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RESEARCH

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Comparison of overwintering potential of seeds in laboratory and field conditions for the risk assessment of transgenic plants: a sunflower case study

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Background: An important consideration for the risk assessment of transgenic plants is their overwintering potential in a natural ecosystem, which allows the survival of the seed bank and may lead to seed reproduction. Here, we investigated the overwintering of sunflower (*Helianthus annuus* L.) seeds in the laboratory (temperatures: –5, –1, 5, and 10°C) and in the field (burial depth: 0, 5, 15, and 30 cm) as a case study to examine the invasiveness of transgenic crops.
Results: Sunflower seeds germinated when incubated at 5°C and 10°C for 2, 4, 6, and 12 weeks but not when incubated at –5°C or –1°C. However, the seeds incubated at –5°C or –1°C germinated when they were transferred to the optimal germination temperature (25°C). Up to 16.5% and 15.0% of seeds were dormant when cultured at sub-zero temperatures in a Petri dish containing filter paper and soil, respectively. In the field trial, soil temperature, moisture, and microbial communities differed significantly between soil depths. Germination-related microorganisms were more distributed on the soil surface. Seeds buried on the surface decayed rapidly from 4 weeks after burial, whereas those buried at depths of 15 cm and 30 cm germinated even 16 weeks after burial. No dormancy was detected for seeds buried at any depth.
Conclusions: Although sunflower seeds did not overwinter in situ in this study, we cannot exclude the possibility that these seeds lie dormant at sub-zero temperatures and then germinate at optimal temperatures in nature.

Keywords: Burial seed, Dormancy, Invasiveness, Overwintering, Risk assessment, Transgenic plant

Introduction

The global climate is projected to change dramatically over the next century and is expected to affect various environmental parameters (Folland et al. 2001). In particular, factors such as warming, drought, and elevated CO_2 levels significantly affect plant growth in both agricultural and natural ecosystems (Howden et al. 2007; Walther 2010). Apart from climate, changes in weed dominance and weed interactions are also important factors in crop production (Ramesh et al. 2017; Slingo et al. 2005). Researchers have raised concerns that climate change-related alterations in weed invasion in the United States could affect maize production and cause the northward spread of invasive weed species of tropical and subtropical origin that are currently confined to the south (McDonald et al. 2009; Patterson 1995). These seasonal and long-term changes will also influence the population dynamics of insects and lead to changes in the overall abundance of soil microbial communities (Castro et al. 2010; Karuppaiah and Sujayanad 2012).

The concentration of greenhouse gases in South Korea has increased over the past 10 years (2008–2018), and the warming trend has been evident in most regions (KMA 2020). Accordingly, studies have reported climate warming-related changes in the species composition of cold-tolerant evergreen broadleaved trees in Korea (Koo et al. 2015). The diversity of subalpine plant species in low-altitude areas and low-latitude mountainous national parks is also greatly threatened by climate change (Adhikari et al. 2019). Changes reported in the first flowering date of six early-spring flowering plants from 1920 to 2019 are expected to exacerbate the temporal asynchrony of plant species with climate change in Korea (Lee et al. 2020). Additionally, a study on 16 invasive weed species reported that climate change would shift the climate barrier to invasive weeds in southern regions, exacerbating the risk of weed invasion into northern regions (Hong et al. 2021).

Living modified organisms (LMOs) are considered invasive alien species in certain respects (Shine et al. 2000). To address concerns regarding the potential ecological impacts of LMOs, the Cartagena Protocol discussed the development of risk assessment guidelines and the evaluation of mechanisms for identifying and managing potential ecological damage caused by LMOs (Hill and Sendashonga 2006). The risk assessment of LMOs for invasiveness requires information on their seed germination characteristics, phenotypes under agronomic conditions, reproductive biology, and seed persistence leading to volunteer occurrence (EFSA Panel on Genetically Modified Organisms [GMO] 2010). This is especially important for overwintering, which can lead to seed reproduction of LM plants (EFSA Panel on Genetically Modified Organisms [GMO] 2010). LMOs can survive in a wide range of environments owing to their advantageous traits, and even those that do not overwinter in the producing country can overwinter in the consuming country. Therefore, accurate predictions and preparation are required for managing the spread of LMOs (Cantamutto and Poverene 2007; Liang et al. 2014). Korea does not cultivate LM plants; however, LMOs have been imported over the past 14 years for use as food, feed, and processing (KBCH 2022). As a result, LM corn, rapeseed, and cotton volunteers have been found in the natural environment around transportation routes, import ports, feed factories, and festival sites (Kim et al. 2020; Lim et al. 2021; Park et al. 2010). Therefore, evaluation of the overwintering potential of imported LMOs, which includes their ability to survive in low temperatures during winter and leads to reproduction in the following spring, is crucial in Korea's LMO review.

In the present study, we used commercial cultivars of sunflower (*Helianthus annuus* L.) as a case study to evaluate the viability and persistence of plant seeds at low temperatures in two different scenarios (in laboratory and field conditions). There are several justifications for conducting research on LMOs using non-LMOs as case studies. Concerns about potential impact of LMOs on the environment, human health and biodiversity can be offset by preliminary studies using non-LMO (Andersson and de Vicente 2010; Kouam et al. 2012). In addition, since LMOs are considered to be substantially equivalent to controls such as wild-type or original cultivars in the risk assessment, studies using non-LMOs can be an important consideration in that they present a more practical option (Hill and Sendashonga 2003). Moreover, regulatory considerations may also come into play, as some countries or regions may have strict regulations on the use of LMOs and using non-LMOs for initial studies may be a more feasible option (Davison 2010).

Here, we recorded the viability and time to germination of sunflower seeds at different low temperatures for 12 weeks in a laboratory. We also recorded the germination and dormancy of sunflower seeds buried at various soil depths in the field for 48 weeks. The results of this study may help evaluate the overwintering performance of LM crops newly introduced in Korea. In addition, this case study can provide insights that should be useful for investigating whether the introduction of LM crops in Korea has the potential to change biodiversity within natural ecosystems.

Materials and Methods

Plant materials

Seeds of the following five commercially available sunflower cultivars purchased from Danong Co. (Namyangju, Korea) were used in this study: Jaeraejongja (standard type), Jaeraejong (semi-dwarf type), Jaeraejong1 (dwarf type), Jaeraejong2 (extreme dwarf type), and Jaeraejong3 (extreme dwarf type). The physical characteristics of these seeds have been reported in a previous study (Nam and Han 2020).

