Heliyon 10 (2024) e27975

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Genetic study for seed germination and shattering in *Euphorbia lagascae* in response to different seed treatments

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ARTICLE INFO

Keywords:
Euphorbia breeding
Genetic control
Gibberellic acid
Industrial crop
Vernolic acid

ABSTRACT

Euphorbia lagascae Spreng is a promising emerging oilseed crop, with its seed oil accounting for approximately 50% of the seed weight. *Euphorbia* oil contains a significant amount of vernolic acid, comprising two-thirds of its composition, which boasts various industrial applications, including acting as a stabilizer-plasticizer and natural dye. However, this species was known to have a high degree of seed-shattering and a low germination rate, which act as two important barriers to large-scale production and exploitation. Therefore, the objective of this study is to determine the genetic control of seed germination and seed-shattering traits in order to develop a reliable pipeline that would be applicable for industries and breeders to select superior *E. lagascae* lines and design a robust breeding scheme in a short time at reduced labor costs. For this objective, five different wild-type genotypes of *E. lagascae* that demonstrated high germination potential were crossed with an ethyl methanesulfonate (EMS) mutant genotype that produces non-shattering capsules. The F_2 populations from two successful crosses (A and B) were separated into three different treated groups for seed germination evaluation and to study the segregation of 200 individuals per F_2 population. The three treatments were: light, gibberellic acid (GA_3), and control treatment. Consequently, plants treated with approximately 250 μmol/m^2/s of light showed significant improvement in germination up to 75% in cross A and 82.4 % in cross B compared with the control plants and the group treated with 0.05% GA_3. According to the chi-square test results, the inheritance pattern of seed germination in response to light treatment follows a 3:1 segregation ratio between germinated and non-germinated seeds, indicating a dominant gene action in the F_2 generation. The same conclusion was followed for the shattering trait in the group treated with light, which was also simply inherited as a 3:1 ratio for shattering vs. non-shattering capsules. Our results emphasize the importance and significance of light treatment in producing uniform populations through acceptable germination and shattering resistance of the mutant genotypes of *E. lagascae*. This is the first report of light treatment that significantly improved seed germination of *E. lagascae*, which may enhance efforts in the development of this new industrial crop as a feedstock for vernolic acid production.

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https://doi.org/10.1016/j.heliyon.2024.e27975
Received 30 April 2023; Received in revised form 24 February 2024; Accepted 8 March 2024
Available online 20 March 2024

1. Introduction

Euphorbia lagascae is known as a novel alternative oilseed crop with a high amount of vernolic acid, constituting around 60% of the seed oil [1,2]. As such, it provides the agricultural industry with additional and new market diversification opportunities with a naturally sourced, renewable, and environmentally friendly chemical feedstock [3]. Traditionally, the most frequent oilseed crops used for non-food products, such as epoxy esters, are soybean, olive, rapeseed, and linseed [4]. The importance of vernolic acid comes from its several applications in chemical industries, such as stabilizers, plasticizers, coatings, paints, and the synthesis of natural dyes [3,5]. The epoxy fatty acid vernolic acid derived from *E. lagascae* has low viscosity and good flow characteristics compared to the epoxy fatty acid synthesized from other sources [6].

The extreme yield loss, the potential to spread the crop as a weed, and the challenges associated with mechanical harvesting are the major constraints of the shattering genotypes such as *E. lagascae* [7]. The mutant genotypes in *E. lagascae* induced by ethyl methanesulfonate (EMS) have a thin mid-layer mesocarp which results in capsule indehiscence [8]. The mutant genotypes in other crops, such as sesame, resulted from gamma-ray irradiation with the affected gene identified as a single recessive [9,10]. However, different trade-off behaviors are associated with the non-shattering mutant genotypes in sesame, including low yield, semi-sterility, short capsules, cupped leaves, and twisted stems, which may occur because of several mutations and pleiotropy behavior in the mutant genotypes [10,11]. The mutant genotypes of *E. lagascae* have less seed shattering compared to wild accessions linked to trade-off irregular and poor germination that reduces plant stand count and results in less seed and oil yield [12].

For crops such as sesame, rapeseed, and linseed, seed shattering has not been lost through domestication, and work to reduce shattering continues [13]. In 1943, the first non-shattering mutant found in sesame had a monogenic nature with homozygous and recessive genes but with pleiotropic allele effects such as semi-sterility, curly leaves, short pods, twisted stems, and low yield [14]. These characteristics are similar to the sterility present in the non-shattering mutant genotypes of *E. lagascae*. Although more than 99% of sesame worldwide is harvested manually, studies were initiated to improve mechanical harvesting by introducing shattering-resistant genotypes [14]. The cloned gene (*Sicl1*) has reduced the parenchyma cells, adaxial and abaxial, characteristics in the leaf blade, and it was recommended to be implemented in further breeding programs to adapt advanced mechanical harvesting in the sesame variety [15].

Rapeseed is another oilseed crop with shattering pod behavior that contributes to more than 50% of seed yield loss in commercial rapeseed production. This trait has not been targeted during domestication approximately 400 years ago [16]. A rapeseed breeding program (Genome-wide association analysis, GWAS) in the Chinese germplasm was carried out to improve the pod-shattering resistance via marker-assisted selection [17]. The study identified six significant quantitative trait loci (QTL), of which a minimum of two loci mainly contribute to providing shattering resistance alleles across different environments [17]. In soybean breeding, the reduction of pod shattering is associated with two factors, one related to the wall of the pod and the other related to the force generated by the dehiscent characteristics [18]. The gene affecting the shattering behavior of the pod during domestication is *pdh1*, with major effects compared to other genes identified by different GWAS methods [19]. Interestingly, it is the gene for lignification (*pdh1*) that increases the shattering force when stimulating the dry pod wall to twist under low humidity, therefore, losing this gene increases the shattering resistance [18]. The narrow-leafed lupin was domesticated so that two recessive genes were discovered and named lentus and tardus [20]. Another study developed one co-dominant microsatellite-anchored fragment length polymorphism (MFLP) marker named (named as "TaLi") as the first simple PCR-based marker linked to the tardus gene to be used widely for the selection of non-shattering narrow-leafed lupin pod with 94% validation [21]. The presence of the lentus allele was associated with the change of pigmentation in the inner mature pod to yellow and the immature pod to purple [20]. Additionally, environmental conditions, such as humidity and temperature, should be played a very important role in how tardus expresses itself and whether the gene is present or absent [21]. In general, legumes have more than one gene controlling the tension of seeds by which each mutant gene contributing part of the shattering resistance can increase with the increased frequency of non-shattering alleles [22]. However, increasing the shattering resistance behavior by crossing cultivars of various resistance genes can cause transgressive segregation that results in plants with opposite resistance characteristics to enable selection for a higher frequency of shattering resistance alleles [22].

