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RESEARCH

Effect of seeding density on the weediness potential of transgenic plants: a case study on sunflowers

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Background: Newly introduced transgenic plants can outcompete native species in natural ecosystems, threatening the biodiversity of a country. This study ascertained the weediness potential of plants according to the seed amount under the assumption that transgenic seeds were unintentionally spilled. Using sunflowers as the study system, 0, 50, 100, and 150 seeds were sown in 1 m × 1 m and 2 m × 2 m plots, and seed germination, survival, flowering, and competition between the surviving and wild plants were investigated.
Results: There was no significant difference in the germination rate of sunflowers depending on the cultivar, but differences were observed depending on the sowing density and plot size. As the number of seeds sown increased, the flowering and seed maturation of sprouted plants occurred earlier; the plant height and flower length of the surviving plants decreased. In addition, as the number of seeds increased, not only did the early importance of sunflowers increase, but the period of dominance over weeds also improved.
Conclusions: These results suggest that the weediness potential of sunflowers varies with the number of seeds at the time of release, which may affect germination and growth, and compete with weeds in transgenic plants.

Keywords: invasiveness, living modified organism, natural ecosystem, risk assessment, transgenic plant, weediness

Introduction

One of the negative effects of living modified organisms (LMOs) on natural ecosystems is that they may outcompete native species, thereby threatening their biodiversity (Warwick et al. 2009; Wolfenbarger and Phifer 2000). Therefore, when conducting a risk review for the import and production of LMOs, it is vital to assess the possibility that newly introduced LMO may become a weed in the domestic natural environment; this is one of the important evaluation items (Nam and Han 2020; Han and Nam 2022; Han et al. 2023).

Various factors can affect the possibility of becoming a weed, such as survival and reproductive ability, presence or absence of related species, seed bank characteristics, and the vitality and dormancy of the buried seeds. Kim et al. (2020) reported that LM soybeans expressing human epidermal growth factor genes (*egf*, *igf-1*, *trx*) form temporary soil seed banks during winter, but seed viability does not last more than 6 months, making them unlikely to develop into invasive plants or weeds in the ecosystem. Lee et al. (2011) investigated the low-temperature germination rate, post-winter survival rate, and reproductive rate in a natural environment to evaluate the weediness potential of vitamin A-enforced transgenic rice and confirmed that there was no significant difference between transgenic and non-transgenic plants. Moreover, in a study of the ecological invasiveness of potatoes expressing *ABF3*, the transgenic volunteers emerged spontaneously and produced tubers at levels similar to those of untransformed potatoes (Kim et al. 2010).

Meanwhile, the probability that spilled LMO seeds will survive in the natural ecosystem depends on the germination ability of the seeds, where environmental factors such as temperature, soil moisture, and light have a significant impact (Chauhan and Johnson 2010; Penfield et al. 2005). Seed weight, seed density, and burial depth may additionally play an important role in their germination (Tielbörger and Prasse 2009; Yin et al. 2009). In an experiment with 13 common annual species, seedling emergence was positively correlated with seed mass but negatively with burial depth (Limón and Peco 2016). Desert perennial plants grown at high densities were inhibited from subsequent germination because of fierce competition with other plants for limited resources (Inouye 1980). Similarly, the emergence of weeds such as *Veronica arvensis* was reduced at high seed densities, which may be related to traits that have evolved to cope with competition (Grundy et al. 2003).

Previously, we examined the invasive potential of LMOs according to the environmental conditions at the time of release using sunflowers, a candidate plant for LMOs in environmental remediation (Han and Nam 2022). This study investigated whether LM seeds imported into South Korea could survive, reproduce, and sustain populations depending on the amount of unintentional spillage into the environment during transportation or handling, using sunflower, a LMO candidate plant, as in our previous study.

Materials and Methods

Field experiment

Field experiments were conducted in an LMO confined field (36°01′43.0″ N, 126°43′22.5″ E) located at the National Institute of Ecology, Seocheon-gun, South Korea. The sunflower cultivar used for this study was the standard cultivar “Jaeraejongja” and the extreme dwarf type cultivar “Jaeraejong3” (Danong Co., Namyangju, Korea). A total of 48 plots were established in four fully randomized blocks, each with a size of 1 m × 1 m and 2 m × 2 m. The seeds were sown directly into the soil in May 2020 at 0, 50, 100, and 150 seeds. The interior of each plot was left without pest or weed control to ensure that it was identical to the natural environment.