Seed germination tests at controlled low temperatures

To evaluate the effect of low temperature and treatment duration on plant germination and dormancy, the seeds of five sunflower cultivars were cultured on filter paper and soil under controlled temperatures in the laboratory. Fifty seeds were placed in a square Petri dish (125 mm × 125 mm; SPL Life Sciences Co., Ltd., Pocheon, Korea) for the culture on filter paper. For the culture grown in soil, 20 seeds were placed in a cylindrical Petri dish (100 mm × 50 mm; SPL Life Sciences Co., Ltd.) and covered with field soils to a depth of 5 cm. Distilled water (20 mL) was supplied to both cultures, which were transferred to a growth chamber (Sanyo MLR-352H; Panasonic Healthcare Co., Ltd., Oizumi, Japan) set to a constant temperature (–5, –1, 5, or 10°C) in darkness. Incubation at each temperature was performed for 1, 2, 4, 6, and 12 weeks. Seed germination and dormancy were determined in accordance with the International Rules for Seed Testing (ISTA 2010). Seeds were considered germinated when the root length was ≤ 2 mm. Germinated seeds were removed from the Petri dish to measure the initial germination rate, and the remaining seeds were germinated for 7 days at 25°C to measure the final germination rate. The dormancy rate was calculated by counting the number of dormant seeds and dividing it by the total number of tested seeds, using the tetrazolium (TZ) test on the intact seeds that did not germinate even after 7 days of incubation at 25°C.

Field trials to evaluate the overwintering potential of sunflower seeds

To measure the overwintering potential of sunflower seeds, seeds were buried in a confined field (36°01′″43.0″ N, 126°43′23.7″ E; elevation: 20 m) at the National Institute of Ecology in Seocheon-gun, South Korea, according to our previous study (Nam and Han 2020). Two sunflower cultivars—a standard type (Jaeraejongja) and an extreme dwarf type (Jaeraejong3)—were placed in a randomized block with three replicates, each within a 50 cm × 50 cm plot, with 56 plots in each block (7 burial period × 4 depth × 2 cultivar). Fifty seeds were mixed with 50 cm^3 of sterilized sand and put in a nylon mesh (0.3 mm) seed bag (12 cm × 16 cm). The bags were sealed and buried at depths of 0, 5, 15, or 30 cm in November 2020.

The seed bags were exhumed 1, 4, 8, 16, 24, 32, and 48 weeks after burial. The soil and plant material were removed from each seed bag, and the seeds were obtained by sieving and washing with distilled water. The number of (i) germinated seeds with a radical length of more than 2 mm, (ii) dead seeds after germination due to the death of the seedling during the germination process, and (iii) dead seeds that yield to pressure or only have the seed coat remaining was counted. Particularly, in cases where only the seed coat was present, all seeds were considered dead, without differentiation between those that failed to germinate, those that died after germination, or those that could not be identified. The ungerminated intact seeds were further incubated for 7 days at 25°C in the laboratory. For the remaining hard seeds, the TZ test was performed to measure seed dormancy as described above.

Soil physical, chemical, and biological properties

To determine biotic and abiotic variables affecting the germination and survival of seeds, physicochemical characteristics, microbial community, temperature, and moisture at different soil depths were investigated. Soil samples were collected by striping 10 mm-thick layers of soil at each depth prior to burying the seeds. Soil physicochemical properties—including pH, moisture, total nitrogen (TN), organic matter content (OC), phytoavailable phosphate (PP), electrical conductivity (EC), cation exchange capacity (CEC), and texture—were analyzed according to our previous methods (Nam and Han 2020; NIAST 2000). The physical and chemical properties of soil in the experimental field did not differ significantly across soil depths (0, 5, 10, 15, and 30 cm; Table 1). The surface layer of soil (soil depth, 0 cm) was a typical sandy loam soil with a pH of 6.4 ± 0.3, OC of 3.9 ± 0.5 %, TN of 329.0 ± 69.3 mg kg^{-1}, and PP of 21.3 ± 3.8 mg kg^{-1}. The deepest soil layer (soil depth, 30 cm) was also a sandy loam soil with a pH of 6.3 ± 0.3, OC of 3.6 ± 0.4%, TN of 278.0 ± 18.5 mg kg^{-1}, and PP of 25.3 ± 7.9 mg kg^{-1}.

To analyze soil microorganisms, soil 16S ribosomal ribonucleic acid was sequenced using the Genomic Analysis Platform (Macrogen, Seoul, Korea). In addition, to compensate for the short reads obtained from the Illumina Miseq sequencing platform, the soil microbial community was analyzed using the PacBio Sequel II system according to the method described by Lee et al. (2022). Soil temperature and moisture were recorded in a HOBO data-logger (U30-NRC-10-S100; Onset Computer Co., Pocasset, MA, USA). Temperature was measured with Onset S-TMB-M002 (Onset Computer Co.) and moisture was determined using S-TMB-M005 (Onset Computer Co.) sensors at soil depths of 10, 20, 30, 40, and 50 cm. The air temperature and precipitation during the experimental period (from November 2020 to February 2022) were collected from the Gunsan Meteorological Observatory (36°00′19.1″ N, 126°45′40.9″ E; 23.2 m above sea level) in the KMA (2022).

Table 1 Soil physicochemical properties at different soil depths

Soil characteristics	*p*-value	0 cm	5 cm	10 cm	15 cm	30 cm
pH	0.233	6.4 ± 0.3	6.4 ± 0.3	6.7 ± 0.1	6.3 ± 0.3	6.3 ± 0.3
Sand (%)	0.188	59.1 ± 3.4	60.4 ± 6.7	62.5 ± 5.7	64.9 ± 4.9	67.8 ± 5.1
Silt (%)	0.321	26.7 ± 3.0	23.9 ± 5.9	25.6 ± 5.8	21.8 ± 5.0	19.5 ± 2.9
Clay (%)	0.321	14.3 ± 3.2	15.8 ± 3.5	12 ± 1.4	13.3 ± 1.0	12.8 ± 2.8
Bulk density (g cm^{-3})	0.724	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	1.3 ± 0.1	1.3 ± 0.1
Particle density (g cm^{-3})	0.610	2.6 ± 0.0	2.6 ± 0.0	2.6 ± 0.0	2.6 ± 0.0	2.6 ± 0.0
Porosity (%)	0.817	50.6 ± 0.6	51.6 ± 1.3	52.2 ± 1.5	51.2 ± 2.2	51 ± 3.1
TN (mg kg^{-1})	-	329.0 ± 69.3	378.3 ± 80.8	242.7 ± 70.0	187.5 ± 21.9	278.0 ± 18.5
PP (mg kg^{-1})	0.855	21.3 ± 3.8	20.4 ± 7.6	21.5 ± 7.9	22.4 ± 5.0	25.3 ± 7.9
OC (%)	0.625	3.9 ± 0.5	4.2 ± 0.6	3.8 ± 0.6	4.0 ± 0.4	3.6 ± 0.4
EC (μS cm^{-1})	-	25.0 ± 0.0	25.0 ± 0.0	25.0 ± 0.0	25.0 ± 0.0	25.0 ± 0.0
Saline (%)	0.594	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.03	0.02 ± 0.01
CEC (cmol$^+$ kg^{-1})	-	6.1 ± 0.9	11.2 ± 4.0	5.8 ± 0.3	8.2 ± 0.0	7.7 ± 0.0
Moisture (%)	0.606	13.5 ± 1.8	15.2 ± 1.6	12.9 ± 2.7	13.8 ± 1.8	13.2 ± 2.8
Soil texture		Sandy loam	Sandy loam	Sandy loam	Sandy loam	Sandy loam

Values are presented as mean ± standard deviation (n = 4).
p-values were obtained from ANOVA.
TN: total nitrogen; PP: phytoavailable phosphate; OC: organic matter content; EC: electrical conductivity; CEC: cation exchange capacity.