Different breeding efforts are required to develop non-shattering mutant genotypes suited to mechanical harvesting [11]. Mutant genotypes in *E. lagascae* (EU005, EU006, and EU008) have proper growth when transplanted from a greenhouse, which in turn increases the cost of planting [12,23]. Therefore, genetic studies for combining the good germination potential in wild-type genotypes with the high seed retention in the mutant genotypes are needed to develop mutant genotypes to reduce capsule shattering and increase seed and oil yield [24].

One of the most important factors for producing a large number of seeds for *E. lagascae* is understanding the seeds' germination potential, including the reason for the different degrees of sterility found in the different generations [25–28]. Chakraborty, Todd [12] suggested that *E. lagascae* seeds may have some dormancy because they found that few seeds planted in late May stayed viable over winter. Although seed dormancy provides species a way to distribute seeds over time, poor seedling vigor and irregular germination make it an undesirable feature for agricultural cultivation [29]. In addition to regulating germination, seed maturity, and seed dormancy, phytohormones such as Abscisic acid (ABA) and Gibberellic acid (GA) contribute to stem length, hypocotyl growth, and the initiation of roots and flowers [30]. The key role of ABA is maintained across plant species, and the overexpression of DOG1-like genes in *Arabidopsis* [31,32] further reinforces this. ABA reaches its maximum levels during the maturation of seeds; however, the after-ripening of dry seeds requires light signals and other requirements for GA synthesis [31] in order to switch from dormancy to germination. Recently GA_3 was found to contribute to adaptation to adverse conditions via a complex antagonistic network with ABA by establishing stress hormones that allow plants to adapt to abiotic stresses, including low temperature, drought, salinity, and flooding [30].

The improvement of germination and shattering in a new *E. lagascae* cultivar will need to be combined with high seed yield, oil content, and vernolic acid content for the crop to be commercially successful. This study aims to determine the genetic control of seed germination and shattering in *E. lagascae* and to study the dormancy potential in the offspring of the mutant genotype. For this aim, two breeding crosses were made to create two different *E. lagascae* populations and different seed treatments were applied to investigate seed germination and capsule shattering rate in the progenies of the two crosses. The results of this study can shed light on the breeding strategy to improve the overall performance of *E. lagascae* while reducing seed capsule shattering and increasing the germination rate. In addition, the developed pipeline can be applied to facilitate the exploitation of *E. lagascae* in different industries.

2. Materials and methods

2.1. Planting and hybridizations parents

Three seeds per pot from five wild-type (WTs) genotypes (Ames 22903, Ames 22906, PI 607972, PI 607975, and PI 649765), which originated in Spain (Fig. 1) were used as the female parent plants, and three seeds from the non-shattering mutant (M) genotype EU006 were soaked in 0.05 % w/v GA_3 for 24 h were used as a male donor plant. Each set of three seeds was planted in a 5-gallon pot using the Sunshine® soil mix #3 (Sun Gro Horticulture/Agawam, USA) to increase the chance of germination of at least one seed per pot under growing conditions as described by Chakraborty, Cici [23]: The photoperiod was 16 h (16 h of light, 8 h of darkness), and the relative humidity was 50%. A total of six different genotypes were planted as parent plants (five WT and one mutant), and staggered planting was used to match the flowering time between the mutant and the WT genotypes.

The female parent genotypes were emasculated when the male flowers began to develop to facilitate hybridization with the male donor. The F_1 capsules were collected from all the successful crosses. Two crosses, namely, Ames 22906 × EU006 and PI 649765 × EU006, were successful, but the F1 seeds did not germinate when planted in a separate pot. However, the other two crosses, PI 607975 × EU006 and PI 607972 × EU006, were successful in producing viable seeds and, therefore, were chosen to produce F_1 plants.

2.2. Selfing of the F1 plants

The F_1 seeds were soaked in 0.05 % w/v GA_3 for 24 h and then planted in a 5-gallon pot using Sunshine® soil mix #3. The growing conditions were used as described by Chakraborty, Cici [23]. Briefly, a photoperiod of 16 h (16 h of light, 8 h of darkness), 70% relative humidity, and light levels of 350 μEm−2 s−1 with day and night temperatures of 25 °C and 20 °C, respectively [23]. The plants were connected to drip irrigation (watered at 50 mL/pot/day) three times per day and were grown to obtain F_1 plants. The irrigation system was made up of water pipes that were inserted into the potting mix in individual pots and fully automated and controlled by a computer. The plants were covered with pollination bags when they started flowering to enable selfing and prevent cross-hybridization with foreign pollen.

Fig. 1. Distribution of the wild-type *E. lagascae* accessions collected in Spain.

2.3. Seed treatment

Each cross was subdivided into three treatment groups, consisting of 200 F_2 seeds for the group that was treated as follows: (i) each group was treated with 250 μmol m^{-2} $s^{-1}$of light for one month in cross A, and two months in cross B; (ii). The seeds were exposed to the light conditions of 16-h light and 8-h dark period daily for one month in growth room in the Crop Science building at the University of Guelph Campus. The growth room was lit with both fluorescent lamps (Sylvania F96T12/CW/VHO 215 W bulbs) and incandescent bulbs (Sylvania 40 W, clear A19 bulb) with an intensity of 270 μmol m^{-2} s^{-1} (measured at 30 cm from lamps), with a range of 225–275 and an average 250 μmol m^{-2} s^{-1}.Both groups were treated with 0.05% GA_3 for 24 h, prior to planting. The percentage of GA_3 was selected as 0.05% based on the earlier study (Chakraborty et al., 2018); (iii) the control plants were used to compare the other groups by soaking the seeds in distilled water for 24 h.

