Spontaneous Gene Flow between Cultivated and Naturalized *Vicia villosa* Roth Populations Increases the Physical Dormancy Seed in a Semiarid Agroecosystem

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Abstract: Hairy vetch (*Vicia villosa* Roth) is the second most cultivated vetch worldwide being used as a forage and cover crop. As it is not domesticated, it displays several wild traits including seed dormancy. The physical seed dormancy (PY) variation could be useful depending on the specific context. High PY is desirable for ley farming systems, while low PY is needed to prevent weediness in subsequent crop rotations. Gene flow between cultivated and naturalized populations has important ecological and agronomic consequences. Experiments were conducted to determine the change in the level of PY in spontaneous crosses between European cultivated accessions (EU) characterized by low PY and naturalized Argentinian population (AR) with high PY. Generations G_0 (initial generation) to G_3 were assessed for their PY in seeds and total dry matter (DM) per plot, at plant maturity. As the result of spontaneous crosses with the AR population, an increase from G_0 to G_3 in PY and DM in EU accessions was observed, while AR maintained its high PY and DM values. In one mating cycle, selecting for PY lower than 14% and high DM can satisfy breeding objectives for cover crops. Isolation during breeding and seed production is necessary to avoid gene flow. This knowledge extends to other legume species with gene flow between wild and cultivated populations.

Keywords: breeding; cover crops; hairy vetch; pasture legume; seed dormancy

1. Introduction

Vicia villosa Roth, commonly known as hairy vetch (HV), is native to Europe and West Asia and was introduced as a crop or weed worldwide to temperate climate regions [1,2]. Generally, it is grown for forage, consumed under direct or indirect grazing or used as green manure. The use of HV as a cover crop in conservation agriculture is increasing [3,4].

HV shows the capacity to form spontaneous populations in ruderal habitats of cultivated areas [4,5]. Argentinian HV was used as forage and escaped from cultivation a century ago and formed naturalized populations [6]. The ability of HV seeds to have physical dormancy (PY) results in soil seed bank formation and pod dehiscence, which are two important factors that influence natural population dynamics and persistence [7,8]. In HV, the acquisition of PY is initiated when the moisture content of the seeds is ≤14% [9,10]. At seed dispersal, the proportion of seeds with PY was 74% on average in the naturalized AR population [11,12]. Despite the effect of the environment, seed dormancy is largely genetically determined and is variable among genotypes, and this is used in breeding selection [8,13–15]. The most important traits for which HV has been selected include biomass production and a low level of seed dormancy [13]. Low PY is a desirable trait for stand establishment [8], while incomplete germination related to PY can result in soil seed bank formation and emergence in subsequent crop rotations as a problematic weed [8,16,17].

On the contrary, in the agroecosystems of semiarid regions, HV's natural reseeding capacity with high PY is a desirable trait. The adoption of HV is associated with its high self-regeneration potential, which is known in ley farming in Australian systems and helps to reduce production costs, minimalizing the seed and sowing operations [17–20].

In Argentina, as in other regions of the world, several HV naturalized populations have been established in ruderal habitats surrounding agricultural areas [4,5]. The semiarid region of Argentina shows favorable conditions for cultivated HV biomass and seed production, as well as suitable habitats for naturalized HV populations [6]. Furthermore, HV is mainly a cross-pollinated species for which bees play an important role [21]; thus, dry and warm weather is favorable for the activity of pollinators [22,23]. It is known that naturalized populations of HV have high degrees of PY [6]. The sympatric occurrence of an HV crop and naturalized populations might increase the frequency of pollinator-mediated gene flow. Consequently, changes in gene frequency can modify traits of interest including the PY level [6,24]. Gene flow-mediated changes in PY may be relevant for breeding and seed production programs, as well as for the study of HV demography from a weed management perspective or ley farming systems in an agricultural environment.

The objective of this study was the evaluation of the changes in PY level as a result of spontaneous crosses between naturalized HV genotype and European (EU) cultivars characterized by the low PY. The change towards low PY could reduce the adaptation of naturalized HV populations or cultivars for ley farming purposes. On the other hand, higher levels of PY could increase the frequency of HV as a problematic weed.