Statistical analysis

All analyses were carried out using SAS Studio (version 3.8; SAS Institute Inc., Cary, NC, USA). Data were subjected to analysis of variance (ANOVA) at a significance of 5%. If the ANOVA results indicated significant differences between means, we used Tukey's honest significant difference test to determine the differences between means. To analyze the effects of cultivar, temperature, duration of incubation, and their interactions on seed germination and dormancy, we performed ANOVA using a general linear model (GLM) module. We also performed a GLM analysis on germination rates to verify the main and interaction effects of cultivar, duration of burial, and depth of burial.

Results

Effects of low temperature on seed germination and dormancy in sunflowers

The initial and final germination rates of sunflower seeds differed significantly among cultivars ($p < 0.001$), incubation temperatures ($p < 0.001$), and incubation durations ($p < 0.001$) in both experimental conditions (filter paper and soil; Table 2). In addition, we found significant interaction effects of temperature × duration ($p < 0.001$), temperature × cultivar ($p < 0.001$), cultivar × duration ($p < 0.001$), and cultivar × temperature × duration ($p < 0.001$) on initial and final germination rates in both culture conditions. For dormancy rate, we found significant differences among cultivars ($p < 0.001$) and incubation temperatures ($p < 0.001$) and significant interaction effects of temperature × duration ($p < 0.001$), temperature × cultivar ($p < 0.001$), and cultivar × temperature × duration ($p < 0.001$) in both experimental conditions.

For the germination experiment on filter paper, sunflower seeds germinated when incubated at 5°C and 10°C but not when incubated at –1°C or –5°C (Fig. 1). The initial germination rate of 'Jaeraejongja' seeds cultured at 5°C and 10°C for 1 week were 14.0% and 6.0%, respectively, and the final germination rates were 98.5% and 98.0%, respectively. There were no germinated seeds when incubated at –1°C and –5°C for all experimental period, but 71.5% to 97.0% germinated over various incubation durations when transferred to the optimum germination temperature of 25°C. The dormancy rate of seeds cultured at –1°C and –5°C was 0.0%–16.5% and 0.0%–19.0%, respectively. The initial and final germination rates of 'Jaeraejong' seeds were significantly lower than those of 'Jaeraejongja' seeds ($p < 0.001$). The dormancy of 'Jaeraejong' was observed at seeds cultured at –5°C, –1°C and 5°C. The germination rate of 'Jaeraejong1' varied widely across various incubation temperatures, and no seeds had germinated after 1 week of incubation. Among seeds cultured at 5°C and 10°C, 2.5% and 46.5% germinated after 2 weeks of culturing, respectively. Dormant seeds were observed in all incubation temperatures, the highest dormancy rate was 12.5% in seeds cultured at –5°C for 6 weeks. For the 'Jaeraejong2' cultivar, the final germination rates of seeds cultured for 12 weeks at all experimental temperature were lower than those of seeds incubated for lower durations ($p < 0.001$). The maximum dormancy rates of seeds cultured at –1°C and –5°C were 5.5% and 6.0%, respectively. For the 'Jaeraejong3' cultivar, the initial germination rate of seeds cultured at 10°C for 1 week was 75.0%, which was significantly higher than

Table 2 Results of the general linear model applied to the germination and dormancy data

Source	Degrees of freedom	Petri dish						Soil					
		Initial germination		Final germination		Dormancy		Initial germination		Final germination		Dormancy	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Cultivar	3	231.2	< 0.001	167.5	< 0.001	16.3	< 0.001	147.4	< 0.001	299.0	< 0.001	9.0	< 0.001
Incubation temperature	4	2,158.7	< 0.001	262.3	< 0.001	74.8	< 0.001	574.9	< 0.001	227.4	< 0.001	97.8	< 0.001
Duration of incubation	4	316.7	< 0.001	150.9	< 0.001	2.0	0.090	137.6	< 0.001	202.2	< 0.001	1.2	0.313
Temperature × duration	12	118.2	< 0.001	21.0	< 0.001	10.2	< 0.001	106.2	< 0.001	65.5	< 0.001	4.7	< 0.001
Temperature × cultivar	12	88.2	< 0.001	51.7	< 0.001	5.0	< 0.001	52.4	< 0.001	90.9	< 0.001	10.0	< 0.001
Cultivar × duration	16	23.0	< 0.001	11.8	< 0.001	4.3	< 0.001	13.2	< 0.001	9.5	< 0.001	2.2	0.005
Cultivar × temperature × duration	48	13.0	< 0.001	7.2	< 0.001	5.6	< 0.001	17.3	< 0.001	7.8	< 0.001	2.3	< 0.001

Values are presented as the mean ± standard deviation (n = 4).
p-values were calculated using a general linear model-based method. Five sunflower cultivars were grown at four different temperatures (–5, –1, 5, and 10°C) during six treatment periods.
F: *F*-statistic; *p*: *p*-value.