Germination and growth characteristics of sunflowers by seeding density

Sunflower germination and phenotypic characteristics were measured once or twice a month, starting from the third week after sowing. Seedlings that emerged above the soil surface and formed cotyledons were considered to be germinated. Growth characteristics were determined by counting the number of individuals at different growth stages (seedling, growth, flowering, and seed-filling) of the sown seeds. Plants above the true leaf stage were labeled individually to distinguish the sown seeds, and it was observed whether sunflower individuals that survived and formed seeds maintained their populations after winter. The growth characteristics of sunflower plants that survived the fourth week after sowing were investigated by measuring the plant height, stem diameter, and head diameter. Plant height was determined at the highest point above the soil surface and stem diameter was measured at the part of the stem closest to the soil surface. Head diameter refers to the width of the head of the first flower that blooms during flowering. The effects of the final germination rate on sunflower cultivars, seeding density, and plot size were analyzed by one-way analysis of variance (ANOVA) using a general linear model (GLM) in SAS Studio (version 3.8; SAS Institute, Cary, NC, USA).

Analysis of plant species composition and vegetation importance

Vegetation surveys were conducted in each square once or twice a month, starting four weeks after sowing. The species list, coverage, frequency, and relative importance of all species were recorded in the experimental plots. Plant species were identified based on visual observations and the National Institute of Biological Resources (NIBR 2020). The importance value (IV) was used as an indicator of the relative dominance of sunflowers and weeds in the plots. The relative dominance rate was obtained by measuring the dominance ranks of sunflowers and wild plants in each plot. The dominance rank was calculated by substituting the median value of Braun-Blanquet (1964). Relative frequencies were obtained from plots where plants were present and calculated as the percentage of the frequency of a species to the sum of the frequencies of all species. The ratio of the IV of sunflowers to that of all weeds was additionally investigated to analyze the competitive period between sunflowers and weeds. The change in the IV of sunflowers and that of all the weeds during the investigation period were investigated, and the period when sunflowers were superior to or at a similar level to the entire weed was judged as a competitive period.

Results

Changes in germination and growth characteristics depending on seeding density of sunflowers

The germination and growth of sunflowers varied depending on the seed amount, plot size, and cultivar (Fig. 1). When 50, 100, and 150 seeds of “Jaeraejongja” were sown in the 1 m × 1 m plots, a maximum of 7.5, 22.5, and 31.0 individuals survived, respectively. When 50, 100, and 150 seeds of “Jaeraejongja” were sown in the 2 m × 2 m plots, a maximum of 10.3, 22.0, and 35.3 individuals survived, respectively. For “Jaeraejong3” in the 2 m × 2 m plots, when 50, 100, and 150 seeds were sown, a maximum of 8.5, 21.0, and 34.0 individuals survived, respectively. GLM analysis results demonstrated that the final germination rates were statistically significantly different according to seeding density ($p < 0.001$) and plot size ($p < 0.001$), but not between cultivars ($p = 0.2538$) (Table 1).

The developmental stages of sunflowers, including germination, growth, flowering, and seed maturation, differed

