



Evaluating herbicide resistance and cold tolerance in interspecific F_1 hybrids of *Zoysia Japonica* and *Zoysia Matrella* using NDVI and percentage green cover

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Abstract

Excessive application of synthetic herbicides in turfgrass raises environmental concerns, including the development of herbicide-resistant populations. Although herbicide-resistance is a challenge in agronomic crops, it offers a promising weed management strategy in *Zoysia* turfgrass, reducing herbicide usage, manual weeding, and ecological impacts. Developing herbicide-resistant *Zoysia* grass cultivars is vital for expanding and managing *Zoysia* lawns in diverse environments. This study screens herbicide-resistant and cold-tolerant *Zoysia* grass using multispectral image analysis for efficient resistance assessments. Artificial cross-pollination between cold-tolerant *Zoysia matrella* (San208) and herbicide-resistant *Zoysia japonica*, produced 30 F_1 hybrid individuals. A 0.545% working solution of glufosinate ammonium (18% SL basta[®]) was applied to 56-day-old F_1 individuals under controlled conditions in the greenhouse. Multispectral sensors captured images to assess growth, vigor, and herbicide response and cold tolerance through Percentage Green Cover (GCP) and NDVI-based evaluations. Within 40 days post-herbicide application, 50% of the hybrid plants perished, and one did not survive the winter of 2023. GCP and NDVI of F_1 hybrid plants differed significantly ($p \leq 0.0001$). At the study end, four out of 30 F_1 hybrids demonstrated superior cold tolerance and herbicide resistance. Cold tolerance in F_1 hybrids may be inherited from *Z. matrella* (San208), while herbicide resistance was derived from *Z. japonica*. Multispectral image analysis effectively screened *Zoysia* grass under herbicide and low-temperature stresses. Further F_2 generation development and molecular genetic studies will refine selection strategies for optimizing *Zoysia* grass under these stresses. These findings enhance *Zoysia* breeding programs by integrating genetic advancements and phenotyping technologies for enhanced turfgrass resilience.

Keywords Cross pollination · *Zoysia* grass hybrids · Glufocinate ammonium · Herbicide resistance

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Introduction

In natural settings, lawn grasses frequently compete with invasive weeds, which significantly diminish the aesthetic and functional value of turfgrass (Reynolds 2022). The presence of several different types of grass species in one location often leads to poor lawn quality due to variations in leaf texture and color, resulting in an uneven and visually unappealing turf. This lack of uniformity has economic consequences, particularly for golf courses, athletic fields, sports grounds, sod farms, and residential lawns (Brosnan et al. 2020; Johnson and Carrow 1999; Wang et al. 2024). To maintain a high-quality, uniform turf stand, effective weed management is essential. This requires an integrated approach that combines cultural, mechanical, biological, and chemical control methods. Among these, chemical control using herbicides remains the most efficient and widely utilized strategy due to its effectiveness and ease of application (Johnson and Carrow 1999).

For effective weed management in turfgrass, herbicides must control or suppress weeds without causing harm to the desired grass species. Although that is that, their overuse can lead to the development of herbicide-resistant weed populations and raise environmental concerns (Bae et al. 2008). For instance, a widespread resistance to glyphosate has rendered it less effective for weed control in agronomic crops (Vencill et al. 2012). As an alternative, glufosinate ammonium has been introduced, offering continued effectiveness in weed suppression (Lajmanovich et al. 2022). Herbicide resistance in weeds refers to the inherited ability of a weed plant to survive and reproduce after exposure to a herbicide dose that would typically be lethal to wild-type plants (Vencill et al. 2012). While resistance is a challenge in agronomic crops, it can be advantageous in turfgrass management. Herbicide resistance results from natural mutations or genetic modifications that enables grass species to endure herbicide applications without adverse effects. Hence, herbicide resistance in turfgrass species is a promising approach in weed management strategies in turfgrass systems as it reduces reliance on manual weeding, and minimize the ecological and economic impact of excessive chemical applications. By integrating herbicide-resistant turfgrass into weed management strategies, turf managers can improve efficiency while promoting sustainable lawn care practices (Brosnan et al. 2020; Vencill et al. 2012).

Developing herbicide resistant *Zoysia* grass varieties is particularly valuable for modern turfgrass management. *Zoysia* species are highly favored by lawn managers and consumers not only for their dense, mat-forming growth and vibrant green appearance but also their exceptional durability, low maintenance requirements, and adaptability to various climates and soil conditions (Wang et al. 2024).

Renowned for its superior stress tolerance, *Zoysia* grass exhibits greater cold tolerance than other C_4 turfgrass species (Fan et al. 2020; Hinton et al. 2012). *Zoysia japonica* (Al-Khayri et al. 1992) and *Zoysia matrella* (Loch et al. 2017) are two of three widely used species in genus *Zoysia* (Loch et al. 2017). Their increasing popularity stems from their ability to establish dense turf, and tolerate drought, salinity, and low nutrient levels. However, one of the major challenges in large-scale cultivation of them is its susceptibility to herbicides commonly used for weed control in turfgrass systems (Craft et al. 2023). The development of herbicide-resistant *Zoysia* grass varieties offers a transformative solution, allowing for effective weed management, while preserving turf quality and minimizing the need for intensive manual maintenance.

The development of herbicide-resistant *Zoysia* grass requires the integration of advanced technologies, including molecular biology, genetic engineering, and precision agriculture. Hybridization is a key breeding technique used to develop improved *Zoysia* grass cultivars with desirable traits such as enhanced herbicide resistance, cold tolerance, disease resistance, and superior aesthetic qualities. This process involves crossing genetically distinct parent plants to combine beneficial characteristics, resulting in new hybrid cultivars with improved adaptability and performance (Haghverdi et al. 2021; Labroo et al. 2021; Raybould and Gray 1993). Several studies have developed transgenic or hybrid *zoysia* grass varieties for herbicide resistance and cold tolerance (Huang et al. 2014; Johnson and Carrow 1999; Lim et al. 2004, 2004; Toyama et al. 2003).

The fundamental principle behind hybridization is the combination of genetic material from two different species or varieties to create offspring with enhanced or novel traits. By utilizing these hybridization techniques, breeders can develop *Zoysia* cultivars that possess both resilience and functionality, making them well-suited for a range of applications in turf management. Hybrid vigor (heterosis) is a key outcome of hybridization, which often leads to enhanced growth rate, stress tolerance, and overall turf quality (Ellstrand and Hoffman 1990; Katche et al. 2019; Raybould and Gray 1993). Various techniques are employed in *Zoysia* hybridization including Controlled Cross-Pollination (manual transfer of pollen between selected parent plants to produce hybrid offspring), Embryo Rescue (a technique used when interspecific hybrids face viability issues, involving the excision and culture of immature embryos), Protoplast Fusion (Somatic Hybridization), Mutation Breeding, Backcrossing (Repeated crossing of hybrid progeny with a parent species to stabilize desirable traits) (Harris-Shultz and Jespersen 2018; Kempe and Gils 2011; Labroo et al. 2021; Pathirana 2011; Turpin 1986; Waara and Glimelius 1995; Yang et al. 1989).

In addition to genetic advancements, phenotyping technologies are essential for evaluating the performance of herbicide-resistant grass varieties. High-throughput phenotyping platforms equipped with imaging sensors, hyperspectral cameras, and machine learning algorithms enable researchers to assess growth, health, and herbicide response in experimental plots. These tools facilitate the identification of optimal traits and improve the accuracy of selection processes (Danilevicz et al. 2021; Tattaris et al. 2016). In this study, we utilized multispectral sensors to enhance the efficiency and accuracy of herbicide resistance assessment in *Zoysia* grass. Images with multispectral sensors have revolutionized precision agriculture, offering a non-invasive, cost-effective, and high-throughput method for monitoring large-scale experimental plots (Karunathilake et al. 2023). One of the key advantages of image analysis with multispectral sensors is its ability to detect early physiological and biochemical responses to herbicide treatments (Das Choudhury et al. 2019). Image analysis with multispectral sensors facilitates the calculation of vegetation indices such as Normalized Difference Vegetation Index (NDVI), Normalized Difference Rededge Index (NDRE), Green Leaf Index (GLI), and Excess Green Index (ExG), which provide insights into photosynthetic activity, chlorophyll content, and stress levels, which are critical indicators of herbicide tolerance (Kim et al. 2020; Santana et al. 2021; Trenholm et al. 1999). Additionally, this method enables high-throughput screening, significantly reducing the labor and time required for manual evaluations by rapidly assessing multiple genotypes across different treatments (Tattaris et al. 2016). It also supports precise phenotyping, allowing for accurate measurements of canopy cover, growth rate, and weed suppression efficiency. Moreover, long-term monitoring using temporal data enables researchers to evaluate the durability of herbicide resistance traits across different growth cycles and environmental conditions (Büchi et al. 2018; Cabrera-Bosquet et al. 2011; El-Shikha et al. 2008; Jia et al. 2014).

The objective of this study is to screen herbicide-resistant and cold-tolerant *Zoysia* grass using image analysis based on multispectral images. To achieve it, F_1 hybrids were generated by crossing *Zoysia matrella*, known for its cold tolerance, with *Zoysia japonica*, recognized for its herbicide resistance.