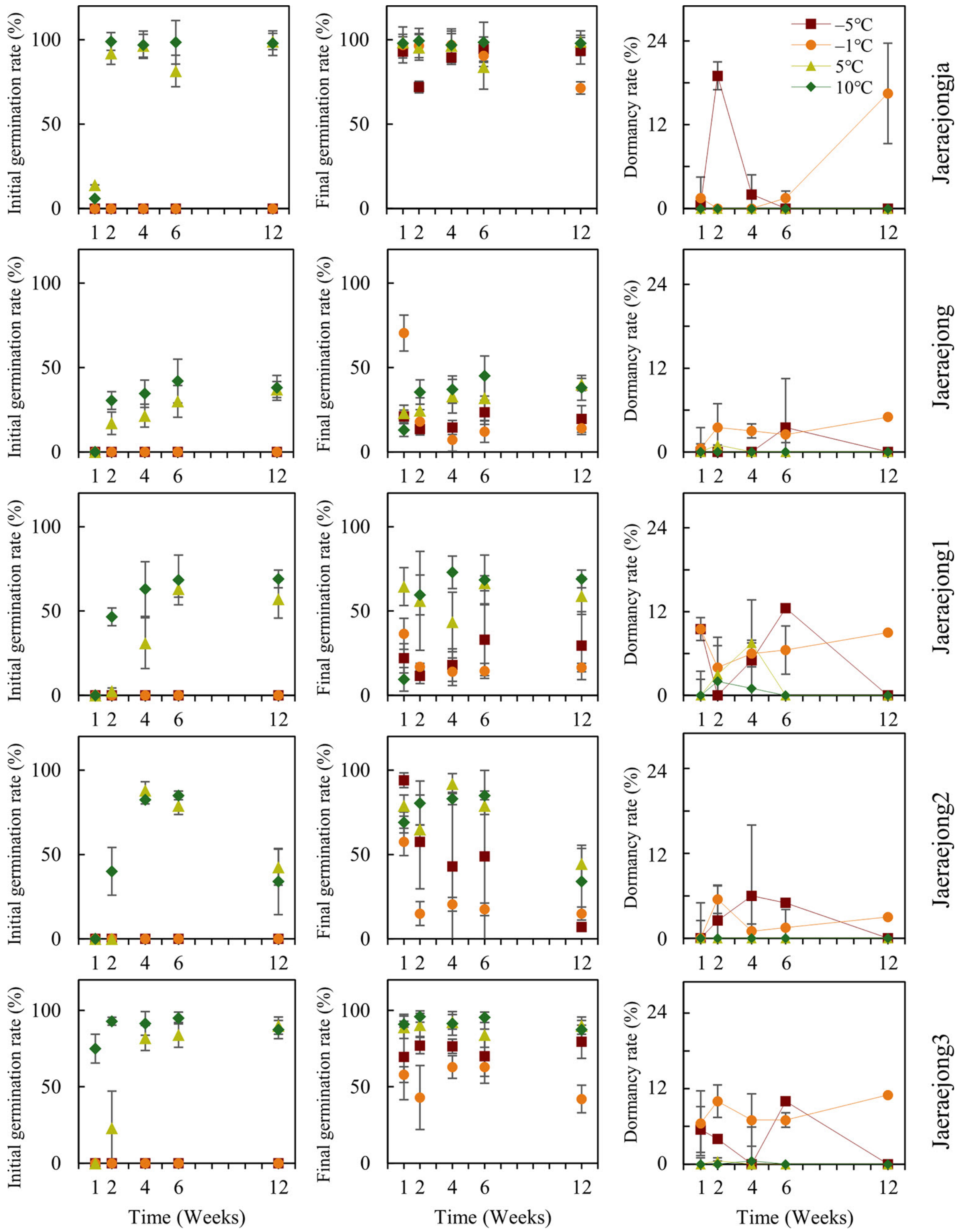

Fig. 1 Initial and final germination rates and dormancy rates of sunflower seeds in a filter paper. Seeds were cultured in various low-temperature regimes (–5, –1, 5, and 10°C) for different durations (1, 2, 4, 6, and 12 weeks). Values are presented as mean ± standard deviation (n = 4). The temperatures are color-coded (dark red, –5°C; orange, –1°C; light green, 5°C; green, 10°C).

that of seeds from other cultivars ($p < 0.001$). The dormancy rates of 'Jaeraejong3' seeds cultured at –1°C and –5°C were 6.5%–11.0% and 0.0%–10.0%, respectively.

No germinated seeds were observed for any sunflower cultivars when cultured for 1 week at –5°C, –1°C, 5°C, and 10°C in Petri dishes containing soil (Fig. 2). In addition, seeds cultured at –1°C and –5°C did not germinate during the entire experimental period. For the 'Jaeraejongja' cultivar, germinated seeds were observed after 2 weeks when cultured at 10°C and after 4 weeks when cultured at 5°C. There were no dormant 'Jaeraejongja' seeds in the soil culture experiments. For the 'Jaeraejong' cultivar, seeds cul-

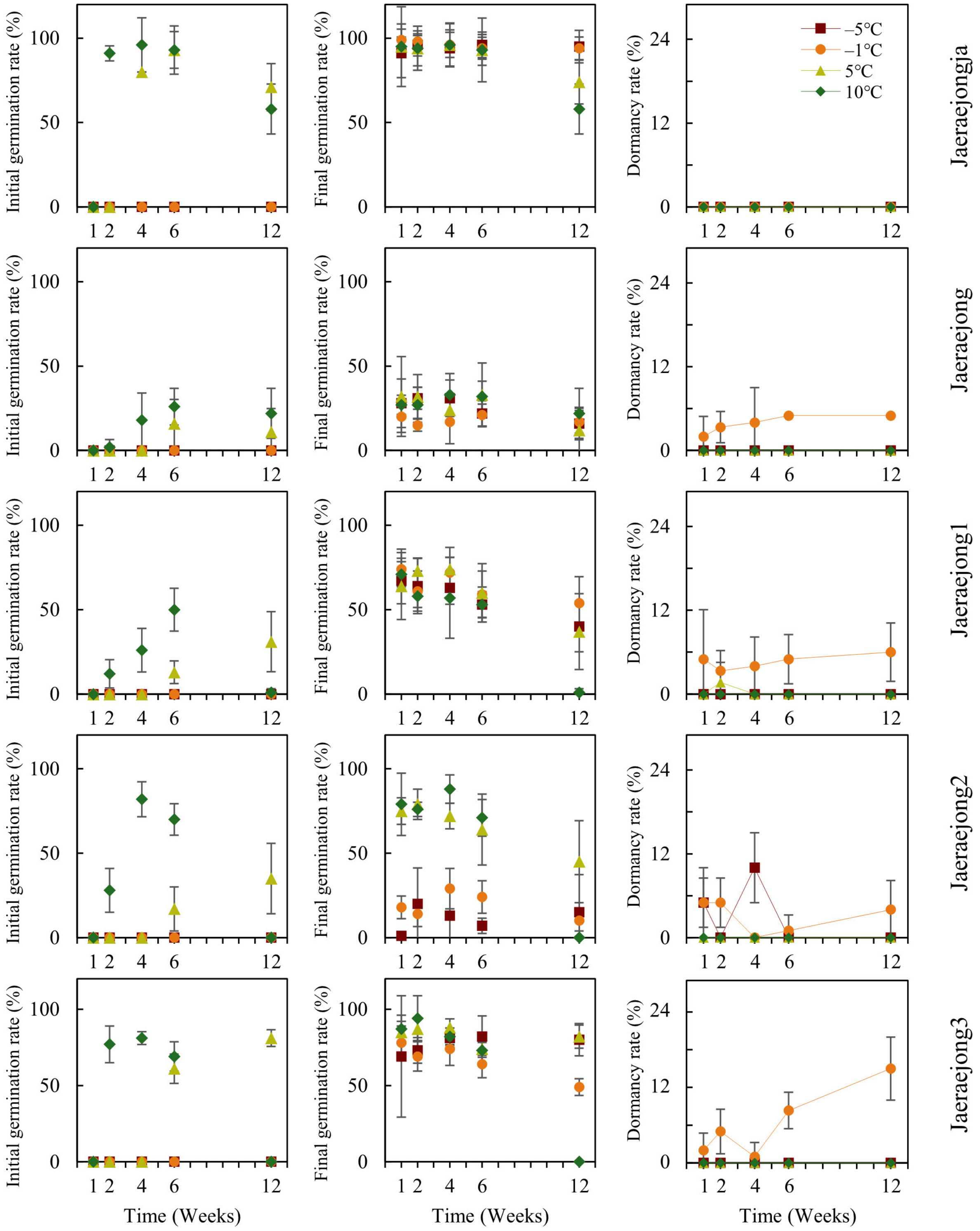

Fig. 2 Initial and final germination rates and dormancy rates of sunflower seed in the soil. Seeds were cultured in various low-temperature regimes (–5, –1, 5, and 10°C) for different durations (1, 2, 4, 6, and 12 weeks). Values are presented as the mean ± standard deviation (n = 4). The temperatures are color-coded (dark red, –5°C; orange, –1°C; light green, 5°C; green, 10°C).