2.4. Genetic study of the F_2 populations

The F_2 seeds were collected from capsules on the F_1 plants and separated into three different treatment groups for each cross. The F_2 seeds were sown in 50 cell seedling trays with a total of 600 seeds (individuals) per cross (F_2 population). The two crosses were named: Cross A for cross between the WT genotype PI607975 and the M genotype EU006; and Cross B for cross between the WT genotype PI607972 and the M genotype EU006. Each cross was subdivided into three treatment groups, consisting of 200 F_2 individual plants. The seeds were planted randomly for the genetic study, which tested several segregation ratios to determine genetic control in the F_2 populations.

2.5. Germination data collection

Phenotypic data collection began the first week after sowing the F_2 seeds by individual monitoring and recording the number of germinated seeds divided by the entire number of seeds as the germination percentage (%). As single F_2 plants were used, replicates were not possible; therefore, error bars were estimated by upscale and downscale each treatment by 5%. The percentage of germination for each treated group and for the two different crosses was calculated upon data collection. The same data were collected starting from the second week until the seventh week for the three different treatment groups and for the two different crosses (Supp. Tables 1–6). Similarly, germination rate and phenotypic data were collected for seven different stem lengths categories (0, non-germinated; Emerge, merged seeds; Short, up to 5 cm; Medium, 5–10 cm; Tall, 10–15 cm, X-tall,15–20 cm; XX-tall$>$ 20 cm). However, to simplify the comparison, the categories were reduced to four rather than seven, excluding the seeds that did not germinate. Plants that were $\geq$10 cm tall were used to denote the "tall" category.

2.6. Shattering data collection

The first data collection on seed shattering started from the first week of producing the capsules and continued for 7–10 days, with data sets collected for three different periods. From the three different periods, the most frequent phenotypes were recorded and considered to represent each individual (Supp.Tables 7–8). There were three different categories used for phenotyping the shattering behavior: 1) plants having one or more indehiscent capsules that did not shatter; 2) plants having five or more indehiscent capsules that did not shatter (Fig. 2A); 3) plants having ten or more indehiscent non-shattering capsules (Fig. 2B).

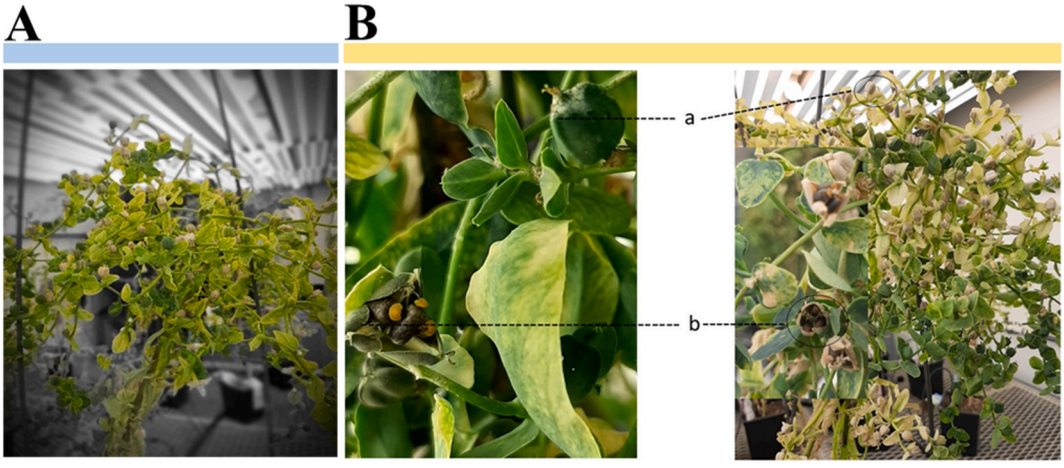

Fig. 2. A) Non-shattering (indehiscent) phenotype of the capsules. B) a. non-shattering phenotype of the hybrid plant, b. different types of shattering (seeds remain in capsule) for the hybrid plant.

2.7. Statistical analysis

The genetic model was subjected to a chi-square test by inspecting the segregation ratios for the observed vs. expected ratio in each of the three treatments for the cross A and B, respectively (Supp. Tables 1–3) for cross A and (Supp. Tables 4–6) for cross B. Data were analyzed using SAS software, version 9.4. A population size of 200 F_2 individuals was used to test all the ratios as it was deemed sufficiently large for testing simple segregation ratios at $\alpha = 0.05$.

Assuming the following Mendelian genetics classes in F_2:

The minimum number of individuals per population used was (n) = 3 × (perfect population) as follows.

1 locus/2 alleles (perfect population = 4) n = 12
2 loci/2 alleles (perfect population = 16) n = 48
3 loci/2 alleles (perfect population = 64) n = 192;

Therefore, the number of genotypes used in this study was 200, which is more than the minimum population size required for testing a diploid plant with two loci per trait. Frequencies were calculated, and chi-square analysis for all categorical variables was conducted to test the hypothesis for germination and shattering behavior separately. Furthermore, the frequencies ranged from 4 to 7 were used to estimate germination uniformity from each treated group from the two crosses.

3. Results

3.1. Germination percentage

Based on the results from 600 individuals from Cross A, the germination percentages for the control group of 200 individuals from the first to the seventh week were calculated as 14.5%, 44.5%, 57%, 59%, 62.5%, 62.5%, and 63%, respectively (Fig. 3A). The germination percentages for the group of 200 individuals treated with 0.05% GA_3 were 25%, 49.5%, 58%, 65%, 68%, 69%, and 69%, respectively, while for the third group of 200 individuals treated with 250 μmol light were 67.5%, 73%, 73.5%, 74.5%, 75%, 75%, and 75% respectively (Fig. 3A). For the 600 individuals from Cross B, the germination percentages for the control group were 11.06%, 31.6%, 31.6%, 36.18%, 47.75%, 49.79%, and 52.26%, respectively (Fig. 3B). The germination percentages for the individuals treated with 0.05% GA_3 were 35.18%, 54.5%, 55.78%, 57.29%, 58.79%, 59.8%, and 61.5%, respectively, while for the individuals treated with 250 μmol light were 62%, 74%, 74.5%, 75%, 79%, 81.5%, and 82.5% respectively (Fig. 3B). Overall, the proportion of germination displayed a positive trend over time across all treated groups for cross A and B, with the highest percentage observed under light treatment as shown in Fig. 4. The germination response over time for the three different treatment groups showed an exponential increase in the germination potential until reaching a plateau as illustrated in Fig. 3A for cross A and Fig. 3B for cross B.