2. Materials and Methods

2.1. Seed Collection Sources

Hairy vetch at the Experimental Agricultural Station (EEA) Hilario Ascasubi was selected as the representative of the naturalized populations of Argentina (AR) [12]. The Ascasubi population was located in the southern area of the semiarid region of Argentina, in a sandy loam soil (pH = 7.5, soil organic matter = 1.2%, available P Bray and Kurtz = 24 mg kg^{-1}) along an internal road margin of EEA H. Ascasubi (62°37′ W, 39°23′ S). The predominant climate in this habitat is semiarid temperate with 489 mm if mean annual precipitation and a mean annual temperature of 14.8 °C (1966–2016; http://inta.gob.ar/documentos/informes-meteorologicos, accessed on 16 April 2021). This population was spontaneously crossed with other HV accessions, including four cultivated accessions from Europe–Asia (EU) that were characterized by low physical seed dormancy (PY) in relation to the naturalized genotypes (Table 1) [6]. Passport data of each EU accession were provided by the Research Institute of Crop Production (CRI), Czech Republic (Table 1; for more information, see https://grinczech.vurv.cz/gringlobal/search.aspx, accessed on 20 March 2021) [12].

Table 1. Country, name of hairy vetch accessions included in the study and average level of seeds' physical dormancy (PY).

Country of Origin	Accession/Locality	Number *	PY Level **
Poland	Sielecka	05L1700015	<12%
Turkey	617 81	05L1700046	<12%
Germany	Oregon	05L1700066	<30%
Russia	Stavchanka	05L1700061	<23%
Argentina	Ascasubi	——	>72%

* EVIGEZ: Plant Genetic Resources Documentation of the Czech Republic. ** From [6].

2.2. Seed Production for Comparative PY Experiments

Seeds from each accession (Table 1) were cultivated at the EEA of Hilario Ascasubi during the 2017–2020 growing seasons. The soil was an entic haplustoll, sandy loam, slightly alkaline (pH ≈ 7.5), high in phosphorus (P) content (≈22 ppm P Bray and Kurtz)

and low in organic matter content (≈1.6%) at 20 cm. Weather data from each year were registered with a meteorological station located nearby (less than 700 m).

The accessions were arranged in row plots in a randomized complete block design with three replications. Each experimental unit was a row of 2 m sown with 20 seeds during May. Seeds were inoculated with commercial inoculum (*Rhizobium leguminosarum* bv *viciae*) immediately before sowing. Original seeds (G_0) were used in 2017–2020, and seeds from spontaneous crosses of G_1 were used in 2018–2020, G_2 in 2019 and 2020 and G_3 in 2020. Regarding the crossing plots design, the relations of G_0–G_1–G_2–G_3 were as follows: 100% G_0 in 2017, 50% G_0–50% G_1 in 2018, 33% G_0–33% G_1–33% G_2 in 2019, and 25% G_0–25% G_1-25% G_2–25% G_3 in 2020. The AR naturalized HV population occupied one-fifth of the experimental trials.

Honey bees (*Apis mellifera* L.) were used as pollinators. The nearest apiary containing 20 colonies was <400 m from the study site. Colonies were moved into the experimental site in early November at full-bloom stage. In addition, an original (G_0) Stavchanka cultivar was multiplied in exclusion cages (4 × 6 m) with honey bees (one nucleus) as a control genotype during the three years (2018–2020) in order to maintain genetic purity and avoid contamination.

Seeds for PY testing were immediately harvested from mature pods (mid-December) and threshed by hand. The seeds collected as G_0 were used in the next growing season as G_1, with G_1 as G_2 and G_2 as G_3 in subsequent seasons. Above-ground total dry matter (DM) was measured at harvest by cutting plant shoots at ground level in a 2 m row in each plot in 2018 and 2020. The seed moisture content at harvest was 9.2 ± 1.1%.

2.3. PY-Dormancy Testing

Immediately after harvest from the common garden, seeds were cleaned and their seed weight was estimated in a sample of 100 seeds (n = 3). PY seeds (i.e., "hard" or impermeable) were determined by an imbibition test performed at 20 ± 2 °C for 3 days [12]. Intact seeds were placed on moist filter paper in Petri dishes in replicas and watered daily with tap water. Imbibed seeds showed a visible change in their size/volume ratio and were easily distinguished [11]. Seed viability was assessed for remaining non-germinated seeds by slicing them longitudinally and immersing them in a 0.5% (wt/vol) tetrazolium chloride (2,3,5-triphenyltetrazolium chloride) solution for 24 h at 30 °C in the dark [25]. Seeds with pink or red stained embryos were considered viable. The total number of viable seeds consisted of germinated and stained seeds.