tured at 10°C and 5°C started to germinate after 2 and 6 weeks of incubation, respectively. Dormant seeds (2.0%–5.0%) were found only among seeds cultured at –1°C. For the 'Jaeraejong1', 'Jaeraejong2', and 'Jaeraejong3' cultivars, seed germination was observed from 2 weeks after incubation at 10°C and from 6 weeks after incubation at 5°C. However, most seeds did not germinate when cultured for 12 weeks at 10°C. The dormancy rates of 'Jaeraejong1' seeds were 3.3%–6.0% when cultured at –1°C and 0.0%–1.7% when cultured at 5°C. The highest dormancy rate of 'Jaeraejong2' seeds were 5.0% when cultured at –1°C for 1 and 2 weeks and 10.0% when cultured at –5°C for 4 weeks. Dormant seeds of 'Jaeraejong3' were observed only when cultured at –1°C, and the maximum dormancy rate was

15.0% after 12 weeks of incubation.

Comparison of biological properties between soil depths

A total of 2,819 operational taxonomic units (OTUs) were clustered at 99% similarity (Fig. 3). Approximately 12% of sequences were identified to the species level. However, most OTUs were identified to the genus or family level and categorized as unclassified species (Fig. 3A). OTUs belonging to the phyla Acidobacteriota, Planctomycetota, Chloroflexi, and Verrucomicrobiota showed relatively low sequence similarity compared those belonging to Proteobacteria, Bacteroidota, and Actinobacteria (Fig. 3A). Soil bacterial communities also differed according to soil depth (Fig. 3B). For instance, OTUs belonging to the genera *Bradyrhizobium*, *Ramlibacter*, and *Arthrobacter* were enriched in the top layer of soil, whereas those belonging to the genera *Ellin6067* and *Pseudolabrys* were enriched in the soil samples collected at depths of 15–30 cm.

During the experimental period, the lowest air temperature was −8.9°C at 7 weeks after burial (January 2021), and the highest air temperature was 32.9°C at 33 weeks after burial (July 2021) (Fig. 4A). The lowest surface temperature was 0.0°C at 6 weeks (January 2021) after burial, and the highest surface temperature was 36.5°C at 33 weeks (July 2021). The total annual precipitation was 1,146.3 mm. The highest precipitation during the experiment period was 31.0 mm and 185.8 mm in weekly average and total, at 30 weeks after burial (July 2021). Soil temperature and moisture differed significantly between samples collected at different depths (Figs. 4C and 4D). After 1 week of seed burial, the soil temperature at 10 cm was 9.8°C and that at 50 cm was 12.3°C. Soil moisture content was 29.9% at 10 cm and 34.0% at 50 cm after 1 week of burial. The atmospheric temperature decreased 4 week after burial, at which point the soil temperature at 10 cm (close to the soil surface) was 1.9°C and that at 50 cm was 4.8°C. Soil moisture ranged from 26.5% to 28.2% across different soil depths. After 8 weeks of burial, there was little difference in soil temperature between soil layers (within 0.7°C). However, after

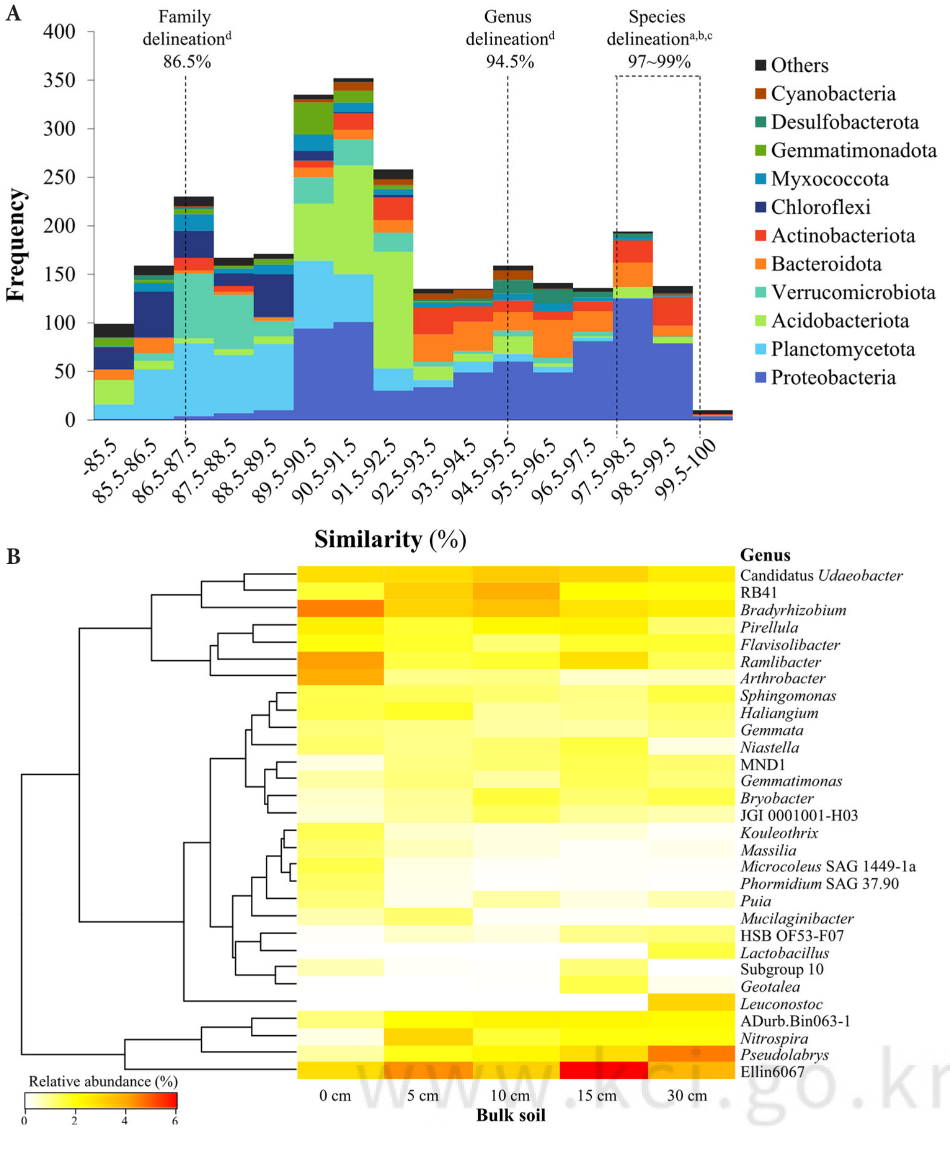

Fig. 3 Sequence similarity and bacterial community structure of burial seed in the confined field. (**A**) The sequence similarity of full-length 16S rRNA gene of operational taxonomic units to type strains by using National Center for Biotechnology Information 16S rRNA databases. Delineation cut-offs were derived from [a]Barco et al. (2020); [b]Kim et al. (2014); [c]Stackebrandt and Goebel (1994); and [d]Yarza et al. (2014). (**B**) Heatmap of hierarchical clustering of bacterial groups at the genus level. The dendrogram was calculated using Euclidean distances and Ward's method. RNA: ribonucleic acid; 16S rRNA: 16S ribosomal RNA.