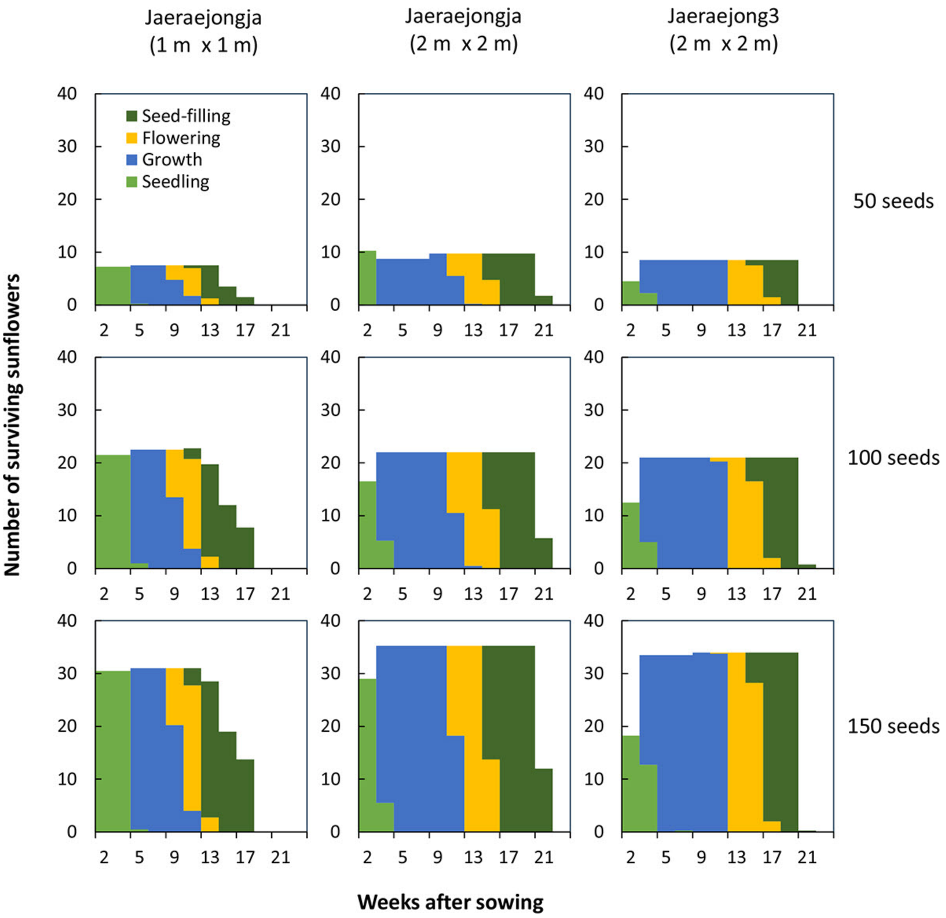

Fig. 1 The number of sunflowers that survived from seeds sown in different quantities (0, 50, 100, and 150 seeds) in two different-sized experimental plots (1 m × 1 m and 2 m × 2 m). Data represent the mean number of surviving individuals from four replicates. The developmental stages of sunflower plants are color-coded (yellow-green, germination; blue, growth; yellow, flowering; dark green, seed-filling).

Table 1 Results of a general linear model for final germination percentage

Source	SS	DF	MS	F	*p*-value
Sowing density	2904.056	2	1452.028	36.55	< 0.001
Cultivar	54	1	54	1.36	0.2538
Plot size	696.8889	1	696.8889	17.54	< 0.001

SS: sum of squares; DF: degrees of freedom; MS: mean square; F: F-statistic.

depending on seeding density, cultivar, and plot size. In all the experimental plots, germinated plants were observed two weeks after sowing. In addition, all the surviving individuals flowered. Germinated individuals were observed for up to 17 weeks after sowing in the 1 m × 1 m plots. In the case of the 2 m × 2 m plots, germinated individuals were observed for up to 21 weeks, except when 150 seeds of "Jaeraejong3" were sown. In the case of "Jaeraejongja" seeds sown in a 1 m × 1 m plot, flowering began nine weeks after sowing, and seed maturation began 11 weeks after sowing. On the other hand, when sown in the 2 m × 2 m plot, flowering, and seed filling started 11 and 15 weeks after sowing, respectively. The flowering period was three weeks in the 1 m × 1 m plots and 3–4 weeks in the 2 m × 2 m plots.