Materials and methods

Selection of parent plants

Zoysia matrella (L.) Merr., named as San208, which exhibited good cold tolerance in the previous study (Ku et al. 2023) and *Zoysia japonica* Steud., has modified for its

herbicide resistance were selected as parent lines for hybridization, as shown in Fig. 1A and B. The study was conducted in a controlled environment in the GMO greenhouse (No. LML 14–501) at Jeju National University, Aradong, Jeju City, Jeju Self-Governing Province, Republic of Korea, situated at 126°56' E longitude and 33°46' N latitude. Parent plants were planted in two separate rectangular pots (size: 550 mm x 410 mm x 240 mm; manufacturer: Bum Nong Co. Ltd., South Korea). *Z. matrella* (L.) Merr., was designated to receive pollen and facilitate seed production. *Z. japonica* Steud., was specifically chosen to provide pollen and was sourced from the Sustainable Agriculture Research Institute, Jeju National University, Aradong, Jeju City, Jeju Self-Governing Province, Republic of Korea. Plants were watered as needed and fertilized with compound fertilizer (Granular 8-8-9+2+0.2; Eco Farming Co., Ltd., located in Jeonggwan-eup, Gijang-gun, Busan, Republic of Korea) according to standard horticultural practices for the species.

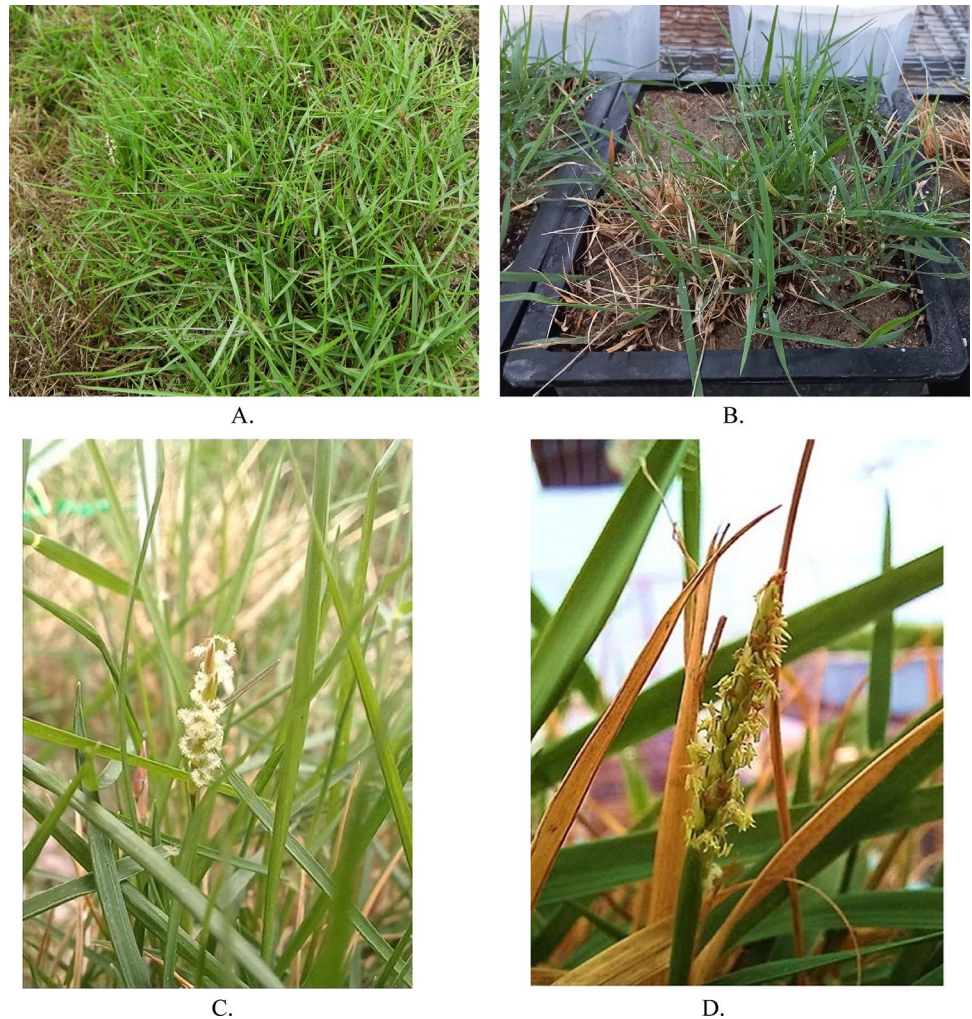
Artificial cross pollination

Parent plants were observed daily until the onset of anthesis from April to mid-May, 2023, ensuring synchronization of blooming stages between both parent plants to facilitate successful cross-pollination. To avoid unwanted pollination, the designated parent plants were spatially isolated from neighboring plants. Upon the observation of mature pollen in mid-May (Fig. 1), artificial pollination procedures were conducted over a span of 10 days, starting from May 8, 2023, coinciding with the flowering stage.

Zoysia grass (*Zoysia* spp.) flowers are bisexual, containing both staminate (male) and pistillate (female) structures within the same floret. Typically, male flowers reside near the apex of the spike, while female flowers are positioned lower on the same spike, both adapted for anemophily (wind pollination). During anthesis, the staminate function occurs first, followed by the pistillate function, facilitating cross-pollination (Al-Khayri et al. 1992; Smith Jr 2005). The anthers appear yellowish, partially open, at the stage of maturity and ready to actively releasing pollen (Fig. 1C). The stigma of female flowers, a minute, elongated structure, that becomes distinctly visible when receptive, characterized by long, white, feathery projections protruding from the flower as illustrated in Fig. 1D (Choi 2010).

Artificial cross-pollination was performed using standard hand-pollination techniques. For successful cross pollination, pollen should be collected at the optimal stage, when the anthers, structures that hold the pollen grains, commence to dehisce. The best outcomes are typically achieved when cross-pollination is performed in the morning, under the cool temperatures and high humidity levels (Kang et al. 2021). Accordingly, pollen was harvested in to Eppendorf

Fig. 1 Parent plants and their spikes: **A** *Zoysia matrella*, **B** *Zoysia japonica*, **C** Spike of *Z. matrella* and **D** Spike of *Z. japonica*



tubes (Natural polypropylene, 1.5 mL, Heathrow Scientific HS4323, 440 Fairway Drive, Vernon Hills, IL 60061 USA) between 8 a.m. and 10 a.m. using sterilized forceps, by delicately extracting anthers from the flowers of *Z. japonica* male parent. Extracted pollen was carefully applied to the receptive stigma of emasculated flowers on *Z. matrella*. Emasculation was performed by carefully removing the anthers with forceps before they reached maturity. During the pollination process, female flowers in stages either too premature or too senescent were deliberately bypassed, as they might not yield viable seeds. Once pollination was completed, pollinated flowers were tagged for identification. This phase involved the use of sterile tools (small camel hair brushes, scissors, tweezers, etc.) and necessary precaution measures were implemented to prevent pollen contamination throughout the procedure. Unlike conventional hybridization techniques, this process was conducted without emasculation and bagging.

Harvesting and storing F_1 seeds

The female plant (the one was planted in a large square pot) was monitored for seed formation while receiving standard horticultural care throughout the seed development period. When the seeds reached maturity, approximately 30 days after, in late-June 2023, they were carefully harvested using pruning shears (Cutting capacity: 1", Ningbo Yinzhou Guoten Industrial Co., Ltd., China) and placed them in a labeled brown paper bag. The brown paper bag is important, as it will allow the seed to 'breathe', ensuring that the moisture leaves. Then seeds were placed in a cool dry place for a month in the laboratory usually maintaining the humidity level of below 50% preventing mold.

Raising the F_1 Generation: seed dormancy breaking and planting

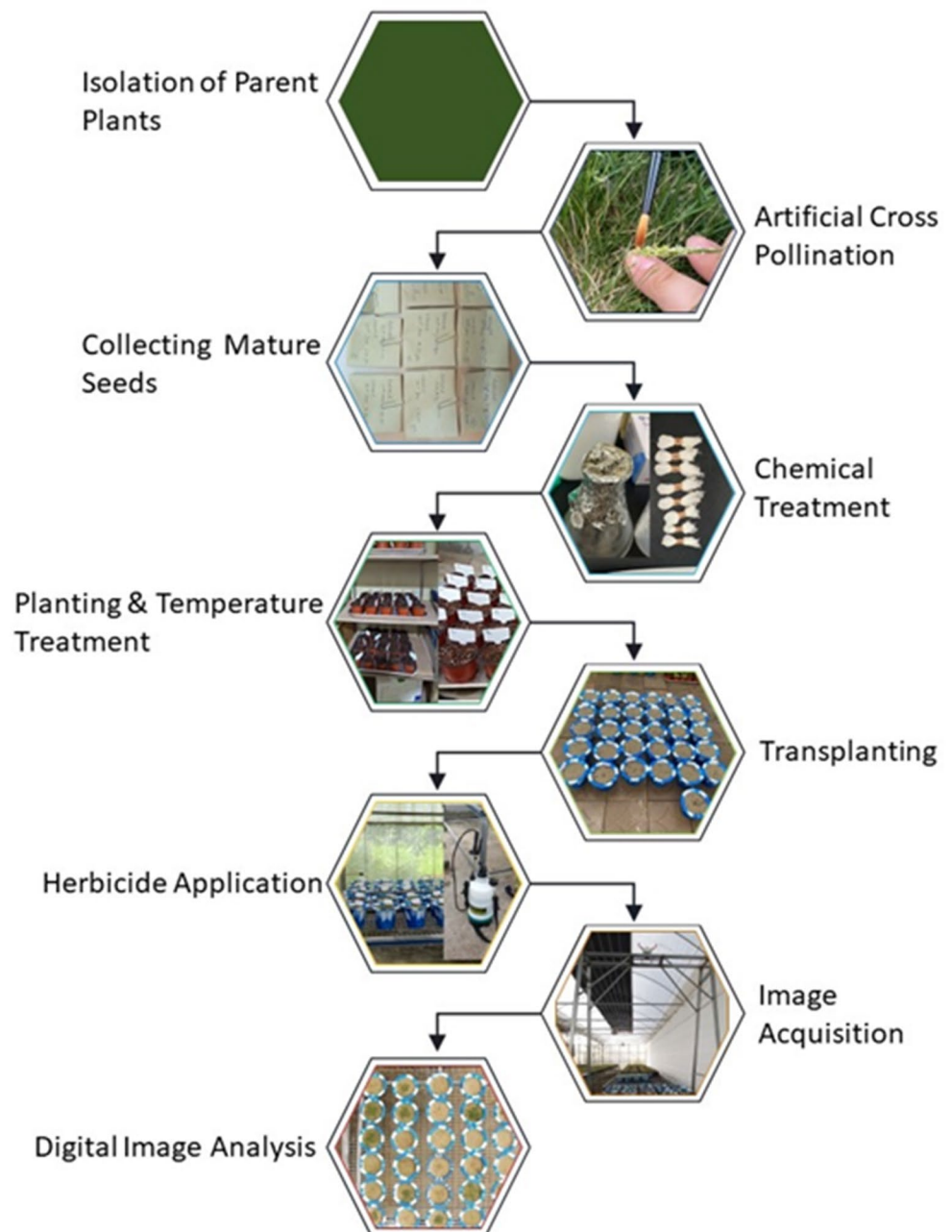
A total of 60 collected F_1 seeds were planted at the end of July, 2023. Prior to planting, seeds were chemically treated with the procedure of potassium hydroxide (KOH)