2.4. Seed Burial Experiments

During January 2019, at the onset of the summer season after natural seed dispersal, batches of 100 seeds (n = 3) of five genotypes (Table 1) of original (G_0) and spontaneous crosses (G_1 and G_2) were placed inside permeable nylon mesh bags (10 × 10 cm) and buried in the field at a depth of 3 cm in a bare-soil site (without vegetation or litter) in order to conduct testing under natural soil conditions (diffusion of air and water, micro-organisms, etc.). A completely randomized factorial design was used. Seeds were exhumed after 0, 120, 190, 280 and 380 days of burial (DAB). Hard, non-imbibed seeds were counted and the proportion of PY seeds was assessed. The remaining hard seeds (with PY) in each exhumation time were returned to the mesh pouches in the field, and the process was repeated to generate cumulative hard seed breakdown curves [26]. The fraction of PY seeds was plotted as a function of burial time.

2.5. Statistical Analysis

A linear mixed model (LMM) was performed to test if the accessions differed in the proportion of PY after harvest, breaking (only 2019), seed weight and biomass DM (2018 and 2020), using the accessions and generations (G_0–G_3) as fixed factors and the year as a random factor. The LMM was performed with R version 3.6.3 (R Development Core Team 2019) using the Infostat software [27] interface to R. Accessions and generations means

were compared by Fisher's Least Significant Difference test ($p < 0.05$). For analyses, the PY data were arcsin-square root-transformed; the untransformed data are presented here for clarity. The relationship between PY level, generations and DM was plotted. A regression analysis was performed using GraphPad Prism Software version 8.0 (GraphPad, San Diego, CA, USA).

3. Results

Registered rainfall in EEA Hilario Ascasubi during the 2017, 2018 and 2020 HV growing season (from April to December) was within the range of conditions for the semiarid region of Argentina (~335 mm), and the level was 27% lower (241 mm) in 2019 compared to historical long-term means (331 mm). Mean daily air temperature values were slightly higher in 2018–2020 (13.4 °C) compared to 2017 (12.9 °C) (Figure S1). All HV accessions performed well, and it was possible to collect seeds for PY testing.

At harvest, the seed moisture content of all HV genotypes was less than 10%. Seed viability was over 95% in all cases. No significant interaction between genotypes and generations was found in the seed weight ($p > 0.05$). Stavchanka and Sielecka cultivars had a slightly smaller seed weight (31.8 ± 4.4 mg) in comparison to 617 81, Oregon and AR (35.1 ± 3.5 mg).

Regarding seeds of the first generation (G_0), AR showed higher PY values than EU cultivars. The influence of the year modified the PY level only in Sielecka and Oregon cultivars (Figure 1). The PY in Stavchanka cultivar within the exclusion cage and seeds that were freely pollinated did not differ significantly due to the fact that seed coats are constituted of maternal tissue ($p > 0.05$; Figure 1).
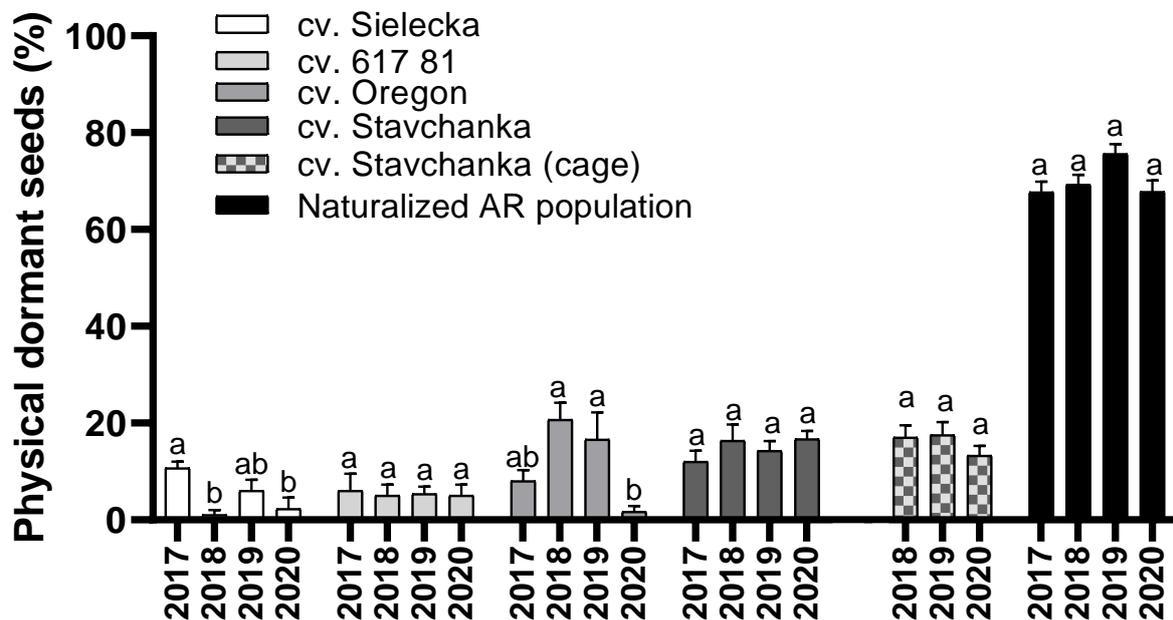