Changes in the phenotypic characteristics of surviving individuals by seed amount were compared by measuring the plant height, stem diameter, and flower length (Fig. 2). The plant height, stem diameter, and flower length of surviving sunflower individuals were greatest between weeks 15th and 17th weeks in all the experimental plots. When 50, 100, and 150 "Jaeraejongja" seeds were sown in the plot of 1 m × 1 m, the highest plant heights were 57.0 cm, 59.3 cm, and 55.5 cm, respectively. In the plot of 2 m × 2 m, the maximum plant height was 65.3 cm, 78.0 cm, and 78.8 cm, respectively. For "Jaeraejong3" seeds, the highest plant heights in the 2 m × 2 m plots were 30.8 cm, 37.0 cm, and 33.3 cm, when 50, 100, and 150 seeds were sown, respectively. The maximum stem diameter of the "Jaeraejongja" was similar at 0.98 to 0.99 cm in both the 1 m × 1 m plots and the 2 m × 2 m plots, while the maximum stem diameter of the "Jaeraejong3" was measured at 0.78 cm. The longest flower length was 6.8 cm for "Jaeraejongja" in the 1 m × 1 m plots, whereas it was 9.3 cm in the 2 m × 2 m plots, which was significantly different. The maximum flower length of "Jaeraejong3" was measured at 6.9 cm. Newly