scarification to disrupt the dormancy (Kalsa et al. 2016; Qian et al. 2013). The seeds were encased in cloth sacks and appropriately labelled. These seed-containing sacks were immersed in a 30% solution of (KOH) (Sigma-Aldrich Inc., Haesung-2-Bldg, 508 Teheran-ro, Gangnam-gu, Seoul, South Korea) at ambient temperature for 30 min. Subsequently, the seed-containing sacks were thoroughly rinsed under running tap water to eliminate any residual KOH (Xu et al. 2005).

Chemically treated seeds were individually planted in 60 small pots (9 cm in diameter and 7 cm in height, UNI International Inc., Taeneck, New Jersey, United States) filled

with the soil medium (Top soil No. 02/Ultra-light, Nongwoo Bio Co., Ltd., Jeongeop-si, Jeollabuk-do, Republic of Korea) and labelled. Following planting, the pots containing the seeds were placed in a cold processing refrigerator at approximately 4 °C for a duration of 48 h available at Sustainable Agriculture Research Institute, Jeju National University, Aradong, Jeju City, Jeju Self-Governing Province, Republic of Korea. After 48 h, the pots were relocated to a greenhouse environment, where they were shielded with a net to regulate shade and maintain an internal temperature of 20 °C conducive to germination (Fig. 2) (Al Sakran et al. 2020). The photoperiod was maintained at approximately

Fig. 2 Major steps in evaluating herbicide resistance of F_1 hybrid generation: from cross pollination to image acquisition



14 h of light and 10 h of darkness, consistent with natural day length during the growth period (Xu et al. 2005). Scouting and maintaining were conducted with watering as needed, until the seedlings developed at least one set of true leaves.

In August, seedlings that had been growing for one month were transplanted into larger pots (20 cm in diameter and 15 cm in height, UNI International Inc., Taeneck, New Jersey, United States) to facilitate uninterrupted continuous growth. The same soil medium utilized for initial pots was prepared for larger pots, with an additional layer of sand applied to the soil surface following the careful transfer of the seedlings. Up until September, regular weeding and watering practices were implemented to ensure optimal growth conditions.

Herbicide application

After 56 days of planting, a total of 30 seedlings of F₁ hybrids were successfully grown individually in pots (one plant per pot). To assess the herbicide resistance of those hybrids, the herbicide glufosinate-ammonium (18% SL basta[®], CAS No.77182-82-2, Bayer's Crop Science Division, Bayer China Ltd., Pudong, Shanghai 200126, China) was applied. The 18% glufosinate ammonium formulation was diluted 33-fold to obtain a 0.545% working solution. This was applied to those 30 hybrids, covering approximately 0.942 m² (as the pot diameter was 20 cm). Based on the estimated spray volume of 100 mL per pot, the equivalent field application rate was approximately 3185 L/ha. Herbicide was sprayed only one time using a sprayer equipped with a flat fan nozzle (Clover compressed automated sprayer, Model No. TH-33, capacity 5 L, Tae Hwan Co. South Korea and nozzle size is 06). No untreated control or replicated treatment groups were included, as this study was designed as a preliminary screening to identify potential herbicide-resistance individuals for subsequent detailed evaluation. Glufosinate ammonium is a non-selective, broad-spectrum herbicide belonging to the phosphinic acid chemical family (Lajmanovich et al. 2022). It acts as a contact herbicide and inhibits the enzyme glutamine synthetase, disrupting nitrogen metabolism. This leads to accumulation of ammonia inside plant tissues in toxic levels resulting the death of foliage and other green parts (Donthi and Kumar 2022). Glufosinate ammonium is classified under WSSA Group 10 and HRAC Group H, and has been utilized extensively since 1984 for weed control across more than 100 crops (Takano and Dayan 2020a; University of California–Davis 2024). Moreover, there is no soil activity with this herbicide. Serving as a broad-spectrum herbicide, glufosinate-ammonium is an active ingredient in several non-selective herbicide

products, including basta[®] (Bayer Crop Science), rely[®], and liberty[®] (BASF) (Kang et al. 2021).

Image acquisition

Prior to herbicide application, images of the F₁ hybrid plants were captured to establish baseline vegetation conditions. Following herbicide treatment, images were acquired daily to monitor the response of the treated seedlings until the susceptible individuals had completely deteriorated. From September 18 to October 31, 2024, images of herbicide-treated hybrid plants were collected over a 44-day period, ensuring comprehensive documentation of their response. After this initial phase, surviving individuals were continuously monitored to assess their long-term resilience as well as tolerance of cold temperatures. This step was conducted under natural winter conditions in the same greenhouse without supplemental heating stimulate field-like winter stress responses. Images of these surviving plants were captured weekly, every Friday, from November 1, 2024, until January 19, 2024, providing valuable insights into their recovery and adaptation to environmental conditions.

The same Phantom 4 Multispectral UAV Phantom 4 Multispectral UAV (Camera model: WM336, DJI, DJI GmbH, Niederauer, Germany) equipped with six 1/2.9" CMOS sensors was used to capture the images, with Table 1 providing wavelength range data for each sensor. Preparatory measures such as weeding and sanding of moss to obscure unwanted plant presence in the images were undertaken prior to image capturing. The DJI GO Pro software (SZ DJI Technology Co. Ltd., China), facilitated the photography process, automatically adjusting white balance and setting the ISO value to 400. Imaging occurred within a specified time frame of 11 a.m. to 1 p.m. to minimize lighting variations affecting the data quality. Utilizing a drone equipped with a sensor and affixed to a specialized apparatus, systematic data collection was ensured, maintaining a fixed altitude of 2 m above the grass surface. To address radiation discrepancies, the Type 882 Woven polyester fabric target plate from Group 8 Technology in the United States was used. Image storage protocols varied based on the sensor in use, with RGB images saved in JPG format and multi-spectral images in TIF format, all maintaining a uniform resolution of 1600 × 1300 pixels across all sensors. Over a span of 44 days, comprehensive imagery documenting the response of the herbicide-treated grasses was meticulously gathered.

Image preprocessing

The preprocessing stages, including distortion correction, radiometric calibration, histogram equalization, and image alignment, were carried out to facilitate image analysis (Ku

Table 1 Means ($\pm$ standard deviation) Green Cover Percentage (GCP) of F1 hybrid plants of *Zoysia matrella* x *Zoysia japonica* from September, 2023 to October 2023. Statistical analysis was performed using the Kruskal–Wallis test ($p < 0.05$), followed by the Bonferroni post hoc test for multiple comparisons. Non-parametric rank data were used for statistical analysis, with comparisons based on medians

