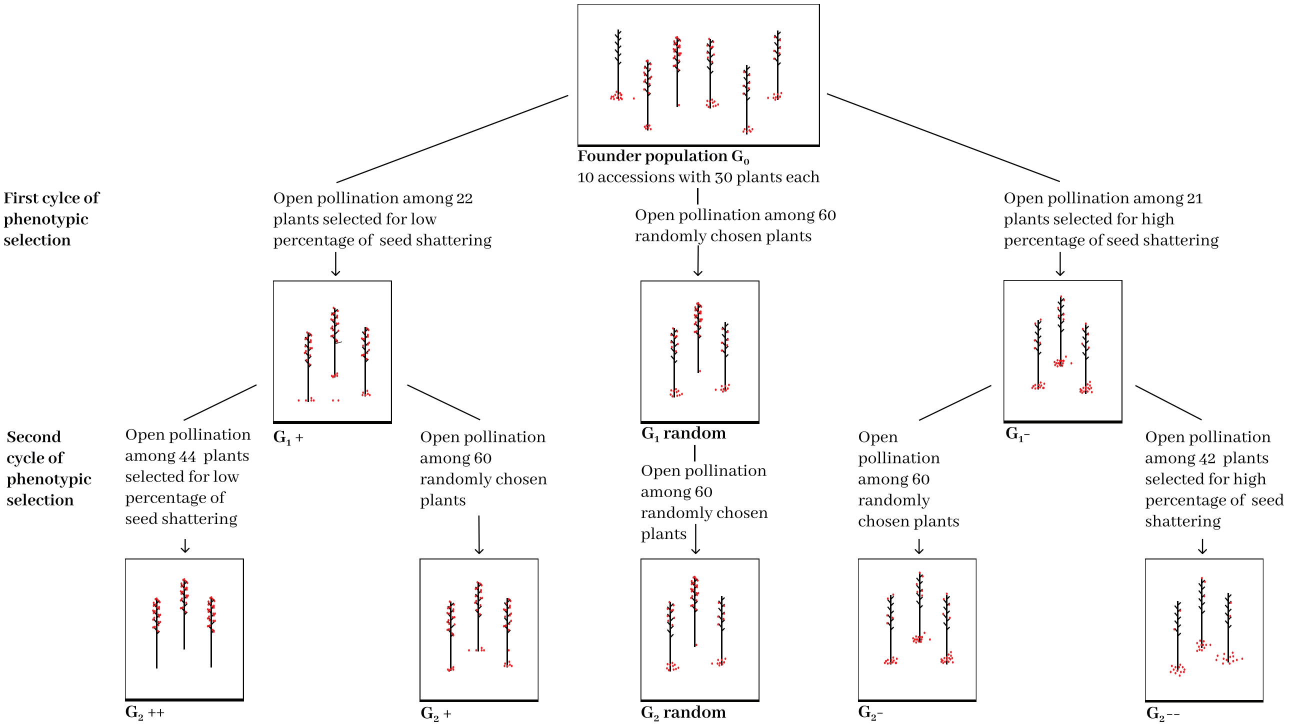

FIGURE 1 Scheme of the selection experiment. Starting from a founder population (G_0), a first cycle of phenotypic selection was made based on spaced plants in the field, and plants with contrasting levels of seed shattering were open pollinated to produce three populations of generation 1 (G_1+, G_1−, and G_1 random). G_1 plants were again selected for low and high seed shattering, giving rise to two populations of generation 2 selected twice for low (G_2++) and high seed shattering (G_2−−). respectively. In addition, plants were randomly chosen from the two selected G_1 populations, resulting in two additional G_2 populations with only one cycle of positive or negative selection (G_2+ and G_2−). An unselected G_2 population (G_2 random) was obtained by open pollination of randomly chosen plants over two generations.

TABLE 1 Synthetic populations contributing to the G_0 generation, including description (cultivar (cv.) name or breeding material), number of genotypes contributing to the polycross (PC size), and number of G_0 plants selected as parents for the positive (low seed shattering, G_0+) and negative (high seed shattering, G_0−) selection in the first cycle.

			Number of plants	
Population	**Description**	**PC size**	**G_0+**	**G_0−**
LI0615	cv. ‘Xanthia’	23	2	2
LI0835	Breeding material	18	3	2
LI1015	Breeding material	11	3	2
LI1105	Breeding material	21	2	2
LI1115	Breeding material	13	1	3
LI1135	cv. ‘Bipes’	8	3	2
LI1235	breeding material	14	2	2
LI1245	Breeding material	10	2	3
LI1255	Breeding material	11	3	2
LI1265	Breeding material	12	2	3

To establish G_0 plants, seeds were pre-germinated on moistened filter paper for 2 days and then sown in plastic trays (40 cm × 30 cm, 14 cm height) filled with soil substrate at a distance of 2 cm next to each other. Plants were grown in the greenhouse (greenhouse hereafter referred to as a glasshouse with a heating system to guarantee at least 16°C during night and 24°C during day, additional light supply for a daylength of 12 h, and no active cooling during summer) for 3 months and fertilized with 0.1% Wuxal Profi (Maag) fertilizer solution every 4 weeks. For each of the 10 Syn 2, 30 genotypes, each one represented by one plant, were transplanted to the field in August 2016 at the Agroscope research station in

Zurich-Reckenholz, Switzerland. A typical breeding nursery design was used, with 10 plants of the same Syn 2 being planted in a row (hereafter denoted as nursery-row). The planting distance was 40 cm within and 50 cm between nursery-rows. Three blocks of 10 nursery-rows (one per Syn 2) were formed. The standard management practice of the nursery trials included a fertilizer application at the beginning of the season and after every cut at a rate of 50 kg nitrogen ha^{-1}. In 2017, plants were cut once at the beginning of May. During the second growth of 2017, plant vigor was visually rated on a scale from 1 (very vigorous growth) to 9 (very poor growth), and seed shattering was determined as outlined above with the exception that seeds were not harvested according to temperature sum but on two separate dates only.

