

Article

Seed Germination and Responses of Five Native *Veronica* Species Under Salinity Stress in Korea

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Abstract: This study analyzed the seed germination characteristics and physiological responses of five Korean *Veronica* species (*V. daurica*, *V. nakaiana*, *V. kiusiana* var. *glabrifolia*, *V. pusanensis*, and *V. pyrethrina*) under salinity stress. Preliminary experiments on five *Veronica* species using various NaCl concentrations revealed that treatment with 150 mM NaCl almost completely inhibited seed germination, whereas treatment with >50 mM NaCl significantly decreased seed germination rate and index. Therefore, this study focused on the effects of treatment with 0, 50, and 100 mM NaCl for 7 days on the germination rate, germination index, germination energy, germination vigor index, water content, fresh weight, dry weight, and root length of the plants. When treated with 100 mM NaCl, most species had few survivors after 5 days, even if germination had occurred. Almost all parameters significantly decreased with increasing NaCl concentration. Especially, fresh weight and water content decreased with increasing NaCl concentration, while dry weight did not show a significant response to NaCl concentration, suggesting that salinity stress inhibited water uptake, which is crucial for seed germination. Hormonal analysis revealed the presence of indole-3-acetic acid (IAA) and abscisic acid (ABA) and the absence of gibberellic acid. Most species showed no significant changes in IAA and ABA levels with varying NaCl concentrations. However, *V. pusanensis* showed significantly increased ABA levels with increasing NaCl concentration, and *V. daurica* showed significantly higher IAA levels at 100 mM NaCl than at other NaCl concentrations. This study demonstrates that salt stress negatively affects *Veronica* seed germination, with varying intensities among species.

Keywords: hormonal analysis; NaCl; salinity stress; salt tolerance; seed germination; *Veronica*



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1. Introduction

In Korea, many reclaimed lands have been established along the southwestern coastal regions, and soil salinization is worsening due to rising sea levels caused by climate change [1,2]. Additionally, various scale gardens are being developed in these reclaimed areas, making it necessary to investigate the salt stress tolerance of domestic garden plants.

Salinity stress is an important abiotic factor that negatively affects the growth and development of plants worldwide [3]. Approximately 19.5% of all irrigated lands and 2.1% of dry lands are affected by salinity stress [4,5]. Salinization occurs in urban areas, gardens, and arid and semi-arid regions because of overdoses of deicer and fertilizer and irrigation of reclaimed water, damaging plant ecology [6–8]. Soil salinization is intensified by high temperatures and increased evaporation caused by climate change [9]. Salinity

stress causes osmotic stress, ion stress, and mineral deficit, which damage the physiological and biochemical systems of plants; consequently, this stress inhibits plant growth and development [10,11], reduces ornamental value by destroying chlorophyll [12,13], and promotes reactive oxygen species production [14]. Oxidative stress induced by reactive oxygen species causes defects in cell membranes, nucleic acids, and cell structures, thereby disrupting normal plant growth [15].

Various stages of the plant life cycle are affected by salinity stress, but seed germination and seedling development are particularly sensitive [16]. Seed germination and seedling development are crucial for successful plant establishment [17]. Resistance to salinity stress at these stages suggests a plant's adaptability to saline soil [18,19]. Studying salinity stress responses during these stages can help predict plant growth responses to salt [3,16,20,21]. It also aids in identifying resistance factors and developing salt-tolerant cultivars [3,22–25]. Salinity stress significantly reduces germination rates (GRs), seedling growth and establishment, and root and shoot development [26,27]. Seed germination and seedling development involve complex pathways that are regulated by numerous hormones and proteins. For instance, seed germination is promoted by hormones such as gibberellic acid (GA), ethylene, cytokinins, and brassinosteroids (BRs) but inhibited by abscisic acid (ABA) [28]. Increased ABA levels help plants adapt to stress [29,30]. A key factor regulating germination is the balance between ABA and GA [31–34]. Salinity stress directly and indirectly disrupts these pathways, thereby suppressing germination and seedling development [35].