3.2. Germination diversity

The germination diversity represented in the stem lengths for the seven different categories, including the non-germinated seeds, resulted in three different histograms for the three different treatments for cross A (Fig. 5(1–3)) and for cross B (Fig. 6(1–3)) in control, GA_3, and light-treated groups, respectively. The earlier histograms clarify the importance of light to produce a uniform stem length,

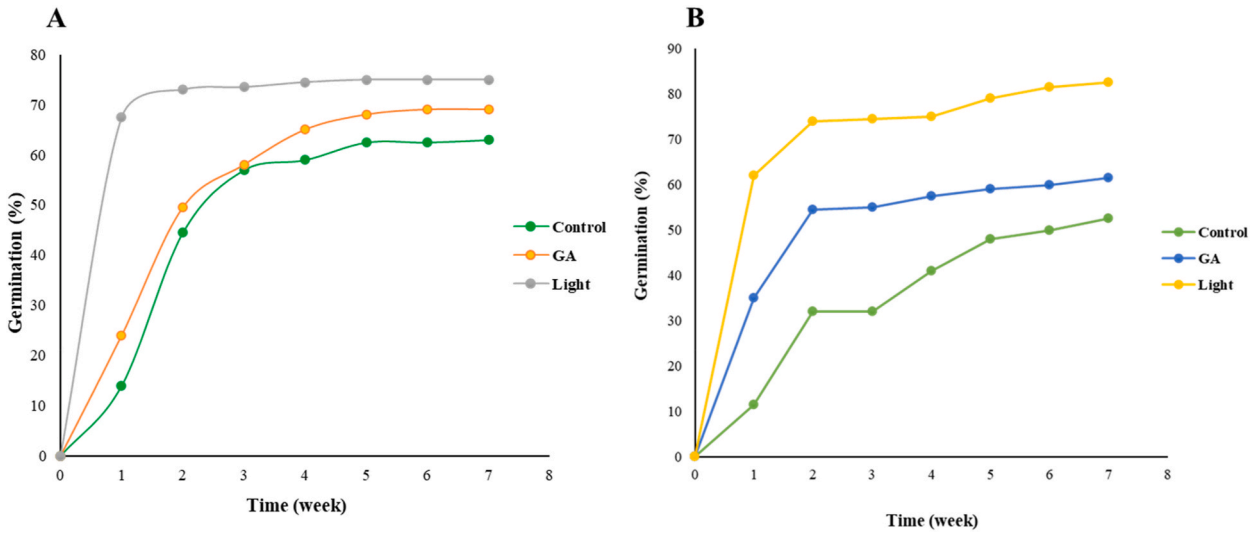

Fig. 3. The germination response by time for three different treated groups in A) cross A, and B) cross B.

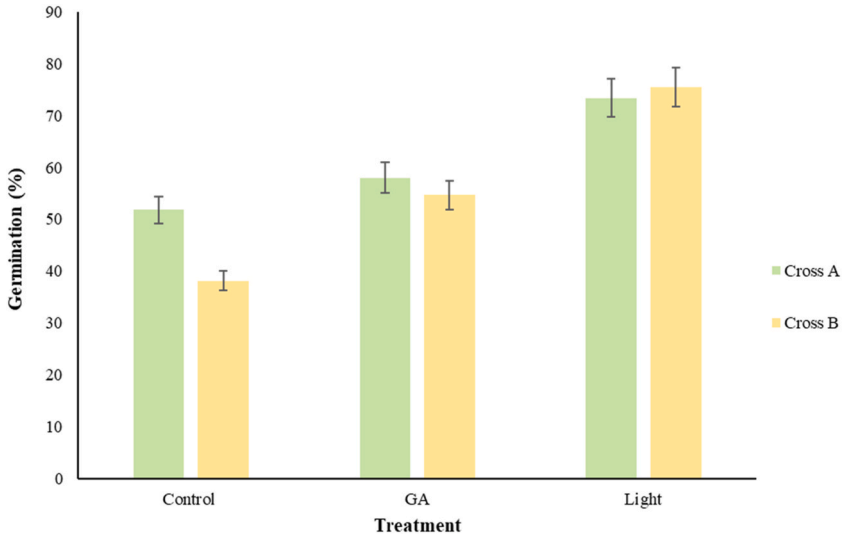

Fig. 4. Germination percentages in an F_2 population for three different treated groups in cross A and B. Error bars were estimated using 5% upscale and downscale of each treatment.

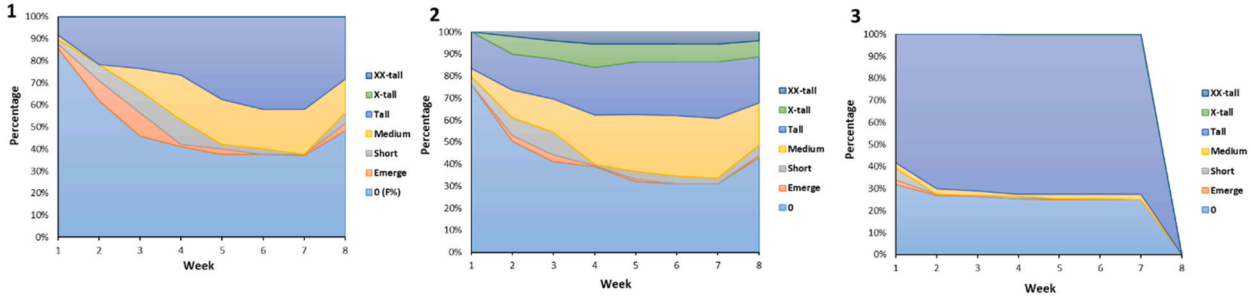

Fig. 5. The germination diversity in the stem lengths for cross A: 1) control plants, 2) treated with GA, and 3) treated with light. Short, up to 5 cm; medium, 5–10 cm; tall, 10–15 cm, x-tall,15–20 cm; xx-tall> 20 cm.