Plant ID	Day0	Day10	Day20	Day30	Day40					
P1	24.35	± 0.14	53.00	± 0.15	102.81	± 1.44	110.88	± 1.77	80.73	± 0.43
P2	47.95	± 0.17	15.58	± 0.13	10.62	± 0.77	0.00	± 0.00	0.00	± 0.00
P3	15.45	± 0.13	1.72	± 0.04	1.64	± 0.39	0.73	± 0.23	0.00	± 0.00
P4	23.17	± 0.12	7.19	± 0.11	5.41	± 0.18	3.06	± 0.16	2.66	± 0.37
P5	43.88	± 0.18	66.41	± 0.21	102.37	± 1.56	160.49	± 2.16	120.25	± 0.70
P6	24.46	± 0.15	10.22	± 0.12	8.50	± 0.61	0.00	± 0.00	0.00	± 0.00
P7	30.68	± 0.18	56.97	± 0.19	88.69	± 1.42	108.12	± 1.29	82.42	± 0.43
P8	2.15	± 0.07	2.90	± 0.05	9.02	± 0.53	4.29	± 0.37	9.95	± 0.58
P9	7.94	± 0.09	14.70	± 0.12	20.59	± 0.84	16.61	± 0.75	15.49	± 0.49
P10	79.60	± 0.35	3.00	± 0.08	2.92	± 0.45	1.90	± 0.25	0.00	± 0.00
P11	49.13	± 0.21	77.56	± 0.26	127.17	± 2.14	177.43	± 2.23	176.24	± 0.53
P12	25.32	± 0.17	1.93	± 0.05	0.36	± 0.14	0.22	± 0.13	0.00	± 0.00
P13	17.49	± 0.13	26.39	± 0.17	50.87	± 1.33	46.35	± 1.24	40.40	± 0.62
P14	47.63	± 0.18	71.45	± 0.23	105.93	± 1.19	125.39	± 1.66	88.98	± 1.45
P15	4.72	± 0.08	2.47	± 0.07	2.33	± 0.32	0.00	± 0.00	0.00	± 0.00
P16	43.34	± 0.17	95.69	± 0.29	104.06	± 0.17	107.89	± 1.53	195.58	± 2.12
P17	40.34	± 0.19	75.20	± 0.25	103.32	± 1.35	132.92	± 1.88	184.55	± 1.97
P18	29.61	± 0.16	4.83	± 0.08	0.32	± 0.18	0.29	± 0.20	0.00	± 0.00
P19	32.93	± 0.17	57.61	± 0.23	85.64	± 1.30	91.60	± 1.31	88.00	± 0.46
P20	31.54	± 0.16	66.83	± 0.23	104.42	± 1.32	104.46	± 1.55	111.46	± 0.64
P21	17.16	± 0.12	11.36	± 0.12	4.81	± 0.46	1.81	± 0.31	0.00	± 0.00
P22	49.67	± 0.22	9.33	± 0.11	0.84	± 0.22	0.12	± 0.08	0.00	± 0.00
P23	58.04	± 0.26	8.15	± 0.09	4.85	± 0.26	3.66	± 0.44	0.00	± 0.00
P24	53.64	± 0.24	6.65	± 0.10	3.96	± 0.31	0.84	± 0.23	0.00	± 0.00
P25	11.48	± 0.11	5.79	± 0.09	1.06	± 0.26	1.15	± 0.29	0.00	± 0.00
P26	61.36	± 0.26	115.32	± 0.32	141.51	± 0.74	162.73	± 2.03	198.53	± 3.35
P27	4.90	± 0.08	3.86	± 0.08	2.28	± 0.42	0.00	± 0.00	0.00	± 0.00
P28	4.29	± 0.07	3.66	± 0.08	0.31	± 0.20	0.00	± 0.00	0.00	± 0.00
P29	33.36	± 0.18	66.08	± 0.23	102.75	± 1.62	110.11	± 1.38	102.05	± 1.41
P30	36.05	± 0.18	4.83	± 0.08	0.43	± 0.18	0.00	± 0.00	0.00	± 0.00

et al. 2023). The primary objectives of these steps were to enhance image quality, correct distortions, standardize radiometric attributes, and ensure spatial alignment for precise analysis, as highlighted by (Wang et al. 2022). To ensure an accurate representation of the scene, radial distortion brought about by the properties of the camera lens is corrected during initial processing followed by histogram equalization, radiometric calibration and alignment using a program written with Python (Version 3.11.9, Programming Language, Python Software Foundation, Wilmington, Delaware, The United States), ‘OpenCV2’ (Version 4.10.0, Open-source Computer Vision Library, Intel Corporation, Santa Clara, California, The United States), ‘Pandas’ (Version 2.2.3, Software Library for Python programming Language), ‘Matplotlib’ (Version 3.10.0, Plotting Library for Python programming Language). Written Codes were run using Visual Studio Code (VS Code), a free and open-source integrated development environment (IDE) developed by Microsoft (Microsoft Corporation, 1 Microsoft Way, Redmond, Washington, The United States).

Histogram equalization and radiometric calibration were performed using the reference plate with a constant reflectance, radiometric calibration balances the light conditions throughout captured photos. Through the conversion of digital numbers in images to reflectance values, this procedure guarantees analytical uniformity even under different lighting circumstances. Histogram equalization was also used to correct radiometric correction-related dimming and enhance the contrast of RGB photographs. Alignment was performed on multispectral images, particularly between the bands NIR and Red, to ensure spatial coherence for subsequent analysis. This involved shifting one image based on metadata indicating physical differences in camera positions during image capture. For metadata extraction and manipulation to facilitate this alignment process, ‘pyexiv2’, python library binding to exiv2, a C++ library for reading and writing various image metadata was utilized. In distortion correction, radial distortion, which appeared in the acquired images as a convex bulge in the center due to the characteristics of the camera lens, was digitally rectified, ensuring an accurate representation of the scene (Yin et al. 2024).

Image analysis

Aligned and undistorted multispectral images were used to compute GCP and NDVI indices (Eqs. 1 and 2), to monitor the degree of stress in the grass for low temperatures over time.

$GCP = (\text{Green Cover Area of each grass} / \text{Total Canopy Area}) \times 100$ (Eq. 1) (Ku et al. 2023).

$NDVI = (NIR - Red) / (NIR + Red)$ (Eq. 2) (Kim et al. 2019).

Corresponding VI image files for each VI were generated using preprocessed images. For NDVI and SAVI image files, preprocessed NIR and Red band images were used in Eq. 1 and Eq. 3 respectively. For NDRE image files, preprocessed images of NIR and RE bands were used in Eq. 2. VI values for each germplasm were extracted using the data stored in those VI image files as Input. VI values were computed by counting the number of white pixels in each rectangular Regions of Interest (ROI) with a size of 200×130 pixels. Those ROI were created specifying the green area of the grass germplasms of the image. This process was performed using an in-house macro, a software module designed for automated batch processing, with Python (Version 3.8.12, Programming Language, Python Software Foundation, Wilmington, Delaware, The United States), PyQt5 (Version 5.15.4, a comprehensive set of Python bindings for Qt v5), Opencv-contrib-python (version 4.5.2.54, Wrapper package for OpenCV python bindings), XlsxWriter (Version 1.2.6, a Python module) and Pandas (Version 1.2.4, Software Library for Python programming Language). This macro enables batch image processing and data transformation, ultimately streamlining the series of tasks involved and providing a more efficient method for managing and analyzing grass bed VI data, which is publicly accessible via GitHub (‘VI-RoI-Computation-Macro’, GitHub repository link: <https://github.com/kibonku/VI-RoI-Computation-Macro>).

To calculate the GCP, green pixels were quantified using preprocessed RGB images, while the total number of pixels was derived from preprocessed NIR images. For this a program written with Python (Version 3.11.9, Programming Language, Python Software Foundation, Wilmington, Delaware, The United States), ‘OpenCV2’ (Version 4.10.0, Open-source Computer Vision Library, Intel Corporation, Santa Clara, California, The United States), ‘Pandas’ (Version 2.2.3, Software Library for Python programming Language), ‘Matplotlib’ (Version 3.10.0, Plotting Library for Python programming Language) and NumPy (Version 2.1.2, a python library for multidimensional arrays and matrices) was used. Written Codes were run using Visual Studio Code (VS Code), a free and open-source integrated development environment (IDE) developed by Microsoft (Microsoft Corporation, 1 Microsoft Way, Redmond, Washington, The United States). A segmentation method combining the Excess Green Index (ExG) and Otsu’s automatic thresholding was employed to distinguish the green canopy from the background in the RGB images. Given that ExG effectively assesses canopy variation in green crop biomass based on RGB imagery, it was computed using reflectance values obtained from RGB sensor imagery, as defined by Kim and Chung (2021) (Kim and Chung 2021):

$ExG = 2g - r - b$ (Eq. 3).

Where, $r = R/(R+G+B)$, $g = G/(R+G+B)$, $b = B/(R+G+B)$, and R, G, and B represent the reflectance values of the red, green, and blue bands in the radiometrically calibrated images, respectively.

Otsu's thresholding, an automatic thresholding technique that assumes the image comprises two-pixel classes (e.g., vegetation and background), was applied to determine the optimal threshold by minimizing interclass variance and maximizing intraclass variance (Otsu, 1979; Zhang and Hu, 2008). Using this method, ExG images from RGB imagery and the NIR band were converted into binary images, categorizing pixels into either vegetation or background (Kim et al., 2023). Subsequently, the number of green pixels and the total number of pixels were computed using an in-house macro program. The final GCP value was then calculated using Microsoft Excel (Version 2108, Free Online Spreadsheet Software by Microsoft 365).

Statistical analysis

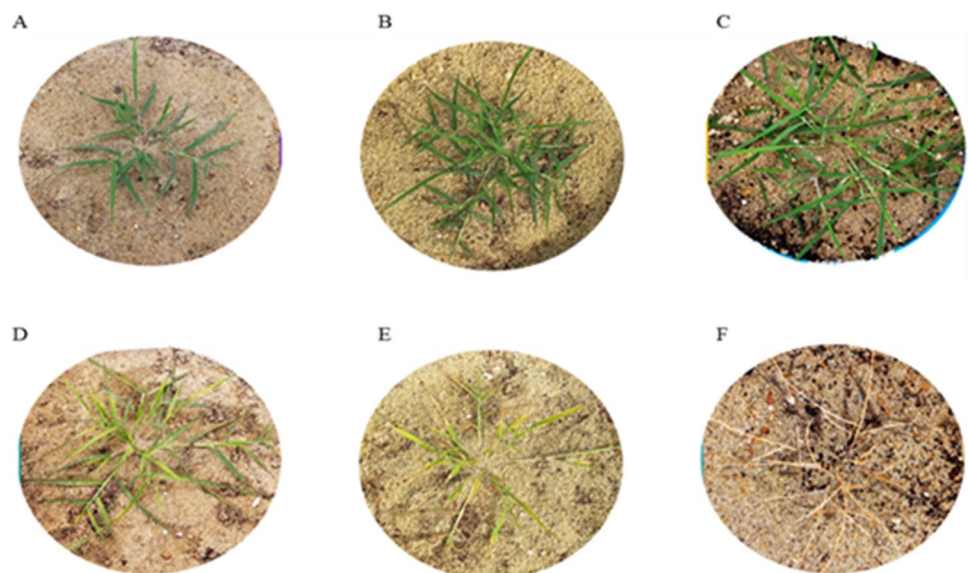
The Green Cover Percentage (GCP) was calculated using Microsoft Excel (Version 2108, Free Online Spreadsheet Software by Microsoft 365) based on previously computed pixel counts. All statistical analyses were conducted in RStudio (Version 2024.09.1+394, “Cranberry Hibiscus” Release, RStudio Team, 2024) running on Windows 10, utilizing R Software (Version 4.4.2, R Foundation for Statistical Computing, Vienna, Austria). Statistical analyses were performed on the temporal NDVI and GCP data for each individual at key time points: 0, 10, 20, 30, and 40 days after herbicide application (DAA). At the same time, weekly NDVI and GCP values were analyzed separately for F_1 hybrids and their parental lines. The Shapiro-Wilk test was used to assess data normality, while Levene's test

was employed to evaluate homogeneity of variance. The data did not meet the assumption of normality ($p \leq 0.05$); however, variance homogeneity was confirmed. Given the non-normal distribution of the data, the Kruskal-Wallis test was used to determine statistical significance, followed by Dunn's test ($p \leq 0.05$) with Bonferroni correction to account for multiple comparisons and control the family-wise error rate. The relationship between GCP and NDVI was assessed using the Pearson correlation coefficient at a significance level of $p \leq 0.05$.