Previous studies explored how individual hormones affect seed germination and development under salinity stress. For instance, auxin accumulation and distribution are altered by salinity stress, causing root structure changes and growth reduction [35–37]. Therefore, the auxin signaling pathway is closely related to salinity stress [38], and pre- and post-treatment with indole-3-acetic acid or α -naphthaleneacetic acid mitigates the negative effects of salinity stress on seed germination and seedling establishment [39]. Additionally, treatments with GA [40] and BR [41] significantly improve germination, whereas ethylene enhances salt tolerance during germination and development [42,43].

Veronica belongs to Plantaginaceae and includes about 500 species worldwide [44], with 17 native species in Korea. Recently in Korea, the genus *Veronica* was changed to *Pseudolysimachion* belongs to Scrophulariaceae [45]. In this paper, it is written as *Veronica* according to the internationally accepted classification system. *Veronica* species are perennials valued for their horticultural potential owing to their diverse flower colors, extended flowering period, and easy maintenance [46]. From 2018 to 2019, *Veronica* species saw an eightfold increase in trade volume and a 50% increase in price [47]. Despite the horticultural significance of *Veronica* species, most studies focused on their antioxidant potential, and few analyzed their stress tolerance [48–50]. Thus, the present study investigated how salt stress affects seed germination and seedling development in five native *Veronica* species: *V. daurica*, *V. pusanensis*, *V. nakaiana*, *V. pyrethrina*, and *V. kiusiana* var. *glabrifolia*. Especially, we attempted hormone analysis with LC/MS-MS, which was difficult to perform due to the small size of the seed of *Veronica*. We set the following hypothesis: (1) As the concentration of NaCl treatment increases, the germination of *Veronica* seeds will be adversely affected; (2) there will be common and significant NaCl concentration that affects the seed germination of five *Veronica* species; and (3) NaCl treatment will cause changes in the endogenous hormones of the seeds of *Veronica*. The results of this study may provide basic data on seed salt stress for breeding salt-tolerant varieties of *Veronica*, which is widely distributed and useful worldwide, but has had limited research on salt tolerance.

2. Results

2.1. Preliminary Experiment for Determining NaCl Concentration

To determine the specific NaCl concentration that induces salinity stress responses in the seeds of the five *Veronica* species, we measured the GR and GI under various NaCl concentrations (Figure 1). Although the responses varied among the species, both GR and GI decreased with increasing NaCl concentration. A significant difference was observed at the NaCl concentration of 100 mM compared with the control group. However, germination was not observed in any species at relatively high NaCl concentrations of 150 mM and above. Specifically, germination was not observed in *V. nakaiana* and *V. pyrethrina* even at a lower concentration of 150 mM. Furthermore, the average GR compared to the control group showed decreases of 45.5, 13.8, 8.4, 49.2, and 38.2% in *V. daurica*, *V. nakaiana*, *V. kiusanum* var. *glabrifolia*, *V. pusanensis*, and *V. pyrethrina*, respectively, at a NaCl concentration of 50 mM, albeit not significantly for all species. Therefore, the NaCl concentration for investigating the effects of salinity stress on germination was determined to be 100 mM NaCl. An additional investigation of seed responses was conducted using 50 mM NaCl to observe the variation in responses.