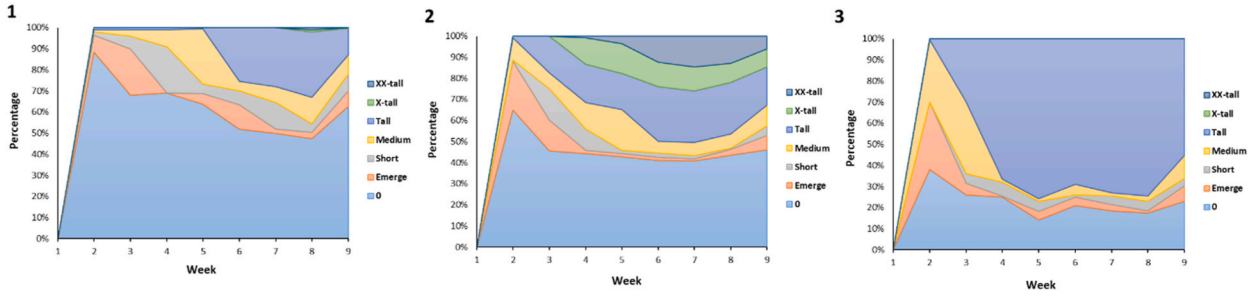

Fig. 6. The germination diversity in the stem lengths for cross B: 1) control plants, 2) treated with GA, and 3) treated with light. Short, up to 5 cm; medium, 5–10 cm; tall, 10–15 cm, x-tall,15–20 cm; xx-tall> 20 cm.

which plays an important role in facilitating mechanical harvesting. Overall, the results showed that the most uniform stem lengths were the groups treated with light in both crosses A and B.

3.3. Genetic study for the germination data

Regarding the genetic study of the F_2 population developed from cross A, all the tested treatments (Control, GA_3, and light) were significantly different than the expected ratios. The exception was for the duplicate recessive epistasis/complementation 9:7 germinated vs. non-germinated seeds for the control and GA_3 treatment (p-value $\geq$0.05) (Tables 1 and 2). The null hypothesis for a single

Table 1
Chi-square test of observed and expected segregation ratios for seed germination in the F_2 population (Cross A) for the control group.

Hypothesis	Ratio	Expected		Observed		Chi-square	Pr > Chi-square
	G: non-G[a]	G	non-G	G	non-G		
Single gene	3:1	150	50	114	86	34.5600	<0.0001
Duplicate dominant epistasis/duplication	15:1	188	13	114	86	460.9920	<0.0001
Duplicate recessive epistasis/complementation	9:7	113	88	114	86	0.0457	0.8307
Dominant and recessive epistasis/suppression interaction	13:3	163	38	114	86	77.2021	<0.0001

[a] G: Germinated seeds; non-G: non-germinated seeds.

Table 2
Chi-square test of observed and expected segregation ratios for seed germination in the F_2 population (Cross A) for the group treated with GA.

Hypothesis	Ratio	Expected		Observed		Chi-square	Pr > Chi-square
	G: non-G[a]	G	non-G	G	non-G		
Single gene	3:1	150	50	116	84	30.8267	<0.0001
Duplicate dominant epistasis/duplication	15:1	188	13	116	84	436.2453	<0.0001
Duplicate recessive epistasis/complementation	9:7	113	88	116	84	0.2489	0.6179
Dominant and recessive epistasis/suppression interaction	13:3	163	38	116	84	70.9662	<0.0001

[a] G: Germinated seeds; non-G: non-germinated seeds.

dominant gene failed to be rejected for a segregation ratio of 3:1 germinated vs. non-germinated seeds for the group treated with light (p-value ≥0.05), while it was rejected for all other hypotheses (Table 3).

For the F_2 population developed from cross B, all the tested treatments were significantly different than the expected ratios for the control treatment (Table 4). For the GA_3 treatment, all tested hypotheses were rejected for being significantly different except the one that was observed between duplicate recessive epistasis/complementation 9:7 germinated vs. non-germinated seeds (Table 5). Similarly, the group treated with light in cross B showed a single dominant gene phenotypic segregation ratio of 3:1 germinated vs. non-germinated seeds, and was rejected for all other hypotheses (Table 6).

3.4. Genetic study for the shattering data

Regarding the genetic study of the F_2 population developed from cross A, significant differences were observed from the expected ratios for the control and GA_3-treated groups except for the dominant and recessive epistasis/suppression interaction ratio of 13:3 shattered vs. non-shattering capsules (Tables 7 and 8). The null hypothesis for a single recessive gene failed to be rejected for a segregation ratio of 3:1 shattered vs. non-shattered capsules for the group that was treated with light with a P-value ≥0.05; Meanwhile, it was rejected for all other hypotheses (Table 9).

For the F_2 population developed from cross B, the results indicated that all tested hypotheses were rejected for the control treatment (Table 10). Considering that individuals with more than ten non-shattering capsules were the indehiscent phenotype in the control group, with a p-value of 0.2451 resulting from a duplicate recessive epistasis/complementation ratio of 9:7 shattered vs. non-shattered capsules (data not shown). Additionally, all tested hypotheses were rejected for the GA_3 and light-treated groups, except for the duplicate recessive epistasis/complementation ratio of 9:7 shattered vs. non-shattered capsules (Tables 11 and 12).

The F_2 segregation ratios for the shattering potential in cross A fit a dominant and recessive epistasis/suppression interaction model with a ratio of 13:3 shattered vs. non-shattered capsules. The latter indicated the presence of two genes controlling the seed-shattering behavior in control and GA_3-treated groups. However, the group treated with light resulted in a single gene with a dominance model with 3:1 shattered vs. non-shattered capsules, indicating that one of the genes had been masked and did not express itself in the presence of light. These results showed for the first time that exposure to light not only affects germination and dormancy but also works on the pathway of shattering response. This may indicate that there could be one main gene deactivated in the presence of light that has more than one phenotypic expression.

The F_2 segregation ratios for the shattering potential in cross B that was treated for an extra month with 250 μmol light fit a

Table 3
Chi-square test of observed and expected segregation ratios for seed germination in the F_2 population (Cross A) for the group treated with light.

Hypothesis	Ratio	Expected		Observed		Chi-square	Pr > Chi-square
	G: non-G[a]	G	non-G	G	non-G		
Single gene	3:1	150	50	146	54	0.2400	0.6242
Duplicate dominant epistasis/duplication	15:1	188	13	146	54	139.9680	<0.0001
Duplicate recessive epistasis/complementation	9:7	113	88	146	54	24.1829	<0.0001
Dominant and recessive epistasis/suppression interaction	13:3	163	38	146	54	7.8851	0.005

[a] G: Germinated seeds; non-G: non-germinated seeds.

Table 4
Chi-square test of observed and expected segregation ratios for seed germination in the F_2 population (Cross B) for the control group.