Results

A total of 60 seeds of F_1 generation were harvested following artificial cross-pollination between two *Zoysia* grass species. After drying for approximately one month, the seeds were planted. The first instance of seed germination was observed nine days after planting, marked by the emergence of the first green shoot. Over the subsequent ten days, 31 additional seeds germinated, bringing the total number of germinated seedlings to 32. Transplanting was conducted when the seedlings were 29 days old. By the time the seedlings reached 56 days of age, only 30 remained viable, as two fragile seedlings were damaged and did not survive during the transplantation process. To the 56th days old individuals of F_1 generation plants, the herbicide (Glufosinate ammonium Basta R) was applied. Three days after application, 15 individuals exhibited visible symptoms of yellowing (Fig. 3) and eventually died by the end of October 2023. As the season transitioned to a colder climate in December 2023, one more seedling did not survive. Ultimately only 14 individuals survived in the experiment.

Fig. 3 Visual representation of F_1 hybrid plant responses to herbicide application over time. Panels (A–C) illustrate the growth progression of herbicide-resistant plants at 3 days (A), 7 days (B), and 35 days (C) after herbicide application, showing sustained growth and healthy green coloration. Panels (D–F) depict herbicide-susceptible plants at corresponding time points: 3 days (D), 7 days (E), and 35 days (F), exhibiting progressive symptoms of herbicide damage, including yellowing, wilting, and eventual straw-brown discoloration, indicative of complete desiccation



The results of GCP and NDVI of each individual at key time points are statistically significant at the 95% confidence level ($p \leq 0.0001$). As shown in Fig. 4; Tables 1 and 50% of F_1 generation plants survived the herbicide application. Figure 4 illustrates the GCP of individuals of this F_1 generation plants over a 40-day period, from September 18 to October 28, 2022, following herbicide application. The GCP was presented at key intervals: Day 0, Day 10, Day 20, Day 30, and Day 40, providing insights into how each plant responded to the herbicide over time. Before herbicide application (0 Day), GCP values ranged from 2.15 to 79.6%. Ten days after application, GCP of 15 individual plants dropped sharply and then continued to decline, gradually reaching 0% at the end of the observation period. By Day 30, six plants (P2, P6, P15, P27, P28, and P30) had completely died, with GCP falling to 0%. By Day 40, all 15 affected plants had reached 0% GCP. The affected plants exhibited visible yellowing of leaves and stems, characterized by the rapid initial drop in GCP. As these plants turned from yellow to brown (becoming straw-like), their GCP eventually reached zero.

In contrast, over the observation period, 15 plants exhibited a gradual increase in GCP. All these individuals exhibited a steady increase in GCP during the first 20 days, with seven individuals reaching 100% by the end of the 40-day period. Specially, P17 and P26 demonstrated continuous growth over the entire 40 days, maintaining the highest GCP among the unaffected plants. P16 exhibited a gradual increase, followed by a rapid surge in the last 10 days (Fig. 4). Conversely, some plants (P1, P5, P7 and P14) reached their peak GCP on Day 30, and experienced a considerable decline over the subsequent 10 days. While P8 and P9 also showed an increase in GCP, their growth rate was the slowest among the unaffected plants throughout the 40-day observation period. By Day 40 (96 days after germination), P8 had only reached a GCP of 9.98%.

Fig. 4 Temporal changes in Green Cover Percentage (GCP) of F_1 hybrid plants following glufosinate ammonium application. The figure illustrates the GCP dynamics of individual F_1 plants over a 40-day period at key time points (0, 10, 20, 30, and 40 days after herbicide application) from September 18 to October 28, 2022

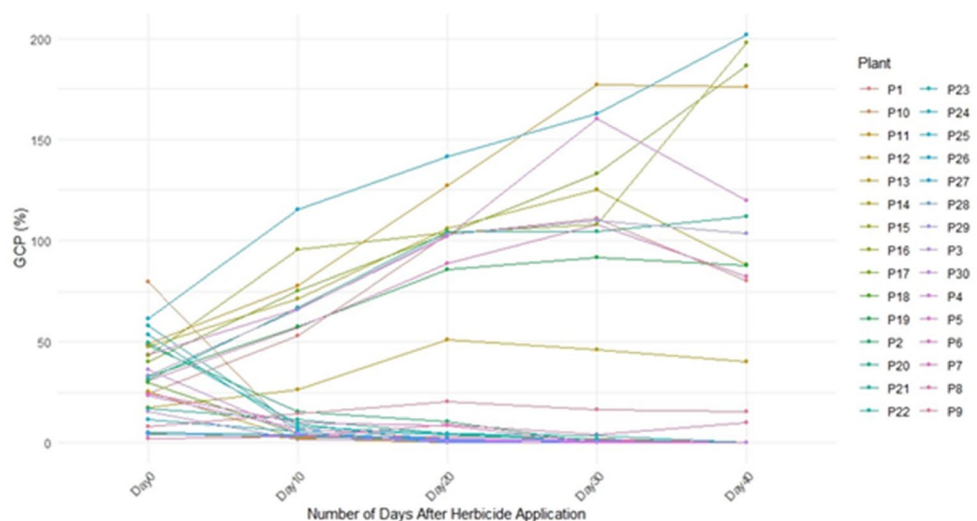


Table 2 presents the NDVI values of each individual at key time points considered throughout the 40-day observation period. Figure 5 illustrates that a significant portion of the plants show a decline in NDVI below 0.150 over time, particularly after Day 10. In that portion, 16 individuals showed a gradual decrease, while other 14 exhibited increasing trends. Initially P4 was notable for having the highest NDVI value of 0.274, but it gradually decreased until the Day 40. In contrast several distinct trends of increasing NDVI were observed (Fig. 5), indicating that the F_1 generation exhibited diverse responses to the herbicide. The NDVI values of P26, P16 and P17 increased steadily and peaked at the end of the observed period. Over the first 20 days, some plants showed a slow increase in NDVI, while others, such as P16 and P17, exhibited a sharp rise. Certain individuals, including P1, P11, and P29, reached their peak NDVI on Day 20, whereas others, such as P5, P7, P13, and P14, peaked on Day 30 before declining in next couple of observation days.

Comparing this NDVI graph to the previous GCP graph, we can see a strong positive correlation ($r = 0.6408$, $p\text{-value} \leq 0.0001$). Plants that showed a decline in GCP also tend to show a decline in NDVI, reinforcing the idea that the herbicide is affecting the plants' green tissue and photosynthetic activity. We continued monitoring the 15 surviving plants from the herbicide application throughout the winter months of 2023. Figures 6 and 7 displays the trends of GCP and NDVI of survived individuals during the cold weather of the winter season. The variability of both GCP and NDVI among plants are statistically significant at $p > 0.05$. The GCP of all 15 plants declined until November 24, 2023, with some continuing to decrease until December 1, 2023. After this period, GCP values gradually increased, indicating plant recovery and growth (Fig. 6). However, P4 did not survive the cold temperatures and died before December 1, as indicated by a GCP of 0%. Some plants, such as P26, P16,

Table 2 Means ($\pm$ standard deviation) normalized difference vegetation index (NDVI) of F₁ hybrid plants of *Zoysia Matrella* x *Zoysia Japonica* from september, 2023 to October 2023. Statistical analysis was performed using the Kruskal–Wallis test ($p < 0.05$), followed by the bonferroni post hoc test for multiple comparisons. Non-parametric rank data were used for statistical analysis, with comparisons based on medians

Plant ID	Day0	Day10	Day20	Day30	Day40
P1	0.131	0.166	0.363	0.346	0.280
P2	0.187	0.144	0.141	0.138	0.090
P3	0.195	0.148	0.128	0.124	0.111
P4	0.274	0.225	0.174	0.135	0.144
P5	0.199	0.242	0.305	0.406	0.371
P6	0.140	0.133	0.124	0.113	0.087
P7	0.134	0.164	0.187	0.335	0.294
P8	0.059	0.057	0.109	0.121	0.138
P9	0.073	0.070	0.147	0.154	0.136
P10	0.259	0.243	0.225	0.173	0.137
P11	0.175	0.192	0.472	0.418	0.405
P12	0.127	0.092	0.081	0.080	0.078
P13	0.083	0.099	0.105	0.224	0.186
P14	0.157	0.224	0.332	0.385	0.315
P15	0.134	0.131	0.131	0.125	0.068
P16	0.174	0.304	0.321	0.409	0.494
P17	0.178	0.184	0.215	0.379	0.480
P18	0.142	0.131	0.124	0.121	0.104
P19	0.139	0.218	0.254	0.291	0.288
P20	0.146	0.247	0.372	0.337	0.364
P21	0.204	0.186	0.172	0.160	0.143
P22	0.187	0.125	0.114	0.093	0.104
P23	0.205	0.136	0.114	0.097	0.070
P24	0.201	0.168	0.147	0.128	0.114
P25	0.186	0.165	0.152	0.144	0.116
P26	0.222	0.330	0.372	0.493	0.533
P27	0.252	0.243	0.197	0.101	0.085
P28	0.155	0.147	0.119	0.111	0.108
P29	0.139	0.214	0.387	0.314	0.319
P30	0.185	0.146	0.143	0.111	0.095