Figure 1. Percentage of seeds with physical dormancy (PY) for each genotype (mean and standard error) evaluated in a common garden during 2017–2020 with the first generation (G_0) from EU produced freely and Stavchanka in exclusion cages with honey bees (cage). Different letters indicate significant differences between years for each genotype (Fisher's LSD test, $\alpha = 0.05$).

PY showed an interaction between genotypes and generations ($p < 0.01$). The AR showed a high and stable PY level (>67%), while EU cultivars showed lower PY (Table 2). However, with the advance of the generations (from G_0–G_3), the PY level increased in all EU cultivars, except for Stavchanka during 2018 and 2019 (Table 2). Spontaneous crosses increased the degree of PY in EU cultivars.

Table 2. Percentage of dormant seeds' PY (mean and standard deviation) of hairy vetch (HV) genotypes for each year and generation.

Generations [#]	European Cultivars				Naturalized AR Population	LSD ($p < 0.05$)
	617 81	Sielecka	Oregon	Stavchanka		
	Physical Dormant Seeds (%)					
2017						
G_0	5.3 ± 6.0	10.6 ± 2.3	8.1 ± 4.0	12.0 ± 3.9	67.8 ± 3.8	8.4 **
2018						
G_0	5.2 ± 4.0	1.0 ± 1.7	20.6 ± 3.0	16.4 ± 5.8	69.3 ± 3.5	8.6 **
G_1	25.0 ± 3.0	29.3 ± 3.2	38.0 ± 1.7	22.4 ± 3.6	66.1 ± 2.0	5.9 **
LSD ($p < 0.05$)	0.14 **	0.18 **	0.12 *	NS	NS	
2019						
G_0	5.3 ± 2.0	6.0 ± 4.1	16.8 ± 9.7	14.4 ± 3.5	75.7 ± 3.6	10.8 **
G_1	32.0 ± 4.4	27.1 ± 2.6	24.0 ± 2.0	32.0 ± 9.1	71.2 ± 13.2	13.9 **
G_2	20.0 ± 4.1	35.2 ± 10.0	48.0 ± 7.2	22.7 ± 12.2	70.3 ± 8.1	18.1 **
LSD ($p < 0.05$)	7.4 **	12.8 **	14.4 **	NS	NS	
2020						
G_0	4.3 ± 4.4	2.3 ± 4.0	1.6 ± 2.1	16.7 ± 2.9	67.8 ± 4.0	7.3 **
G_1	6.7 ± 1.5	15.0 ± 2.0	28.7 ± 1.1	25.0 ± 5.0	72.2 ± 4.3	6.2 **
G_2	22.6 ± 4.5	20.0 ± 2.6	31.0 ± 1.7	36.4 ± 5.8	76.4 ± 14.7	14.7 **
G_3	37.0 ± 0.8	25.3 ± 6.6	41.3 ± 5.1	56.1 ± 3.6	75.7 ± 9.1	11.6 **
LSD ($p < 0.05$)	5.9 **	7.8 **	5.6 **	8.4 **	NS	
Generations × Genotype			**			

This indicates that the seeds tested in G_1, G_2 and G_3 were produced without isolation and likely crossed. * and ** indicates significance at $p < 0.05$ and $p < 0.01$. NS, not significant.