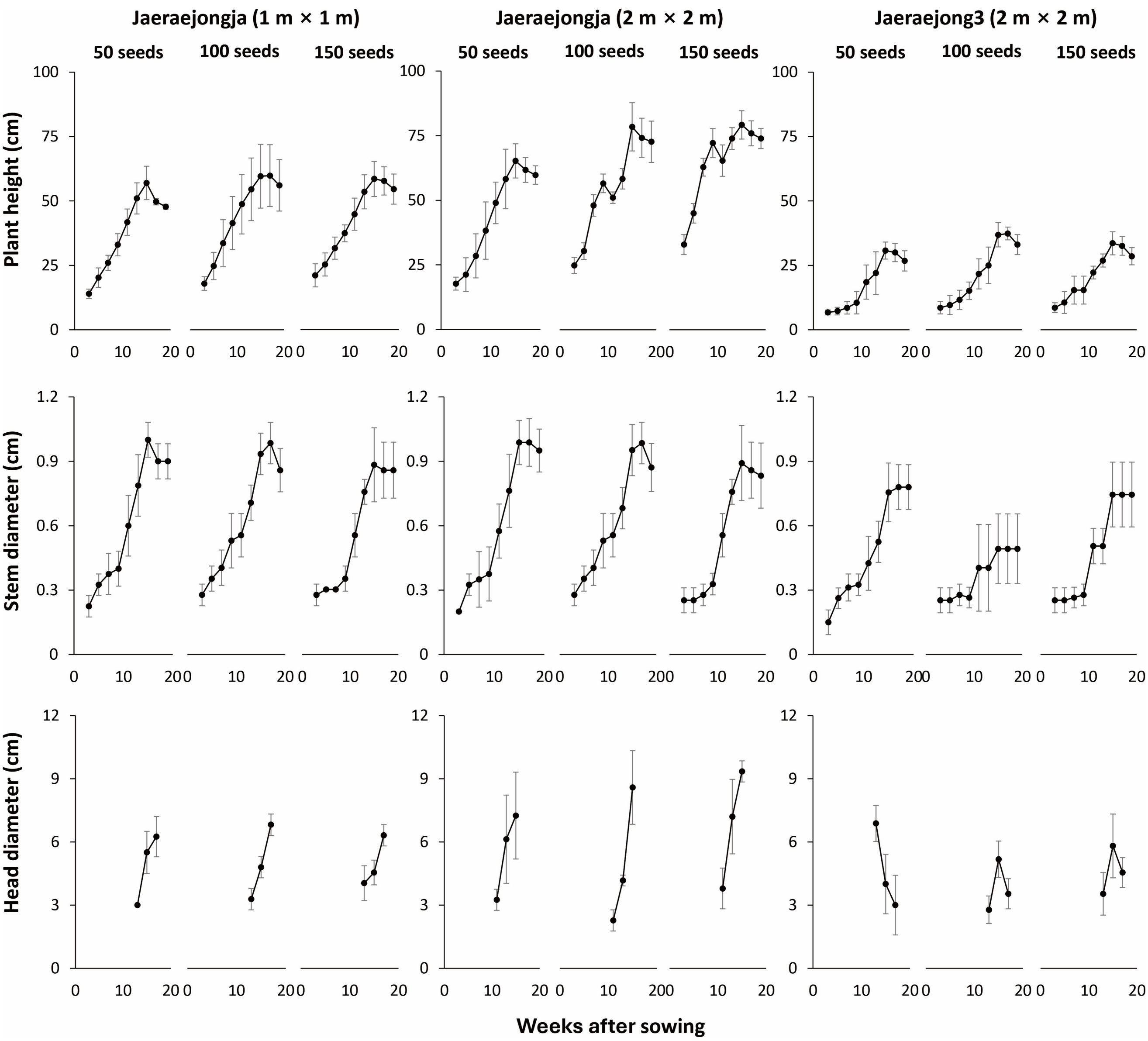

Fig. 2 Changes in the phenotypic characteristics of surviving sunflowers sown at different amounts (0, 50, 100, and 150 seeds) in two plot sizes (1 m × 1 m and 2 m × 2 m). Bar plots represent the means of plant height, stem diameter, and head diameter from four replicates.

emerged sunflowers in the year after winter were not observed in any of the study plots.

Vegetation changes and crop-weed competition depending on seeding density of sunflowers

The plant taxa that appeared in the experimental plots during the study period are listed in Table 2. The total number of species was 27, including sunflowers, which were classified into three classes—eight orders and ten families. Regarding lifestyle type, 63.0% were determined to be annual, 25.9% perennial, and 11.1% biennial.

Figure 3 presents the change in the importance value of plant species occurring in the experimental plot depending on the seed amount, plot size, and sunflower cultivar. In the plots where sunflowers were not sown, the importance value was initially the highest for *Eleusine indica*, and the importance values of *Cyperus iria*, *Echinochloa crus-gall*, *Setaria viridis*, and *Chenopodium album* were also high. The importance value of early sunflowers in the plots where 50 seeds were sown was 46.3% to 47.8% for the 2 m × 2 m plots and 64.0% for the 1 m × 1 m plots. The importance value of sunflowers decreased to less than 10% from eight weeks after sowing in the 2 m × 2 m plots and after 14 weeks in the 1 m × 1 m plots. As the importance of sunflowers decreased in the plots, the importance of *S. viridis*, *E.crus-gall*, *E. indica*, and *C. album* also increased. In the plots where 100 seeds were sown, the importance of early sunflowers was determined to be 51.6% to 57.2%, regardless of cultivar and plot size. However, the importance of sunflower in the plot sown with "Jaeraejongja" decreased

Table 2 List of plants that were observed during the experimental period

Common name	Life cycle	Family	Species
Sunflower	Annual	Asteraceae	*Helianthus annuus*
White clover	Perennial	Fabaceae	*Trifolium repens*
Rice flat sedge	Annual	Cyperaceae	*Cyperus iria*
Indian jointvetch	Annual	Fabaceae	*Aeschynomene indica*
Indian goosegrass	Annual	Poaceae	*Eleusine indica*
Marshpepper knotweed	Annual	Polygonaceae	*Persicaria hydropiper*
Japanese thistle	Perennial	Asteraceae	*Cirsium japonicum*
Black locust	Perennial	Fabaceae	*Robinia pseudoacacia*
Purslane	Annual	Portulacaceae	*Portulaca oleracea*
Common horsetail	Perennial	Equisetaceae	*Equisetum arvense*
Carpetweed	Annual	Molluginaceae	*Mollugo stricta*
Common dandelion	Perennial	Asteraceae	*Taraxacum officinale*
Fireweed	Annual	Asteraceae	*Erechtites hieracifolia*
Baconweed	Annual	Chenopodiaceae	*Chenopodium album*
Canadian fleabane	Annual	Asteraceae	*Conyza canadensis*
Common knotgrass	Annual	Polygonaceae	*Polygonum aviculare*
Barnyard millet	Annual	Poaceae	*Echinochloa crus-galli*
Wild soybean	Annual	Fabaceae	*Glycine soja*
Evening primrose	Biennial	Onagraceae	*Oenothera biennis*
Sonchus-leaf crepidiastrum	Biennial	Asteraceae	*Crepidiastrum sonchifolium*
Groundsel	Annual	Asteraceae	*Senecio vulgaris*
Annual fleabane	Biennial	Asteraceae	*Erigeron annuus*
Green foxtail	Annual	Poaceae	*Setaria viridis*
Chinese bushclover	Annual	Fabaceae	*Lespedeza cuneata*
Indian wormwood	Perennial	Asteraceae	*Artemisia indica*
Barnyard grass sp.	Annual	Poaceae	*Echinochloa* sp.
Chinese plantain	Perennial	Plantaginaceae	*Plantago asiatica*