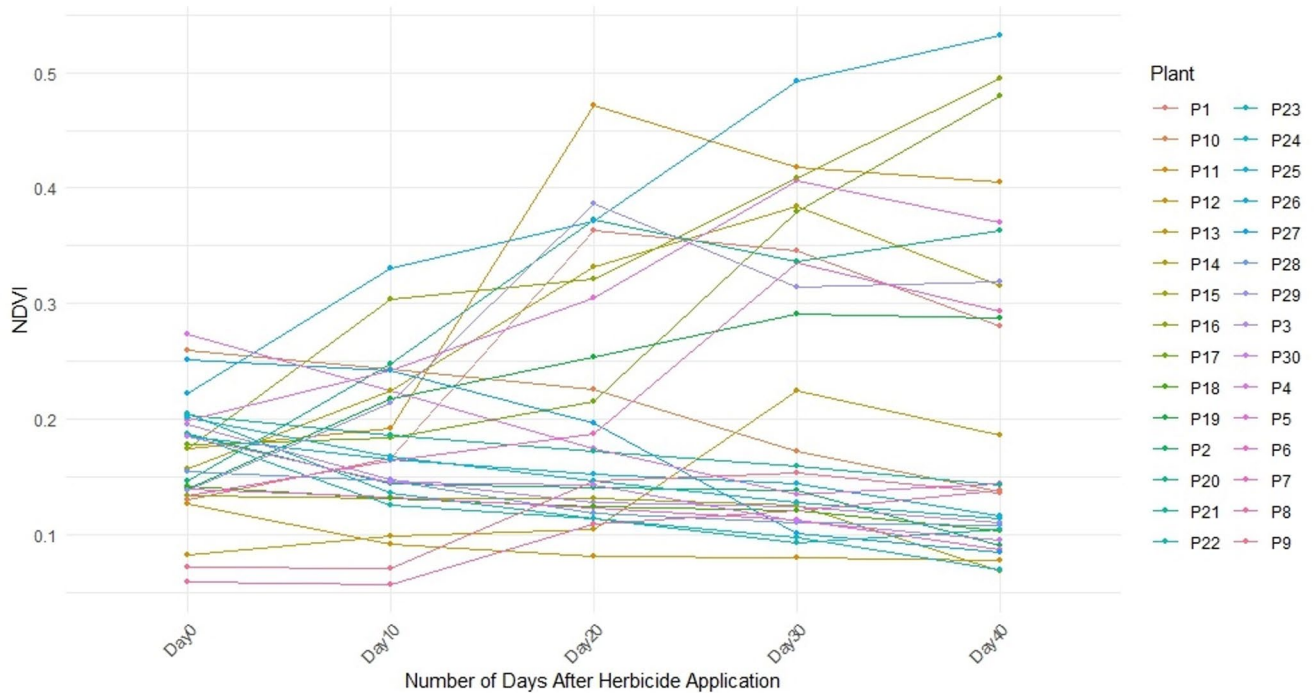


Fig. 5 Temporal changes in Normalized Difference Vegetation Index (NDVI) of F₁ hybrid plants following Glufosinate ammonium application. The figure illustrates the GCP dynamics of individual F₁ plants

over a 40-day period at key time points (0, 10, 20, 30, and 40 days after herbicide application) from September 18 to October 28, 2022

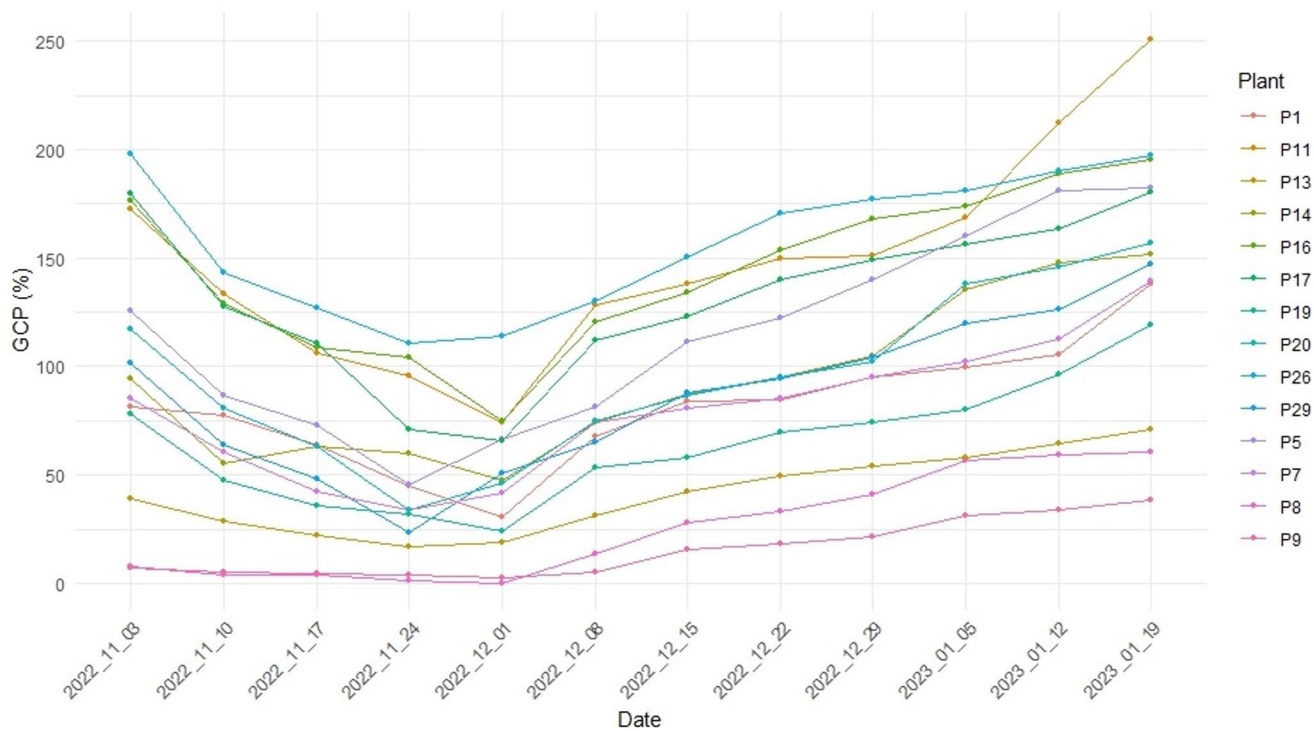


Fig. 6 Temporal variation in Green Cover Percentage (GCP) among herbicide-resistant F₁ hybrid plants during winter (November 3, 2022 – January 19, 2023)

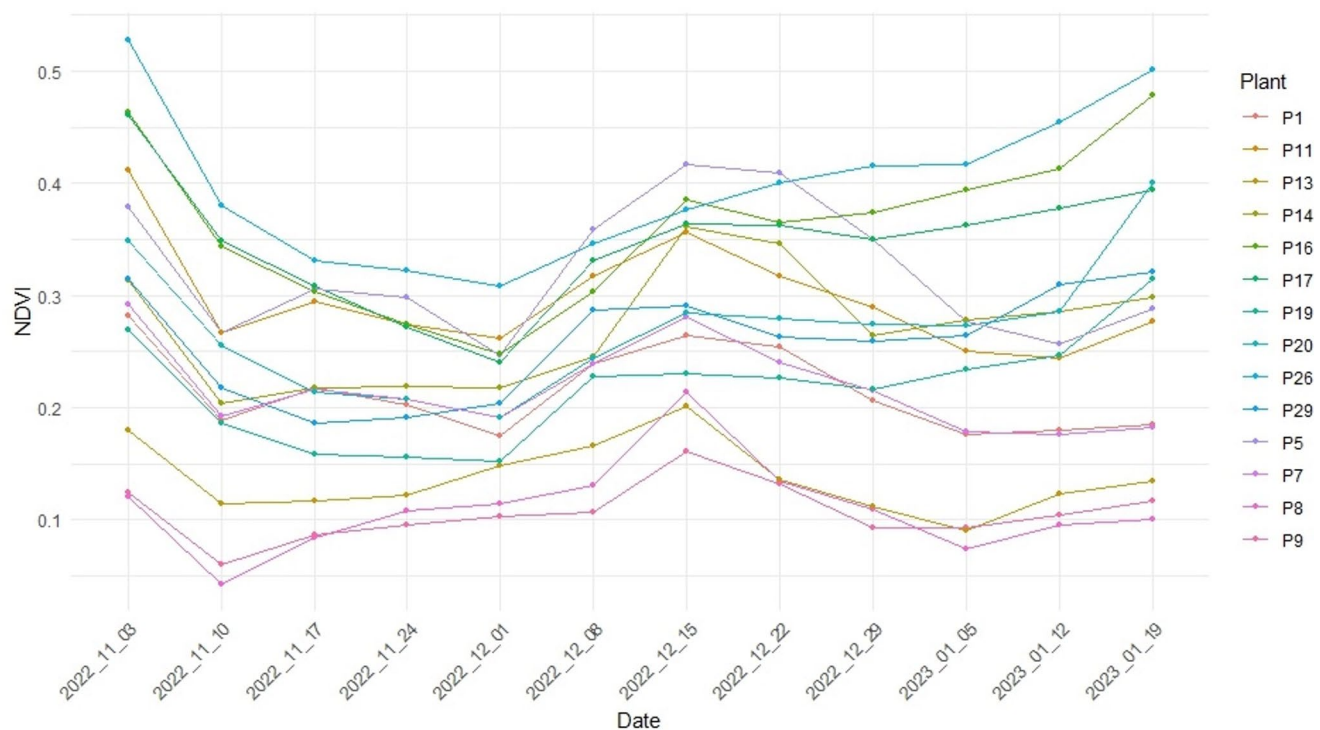


Fig. 7 Temporal variation in Normalized Difference Vegetation Index (NDVI) among herbicide-resistant F_1 hybrid plants during winter (November 3, 2022 – January 19, 2023)

and P11, maintained relatively stable and high GCP levels throughout the observation period. Notably, during the final two weeks of the study, P11 exhibited a rapid increase, ultimately reaching the highest GCP among all individuals. In contrast, P13, P8, and P9 consistently displayed low GCP values. However, P8 and P9, which had the lowest GCP during the initial 40-day observation period, showed significant improvement, with their GCP increasing to approximately 50% by January.