Only plants with an adequate vigor (rating score <5) were considered in the first selection cycle. Per Syn 2 population, one to three genotypes with lowest and highest seed shattering were selected, resulting in a total of 23 genotypes for the positive (G_0+) and 23 genotypes for the negative (G_0-) selection (Table 1). For both selections, culms were cut shortly before flowering during the first growth in 2018, put into jars filled with water, and allowed to openly pollinate in two separate isolations in the greenhouse for seed production. G_1 seeds were harvested separately for each maternal G_0 plant, whereby one genotype of the positive selection and two genotypes of the negative selection did not set seed.

To establish the G_1 random population, 60 randomly chosen plants of the G_0 population (evenly spread over the 10 Syn 2) were cut from the field and open pollinated in a separate isolation in the greenhouse.

2.3 | Second cycle of selection

G_1 plants were established in the greenhouse as described above for G_0 plants. For every half-sib (hs) family (i.e., seeds harvested from the same G_0 genotype), 20 genotypes were cloned into two plantlets that were transplanted to the field at the Agroscope research station in Zurich-Reckenholz, Switzerland, in August 2019. This resulted in 1720 spaced plants in the field (22 hs families of spaced plants for G_1+ and 21 hs families of spaced plants for G_1-, 20 genotypes per hs family, every genotype represented by two clones). The same breeding nursery design as for the first cycle of selection was used with genotypes of the same hs-family planted in two adjacent nursery-rows. An alternating pattern with two rows of G_1+ plants followed by two rows of G_1- plants was used. For the second replicate (i.e., the second plant per genotype), the same layout and randomization was used as for the first replicate. In 2020, plants were cut at the beginning of May. In the second growth, plant vigor was determined as described in Section 2.2 and seed shattering as defined in Section 2.1. In addition, occurrence of stem rust (caused by *Puccinia graminis* subsp. *graminicola*) was rated on a scale from 1 (no rust occurrence) to 9 (very heavy rust infestation) at time of seed harvest according to Schubiger and Boller (2016).

Two genotypes of each G_1+ hs-family (44 in total) with acceptable vigor (<3), no or low stem rust occurrence and lowest seed shattering ($G_1{++}$), and two genotypes of each G_1- hs-family (42 in total), with acceptable vigor, no or low stem rust occurrence and highest seed shattering ($G_1{--}$), were selected as parents for the respective G_2 populations. One plant per selected genotype was dug out from the field in September 2020. Plants were kept outside in pots with a volume of 5.47 L (diameter 180 mm, height 215 mm) for vernalization and were transferred to the greenhouse in January 2021. Every 4 weeks, all the plants were fertilized with 0.1% Wuxal Profi (Maag) fertilizer solution. $G_1{++}$ and $G_1{--}$ plants were allowed to flower in two separate chambers and produced seeds for the $G_2{++}$ and $G_2{--}$ populations, respectively. To generate seeds for the G_2+ and G_2- populations, approximately 60 randomly chosen culms of G_1+ and G_1- plants, respectively, were cut shortly before flowering during the first growth in 2021, put into jars filled with water, and allowed to openly pollinate in two separate isolations in the greenhouse. To generate seed for the G_2 random population, the same method was used to produce seed on culms cut from approximately 60 randomly chosen plants of the G_1 random population. The seeds from the five differently selected populations were harvested manually and stored separately for each G_2 population as seed bulks.

2.4 | Comparative trials

During the field season 2021 and 2022, the populations divergently selected for seed shattering ($G_2{++}$, G_2+, G_2 random, G_2-, and $G_2{--}$; Figure 1) were compared in two field experiments with plots and spaced plants.

2.4.1 | Plot trial

Plot trials with seed bulks of the five G_2 populations were sown in August 2021 at the three locations in Zürich-Reckenholz (47.4301° N, 8.5235° E), Rümlang (47.4380° N, 8.5290° E), and Oensingen (47.2840° N, 7.7321° E). Rows of 2.5 m length were sown by hand with a distance of 0.5 m between rows. Two rows next to each other are defined as a plot, and four plots were sown per G_2 population at each location, arranged in a randomized complete block design. This resulted in 20 plots per location. To avoid border effects, additional rows were sown at the edge of each trial. Trials were fertilized according to standard management practice as defined for the nursery trials.

In 2022, plots were cut in early May before heading, and plastic foil strips of 0.45 m × 2.0 m were placed between the two rows at the center of a plot shortly thereafter. Plots were then allowed to grow, flower, and set seeds. At each location, 10 plots (i.e., two replicates) were harvested at an early harvest time (June 30 for Zürich-Reckenholz and Rümlang and July 4 for Oensingen) and the remaining 10 plots at a late harvest time (July 6 for Zürich-Reckenholz and Rümlang and July 8 for Oensingen). At each harvest date, culms with inflorescences of plots were cut, put into fabric bags, and put to the drying cabinet at constant airflow for 3 days. The seeds that shattered onto the plastic foil strips within plots were collected and dried separately for each plot in the drying cabinet at constant airflow for 3 days. Further determination of seed shattering, that is, manual removal of non-shattered seeds from inflorescences, seed cleaning, and calculation of proportion of shattered seeds, was done as described in Section 2.1. In addition, plant vigor was assessed as described for spaced plants. Heading date in plots was determined by when at least 10 spikes of a plot first emerged (defined in days after first of April).