Hypothesis	Ratio	Expected		Observed		Chi-square	Pr > Chi-square
	G: non-G[a]	G	non-G	G	non-G		
Single gene	3:1	150	50	63	137	199.37	<0.0001
Duplicate dominant epistasis/duplication	15:1	188	13	63	137	1309.39	<0.0001
Duplicate recessive epistasis/complementation	9:7	113	88	63	137	48.90	<0.0001
Dominant and recessive epistasis/suppression interaction	13:3	163	38	63	137	321.25	<0.0001

[a] G: Germinated seeds; non-G: non-germinated seeds.

Table 5
Chi-square test of observed and expected segregation ratios for seed germination in the F_2 population (Cross B) for the group treated with GA.

Hypothesis	Ratio	Expected		Observed		Chi-square	Pr > Chi-square
	G: non-G[a]	G	non-G	G	non-G		
Single gene	3:1	150	50	111	89	39.2111	<0.0001
Duplicate dominant epistasis/duplication	15:1	188	13	111	89	44.22	<0.0001
Duplicate recessive epistasis/complementation	9:7	113	88	111	89	0.0179	0.8934
Dominant and recessive epistasis/suppression interaction	13:3	163	38	111	89	84.7469	<0.0001

[a] G: Germinated seeds; non-G: non-germinated seeds.

Table 6
Chi-square test of observed and expected segregation ratios for seed germination in the F_2 population (Cross B) for the group treated with light.

Hypothesis	Ratio	Expected		Observed		Chi-square	Pr > Chi-square
	G: non-G[a]	G	non-G	G	non-G		
Single gene	3:1	150	50	149	51	0.0267	0.8703
Duplicate dominant epistasis/duplication	15:1	188	13	149	51	126.4853	<0.0001
Duplicate recessive epistasis/complementation	9:7	113	88	149	51	27.0679	<0.0001
Dominant and recessive epistasis/suppression interaction	13:3	163	38	149	51	5.9815	0.0145

[a] G: Germinated seeds; non-G: non-germinated seeds.

Table 7
Chi-square test of observed and expected segregation ratios for seed shattering in the F_2 population (Cross A) for the control group (Total 125 individuals germinated).

Hypothesis	Ratio	Expected		Observed		Chi-square	Pr > Chi-square
	Sh: non-Sh[a]	Sh	non-Sh	Sh	non-Sh		
Single gene	3:1	94	31	107	18	7.49	0.0062
Duplicate dominant epistasis/duplication	15:1	117	8	107	18	14.17	0.0002
Duplicate recessive epistasis/complementation	9:7	70	55	107	18	43.75	<0.0001
Dominant and recessive epistasis/suppression interaction	13:3	102	23	107	18	1.55	0.2127

[a] Sh: Shattered capsules; non-Sh: non-shattered capsules.

Table 8
Chi-square test of observed and expected segregation ratios for seed shattering in the F_2 population (Cross A) for the group treated with GA_3 (Total 115 individuals germinated).

Hypothesis	Ratio	Expected		Observed		Chi-square	Pr > Chi-square
	Sh: non-Sh[a]	Sh	non-Sh	Sh	non-Sh		
Single gene	3:1	86	29	97	18	5.36	0.0206
Duplicate dominant epistasis/duplication	15:1	108	7	97	18	17.35	<0.0001
Duplicate recessive epistasis/complementation	9:7	65	50	97	18	36.89	<0.0001
Dominant and recessive epistasis/suppression interaction	13:3	93	22	97	18	0.72	0.3947

[a] Sh: Shattered capsules; non-Sh: non-shattered capsules.

duplicate recessive epistasis model with a ratio of 9:7 shattered vs. non-shattered capsules for all treated groups. Accordingly, the existence of two genes was associated with shattering behaviors that appeared in the GA_3-treated group accepting the alternative hypothesis (p-value = 0.95)

Table 9
Chi-square test of observed and expected segregation ratios for seed shattering in the F_2 population (Cross A) for the group treated with light (Total 163 individuals germinated).

Hypothesis	Ratio	Expected		Observed		Chi-square	Pr > Chi-square
	Sh: non-Sh[a]	Sh	non-Sh	Sh	non-Sh		
Single gene	3:1	122	41	119	44	0.35	0.5566
Duplicate dominant epistasis/duplication	15:1	153	10	119	44	119.7	<0.0001
Duplicate recessive epistasis/complementation	9:7	92	71	119	44	18.59	<0.0001
Dominant and recessive epistasis/suppression interaction	13:3	132	31	119	44	7.27	0.0070

[a] Sh: Shattered capsules; non-Sh: non-shattered capsules.

Table 10
Chi-square test of observed and expected segregation ratios for seed shattering in the F_2 population (Cross B) for the control group (Total 100 individuals germinated).

Hypothesis	Ratio	Expected		Observed		Chi-square	Pr > Chi-square
	Sh: non-Sh[a]	Sh	non-Sh	Sh	non-Sh		
Single gene	3:1	75	25	55	45	48.00	<0.0001
Duplicate dominant epistasis/duplication	15:1	94	6	55	45	405.6	<0.0001
Duplicate recessive epistasis/complementation	9:7	56	44	55	45	5.14	0.0233
Dominant and recessive epistasis/suppression interaction	13:3	81	19	55	45	86.25	<0.0001

[a] Sh: Shattered capsules; non-Sh: non-shattered capsules.

Table 11
Chi-square test of observed and expected segregation ratios for seed shattering in the F_2 population (Cross B) for the group treated with GA_3 (Total 150 individuals germinated).

Hypothesis	Ratio	Expected		Observed		Chi-square	Pr > Chi-square
	Sh: non-Sh[a]	Sh	non-Sh	Sh	non-Sh		
Single gene	3:1	113	38	89	61	19.6	<0.0001
Duplicate dominant epistasis/duplication	15:1	141	9	89	61	303.2	<0.0001
Duplicate recessive epistasis/complementation	9:7	84	66	89	61	0.58	0.4465
Dominant and recessive epistasis/suppression interaction	13:3	122	28	89	61	47.3	<0.0001

[a] Sh: Shattered capsules; non-Sh: non-shattered capsules.

Table 12
Chi-square test of observed and expected segregation ratios for seed shattering in the F_2 population (Cross B) for the group treated with light (Total 166 individuals germinated).