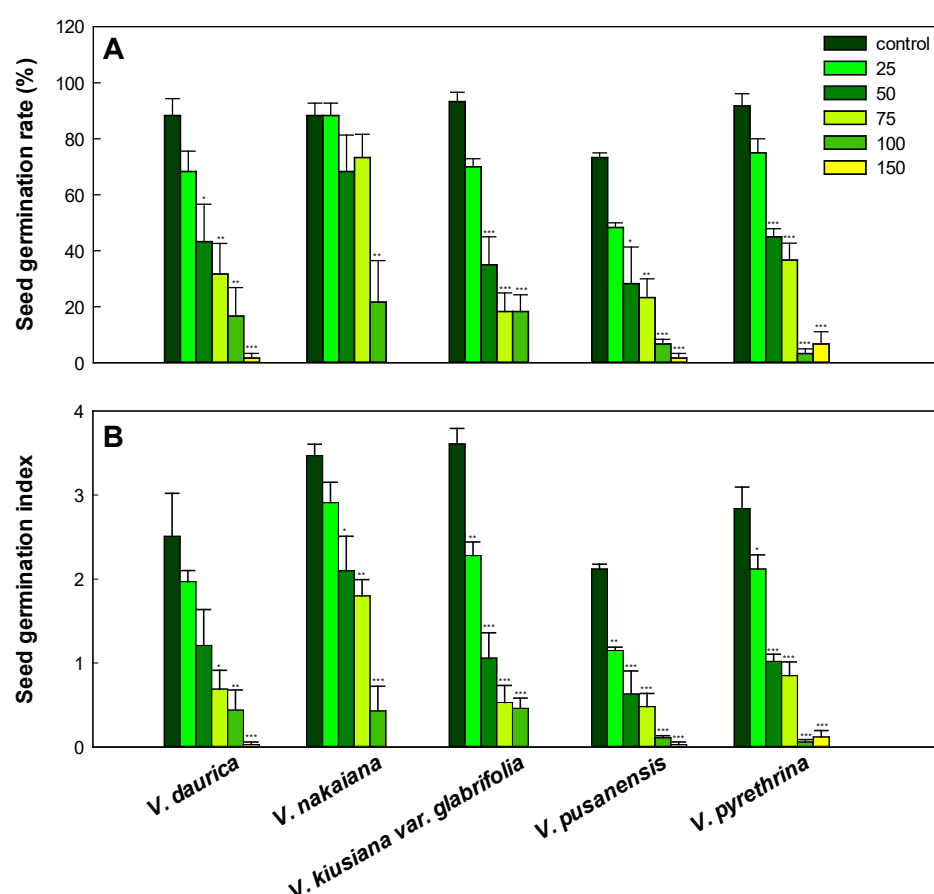


Figure 1. Seed germination rate (A) and germination index (B) of *Veronica daurica*, *V. nakaiana*, *V. kiusiana* var. *glabrifolia*, *V. pusanensis*, and *V. pyrethrina* treated with different NaCl concentrations for 14 days. Vertical bars represent the standard error. *, **, and *** indicate significant differences at $p < 0.05$, 0.01, and 0.001, respectively, between the control and treatment groups.

2.2. Germination Experiment

The GR, GI, GE, GVI, WC, FW, DW, and RL of seeds from the five *Veronica* species treated for 7 days with NaCl concentrations of 0, 50, and 100 mM were investigated (Table 1). The G_p , which is used in calculating GE, is G_4 and G_5 in *V. nakainaum*, *V. kiusiana*

var. *glabrifolia*, and *V. pyrethrina* and in *V. daurica* and *V. pusanensis*, respectively. The RL data from the 100 mM NaCl treatment groups were excluded from further analysis because in the 100 mM NaCl treatment group, almost no individuals from any species survived beyond 5 days post-germination. The analysis revealed that NaCl treatment significantly affected most parameters, except for DW, which showed no significant effect. Notably, the GI decreased significantly ($p < 0.001$) across all species with increasing NaCl concentration.

Table 1. Results of the germination experiment of seeds of five Veronica species treated with 0, 50, and 100 mM NaCl.