to less than 10% after 16 weeks, while in the plot sown with "Jaeraejong3," the importance of sunflower declined to less than 10% after 14 weeks. When 150 seeds were sown in the 1 m × 1 m and 2 m × 2 m plots, the initial importance values of sunflowers were 66.3% and 50.9% to 52.9%, respectively. When "Jaeraejongja" seeds were sown in the 1 m × 1 m plots, the importance of sunflower fell below 10% from 22 weeks after sowing, but when "Jaeraejongja" seeds were sown in the 2 m × 2 m plots, it fell to 10% after 14 weeks. Excluding the plots where 50 and 100 seeds of "Jaeraejong3" were sown, sunflowers were not observed from 22 weeks after sowing in all the experimental plots. In the plots where 50 and 100 seeds of "Jaeraejong3" were sown, no sunflowers were observed for 16 and 18 weeks, respectively, after sowing.

The competition between sunflowers and wild plants is presented in Figure 4. In this study, when more than 100 seeds were sown in the 2 m × 2 m plots, competition between sunflowers and wild plants was possible until four weeks after sowing. In particular, in the 1 m × 1 m plots where 150 seeds were sown, sunflowers demonstrated superiority in competition with wild plants until six weeks later. However, the dominance of sunflowers decreased in other plots after six weeks. Therefore, when the number of sunflower seeds was more than 100 seeds per 4 m^2, they were initially superior to wild plants, but decreased after six weeks, making it difficult for sunflowers to dominate the natural ecosystem.

Discussion

For newly introduced transgenic plants to have ecological advantages in their natural environments, they must have improved survival and reproductive abilities. It is, therefore, necessary to verify whether newly introduced transgenic plants are equivalent to existing non-transgenic plants in terms of phenotypic performance such as survival, growth, and reproductive ability. Several studies have reported that when the genes of transgenic plants that have acquired tolerance to biotic and abiotic stresses are transferred to wild plants, the competitive fitness and invasiveness of the hybrids may increase, further increasing the ecological risk (Nam et al. 2020; Pilson and Prendeville 2004).

Various factors, such as environmental parameters (including temperature and soil moisture), seed size, and seeding depth affect the germination and growth of plants (Fernandez-Quinantilla et al. 1990; Han et al. 2023; Lamb and Johnson 2004; Nam and Han 2022). Javaid et al. (2022) reported that the seeds of *Lolium perenne* L. did not germinate on the soil surface or at a depth of 6–7 cm but ger-