Hypothesis	Ratio	Expected		Observed		Chi-square	Pr > Chi-square
	Sh: non-Sh[a]	Sh	non-Sh	Sh	non-Sh		
Single gene	3:1	125	42	93	73	31.8795	<0.0001
Duplicate dominant epistasis/duplication	15:1	156	10	93	73	403.2145	<0.0001
Duplicate recessive epistasis/complementation	9:7	93	73	93	73	0.0034	0.9532
Dominant and recessive epistasis/suppression interaction	13:3	135	31	93	73	69.3389	<0.0001

[a] Sh: Shattered capsules; non-Sh: non-shattered capsules.

4. Discussion

The development of plant-based sources of vernolic acid would provide farmers with a new market diversification opportunity and industrial manufacturers with a source of renewable, environmentally friendly chemical feedstock [33]. Recent agronomic research demonstrated that E. *lagascae* can grow well in southern Ontario and that the seeds can be harvested using conventional farming equipment [1,25].

In the current study, the null hypothesis for a single recessive gene failed to be rejected for a segregation ratio of 3:1 shattered vs. non-shattered capsules for the group that was treated with light. Similar findings were obtained in a study investigating soybean in Ghana, wherein it was revealed that the shattering trait exhibited a bidirectional qualitative pattern, with an estimated broad-sense heritability ranging from 0.90 to 0.95 due to duplicated dominant and recessive epistatic effects [34]. In the study on lupins, Li, Renshaw [21] found that environmental conditions such as temperature and humidity affected the tardus gene expression, which varied depending on the presence or absence of the lentus gene [21]. This finding is consistent with the outcomes of our study of

E. lagascae flower morphology, which revealed two distinct groups of shattering behaviour. One of these groups was correlated with external pressures, and a 10 °C escalation in temperature resulted in a significant enhancement of seed retention. Furthermore, this trend bears resemblance to the findings of a contemporary investigation on the genetic determinants of leguminous plants' shattering resistance, which demonstrated a positive relationship between arid growing conditions and heightened resistance, implying a significant interplay between genotypic traits and environmental factors [22]. Moreover, certain phenotypic traits, including varying inner pod colour, have been suggested as indicative of the presence of lentus alleles in lupin, as noted by Gladstones [20]. Similarly, in *E. lagascae*, the emerging "partially indehiscent capsule" trait, exemplified in the accompanying image in the morphological analysis (Fig. 2), can serve as an indicator for the presence of a specific shattering gene. Similarly, in *E. lagascae*, certain phenotypes, such as the new shattering phenotype (partially indehiscent capsule) found in the hybrid capsules illustrated in the picture provided in the morphology study (Fig. 2), can be used to monitor the presence of a certain shattering gene.

To obtain the maximum seed retention in lupin, the two recessive genes, tardus and lentus, should both be present for a completely non-shattering phenotype. The effect of the shattering resistance allele on pod shattering and torsion of the pod wall in soybean NILs for the QTL pdh1 was investigated in a study where contrasting outcomes were observed under conditions of low humidity. Specifically, differences in shattering levels were observed between heterozygous plants with and without the presence of the resistance allele [18]. The complete shattering behaviour observed in *E. lagascae* can be attributed to the occurrence of the initial variant of the indehiscent capsule, which is independent of external factors, but may be indicative of the potential existence of inwardly heritable recessive homozygous alleles regulating the shattering behaviours associated with sterility.

Increasing germination rate is another important factor in *E. lagascae* that is not sufficiently well documented. Several studies suggested a strong correlation between dormancy and germination rate. As an example, a study of three ABA mutants (ABI1, ABI2, and ABI3) in *Arabidopsis* suggested that at least two parallel paths in mature seeds control the response for ABA by which one of ABI is not seed-specific while the other two are not responsible for all ABA-inducible reactions [35]. Another study that isolated ABA-deficient mutants in *Arabidopsis* showed that only one to two loci were identified as the possible homozygous recessive genes that affect the level of endogenous ABA [32]. ABA levels mainly regulate the release of dormancy and the germination process from dormant seeds to seedlings via complex interactions related to different hormonal signals that affect downstream gene expression [29]. The term "after-ripening" refers to changes that occur to dry seeds as a result of storage conditions such as relative humidity and temperature that reduce or remove the germination dormancy characteristic over time (Bewley et al., 2013). The low level of ABA found in dry seeds as well as the DOG1 protein (first discovered in *Arabidopsis*), are responsible for the delay of germination and can be used as a timer to release dormancy [36]. In rehydrated seeds, the dormancy and germination pathways' response and sensitivity to GA_3 are stimulated, altered, and affected more by light and temperature [37]. After primary dormancy is overcome, the secondary dormancy seasonal cycle is released, which is not well studied at the molecular level. However, it is better documented in terms of the light effect on a model species like *Arabidopsis* [38–41]. Nevertheless, the ABA levels in *Arabidopsis* have been linked to the expression of DOG1, ABI3, and SnRK2 and were associated with strong dormancy in low winter temperatures [36,38]. Further genetic study is recommended to dissect the marker trait associations with germination rate in *E. lagascae* using different genetic tools such as genome wide association study or quantitative trait loci mapping [42,43].

The key role of ABA is maintained among species [32,44]. Overexpression of the DOG1-like genes in *Arabidopsis* reaches a maximum during the maturation of seeds, but the expression is reduced in after-ripening dry seeds, in which the switch turning off dormancy depends on light signals and the other requirements for GA_3 synthesis [32,44]. In this study, the results from cross A and cross B (taking the first week to compare the germination percentages) showed that the group treated with light germinated at higher percentages than either the control plants or the plants treated with GA_3. The higher germination numbers for cross B treated with light may have been due to cross B being exposed to light for 2 months (environmental condition) compared to the one-month exposure given to cross A. Environmental conditions can affect the level of dormancy as well as the rate of germination under certain light and temperature signals [38]. However, GA_3 synthesis is associated with light signal requirements to complete germination that works like a sensor when seeds reach the soil surface [32,45]. The uptake of water in seeds with physical dormancy is affected by temperature [46]. Accordingly, further studies to ensure the absence of this kind of dormancy in this species are necessary.