2.4.2 | Spaced plant trial

To compare plot data with spaced plants, a field trial was established at the Agroscope research station in Zurich-Reckenholz, Switzerland. From seed bulks of each of the five G_2 populations, plants were established in the greenhouse as described for G_0 plants (see Section 2.2). Then, 80 genotypes, each one represented by one plant, were transplanted to the field in August 2021 for each G_2 population, resulting in 400 plants in total. The same planting scheme as for the first selection cycle was used. Nursery-rows, each one containing 10 plants of the same population, were randomly distributed in the field.

Phenotyping was performed on single-spaced plants. In 2022, heading date and plant vigor of the first growth were assessed. Heading date in spaced plants was determined by the first spike of a plant that emerged (defined in days after first of April). After completion of heading, plants were cut. For the second growth, plant vigor and heading date were again determined. Additionally, beginning of flowering (also determined in days after first of April) was assessed. The occurrence of stem rust was rated on a scale from 1 to 9 (see above). The occurrence of late culms, that is, culms that appear later and grow much shorter, was also visually assessed on a scale from 1 (no late culms) to 9 (numerous late culms). Seed shattering was determined as defined in Section 2.1.

2.5 | Data analysis

All statistical analyses were conducted with R v4.1.2 within RStudio v4.0.5 (R Core Team, 2020; RStudio Team, 2021) using standard functions for different calculations, functions "lm" and "anova" for classical analysis of variance (ANOVA) and function "lmer" from the package "lme4" for mixed model analyses (Bates et al., 2015).

2.5.1 | Plot trial

ANOVA for the traits assessed in the plot trial was performed using general linear models. In the analysis, all factors, that is, population, location, and harvesting, as well as all of their possible interactions, were tested in a fully factorial model. Therefore, the following model was used:

$$y_{tnm} = \mu + p_t + l_n + h_m + lh_{nm} + pl_{tn} + ph_{tm} + plh_{tnm} + \varepsilon_{tnm}, \quad (2)$$

where y_{tnm} represents the observation for trait y on a plot basis, μ denotes the overall mean, p_t is the effect of population t, l_n is the effect of location n, h_m is the effect of harvesting timepoint m, lh_{nm} is the interaction between location n and harvesting timepoint m, pl_{tn} is the interaction between population t and location n, ph_{tm} is the interaction between population t and harvesting timepoint m, plh_{tnm} is the triple interaction between population, location, and harvesting, and ε_{tnm} is the residual error. Estimated marginal means per population were calculated, and $G_2{++}$, $G_2{+}$, $G_2{-}$, and $G_2{--}$ were each compared to G_2 random using Dunnett's test for multiple comparison.

2.5.2 | Spaced plants

The spaced plant trial followed a split-plot design, where nursery-rows with 10 plants of the same selection formed the main-plot stratum and single plants formed the sub-plot stratum. To account for this noncompletely randomized design, the following mixed model was used:

$$y_{tn} = \mu + p_t + r_n + \varepsilon_{tn}, \quad (3)$$

where y_{tn} represents the observation for trait y on a single-plant basis, μ denotes the overall mean, p_t is the effect of population t, r_n is the effect of nursery-row n, and ε_{tn} is the residual error. Factor p_t was taken as fixed, whereas r_n was random. Mean values per population were calculated, and $G_2{++}$, $G_2{+}$, $G_2{-}$, and $G_2{--}$ were each compared to G_2 random using Dunnett's test for multiple comparison. Realized heritability (h^2) was calculated based on the breeder's equation as follows:

$$h^2 = R/S, \quad (4)$$

where R is the realized response to selection and S is the selection differential. Realized heritability could only be calculated for the second selection cycle, that is, from $G_1{+}$ to $G_2{++}$ and

TABLE 2 Mean squares from analysis of variance (ANOVA) for different traits assessed in plots at three locations.

Source of variation	df	Seed shattering (%)	Seed total (g)	Seed shattered (g)	Seed non-shattered (g)	Vigor
Population	4	254***	15,316***	558***	21,633***	0.06
Location	2	36*	26,245***	916***	17,354***	9.8375***
Harvesting	1	2802***	115,397***	7988***	184,107***	1.35*
Location:harvesting	2	89***	3785**	429***	6237***	0.24
Population:location	8	9	766	78	677	0.15
Population:harvesting	4	71***	1648	44	1683*	0.24
Population: location: harvesting	8	4	403	30	326	0.24
Residuals	30	8	716	50	695	0.20

*, **, and *** denote significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

from G_1- to G_2--. *S* was calculated based on data assessed from the trial with G_1 plants. For the second positive selection step, *S* was defined as the difference between the mean performance of all G_1+ plants and the mean performance of the G_1+ selected as parents for the G_2++ population (i.e., G_1++ plants). For the second negative selection step, *S* was defined as the difference between the mean performance of all G_1- plants and the mean performance of the G_1- plants selected as parents for the G_2-- population (i.e., G_1-- plants). *R* was calculated based on data assessed from the comparative spaced plant trial with G_2 plants in 2022. For the second positive selection step, *R* was calculated as the difference between the mean of all G_2++ plants and the mean of all G_2+ plants. For the second negative selection step, *R* was calculated as the difference between the mean of all G_2-- plants and the mean of all G_2- plants. Correlations among mean performance of populations as assessed on spaced plants, early harvest in plot trials and late harvest in plot trials were calculated as Spearman's rank correlations.