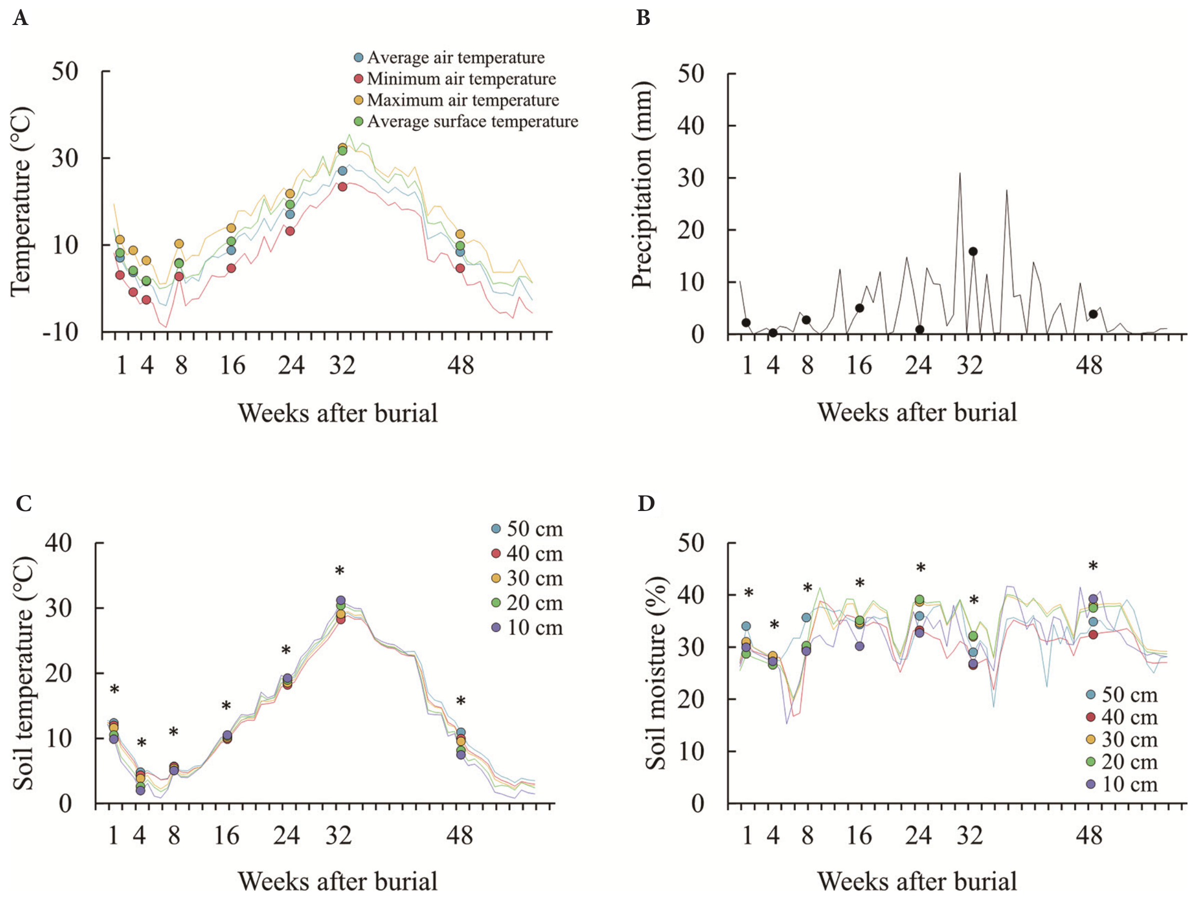

Fig. 4 Meteorological changes at the field during the experimental period. Variation in the (**A**) weekly temperature, (**B**) precipitation, (**C**) soil temperature, and (**D**) soil moisture. Soil temperature and moisture were measured at depths 10, 20, 30, 40, and 50 cm. Asterisks indicate significant differences among soil depths ($p < 0.05$) based on ANOVA.

Table 3 The general linear model results applied to the germination

Source	Sum of squares	Degrees of freedom	Mean square	*F*-statistic	*p*-value
Burial duration	24,0181.67	6	40,030.28	494.35	< 0.001
Cultivar	16.10	1	16.10	0.20	0.657
Depth of burial	9,348.29	3	3,116.10	38.48	< 0.001
Duration × cultivar	2,742.24	6	457.04	5.64	< 0.001
Duration × depth	24,937.38	18	1385.41	17.11	< 0.001
Cultivar × depth	376.48	3	125.49	1.55	0.206
Cultivar × duration × depth	1,299.86	18	72.21	0.89	0.589

Two sunflower cultivars were buried at four different depths (0, 5, 15, and 30 cm) for seven burial durations.

32 weeks of burial, soil temperatures at 10 cm and 50 cm were 31.2°C and 28.8°C, respectively.

At 1 week after burial, the average surface temperature was 13.5°C, with a maximum of 22.6°C. Soil temperature at a depth of 30 cm was 12.7°C, with a maximum of 13.5°C. At 2 weeks after burial, the average surface temperature was 10.0°C, and the minimum air temperature dropped to below zero. The average weekly air temperature decreased to below zero 4 weeks after seed burial. Most of the sub-zero temperatures were observed after 8 weeks of burial. From December 30, 2020, to January 12, 2021, the air temperature remained below zero for 14 consecutive days. During the experimental period, the difference between the maximum and minimum soil temperatures was 41.9°C at the surface and 28.6°C at a depth of 30 cm.

Overwintering potential of sunflower seeds buried at different depths of soil

Among sunflower seeds buried in the field in winter, germination was significantly affected by burial depth ($p < 0.001$) and duration of burial ($p < 0.001$) but did not differ significantly between cultivars ($p = 0.657$) (Table 3). Additionally, we found significant interactions of duration × cultivar ($p < 0.001$) and duration × depth ($p < 0.001$).