Based on the segregation ratio in the two crosses, the duplicate recessive epistasis model with a ratio of 9:7 germinated vs. non-germinated seeds was the accepted model ratio for the Control and GA_3-treated groups of plants. However, the group treated with light in the two crosses resulted in a single gene with a dominance model of 3:1 germinated vs. non-germinated seeds. This may indicate that one of the genes in the control plants, such as endogenous ABA, may have been masked or was not expressed in the presence of light. Another factor, such as endogenous GA_3 is taking the dominant role with the main single gene synesthetic pathway when light signaling breaks dormancy and enhances seed germination. The dormancy of the seeds is usually determined by multiple genes and proteins [29]. This finding could indicate the activity of ABA in the absence of light, which inhibits germination and induces a certain type of dormancy that is represented by a duplicate recessive epistasis model with other pleiotropic effects. All of the above can be simplified by the antagonistic effects between the two main endogenous phytohormones, including ABA and GA_3, with a primary indication for a possibility of a qualitative nature for both of them, one as the main single gene synthetic pathway, and the other having double gene pathways. This requires further molecular and functional genetic studies to determine if this is the case and if so, the specific roles of these genes in dormancy and germination.

Furthermore, variations within the single-gene model have been observed due to the additional month of light exposure for the seeds from cross B. The variation in germination numbers between the different crosses may have occurred due to unexpected environmental stresses, such as the unexpected hot and dry conditions when recording the shattering data in cross B. Variations or biases in phenotyping could occur for the following reasons. First, it was difficult to categorize those plants that looked half shattered (i.e. the seeds remained inside capsules for days although the capsule was opened). Second, in some cases, capsules on the main stem

would shatter differently than those on the branches of the same plant. Third, defining capsules as shattered vs. non-shattered on younger still partially green plant vs. old and completely dry ones was difficult. Fourth, there were differences in the exact dates on which the data was collected. Due to COVID-19, some counts were delayed up to 10 days instead of a week. Therefore, errors that occurred during transplanting (i.e., emerged seedlings were not seen and sometimes died after transplanting), may have caused slight differences in the counts. Therefore, in future studies, it would be beneficial to develop an automated unbiased high throughput phenotyping pipeline in order to reduce errors.

Frequent phenomena that were seen during the breeding study of E. *lagascae* included extreme plant lodging in the plants coming from seeds treated with GA_3 (which was not present in control and light-treated subgroups). Similarly, in a study on the effects of GA_3 inhibitors such as paclobutrazol (PBZ) researchers reported reduced lodging and height of tef and finger millet plants and increased drought tolerance, and this was also found in rice when the biosynthesis pathway and the signaling of GA_3 were altered [47]. The results obtained in this study indicate the importance of conducting further studies to explore the effect of different light wavelengths in different temperature regimes to break seed dormancy that may result in altering germination rates. Both seed germination and shattering appeared to be simply inherited, fitting the one gene model under light treatment and two gene epistatic models for control and GA_3 treatments. Further research using molecular markers associated with genes for seed germination and shattering may shed more light on the genetic control of these two traits and confirm the classical genetic study done here. The selection of new recombinants or mutation genotypes with beneficial characteristics could also add value toward further domestication and adaptation of E. *lagascae* as an industrial crop. This study can help guide the development of stable and simple PCR molecular marker tags to genes of interest (e.g, indehiscent capsule gene) in *E. lagascae* that can be used in molecular selection for any conventional breeding program rather than taking the extra time and effort to do field assessment and would speed up the process of developing commercial lines with enhanced growth. These efforts could result in the successful breeding of *E. lagasce* varieties suited to mechanical harvesting, resulting in increased seed yield.

5. Conclusion

The seeds of F_2 populations from the two crosses made between wild-types and mutant genotypes were subjected to control, GA_3, and light treatments. An exponential increase in the plants' germination potential was observed over seven weeks post-seeding until it reached a plateau with the highest germination levels for light-treated seeds. Moreover, the subdivided groups subjected to light exposure in all breeding experiments resulted in the production of robust and homogenous populations, as it stimulated a faster germination process and healthier seedling growth. This ultimately leads to the development of stronger plants that are more easily harvested, underscoring the critical role of light in enhancing the harvestability of *E. lagascae*. The null hypothesis for a single dominant gene failed to be rejected for a segregation ratio of 3:1 germinated vs. non-germinated seeds for the group treated with light (p-value $\geq$0.05) for both cross A and B, indicating a single gene model with dominance for the trait. The F_2 segregation ratios for the shattering potential in cross A fit a dominant and recessive epistasis/suppression interaction model with a ratio of 13:3 shattered vs. non-shattered capsules. The F_2 segregation ratios for the shattering potential in cross B that was treated for an extra month with 250 μmol light fit a duplicate recessive epistasis model with a ratio of 9:7 shattered vs. non-shattered capsules for all treated groups. Accordingly, the existence of two genes associated with shattering behaviors that appeared in the GA_3-treated group accepting the alternative hypothesis Domestication of a novel crop such as *E. lagascae* is very challenging as few genetic studies have been done on this species. Furthermore, the interaction of these genetic components with environmental conditions warrants further research. The results of this study may help in the development of well-germinating, non-shattering cultivars of *E. lagascae*, ultimately providing a new crop option for farmers. The improvement of germination and shattering in a new cultivar will need to be combined with high seed yield, high oil content, and high vernolic acid content for the crop to be commercially successful.

Funding

The funding for the study was provided by the Ontario Agrifood Alliance, project #030254, titled "Agronomic improvement of Euphorbia lagascae and large-scale oil extraction to produce vernolic acid, an important industrial epoxidized ester.", which was awarded to Dr. Rene Van Acker.

Data availability statement

The raw data supporting the conclusions of this article will be available without undue reservation.

Compliance with ethical standards

This work does not involve any human participation nor live animals performed by any of the listed authors.

CRediT authorship contribution statement

Maram Istaitieh: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis. **Mohsen Yoosefzadeh Najafabadi:** Writing – review & editing, Validation. **A. Michelle Edwards:** Writing – review & editing, Resources, Formal analysis. **Jim Todd:** Writing – review & editing, Resources, Methodology, Funding

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Rene Van Acker reports financial support was provided by Ontario Ministry of Agriculture Food and Rural Affairs. None.

Acknowledgements

The authors thanks current and former staff of Dr. Rene Van Acker's, Dr. Istvan Rajcan's and Dr. James Todd's research teams at the University of Guelph and Ontario Ministry of Agriculture, Food and Rural Affairs. The authors also appreciate the generous funding Ontario Agrifood Alliance without which this project would not have been possible.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27975.

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