3 | RESULTS

Seed shattering in plots was significantly affected by the type of selection (i.e., population), harvest time, location, as well as location-by-harvest time and population-by-harvest time interaction (Table 2). The same was the case for weight of non-shattered seeds, whereas the other two components of seed shattering, that is, weight of shattered seeds and total seed weight, were not significantly influenced by the population-by-harvest time interaction. Plant vigor was significantly influenced by location and by harvest time, but not by the other factors or their interactions. The triple interaction (population-by-location-by-harvesting time) was not significant for any trait. Since variation for heading date was larger within than among plots (i.e., all plots heading at the same time), no statistical analysis could be performed for this trait.

Seed shattering in plots was lower for early compared to late harvesting for all five populations, but ranking of the populations was the same for both harvest times (Spearman rank correlation $r = 1$, $p = 0.017$, Figure 2). The highest percentage of seed shattering was observed in the G_2-- (7.92% early, 28.44% late), followed by the G_2- (7.34% early, 21.56% late), G_2 random (6.12% early, 20.22% late), G_2+ (4.13% early, 16.73% late), and G_2++ (2.41% early, 9.31% late) populations (Figure 2). The difference in seed shattering between early and late harvesting continuously decreased from 20.52% in the G_2-- population to 6.9% in the G_2++ population, indicating that the absolute effect of the population was larger for late compared to early harvesting. Comparing the differently selected G_2 populations to the G_2 random population using Dunnett's test, only G_2++ and G_2-- were significantly different ($p < 0.001$, see Table S2).

The harvestable seed yield in plot trials, that is, weight of non-shattered seeds, was distinctively higher for early compared to late harvesting, but the ranking of the differently selected populations was comparable between the two harvesting timepoints (Figure 3). For late harvesting, harvestable seed yield doubled from the G_2-- (117.5 g) to the G_2++ population (254.9 g), with intermediate values of 149.3, 171.6, and 194 g for the G_2-, G_2 random, and G_2+ selection, respectively. In comparison, differences among the populations were not as pronounced for early harvesting, where G_2--, G_2-, G_2 random, and G_2+ populations all showed similar values for weight of non-shattered seeds (266.6, 264.6, 277.7, and 283.4 g, respectively). Only the G_2++ population showed a distinctively increased weight of non-shattered seeds at early harvest (349.0 g). For early harvesting, the weight of non-shattered seeds was substantially lower for location Oensingen compared to the other sites (see blue-filled circles in Figure 3).

Seed shattering as observed in the spaced plant trial also showed a clear effect of selection, with the same ranking among the five populations as observed for both harvest timepoints in the plot trial (Spearman rank correlation $r = 1$

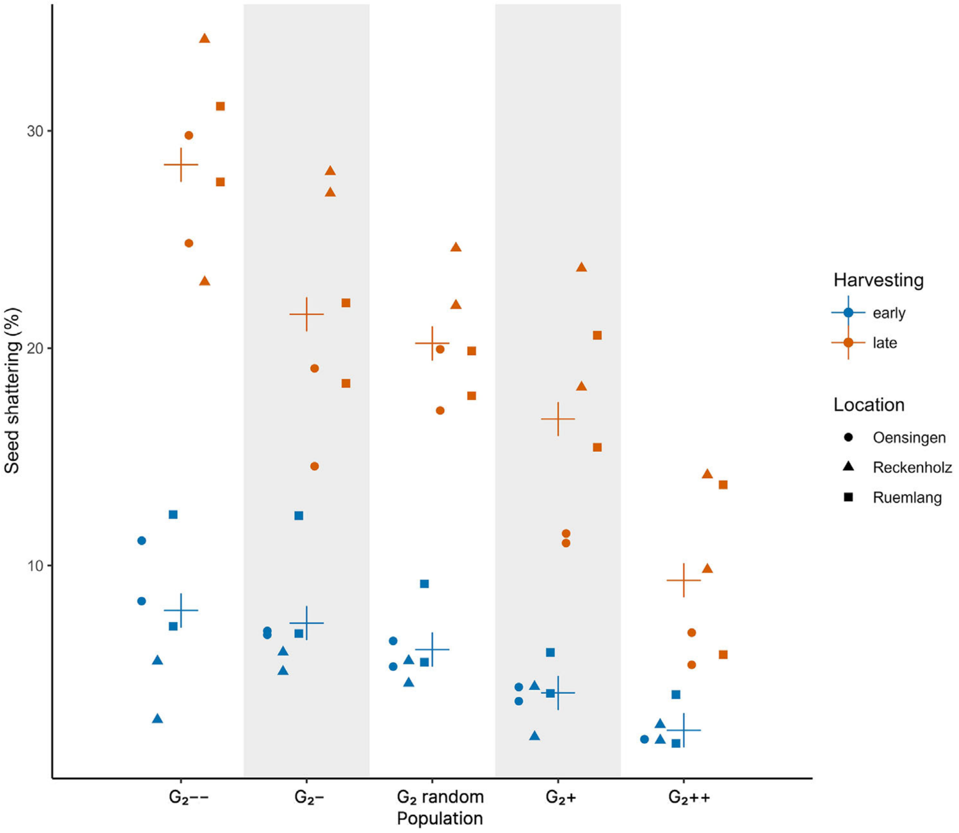

FIGURE 2 Seed shattering of the five differently selected populations as determined in the comparative plot trial. Blue and orange colors indicate early and late harvesting, respectively. Crosses give the average over locations per selection and harvesting time.