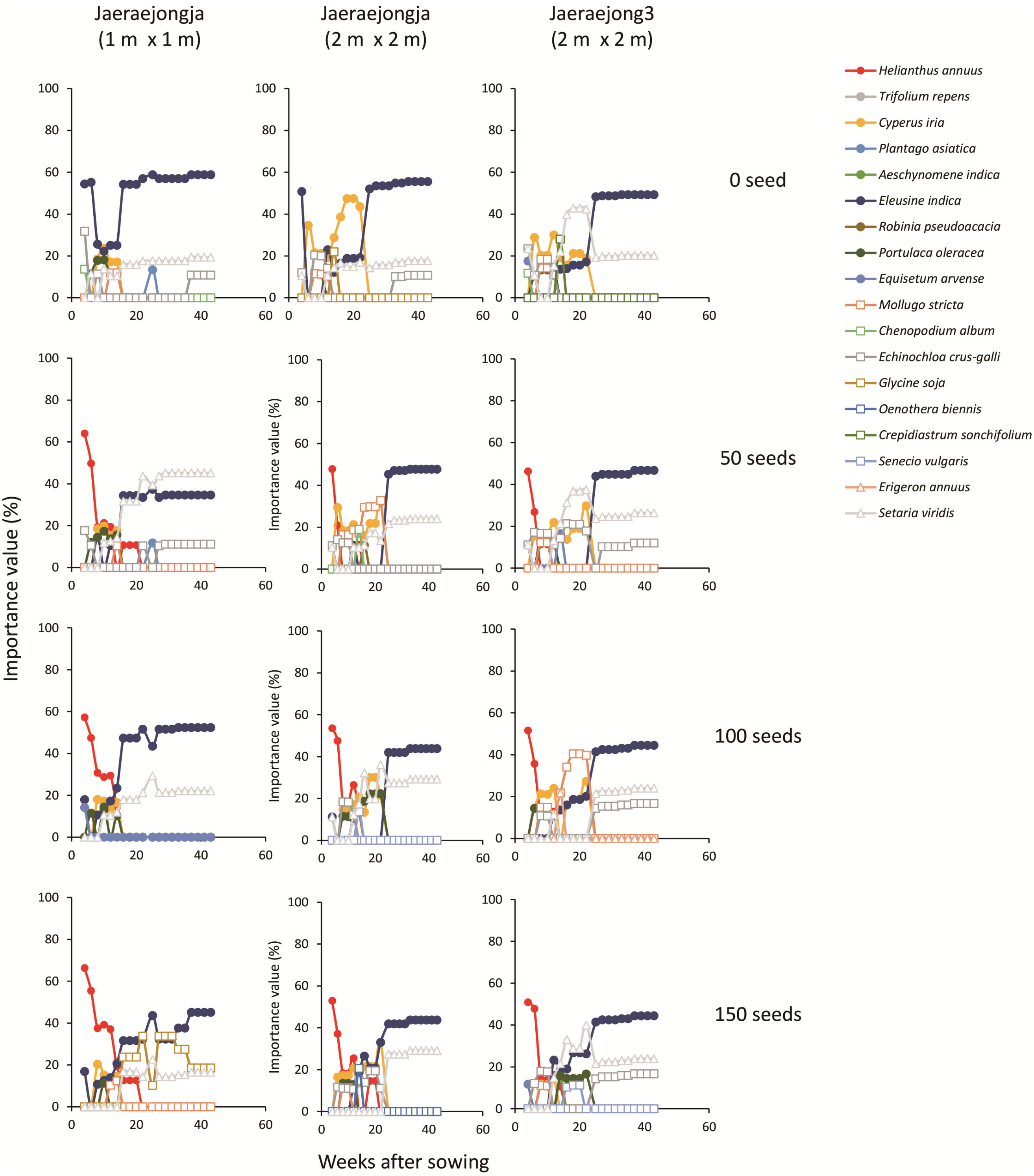

Fig. 3 Changes in the importance value of sunflower and wild plants in experimental plots. Two cultivars ("Jaeraejongja" and "Jaeraejong3") of sunflower seeds were sown at different amounts (0, 50, 100, and 150 seeds) in two plot sizes (1 m × 1 m and 2 m × 2 m).

minated at a depth of 1 cm. Domic et al. (2020) reported that seed mass and size when seedlings first grow are important for the persistence, population statistics, and conservation of *Polylepis tomentella*. In a previous study, it was reported that the invasive potential of sunflowers varied depending on the environmental conditions at the time of release, and especially when sown in March, germination rates were high and they could compete with wild plants for a longer period (Han and Nam 2022).

In this study, the germination rate of sunflowers did not differ significantly by cultivar but demonstrated a difference depending on seeding density and plot size, and the germination rate increased as the number of seeds sown increased. Flowering and seed maturity of germinated