Species	NaCl Concentration (mM)	Germination Rate (%)	Germination Index	Germination Energy	Germination Vigor Index
<i>V. daurica</i>	0	76.2 ± 4.55 ^z a ^y	10.6 ± 0.94 a	41.6 ± 4.80 a	5.8 ± 1.04 a
	50	41.5 ± 2.15 b	4.6 ± 0.77 b	11.2 ± 3.14 b	1.4 ± 0.36 b
	100	7.9 ± 1.0 c	0.4 ± 0.05 c	0.0 ± 0.00 b	0.1 ± 0.01 b
	Significance	***	***	***	**
<i>V. nakaiana</i>	0	95.8 ± 1.04 a	21.4 ± 0.94 a	23.0 ± 3.15 a	15.7 ± 1.22 a
	50	82.6 ± 4.83 a	9.2 ± 1.43 b	6.5 ± 3.28 b	3.4 ± 0.94 b
	100	8.2 ± 4.52 b	0.7 ± 0.35 c	0.0 ± 0.00 b	0.1 ± 0.07 b
	Significance	***	***	***	***
<i>V. kiusiana</i> var. <i>glabrifolia</i>	0	65.9 ± 7.13 a	14.2 ± 1.26 a	51.3 ± 7.42 a	10.9 ± 2.87 a
	50	60.3 ± 3.18 a	6.2 ± 0.22 b	6.8 ± 0.91 b	3.2 ± 0.22 b
	100	14.0 ± 1.61 b	1.1 ± 0.16 c	0.0 ± 0.00 b	0.3 ± 0.05 b
	Significance	***	***	***	*
<i>V. pusanensis</i>	0	42.7 ± 1.78 a	5.9 ± 0.18 a	23.0 ± 1.82 a	2.5 ± 0.02 a
	50	21.7 ± 6.13 b	2.4 ± 0.48 b	6.5 ± 1.89 b	0.7 ± 0.17 b
	100	6.4 ± 1.74 b	0.5 ± 0.18 c	0.0 ± 0.00 c	0.1 ± 0.04 c
	Significance	**	***	***	***
<i>V. pyrethrina</i>	0	78.1 ± 6.95 a	11.6 ± 1.19 a	26.0 ± 5.85 a	7.8 ± 1.56 a
	50	48.3 ± 4.81 b	4.6 ± 0.76 b	4.4 ± 2.94 b	2.0 ± 0.50 b
	100	6.5 ± 4.00 c	0.5 ± 0.26 c	0.0 ± 0.00 b	0.1 ± 0.08 b
	Significance	***	***	**	**
Species	NaCl Concentration (mM)	Water Content (%)	Fresh Weight (mg)	Dry Weight (mg)	Root Length (cm)
<i>V. daurica</i>	0	78.6 ± 1.96 a	0.54 ± 0.034 a	0.11 ± 0.003 a	1.66 ± 0.038
	50	65.6 ± 0.62 b	0.29 ± 0.019 b	0.10 ± 0.007 a	0.38 ± 0.049
	100	54.0 ± 2.37 c	0.21 ± 0.004 b	0.10 ± 0.004 a	- ^x
	Significance	***	**	ns	***
<i>V. nakaiana</i>	0	87.1 ± 0.78 a	0.73 ± 0.014 a	0.09 ± 0.002 a	1.55 ± 0.321
	50	75.9 ± 4.24 a	0.35 ± 0.033 b	0.08 ± 0.001 a	0.43 ± 0.009
	100	46.4 ± 1.39 b	0.16 ± 0.010 c	0.08 ± 0.004 a	-
	Significance	***	***	ns	*
<i>V. kiusiana</i> var. <i>glabrifolia</i>	0	84.0 ± 1.65 a	0.74 ± 0.076 a	0.07 ± 0.020 a	0.83 ± 0.020
	50	75.6 ± 1.87 b	0.51 ± 0.022 a	0.12 ± 0.004 a	0.39 ± 0.108
	100	59.6 ± 0.31 c	0.31 ± 0.008 b	0.13 ± 0.003 a	-
	Significance	***	*	ns	*
<i>V. pusanensis</i>	0	73.6 ± 1.37 a	0.42 ± 0.007 a	0.11 ± 0.005 a	1.34 ± 0.094
	50	57.2 ± 4.56 b	0.28 ± 0.011 b	0.12 ± 0.003 a	0.32 ± 0.014
	100	53.1 ± 1.50 b	0.22 ± 0.002 c	0.11 ± 0.003 a	-
	Significance	**	***	ns	***
<i>V. pyrethrina</i>	0	78.2 ± 1.71 a	0.66 ± 0.035 a	0.14 ± 0.004 a	1.75 ± 0.088
	50	76.0 ± 4.03 a	0.44 ± 0.021 b	0.10 ± 0.009 a	0.60 ± 0.074
	100	51.2 ± 1.74 b	0.29 ± 0.003 c	0.14 ± 0.004 a	-
	Significance	**	**	ns	**

^z Values represent standard error (n = 3). ^y Different letters in the table indicate significant difference by Duncan's multiple range test, $p \leq 0.05$ (n = 3). ^x No data are available for this treatment group as all individuals withered. ns, *, **, and *** indicate non-significance or significance at $p \leq 0.05$, 0.01, and 0.001, respectively, by ANOVA (n = 3).