and $p = 0.017$). The highest mean percentage of seed shattering was observed in the G_2−− (47.2%), followed by the G_2− (41.9%), G_2 random (38.7%), G_2+ (22.5%), and G_2++ (15.3%) population (Figure 4). Analysis of variance indicated a significant effect of the population on seed shattering ($p < 0.001$, data shown in Figure 1 and Table S3). A comparison of the G_2+, G_2++, G_2−, and G_2−− to the G_2 random population with Dunnett's test revealed that already one cycle of selection significantly decreased seed shattering (G_2 random vs. G_2+, $p < 0.001$) and this difference even increased with the second selection cycle (G_2 random vs. G_2++; Figure 4). On the other hand, the selection for higher seed shattering did not significantly increase seed shattering after one cycle of selection (G_2 random vs. G_2−, $p = 0.749$), and the difference was still not significant after a second cycle of selection (G_2 random vs. G_2−−, $p = 0.065$).

Calculation of realized heritability indicated low to moderate values of $h^2 = 0.20$ and 0.32 for the second negative and second positive selection cycles, respectively (Table 3). Although the selection differentials (S) were of nearly equal size (24.4 for positive and 23.0 for negative selection), the lower heritability for the negative selection resulted in a pronouncedly reduced response to selection (R) compared to the positive selection.

Seed shattering showed strongest correlations with the two components it was calculated from, that is, the weight of shattered seeds ($r = 0.85$, $p < 0.001$) and the weight of non-shattered seeds ($r = -0.65$, $p < 0.001$; Table 4). Seed shattering only showed weak correlations with earliness of plants as indicated by heading date of the first ($r = -0.21$, $p < 0.001$) and second growth ($r = -0.16$, $p = 0.002$) as well as beginning of flowering ($r = -0.16$, $p = 0.002$). The negative correlations indicate that later maturing plants tended to show lower seed shattering. Other traits like vigor of plants, the occurrence of late culms or stem rust did have no or only a very small effect on seed shattering. Weight of shattered and non-shattered seeds showed a weak negative correlation ($r = -0.23$, $p < 0.001$; Table 4), indicating that plants with a higher mass of shattered seeds tended to have lower mass of non-shattered seeds. While seed shattering was not correlated to plant vigor, negative correlations of seed shattered as well as of seed non-shattered with plant vigor in the first and second growth indicated that more vigorous plants have, in total, more seeds, which are either being shattered or remaining on the plant. The only low to moderate correlation between heading date of the first and the second growth is largely driven by the reduced variation for this trait in the second growth and indicates that earliness from the first growth does

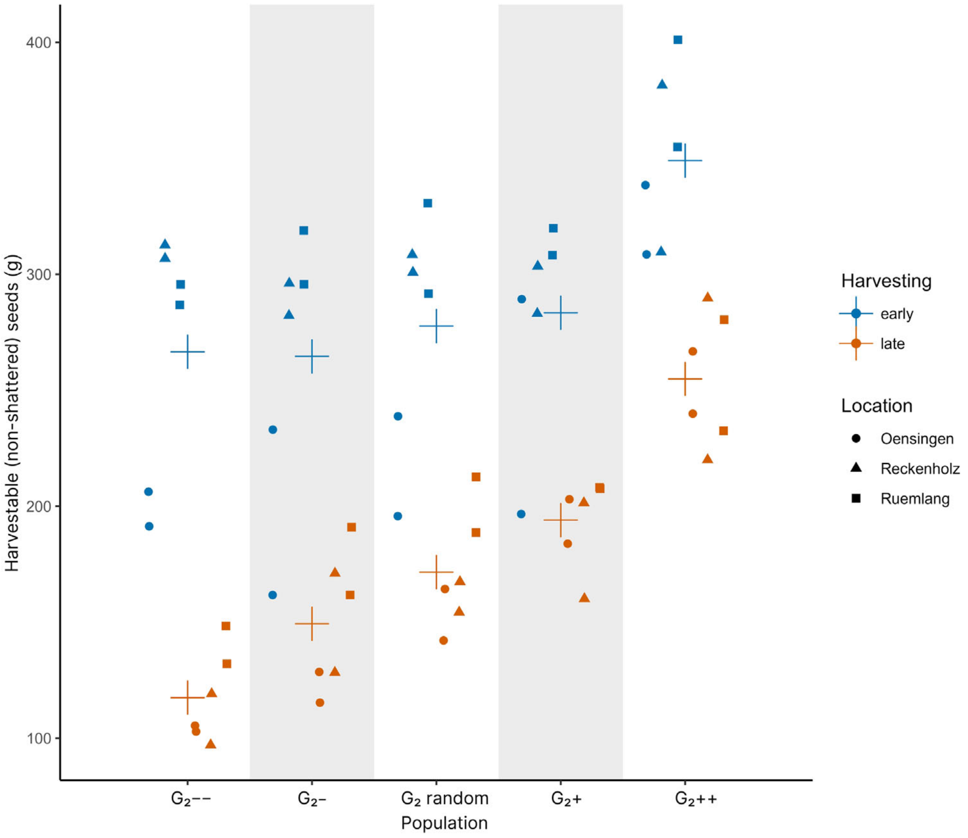

FIGURE 3 Harvestable (non-shattered) seeds of the five differently selected populations as determined in the comparative plot trial. Blue and orange colors indicate early and late harvesting, respectively. Crosses give the average over locations per selection type and harvesting time.