According to the mean projections (Figure 2), it could be expected that the PY level of EU cultivars after 6 years of the spontaneous crosses with the naturalized AR population would reach a similar value to the latter population (~70%).
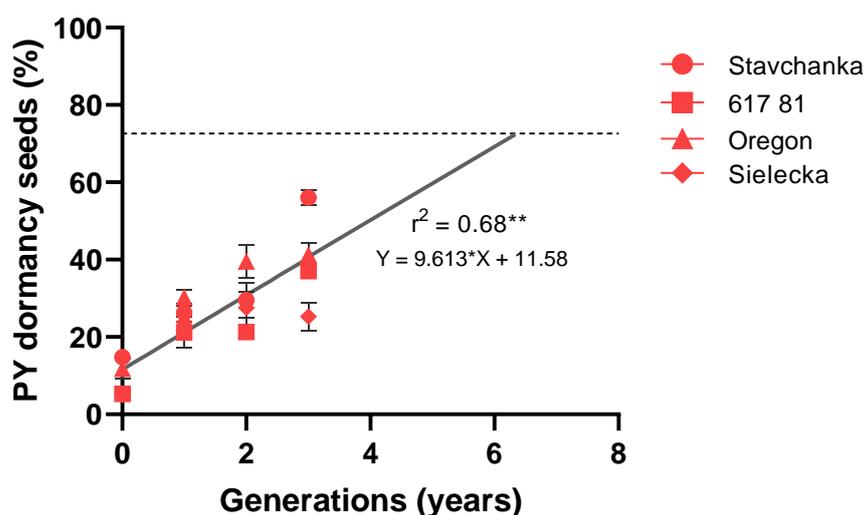
Figure 2. Projection of physically dormant seeds (PY) of EU cultivars (mean and standard error) as a function of generations of spontaneous crosses with a naturalized AR population. (** $p < 0.01$)

A faster rate of PY break was observed under summer compared to winter field conditions, due to the high temperatures (>9.8 °C) [11] increasing PY release (Figure S2). After 6 months and one year of burial, the PY levels ranged from 0–46% and 0–22%, respectively, depending on the initial PY (Figure 3a). Completely eliminated seedbank persistence (0% PY) after one year was observed when the initial PY level was lower than 14% (Figure 3b, Table S1).
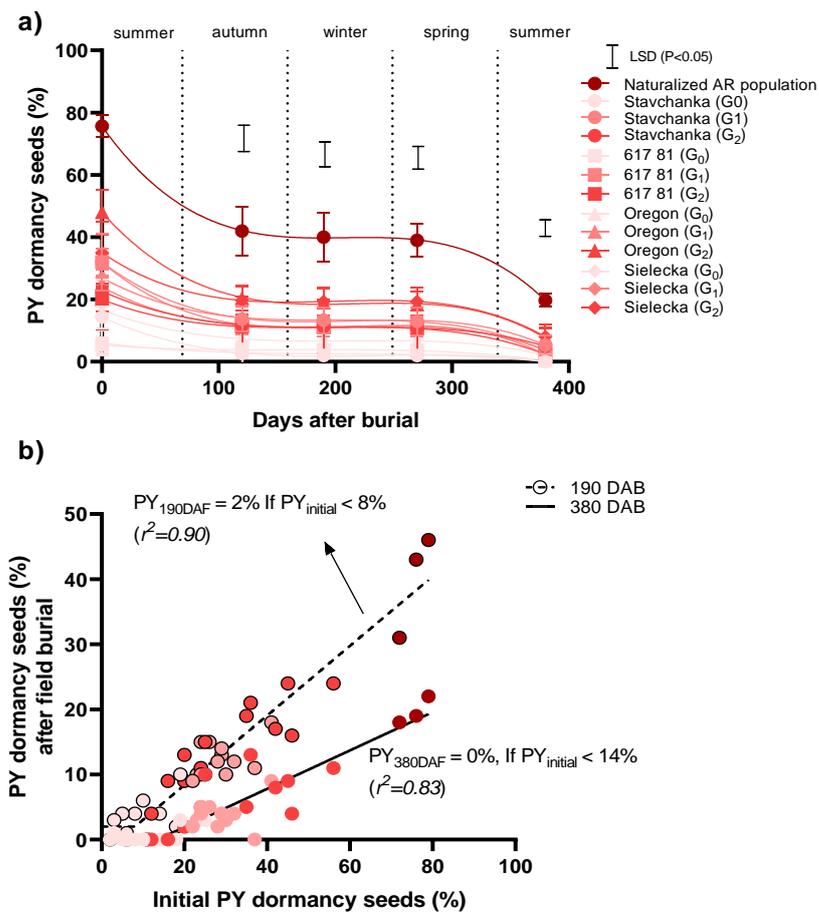
Figure 3. Percentage of physically dormant (PY) seeds for different genotypes and generations of hairy vetch as a function of days after burial (DAB) in field conditions during the 2019 growing season (**a**), and relation between PY at 190 and 380 days after field burial and initial PY (**b**).