2.3. Hormonal Analysis

Hormonal analysis revealed the presence of IAA and ABA and the absence of GA3. For most species, neither IAA nor ABA levels showed a significant difference across NaCl concentrations (Figure 2). However, *V. pusanensis* exhibited significantly higher ABA levels with increasing NaCl concentration, and *V. daurica* showed significantly higher IAA levels at 100 mM NaCl.

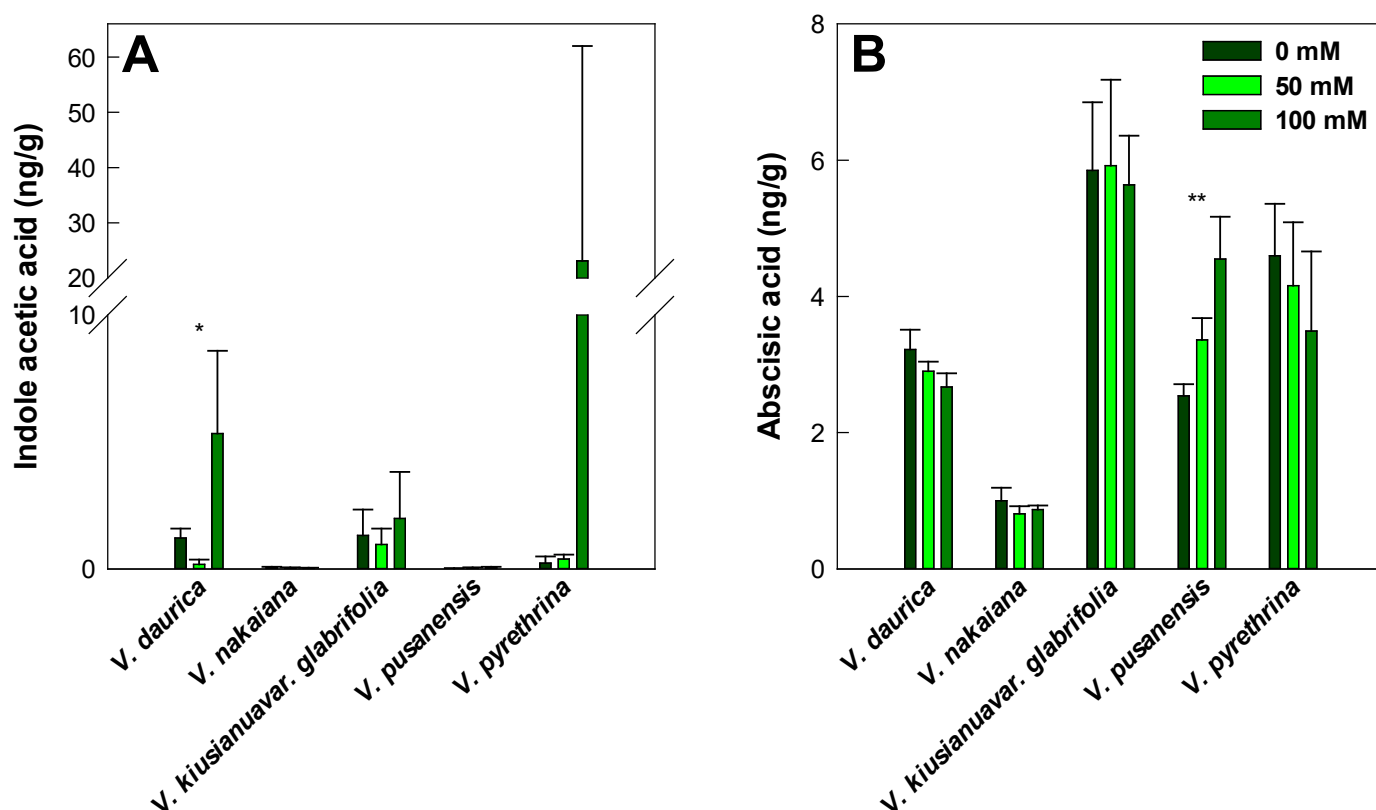


Figure 2. Endogenous indole-3-acetic acid content (A) and abscisic acid content (B) of *Veronica daurica*, *V. nakaiana*, *V. kiusiana* var. *glabrifolia*, *V. pusanensis*, and *V. pyrethrina* treated with 0, 50, and 100 mM NaCl on days 4–5. Vertical bars represent the standard error. * and ** indicated significant differences at $p < 0.05$, and 0.01 respectively.