TABLE 3 Realized heritability (h^2) for the selection steps leading to the G_2 populations G_2++ and G_2−−.

	Seed shattering (%) in G_1 generation			**Seed shattering (%) in G_2 generation**			
Selection cycle	**All**	**Selected**	***S***	**Single**	**Double**	***R***	**h^2**
G_1+ to G_2++	32.3	7.9	−24.4	22.6	14.8	−7.8	0.32
G_1− to G_2−−	53.1	76.1	23.0	41.9	46.6	4.7	0.20

Note: The selection differential (*S*) is calculated as the difference between the mean of all and the mean of the selected genotypes from the corresponding G_1 populations (G_1+ and G_1−). The response to selection (*R*) is calculated as the difference between the means of the G_2++ and G_2+ (or G_2−− and G_2−) population from the comparative spaced plant trial.

not directly translate to the second growth. The moderate to strong correlation ($r = 0.61$, $p = < 0.001$; Table 4) of heading date in the second growth with beginning of flowering in the second growth indicates earlier flowering of early heading plants.

4 | DISCUSSION

In this study, we could show that recurrent phenotypic selection for reduced seed shattering in spaced plants is very effective in Italian ryegrass for the population used in the environments tested and may be generally useful to increase seed yield. The phenotyping of spaced plants is relatively fast and easy, only a small amount of seed is required, and in a second step, selection within and among families is possible (Vogel & Pedersen, 1993). The improvement of 23.4 percentage points after two cycles of recurrent selection, as observed in the comparative spaced plant trial (G_2 random vs. G_2++), is comparable to recurrent phenotypic selection for improving seed yield in perennial ryegrass (A. H. Marshall & Wilkins, 2003). In the first selection cycle, the selection was based on data from only one replicate per genotype grown as spaced plant. Already these phenotypic data were sufficient to realize a decrease of 3.49 percentage points for late harvesting in seed shattering when comparing the progenies of the G_2

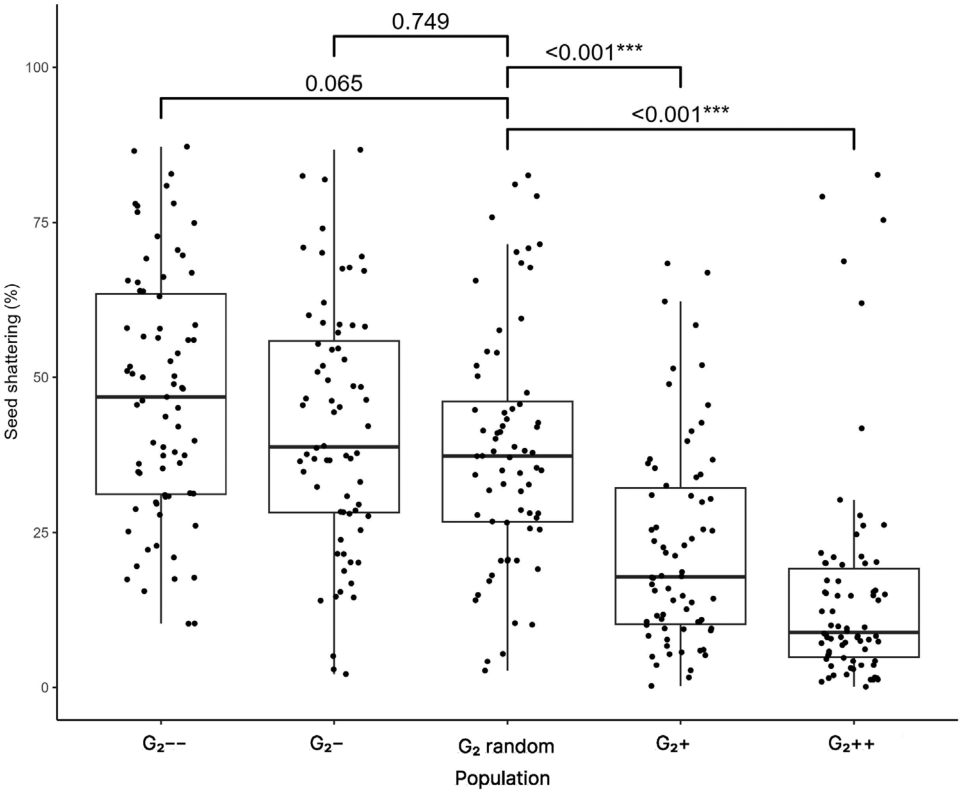

FIGURE 4 Seed shattering of the five differently selected populations as determined in the comparative spaced plant trial. Numbers above bars give the *p*-value of the multiple comparisons with Dunnets contrasts to the G_2 random population.

random and G_2+ in the comparative plot trial. The improvement for late harvesting was even higher in the second cycle of selection (7.42 percentage points from G_2+ to G_2++). This might be partly explained by a better quality of the phenotypic data in the second selection step (two replicates per genotype in the second compared to one replicate in the first selection cycle).