NDVI trends across different plants over time is shown in the Fig. 7. All plants show a sharp decline in NDVI at the beginning until December 1, 2023, suggesting early stress or environmental changes affecting vegetation health. After the initial drop, NDVI values stabilize and even slightly increase for some plants around mid-December, indicating a potential recovery or adaptation phase. Some plants continue to recover, while others experience a second dip in NDVI values around early January, suggesting different responses to external conditions such as temperature or nutrient availability. Most plants show an upward NDVI trend in mid-to-late January, indicating improved vegetation health or a favorable growth phase. Certain plants (e.g., P17, P20, P26) maintain relatively higher NDVI values throughout, while others (P13, P4, P8, P9) remain consistently lower, indicating differences in plant vigor or tolerance.

Discussion

This study was aimed to develop a *Zoysia* grass hybrid by combining herbicide resistance and cold tolerance traits. A F_1 hybrid population was successfully produced by the interspecific hybridization of *Zoysia matrella* (cold tolerance) and *Zoysia japonica* (herbicide resistance). After 40 days of herbicide application, 50% of the F_1 hybrid plants successfully survived, while the remaining plants did not tolerate the herbicide and perished. Among the survivors, only one plant failed to withstand the winter conditions. In this study we used 0.5% glufosinate ammonium (commonly sold under names like Basta), a non-selective herbicide often used to control weeds which have damaging effects on most plants when it comes into contact with (Donthi and Kumar 2022). Glufosinate ammonium inhibits the enzyme glutamine synthetase, which is critical for the assimilation of ammonia, a byproduct of plant metabolism, into amino acids. Without functioning glutamine synthetase, ammonia builds up to toxic levels in plant tissues. This toxicity damages cell membranes, leading to leakage and death of plant cells. The accumulation of ammonia disrupts photosynthesis by inhibiting electron transport in chloroplasts. This leads to energy deficiency within the plant. Furthermore, the herbicide triggers the production of reactive oxygen species (ROS), further damaging proteins, lipids, and DNA within plant cells (Lim et al. 2004; Takano and Dayan 2020b).

The variation in responses observed in the F_1 generation suggests different levels of inherited herbicide resistance. The initial drop in GCP among susceptible individuals can be attributed to chlorophyll degradation, membrane damage, and impaired metabolic activity. The yellowing and browning of leaves and stems indicate that the herbicide effectively disrupted essential physiological processes. The chlorosis (yellowing) followed by necrosis (browning of the grass blades before they wither) resulted the steady decline in both GCP and NDVI (Figs. 4 and 5). This indicate that those F_1 hybrid plants were not resistant to the herbicide. Moreover, this suggests the herbicide had a quick and strong effect on these individuals, likely damaging their green tissues. The individuals that survived maintained or increased their GCP and NDVI values over time, indicating their ability to resist the herbicide stress. The gradual increase in GCP and NDVI among resistant individuals may be due to enhanced antioxidant activity, efficient herbicide metabolism, or the presence of resistant alleles inherited from the herbicide-resistant parent.

The statistically significant differences in GCP and NDVI values over time further emphasize the variation in individual responses to herbicide stress as well as low temperature stress. Hybrid plants P17 and P26 demonstrated consistent growth, suggesting a high level of resistance, while individuals such as P16 exhibited a delayed but rapid increase in GCP, possibly indicating partial resistance. Conversely, individuals who died first (Day 30) is more susceptible to the 0.5% glufosinate ammonium. The GCP of P4 is gradually decreasing, although the plant has not died. This could indicate a delayed response to the herbicide or some level of tolerance or resistance. Alternatively, the herbicide may have taken longer to take effect. However, P4 ultimately failed to recover from low temperatures and died. This may be because the herbicide disrupted the ability of plant to grow or regenerate, or its tolerance to freezing temperatures are not enough to survive.

These findings align with previous research on herbicide resistance in hybrid Zoysia grass, highlighting the crucial role of genetic inheritance in determining survival rates, despite differences in hybrid production techniques. In 2003, Toyama et al. developed *Z. japonica* plants resistant to bialaphos, glufosinate-type non-selective herbicide, using Agrobacterium-mediated transformation (Toyama et al. 2003). Their study demonstrated that the resistance of transgenic plants persisted even after a two-year period. Similarly, in 2004, Lim et al. successfully developed glufosinate (BastaR) resistant *Z. japonica* cv. ‘Zenith’ plants by particle bombardment-mediated transformation. These plants exhibited high resistance under field application conditions (Lim et al. 2004). Moreover, both studies confirmed that the *bar* gene is responsible for herbicide resistance in

transgenic Zoysia grass. Also, the transgenic *Z. japonica* line, Jeju GREEN 21, was developed using the *bar* gene, further demonstrating its effectiveness in conferring herbicide resistance. Herbicide resistance genes, such as 5-enol-pyruvylshikimate-3-phosphate synthase (*EPSPS*), which imparts resistance to glyphosate (Roundup), and Bialaphos resistance (*BAR*) or phosphinothricin acetyltransferase (*PAT*), which provide resistance to glufosinate (Basta), are extensively used in the development of genetically modified plants. (Wang et al. 2024).

The continued monitoring of the 15 herbicide resistant plants throughout the winter of 2023 provided valuable insights into their cold tolerance. Except for P4, which perished during winter, all 14 other herbicide-resistant plants successfully withstood cold temperature stress. This indicates high level of cold resilience within the population. The observed cold tolerance may have been inherited from the female parent (*Z. matrella*), which demonstrated significantly greater cold tolerance than the male parent (*Z. japonica*) during the winter weeks (Fig. 8). However, the single winter mortality of P4 could be attributed to environmental micro-variability, epigenetic factors, or differences in individual plant vigor. It is worth considering whether P4 had lower overall fitness or if specific environmental conditions contributed to its demise. One possibility is that P4 inherently had weaker vigor with insufficient root development, making it less capable of withstanding combined herbicide and cold stress.

Although there are significant variabilities in both GCP and NDVI among individual plants as illustrated in Figs. 6 and 7, all F_1 hybrid plants which showed herbicide resistance experienced a decline in GCP and NDVI until the end of November with some continuing to decrease until first week of December, 2023. This initial decline suggests a period of physiological stress as temperatures dropped. Cold stress adversely affects plant growth by impairing nutrient uptake, reducing photosynthetic efficiency, and disrupting hormone balance. These physiological disruptions result in stunted growth, premature leaf senescence, and decreased yield (Cook et al. 2004; Hinton et al. 2012). The temperature graph indicates that during this period, the average temperature remains consistently low with minimal fluctuations (Fig. 9). However, mid-December marked the beginning of a stabilization or recovery phase for many plants, with some even exhibiting a second dip in early January, likely due to further environmental fluctuations such as freezing. The daily average temperature reaches its lowest point and fluctuates around mid-December (Fig. 9). Also, the general upward NDVI trend in mid-to-late January suggests an overall improvement in vegetation health, likely as temperatures became more favorable. This is reinforcing