Naturalized AR showed higher dry matter production compared to EU cultivars (1229 ± 258 vs. 931 ± 311 g $plot^{-1}$; $p < 0.01$), but this difference was not observed as the generation of spontaneous crosses progressed ($p > 0.05$). DM was slightly related to increases in PY levels (Figure 4).
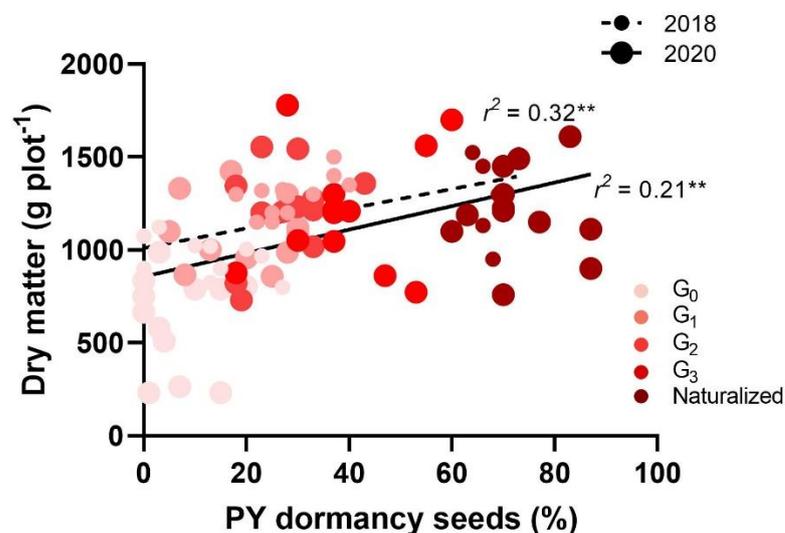
Figure 4. Dry matter per plot (DM) produced by hairy vetch during 2018 and 2020 in relation to the initial PY level of seeds. (** $p < 0.01$)

4. Discussion

The seed coat (testa) surrounding the embryo offers both physical and chemical protection against predation and microbial and fungal decay [28,29]. In particular, phenolic compounds seem to act as antioxidants, antimicrobial and anti-predation compounds [30–32] but also engage in the impregnation of the testa [33], which is involved directly in seed dormancy. Various polyphenols can be found in the seed coat, such as flavonoids, lignins and lignans. Several lines of evidence indicate that polyphenols influence both seed longevity and dormancy [32]. It has been shown that insoluble polyphenolic compounds contribute to seed coat hardness and the resulting seed dormancy [33–35].

Seed dormancy is largely genetically determined; however, it is also influenced by the environmental conditions experienced by the mother plant (maternal effect) and the subsequent degree of seed dehydration [8,14,36]. In our study of HV seeds growing under a semiarid environment, we observed that the environmental effect between years was lower than the genotypic effect (Figure 1). Appropriate conditions for seed development and drying would reduce the inter-annual variability in the acquisition of PY [6]. Our results indicated that the seed production environment under semiarid conditions had little effect on HV seed dormancy and that it is feasible to breed for the improvement of these traits by applying selection. The influence of the maternal environment may be greater in more contrasting year–site scenarios [8].