3. Discussion

Germination experiments indicated that the GIs for all five *Veronica* species decreased with increasing NaCl concentration, except for DW. Although significant differences in GR were observed between the 100 mM NaCl treatment groups and the control group, the GRs of *V. nakaiana* and *V. kiusiana* var. *glabrifolia* treated with 50 mM NaCl did not differ significantly from that of the control. However, the GI, the parameter most significantly affected by NaCl concentration, indicated a prolonged germination time compared with the control. This finding suggests that NaCl-induced salinity stress inhibits water uptake in plant cells and causes ion imbalance, which reduces seed GR and vigor [10]. This conclusion is supported by the significant differences in the GVI, FW, and WC across NaCl concentrations despite the absence of a significant difference in DW.

Similar to other stress responses, salinity stress tolerance in plants is mediated by the complex coordination of various signaling pathways, including stress-recognizing sensor proteins, signaling transducers, transcription factors, and stress-responsive genes and metabolites [20,26]. In the present study, we investigated the effects of salinity stress on seed internal hormone levels beyond germination indices to identify salinity stress-related factors, such as transcription factors and proteins. However, the hormonal analysis results were inconsistent across species and NaCl concentrations. GA induces the synthesis of α -amylase in embryoless seeds [51]. Similar to GA, IAA promotes starch degradation during seed germination and regulates root growth in seedlings [52]. These two phytohormones positively affect seed germination and seedling growth and development, and their levels decrease under salinity stress [31,53–55]. Meanwhile, ABA facilitates plant acclimation to stressful conditions, and exposure to salt stress increases endogenous ABA levels [20,56,57].

The absence of detectable GA, the relatively high IAA levels in the 100 mM NaCl treatment group, and the increase in ABA levels with increasing NaCl concentration in *V. pusanensis* remain unexplained by our hormonal analysis. Errors in the timing of the hormonal analysis may have occurred. We hypothesized that endogenous hormone production would be highest during the peak germination period. However, the limitations of our hormonal analysis suggest the need for further investigations into the temporal dynamics of endogenous hormone levels in NaCl-treated seeds.

This study aimed to gather fundamental information for breeding salt-tolerant *Veronica* cultivars. Although this study investigated various indicators of salt stress response to identify the most influential factors and elucidate the underlying mechanisms, consistent correlations between all measured indicators and salt stress response were not found. Nevertheless, the significant correlation between the GI and NaCl concentration across all species aligns with the findings of Li et al. [58]. Therefore, further research should identify reliable indicators associated with the GI for a more efficient assessment of salt stress response and salt tolerance and investigate temporal changes in hormone, protein, and transcription factor levels.

4. Materials and Methods

4.1. Plant Materials

Seeds of the five *Veronica* species were obtained from the Useful Plant Propagation Center and gathered from Namyang Valley (Table 2). The seeds had been stored immediately within one month from the date of gathering in the refrigerator at 5 °C. Seeds with dormancy broken and high germination rates (GRs) were selected under optimal germination conditions to ensure accurate assessment of salinity stress responses. The optimal germination condition of the native *Veronica* in Korea is a 25/20 °C day/night temperature cycle with a 16/8 h light/dark photoperiod [59].

Table 2. Scientific name, source, and date of seed gathering of the five *Veronica* species used in this study.

Scientific Name	Source	Date of Seed Gathering
<i>Veronica daurica</i>	Useful Plant Propagation Center (Dudam-gil, Yangpyeong, Republic of Korea)	1 November 2018
<i>Veronica nakaiana</i>	Namyang valley (Namyang-gil, Ulleung island, Republic of Korea)	16 October 2016
<i>Veronica kiusiana</i> var. <i>galbrifolia</i>	Useful Plant Propagation Center (Dudam-gil, Yangpyeong, Republic of Korea)	31 October 2016
<i>Veronica pusanensis</i>	Useful Plant Propagation Center (Dudam-gil, Yangpyeong, Republic of Korea)	20 October 2020
<i>Veronica pyrethrina</i>	Useful Plant Propagation Center (Dudam-gil, Yangpyeong, Republic of Korea)	1 November 2018