The germination rate of 'Jaeraejongja' seeds at different burial depths was not significantly different between most burial durations 1, 8, 16, 24, 32, and 48 weeks after burial, except at 4 weeks after burial (Fig. 5A). The germination

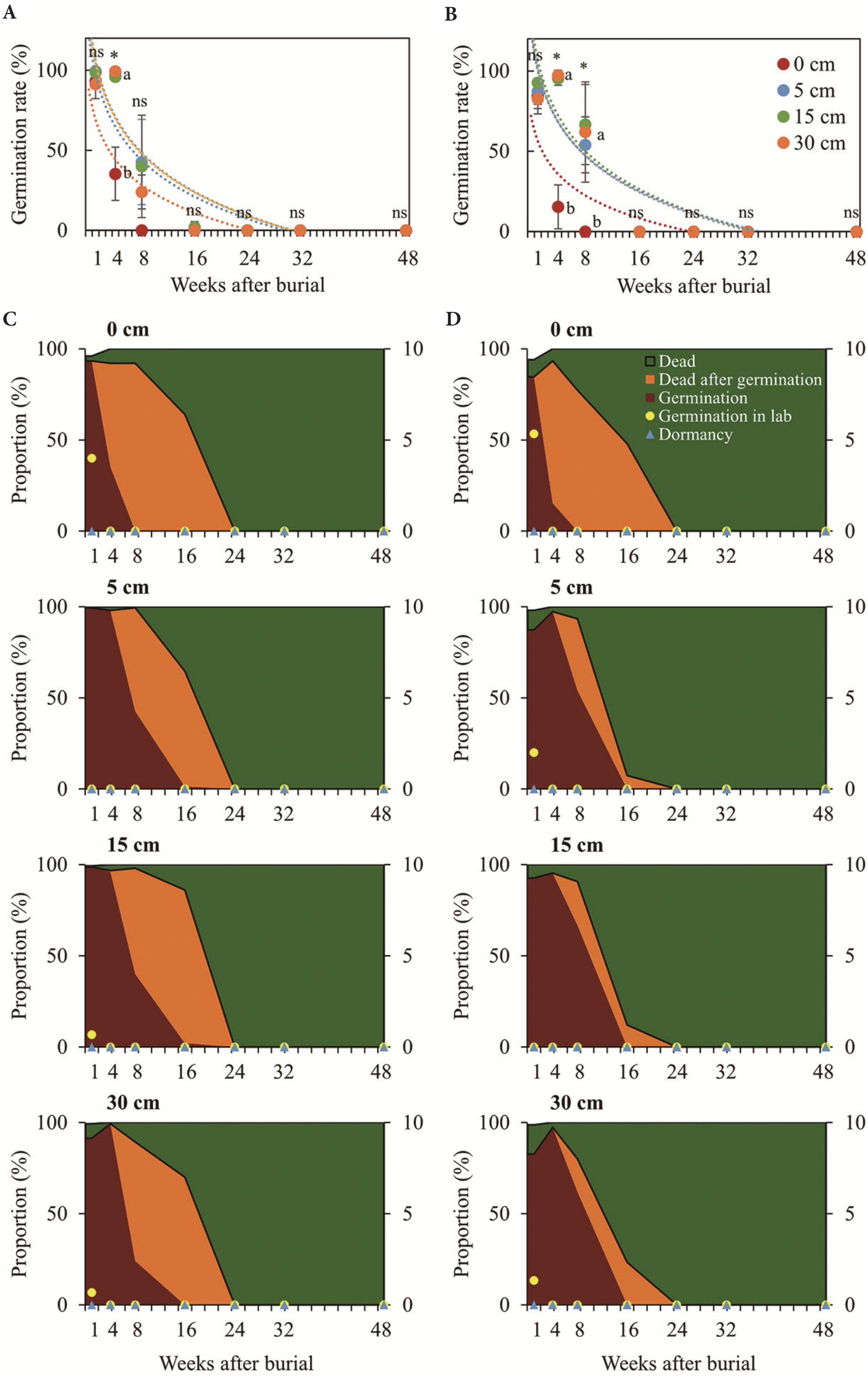

Fig. 5 Seed germination for Jaeraejongja (**A**) and Jaeraejong3 (**B**) seeds during 48 weeks at four soil depths. Proportions of Jaeraejongja (**C**) and Jaeraejong3 (**D**) seeds that were dead, dead after germination, germinated, germinated in the lab, and dormant during 48 weeks at soil depths of 0, 5, 15, and 30 cm. Values are presented as mean ± standard deviation (n = 3). The colored areas and symbols indicate the seed state (green, dead; orange, dead after germination; dark red, germinated; yellow, germinated in the lab; blue, dormant). ns: not significant. Asterisks indicate significant differences among soil depths ($p < 0.05$) based on ANOVA. Different letters indicate significant differences between means ($p < 0.05$).

rate after 1 week of burial was >91.3% at all burial depths. The germination rate of ‘Jaeraejongja’ seeds 4 weeks after burial was 35.3% at a depth of 0 cm and 99.3% at 30 cm. At 8 weeks after burial, the germination rate was 0% at a depth of 0 cm, while it was 24.0%–42.7% at 5–30 cm. After 16 weeks of burial, 1.3% and 2.0% seeds had germinated at depths of 10 cm and 15 cm, respectively; however, no seeds had germinated at the other burial depths. After 24 weeks of burial, all seeds were dead with only the seed coat remaining; no germination was observed at any depth. For ‘Jaeraejong3’ seeds, we found significant differences in germination rate between burial depths at 4 and 8 weeks after burial (Fig. 5B). The germination rate after 1 week of burial was 82.7%–92.7% at all burial depths. After 4 weeks of burial, the germination rate decreased to 15.3% at a depth of 0 cm but was maintained 95.3%–97.3% at depths of 5–30 cm. After 8 weeks of burial, no seeds had germinated at a depth of 0 cm; however, the germination rates at 5–30 cm were 54.0%–66.7%. After 16 weeks of burial, all seeds were dead, and no seed germination was observed at any depth.

When the buried ‘Jaeraejongja’ and ‘Jaeraejong3’ seeds were incubated at 25°C in the laboratory, only 0.7%–5.3% of seeds retrieved after 1 week of burial were able to germinate (Fig. 5C and 5D). There were no dormant seeds of ‘Jaeraejongja’ or ‘Jaeraejong3’ at any depth or duration of burial. After 4 weeks of burial, 56.7% of ‘Jaeraejongja’ seeds buried at a depth of 0 cm died after germination, whereas at 5–30cm, 0.0%–0.7% died after germination. After 16 weeks of burial, the proportion of ‘Jaeraejongja’ seeds dead after germination at depths of 0–5 cm and 15–30 cm were 63.3%–64.0% and 70.0%–84.0%, respectively. After 4 weeks of burial, 78.0% of ‘Jaeraejong3’ seeds buried at a depth of 0 cm died after germination, whereas there were no seeds died after germination at depths of 5–30 cm. The proportion of ‘Jaeraejong3’ seeds that died after germination at a depth of 5–30 cm were 7.3%–23.3% at 16 weeks after burial.