EU cultivars had lower PY values compared to naturalized AR populations. Observed differences between genotypes are likely explained by human selection during the improvement process [12,37,38]. Thus, special care must be taken when multiplying these improved genotypes to avoid contamination by gene flow. In our study, we observed that the PY level increased over the generations as the result of spontaneous crosses with high PY populations (Figure 1).

The naturalized AR population showed a higher biomass compared to original EU cultivars, probably due to the adaptation to Argentinian ecological conditions. It was shown that AR populations constitute an important reservoir of genes for high biomass production [6], but spontaneous crosses between EU cultivars and AR throughout the cycles (G_1 to G_3) did not show difference in biomass production.

In our study, we observed that the total reduction of the seedbank persistence is unlikely within 6 months of the incorporation of seeds into the soil, and after 12 months, it is possible only when the initial PY was lower than 14% (Figure 3). However, these genotypes would not be suitable in ley farming practice, in which the success of annual crop rotation depends on the ability to provide an adequate plant density for seed/forage production by self-regeneration from the soil seedbank. On the contrary, from a weed management perspective, we observed that in HV genotypes with more than 70% of initial PY, the problem of "volunteer plants" can be avoided if management practices avoid seed production and seedbank replenishment for at least three consecutive years [17]. The selection breeding of cultivars with <14% and <8% of initial PY could reduce this period further to one year and 6 months, respectively.

Managing volunteer HV is particularly problematic in organic cereal production, where HV invasion could not be controlled by herbicides (synthetic auxins or sulfonylureas) in the winter cereal crop phase [39,40].

The mechanical scarification process could reduce the proportion of PY seeds but also includes potential risks, such as low seed viability and associated operational costs [41,42]. Likewise, this management approach would not reduce the potential risk of weedy HV. Seed production and dispersal followed by a cereal crop or the incomplete termination of HV cover crop with plant regrowth and seed setting could result in the formation of seedbank and HV volunteer problems in successive crops [43,44].

In this sense, dedicated crosses between EU (G_0) and AR accessions and subsequent selection would serve to increase DM and reduce PY. With only one mating cycle, selecting (EU G_1) with <14% of PY immediately after seed harvest and high DM in individual HV plants can satisfy both breeding objectives. It is important that the selection by low PY is

carried out immediately after the harvest with careful threshing of the mature pods (<14% of seed moisture). After harvest, PY release occurs rapidly at >9.8 °C, and post-harvest conditions could impact PY [8,11]. Additionally, mechanical harvest reduces PY by 78%, and it is easy to underestimate this trait considering natural seed dispersal [45].

Under natural conditions, dormancy has evolved as a bet-hedging strategy and is favored in temporally variable environments [46,47]. The prevention of the germination of a certain proportion of seeds reduces the risk of extinction once conditions become unfavorable. The physical dormancy of seeds of annual legumes is proposed to be broken down during fluctuating summer temperatures [48]. This mechanism prevents inopportune germination by protecting false germination before the onset of the autumn–winter rainy season [49], resulting in selection operating in natural habitats. Similarly, higher PY is favored in naturalized populations, and the gene flow to genotypes with low PY leads also to this adaptation [50].

Since high PY is not compatible with agronomical practices, it has been reduced during the domestication of crops and, in the case of less domesticated forages such as HV, by breeding selection [8]. However, particularly in cases when wild relatives grow sympatrically and there are no crossing barriers, subsequent gene flow results in the occurrence of mostly genetically dominant wild traits in the resulting hybrid offspring, including seed dormancy [51].

5. Conclusions

Special care of the prevention of gene flow is required during the breeding and seed production of HV. This is especially relevant in situations such as the semiarid agroecosystem of Argentina where naturalized HV populations are well adapted. Spontaneous crosses with naturalized populations increase the degree of PY of the EU cultivars over breeding cycles. The effect of gene flow to the crop is not only of breeding and agronomical importance but can lead to the evolution of more aggressive invasive species [52]. Further studies of gene flow and isolation distance are needed.

The situation analyzed in this study of HV, which is increasingly used as a cover crop, and respective naturalized populations widely distributed worldwide [5,13,53] can be extended to other crops and particularly to forage species.

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