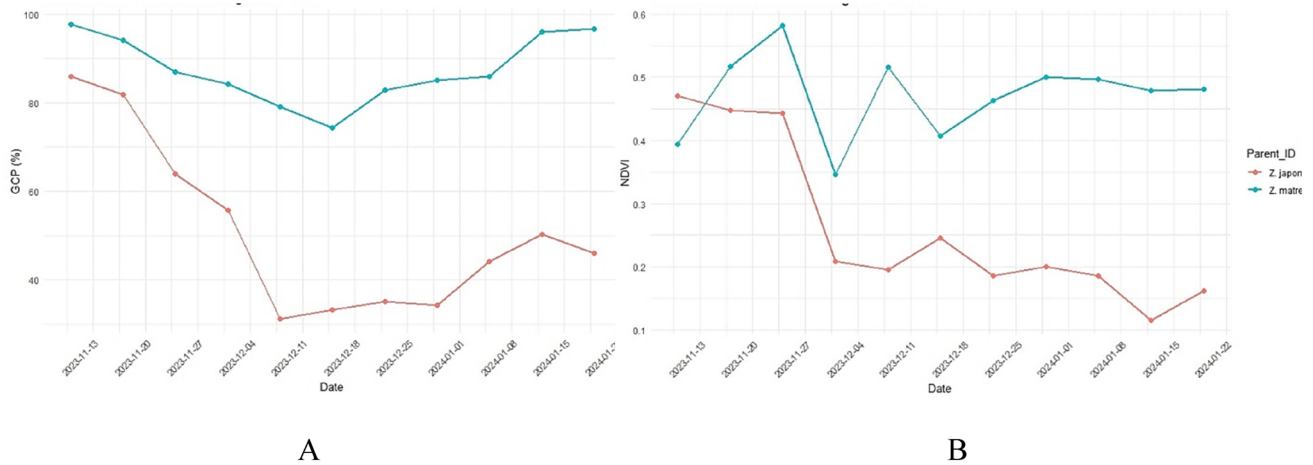
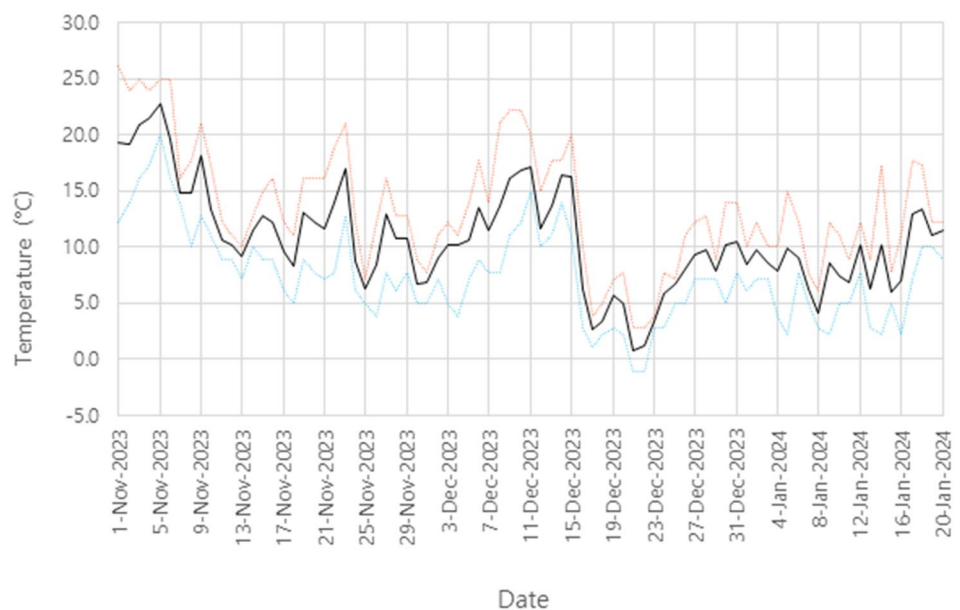


Fig. 8 Temporal variation in (A) Green Cover Percentage (GCP) and (B) Normalized Difference Vegetation Index (NDVI) of parent *Zoysia* plants during winter. The figure illustrates GCD and NDVI trends for

Zoysia matrella (blue line), female parent and *Zoysia japonica* (red line), male parent from November 3, 2022, to January 19, 2023

Fig. 9 Temperature graph presenting temperature data over a time period from November 1, 2023, to January 20, 2024. The x-axis represents the date, while the y-axis shows the temperature in degrees Celsius (°C). The black solid line represents the main average temperature trend over time. The red dotted line represents the upper temperature range (daily highs). The blue dotted line represents the lower temperature range (daily lows)



the hypothesis that the cold tolerance trait from *Z. matrella* was inherited.

Plants such as P26, P16, and P11 maintained relatively high and stable GCP levels as well as NDVI values throughout the winter, indicating a higher degree of cold tolerance. Interestingly, P11 exhibited a rapid increase in GCP during the final two weeks of the study, ultimately reaching the highest GCP among all individuals. This could suggest either superior adaptability to cold stress or a delayed compensatory growth phase following initial decline. On the other hand, plants like P13, P8, and P9 displayed consistently low GCP values, but their significant improvement by January suggests an ability to recover, albeit at a slower rate. However, mid-December marked the beginning of a stabilization or recovery phase for many plants, with some

even exhibiting a second dip in early January, likely due to further environmental fluctuations such as freezing. Numerous studies have demonstrated significant variation in cold tolerance among *Zoysia* species, with *Z. japonica* exhibiting greater cold tolerance than *Z. matrella* varieties nevertheless freezing tolerance varies widely among *Zoysia* genotypes (Dunn et al. 1999; Hinton et al. 2012; Patton et al. 2007; Patton and Reicher 2007; Toyama et al. 2003; Xuan et al. 2013). In contrast, between parental *Zoysia* germplasms in our study, *Z. matrella* germplasm exhibited greater cold tolerance than *Z. japonica*.

Building on genetic insights, our findings can be explained as follows. The genetic background of the hybrid F₁ *Zoysia* plants originates from cross-pollination between a cold-tolerant female parent, *Z. matrella* and an herbicide-resistant

male parent, *Z. japonica*. Our breeding strategy aimed to combine both desirable traits, herbicide resistance and cold tolerance, within the F_1 generation. The variability in herbicide tolerance observed among the F_1 hybrids can be attributed to genetic segregation and inheritance patterns. Since only 50% of the hybrid plants survived herbicide application, it suggests that herbicide resistance in this population may follow Mendelian inheritance, potentially as a single-gene trait. Depending on whether the resistance trait is dominant or recessive, the observed survival rate aligns with expected segregation ratios. If herbicide resistance is controlled by a single dominant allele (R), and the herbicide-sensitive allele (r) is recessive, a heterozygous (Rr) F_1 population would be expected to show 100% survival. However, if the resistance trait is recessive (rr for resistance and Rr or RR for sensitivity), then a 1:1 segregation ratio in the F_1 generation (50% resistant, 50% sensitive) would be expected, which is consistent with the observed survival rate. Furthermore, among the surviving plants, the retention of strong cold tolerance suggests that this trait may be inherited as a dominant or polygenic trait, ensuring its persistence in the F_1 hybrids. This indicates that while herbicide resistance may segregate, cold tolerance remains a stable trait passed down from the female parent. The results highlight the complex nature of trait inheritance in hybrid *Zoysia* populations, emphasizing the need for further genetic analysis to confirm the specific mode of inheritance.

Our findings highlight the potential for selecting dual-resistant hybrids, which could improve adaptability across diverse environmental conditions. Weed management is crucial concern in turfgrass industry, where turf managers often prefer efficient and cost-effective strategies (Neal 2024). Pre-emergence herbicides are commonly used to minimize damage to desirable turf varieties or species during their dormancy period in winter; however, they are expensive, time-consuming, and can affect post-winter green-up. In contrast, post-emergence herbicides are more affordable but often require multiple applications or herbicide mixtures to effectively eliminate unwanted weeds (Craft et al. 2023; Johnson and Carrow 1999; Lajmanovich et al. 2022). Environmental, ecological, and social constraints have led to increasing restrictions on the use of synthetic herbicides (Bae et al. 2008). However, the development of herbicide-resistant turfgrass allows for the precise and efficient application of non-selective post-emergence herbicides, reducing the overall chemical input required. Furthermore, improving cold tolerance and shortening the yellowing period remains a pressing challenge in turfgrass breeding (Xuan et al. 2013). Remarkably, the female parent in our study maintained higher GCP throughout the winter, exhibiting minimal visible effects. Thus, among the hybrid plants, P26, P17, P16, and P11 have shown strong potential for further

breeding efforts, as they maintained or increased their GCP over time. These individuals demonstrate both tolerance to cold climate and resistance to glufosinate ammonium, making them valuable candidates for future research and turfgrass.

This research will continue into the F_2 generation through the self-pollination of F_1 hybrid individuals to delve into their genetic background. Future studies should incorporate molecular analyses to identify resistance-associated genes and metabolic pathways. Additionally, further investigations into secondary stress tolerance mechanisms, such as cold resistance, will be essential for fully understanding the potential of these F_1 hybrids under diverse environmental conditions. This knowledge is important because it is required careful consideration before releasing transgenic plants or hybrids into natural ecosystems (Bae et al. 2008). Since all *Zoysia* grass species are sexually compatible, the potential environmental impact of transgenic plants must be assessed as the spread of herbicide resistance has already become a significant challenge in agronomic crops (Brosnan et al. 2020; Tsuruta et al. 2011). At the same time, the results of this study highlight the potential of UAV-based remote sensing metrics such as GCP and NDVI indices, for evaluating herbicide resistance in F_1 hybrids. These findings provide a foundation for refining selection strategies in breeding programs and advancing precision agriculture approaches for sustainable weed management. By leveraging image analysis, researchers can optimize breeding programs, refine herbicide application strategies, and ensure the long-term viability of herbicide-resistant varieties. The resulted higher GCP over 100%, may be due to the effects of environmental factors, such as variations in light intensity and soil conditions, which may have influenced plant responses. Additionally, when applying the ExG to RGB images, thresholding may have overestimated green pixel counts. This could explain instances where the recorded GCP exceeded 100%.

Conclusion

The development of resistant or tolerant *Zoysia* grass varieties or cultivars is crucial for the expand and efficient management of *Zoysia* lawns in various environmental conditions. This study aimed to screen *Zoysia* hybrids with both cold tolerance and herbicide resistance, and we successfully achieved this objective. We identified four F_1 hybrid individuals exhibiting superior tolerance to cold stress while maintaining resistance to herbicide (glufosinate ammonium Basta) application. The inheritance of these traits aligns with the genetic contributions of the parent plants. Cold tolerance was evidently inherited from the cold-resistant *Zoysia*

matrella germplasm (San208), while herbicide resistance was derived from the *Zoysia japonica* herbicide-resistant parent. To assess plant vigor and greenness, we effectively utilized multispectral image analysis (RGB image-based and NDVI-based evaluations), demonstrating the complementary advantages of these methods for turfgrass assessment. For further validation, additional analyses should be conducted, including the development of an F₂ generation through self-pollination of the F₁ hybrids and molecular genetic studies to identify the key genes associated with these traits. These findings provide a strong foundation for advancing *Zoysia* breeding programs and optimizing turfgrass selection strategies for enhanced resilience in various abiotic stresses.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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