The G_0 generation in our experiment consisted of breeding material that traces back to collections from semi-naturally occurring populations (ecotypes) (Peter-Schmid et al., 2008). This material has so far not undergone targeted selection to reduce seed shattering (data not shown). As high seed shattering in wild populations is rather an advantage to increase dispersal of seeds and, therefore, increase the spread of offspring (Dong & Wang, 2015), the shattering trait is likely to be very abundant in these materials (Piccirilli & Falcinelli, 1989). In general, selection for a trait not selected before will lead to rapid improvements in the first selection cycles, whereas after some cycles, an improvement will be harder to achieve. This would be particularly pronounced if the trait is influenced by dominant genes and the frequency of the advantageous, dominant alleles increases from low initial levels. For example, in red clover, improvement of resistance to the fungal disease southern anthracnose, caused by *Colletotrichum trifolii*, revealed the highest improvement of up to 52% after one cycle of phenotypic selection in susceptible to moderately resistant cultivars (Schubiger & Grieder, 2019). After a second cycle of selection, improvement for resistance to southern anthracnose was already distinctly lower (Jacob et al., 2013). This might also explain why negative selection did not have a significant effect even after two cycles, as the frequency of the alleles leading to high shattering, already at a very high level in the G_0 population, could not be increased any more. The realized heritability (h^2) for the negative selection was lower than for the positive selection (Table 2), indicating that decreasing seed shattering is more effective than increasing seed shattering within these populations. Dry matter yield, one of the most important and most selected traits within forage grasses, displays an improvement of 0.17%–0.80% per breeding cycle, depending on the species (Grieder et al., 2019). This is substantially lower compared to the rates of improvement for seed shattering we observed here. One explanation could be that dry matter yield is described as a complex trait with many small effect genes (Arojju et al., 2020), whereas shattering could be controlled by only few genes, each having large effects on the phenotype (Senda et al., 2006). Thus, recurrent phenotypic selection as performed in our experiment with bagging inflorescences after flowering and harvesting according to sum of temperature is a valid method to decrease seed shattering in spaced plants, which is especially effective when starting from populations with high levels of shattering.

TABLE 4 Spearman's rank correlations among traits assessed in the comparative spaced plant trial.

Trait	Vigor 1	Vigor 2	Heading date 1	Heading date 2	Beginning of flowering	Late culms	Stem rust	Seed shattered	Seed non-shattered
Seed shattering	−0.14*	−0.05	−0.21***	−0.16**	−0.16**	0.07	0.12*	0.85***	−0.65***
Vigor 1		0.42***	0.26***	0.02	−0.02	−0.23***	−0.01	−0.26***	−0.17**
Vigor 2			0	0.09	0.02	−0.24***	−0.02	−0.31***	−0.35***
Heading date 1				0.29***	0.24***	−0.12*	0	−0.22***	0.05
Heading date 2					0.61***	−0.23***	0.01	−0.22***	−0.01
Beginning of flowering						−0.2***	0.06	−0.16**	0.04
Late culms							−0.06	0.13*	0.05
Stem rust								0.13*	−0.04
Seed shattered									−0.23***

Note: Vigor 1 was observed on first growth. Vigor 2 was vigor observed on second growth. Heading date 1 was observed on first growth. Heading date 2 observed on second growth. Beginning of flowering was observed on second growth. Late culms as well as stem rust were phenotyped at harvesting date; seed shattering (%) was determined as the ratio of seed shattered (g) over total seed weight per plant (Equation 1). Seed non-shattered is the weight (g) of the seeds still on the tillers at harvesting date.

*, **, and *** denote significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Indirect selection, where the breeder improves a primary character by selecting for a secondary character, is often used in breeding for traits that are difficult to phenotype (Gallais, 1984). Here, the employed system for phenotyping seed shattering on spaced plants is labor intensive and time consuming. Therefore, another, simpler trait to indirectly select for low seed shattering would be preferable. Several agronomically important traits were assessed and correlated with seed shattering. A moderate negative correlation ($r = -0.65$) was found between seed shattering and the weight of non-shattered seeds (i.e., seed non-shattered), indicating that selection for plants with a high number of seeds remaining on the plant could indirectly reduce seed shattering. Whether this is only the case in this population or a general observation for Italian ryegrass remains to be clarified. Selection for high seed yield (i.e., seed non-shattered) would be an easier phenotyping method to reduce seed shattering as bagging of inflorescences is not necessary. Relative efficiency of indirect selection (Falconer & Mackay, 1996), that is, improvement of seed shattering when selecting for seed non-shattered, increases with the genetic correlation between the two traits and is higher if selection intensity and/or heritability is higher for the directly compared to the indirectly selected trait. Assuming similar heritability for non-shattered seeds as for seed shattering and the phenotypic correlation coefficient of 0.65 as a lower estimate for the genetic correlation, indirect selection would be at least as efficient if selection intensity for seed non-shattered would be 1.54 times higher than selection intensity for seed shattering. Hence, if the faster phenotyping method for seed non-shattered would allow to phenotype more plants and, therefore, allow for a selection intensity that is at least 50% higher, indirect selection would be effective.