Discussion

Overwintering of seeds is crucial for the survival of the species in the following year, as annual plants exhibit seed persistence by securing seed banks through overwintering of seeds (Aikio et al. 2002; Baskin and Baskin 2014). Our experiments in laboratory conditions revealed that although sunflower seeds did not germinate at sub-zero temperatures, they did germinate when transferred to temperatures optimal for germination (25°C). Our low-temperature germination experiments were comparable with the 30-year average winter temperatures in Korea, which generally include the temperatures from November to February (KMA 2022). Previous studies have shown that non-dormant sunflower seeds can germinate in a wide temperature range (5–40°C) (Gay et al. 1991). This indicates that sunflower seeds released before winter can survive in the soil until the following spring.

The maximum dormancy rates of sunflower seeds cultured at −1°C for 12 weeks were 16.5% and 15.0% in the Petri dishes containing filter paper and soil, respectively. Moreover, these seeds germinated steadily when transferred to the optimum germination temperature of 25°C. These results indicate that even if sunflower seeds spend >3 months buried in the soil at sub-zero temperatures, through dormancy, they can germinate and continue to survive when the environment reaches optimal conditions. Seed dormancy allows plants to survive harsh low-temperature environments, such as Arctic and alpine regions (Billings and Mooney 1968). This mechanism explains how seeds buried in soil can survive at sub-zero temperatures for centuries (McGraw et al. 1991). Alexander and Schrag (2003) reported that sunflower seed banks can survive between 1 to 10 years or even more, allowing the seeds to survive as long. Nevertheless, in the present study, the final germination rate of seeds incubated in Petri dishes containing soil decreased from the 6th week as the seeds decayed over time. This suggested that a variety of variables can interfere with sunflower seeds buried in the soil, and that such seeds can exhibit different results than those observed in a controlled laboratory environment. This is because seed germination and the emergence of seedlings in field conditions are affected by several non-biological and biological factors (Lamichhane et al. 2018).

Important abiotic parameters that control germination—including soil moisture, temperature, and light—vary vertically and spatially within the soil (Long et al. 2015). When a seed is located close to the soil surface, it germinates, which negatively affects the maintenance of persistence through the dormancy period (Traba et al. 2004). In this study, the physicochemical properties of soil did not differ significantly between soil depths; however, soil moisture and temperature showed significant differences between the surface soil and soils at the deepest depth. Seeds on the soil surface are subjected to various stimuli related to the control of seed germination and had difficulty surviving through dormancy. In contrast, seeds buried in the soil experience less drastic changes in soil moisture and temperature than seeds at the surface, and thus seemed to survive longer. Previous studies have also reported that buried seeds, including sunflower seeds, survived longer than those on the soil surface (Alexander and Schrag 2003; Burton et al. 2004).

Our analysis of the soil microbial community also revealed differences between the microbial communities at different soil depths. The microbial community of the surface soil was abundant in microorganisms related to seed germination, whereas that of the subsoil was abundant in microbes related to decomposition. Xu et al. (2021) reported that *Arthrobacter* sp. (which was abundant in surface soil samples in this study) was responsible for increasing the germination rate of peanut seeds. When co-inoculated with other microorganisms, *Bradyrhizobium* promotes soybean seed germination and emergence (Queiroz et al. 2018). By contrast, *Ellin6067* (which was abundant in soil samples collected at 15–30 cm in this study) was the most representative bacterium for the decomposition of living organisms and other complex organic compounds. Accordingly, its abundance is also associated with the decomposition of plant vegetation and straw mulch (Lezcano et al. 2017).

The effect of temperature on seed persistence in soil varies across plants (Walck et al. 2011). In the present study, seed viability differed according to the location of the seeds in the soil, and unlike in the laboratory experiments, the seeds did not lie dormant and decay over time. The soil in the field exhibited a difference in temperature between daytime and nighttime, and soil moisture levels also changed rapidly depending on the amount of precipitation. At the time of burying the seeds, the surface temperature had been maintained at 22.3°C at the highest point of the day for one week, indicating conditions favorable for seed germination. Additionally, the seeds buried at 30 cm were exposed to an average soil temperature of 13.2°C and showed similar results to the seeds grown in the laboratory at 10°C. Previous overwintering studies in Korea have surveyed the survival of seeds that had been buried before the occurrence of sub-zero temperatures (Ko et al. 2016; Yook et al. 2021). However, as observed in our experiment, the actual surface and subsoil temperatures were high enough to satisfy the germination conditions before sub-zero temperatures were observed, and the duration of sub-zero temperatures was not as long in the field as it was in the laboratory experiment. In addition, the average temperature at the deepest point was 5°C or higher than that at the surface, even when the surface temperature was maintained below –5°C. Therefore, adjusting the subsoil temperatures in the field to those in the laboratory experiment was difficult and the resulting seed dormancy rate was not observed. Additionally, the surface temperature did not drop below freezing for 2 weeks at the beginning of the experiment, suggesting that seed germination was possible during this period. However, the temperature dropped below freezing at night from the 4th week onward, thus, adversely affecting germination. By the 8th week, the temperature was maintained below zero, which prevented germination. Ooi et al. (2009) reported that in Australia, increasing temperatures were accompanied by increased germination in 3 out of 8 plants, whereas seed viability decreased in another species. By contrast, *Apium*, *Lactuca*, and *Viola* seeds become dormant with rising temperatures, and germination occurs by breaking the dormancy by exposure to low temperatures (Geneve 2003). Therefore, differences in seed dormancy rates between our laboratory and field conditions might be arisen from variations in soil temperature and microbial distribution at different depths in an uncontrolled outdoor environment.

Conclusions

Climate change will ultimately affect the ecosystem vegetation, especially seed germination and dormancy, which are highly susceptible to changes in temperature, patterns of precipitation, and transpiration (Baskin and Baskin 2014). In particular, buried seeds have a seed dormancy mechanism that allows developing and viable seeds to avoid germination at times when temperature and moisture conditions are not conducive to seedling survival (Baskin and Baskin 2004). Our recent report shows that the invasive potential of sunflowers depends on environmental conditions at the time of release (Han and Nam 2022). A gradual increase in domestic temperatures due to climate change may result in the disappearance of sub-zero weather in some areas, increasing the possibility of overwintering of newly introduced LM plants. Therefore, it is necessary to evaluate seed overwintering at multiple sites that can meet various temperature conditions. Our findings could provide important insights into evaluating and reviewing the effects of newly introduced LM plants on the natural ecosystem. However, limitations of this study using non-LMOs may include reduced generalizability of the findings to LMOs and potential confounding effects of other factors.

Abbreviations

LMOs: Living modified organisms
TZ: Tetrazolium
TN: Total nitrogen
OC: organic matter content
PP: Phytoavailable phosphate
EC: Electrical conductivity
CEC: Cation exchange capacity
ANOVA: Analysis of variance
OTUs: operational taxonomic units

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None.

Authors' contributions

SMH performed the experiments and wrote the draft manuscript. SJC analyzed the soil microbial community. KHN designed the research and wrote the manuscript. All authors have read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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