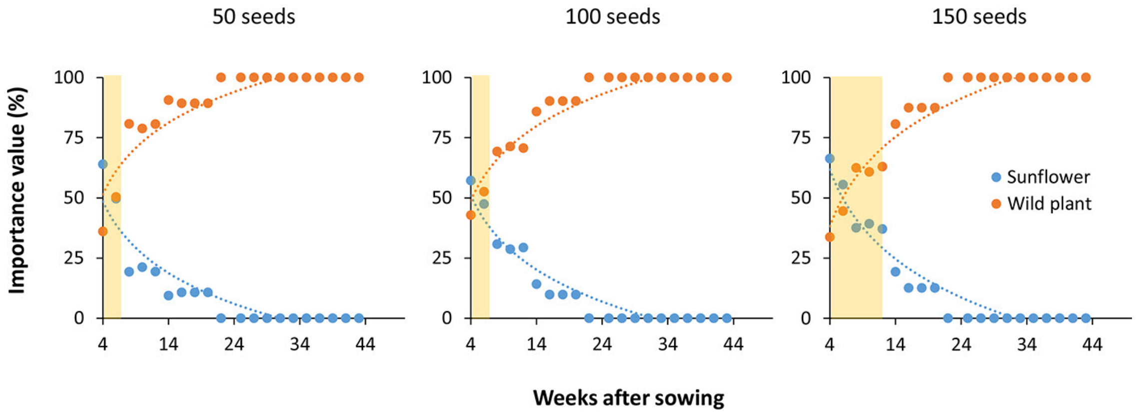

Fig. 4 Critical periods of weed interference and the importance values of sunflower and wild plants. Seeds of the "Jaeraejongja" cultivar were sown in 1 m × 1 m experimental plots at 0, 50, 100, and 150 seeds each. Colored symbols indicate the importance value (red, sunflower; blue, sum of wild plants), and the orange box indicates the critical period for weed interference.

plants did not differ depending on seed density but were faster in 1 m × 1 m plots than in 2 m × 2 m plots. In the case of "Jaeraejongja," regardless of seeding density, the plant height and flower length of surviving plants were 14.6% to 42.0% and 36.8% higher, respectively, in plants grown in 2 m × 2 m plots than in plants grown in 1 m × 1 m plots. These results suggest that seeding density at the time of release does not significantly affect plant germination but does affect the growth and development of germinated plants.

LM crops that grow taller or produce more biomass may have an advantage over other wild plants when using sunlight for photosynthesis or by absorbing water through their roots. The ability of a crop to compete with weeds depends on a variety of physiological and morphological characteristics that allow it to effectively utilize light, water, nutrients, and other limited resources (Jha et al. 2017). Therefore, adjustments in cultural management, such as sowing date, seeding rate, and nutrient management, can enhance crop competitiveness against weeds (Chauhan and Opeña 2013; Swanton et al. 2015). Sunflowers planted in March, when the temperature and humidity are ideal for germination and growth, survive longer and outperform wild plants when planted at other times (Han and Nam 2022).

In this study, the initial importance of sunflowers in the 1 m × 1 m experimental plot was 38.2% higher than that in the 2 m × 2 m area. In addition, the importance of sunflowers was maintained for the longest time in the plot where 150 seeds were sown. When comparing the values where the importance of sunflower fell below 10%, it was maintained 14 weeks longer when 150 seeds of "Jaeraejongja" were sown in the 1 m × 1 m plot than when 50 seeds of "Jaeraejong3" were sown in the 2 m × 2 m plot. These results indicate that a larger number of seeds not only leads to higher initial importance values of sunflowers but also to a longer period of dominance. The results, therefore, suggest that the number of seeds at the time of release can affect germination, growth, and competition with weeds in transgenic plants.

Conclusions

In summary, when the number of spilled seeds is assumed to be small, the growth of sunflowers may be more favorable. However, competition with weeds lasts longer when the number of spilled seeds is large. Although all sprouted sunflowers flowered, no newly emerged sunflowers were observed the following year. The ecological invasiveness of sunflowers was, therefore, not expected to be high in unmanaged natural environments. Nevertheless, the results of this study suggest that it is vital to consider these physical factors when assessing the weeding potential of LM plants because their germination, growth, and competitiveness with weeds may differ depending on the amount of transgenic seeds spilled.

Abbreviations

LMO: Living modified organism
ANOVA: Analysis of variance
GLM: General linear model

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Authors' contributions

KHN designed the study and wrote the manuscript. SMH performed the experiments. SJC, JWL, and JK participated in data analysis. All the authors have read and approved the final version of the manuscript.

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Not applicable.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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