Selection for a particular trait may lead to unintentional selection for another trait. For example, a tradeoff between vegetative and reproductive growth is reported for forage grasses (Humphreys et al., 2010). On the one side, Italian ryegrass as a forage crop needs to have a high vegetative biomass yield; on the other side, seed yield as a reproductive trait is also important for the successful seed multiplication of cultivars (Sampoux et al., 2011). The interaction between these traits is yet unclear, but selection for seed yield should not negatively impact important agronomical traits. Low correlation coefficients observed between vigor and seed shattering indicate that the two traits can be selected independently from each other. There is a very weak correlation between heading date and seed shattering in the comparative spaced plant trial and the missing variation in heading date in the comparative plot trial indicates that the method of determining harvesting time according to temperature sum after start flowering was effective to avoid any indirect selection effects on maturity.

Stem rust usually develops late in the season on the panicle and florets, where it may directly interfere with seed set, resulting in less viable seeds (Barker et al., 2003). Consequently, several studies reported a negative correlation between the occurrence of stem rust and seed yield (Leonard & Szabo, 2005; Pfender, 2001; Rodriguez-Algaba et al., 2020). However, within our field trials, stem rust occurred in each environment and year. Whether seed shattering is negatively affected by stem rust remains yet unclear. In order not to influence the selection by stem rust, only plants with no or very weak rust symptoms (score ≤ 2) were selected from all populations. Based on the correlations in spaced plants (Table 3), occurrence of stem rust only weakly increased seed shattering, mainly via increasing the weight of shattered seeds. This weak effect of stem rust occurrence on seed yield traits might be explained by a limited variation and a preponderance of other factors affecting seed yield traits. Thus, breeding for a low seed shattering cultivar should be possible without compromising vigor, maturity, or other traits. However, to obtain high seed-yielding plants, breeders must select more consistently for stem rust-resistant plants.

Even though seed shattering is genetically controlled, seed shattering is also strongly influenced by agronomical practices, of which timing of harvest is very important (Shirtliffe et al., 2000; Walsh & Powles, 2014). Early harvesting always reduced seed shattering and increased harvestable seed yield, whereby the difference to the late harvest was highest for the G_2−− population and lowest for the G_2++ selected population. Hence, early timing of seed harvest is very suitable to enable high seed yields, regardless of the genetic material (Figure 4; Table 2). However, due to unfavorable weather conditions or other constraints, an early harvest is not always possible. Furthermore, early harvesting could have additional negative effects such as high moisture content in seeds resulting in higher costs for drying or increased frequency of immature seeds resulting in lower germination rates (Hill & Watkin, 1975; Larson et al., 2020). Therefore, cultivars with reduced seed shattering are an important prerequisite for seed growers to ensure high seed yields also under non-optimal conditions.

Forage grass breeding mainly depends on recurrent phenotypic selection of populations (Posselt, 2010). Phenotypic selection is often based on selection among spaced plants and is especially useful for traits having a high heritability and good correlation between spaced plants and sward conditions. In our study, with a high level of seed shattering in the starting material, we could show that phenotypic selection for low seed shattering in spaced plants is effective to reduce seed shattering and increase harvestable yield (i.e., seed non-shattered) under plot conditions, the latter trait being most relevant for seed growers. However, earlier studies in perennial ryegrass showed a low correlation between seed yield in spaced plants and their offspring sown in plots (Bugge, 1987; Elgersma et al., 1994). Especially for populations that already exhibit a low level of seed shattering, other factors than shattering might drive seed yield and be the reason for low correlation between spaced plants and plot trials as observed in these earlier studies.

To conclude, our phenotyping method, which is based on bagging inflorescences after flowering followed by harvesting according to the sum of temperature, proved to be efficient to select for reduced seed shattering in spaced plants. To reduce the efforts needed for phenotyping, indirect improvement of seed shattering via selection for plants with higher mass of non-shattered seeds might be an alternative. Effects of selection were also significant in plot trials. Phenotypic selection in spaced plants is, therefore, effective to create cultivars with reduced seed shattering and increased harvestable yield under plot conditions, allowing farmers to ensure high seed yields also under non-optimal harvest conditions. The population selected for low seed shattering in this study is, therefore, a valuable genetic resource that can be directly used within breeding programs.

AUTHOR CONTRIBUTIONS

Jenny Kiesbauer: Data curation; formal analysis; investigation; visualization; writing—original draft; writing—review and editing. **Roland Kölliker**: Conceptualization; supervision; writing—review and editing. **Maria Hug**: Investigation. **Meril Sindelar**: Investigation. **Linda Helene Schlatter**: Investigation; writing—review and editing. **Jonathan Ohnmacht**: Data curation; investigation; visualization. **Bruno Studer**: Supervision; writing—review and editing. **Christoph Grieder**: Conceptualization; data curation; funding acquisition; methodology; supervision; writing—original draft; writing—review and editing.

ACKNOWLEDGMENTS

The authors thank Peter Tanner, Simone Günter, and Daniel Schmid from the Fodder Plant Breeding group at Agroscope for technical support in the field trials. This project received funding from Agroscope and Delley Seeds and Plants Ltd.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data presented in this study are openly available at zenodo.org (https://zenodo.org/record/10224788).

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How to cite this article: Kiesbauer, J., Kölliker, R., Hug, M., Sindelar, M., Schlatter, L. H., Ohnmacht, J., Studer, B., & Grieder, C. (2025). Higher seed yield through selection for reduced seed shattering in Italian ryegrass. *Crop Science*, *65*, e70002. https://doi.org/10.1002/csc2.70002