4.2. Preliminary Experiment for Determining NaCl Concentration

A preliminary experiment was conducted to determine the specific NaCl concentration for inducing salinity stress. Seeds were surface-sterilized by soaking in 10% NaOCl₂ solution for 1 min, rinsed three times with distilled water. Maximum salt tolerance of halophytes and glycophytes at the seed germination stage has been reported to vary from 1.7 to 0.3 M and from 0.25 to 0.05 M NaCl, depending on species and other environmental conditions such as temperature, moisture, and light [60]. Salt tolerance at the seed germination stages of *Veronica* has not been studied, and the native environment of *V. nakaiana* and *V. pusanensis* is Ulleung Island and the coasts in Busan in Korea, respectively. Therefore, a wide range of NaCl concentrations needed to be treated. Seeds were exposed to 0, 25, 50, 75,

100, 150, 200, 300, and 400 mM NaCl. Twenty seeds were placed on two layers of filter paper in a 9 cm-diameter Petri dish. Germination tests were performed at 25/20 °C day/night temperature cycle with a 16/8 h light/dark photoperiod for 2 weeks, and the GR and germination index (GI) were calculated for each treatment using the following formulas:

$$GR = \frac{G_{14}}{N} \times 100\% \quad (1)$$

$$GI = \sum \frac{G_t}{T}; \quad (2)$$

where G_{14} is the number of seeds germinated on day 14, N is the total number of seeds, T is the number of days after sowing, and G_t is the number of seeds germinated on day T .

4.3. Germination Experiment

On the basis of the preliminary experiment results, 0, 50, and 100 mM were selected as the NaCl concentrations for further analyses. Thirty seeds were placed on each Petri dish, and the experiment was conducted in triplicate. Parameters such as GR, GI, germination energy (GE), germination vigor index (GVI), water content (WC), and root length (RL) were measured over 7 days. GE, GVI, and WC were calculated as follows:

$$GE = \frac{G_p}{N} \times 100\% \quad (3)$$

$$GVI = \sum \frac{G_t}{T} \times AFW; \quad (4)$$

$$WC = \frac{FW - DW}{FW} \times 100\% \quad (5)$$

where G_p is the number of seeds germinated on the day with the highest germination, N is the total number of seeds, G_t is the number of seeds germinated on day T , AFW is the average fresh weight, FW is the fresh weight, and DW is the dry weight.

FW was measured immediately after the experiment concluded, and dry weight was measured after drying the samples at 70 °C for at least 72 h. RL was determined by sowing 20 seeds per treatment and measuring them 5 days after germination.

4.4. Hormonal Analysis

4.4.1. Preparation of Seed Samples

Germination, a period of high hormonal activity, is most active at a specific point during germination. Germination experiments showed that relative to the control group, *V. nakaiana*, *V. pyrethrina*, and *V. kiusiana* var. *glabrifolia* exhibited peak germination on day 4 post-sowing, whereas *V. daurica* and *V. pusanensis* showed peak germination on day 5. Therefore, hormonal analysis was conducted on days 4 and 5 using ≥ 5 g of seeds per Petri dish for each species treated with 0, 50, and 100 mM NaCl.

4.4.2. Chemicals and Reagents

Analytical-grade 1H-indole-3-acetic acid (HPLC $\geq 99\%$), gibberellin A3 (HPLC $\geq 99\%$), and abscisic acid (HPLC $\geq 98\%$) and formic acid, acetonitrile, methanol, and ultrapure water were purchased from Thermo Fisher Scientific Inc. (Massachusetts, USA). All mobile phase manufacturing reagents were of LC-MS/MS grade.

4.4.3. Preparation of Standard Solutions

Primary stock solutions of daidzin, daidzein, genistin, genistein, and glycitein were prepared in DMSO to obtain a nominal concentration of 20 mg/mL (20,000 ppm). Individ-

ual sub-stocks were prepared at 1 mg/mL (1000 ppm) in DMSO, and dilutions of these sub-stocks of 20 ppm (20 µg/mL, in 70% methanol) were used to extract the optimized multiple reaction monitoring (MRM) transition for the MRM method of LC-MS/MS analysis. Each diluted sub-stock (20 ppm, 70% methanol) was mixed and serially diluted to prepare the mixture working solution and obtain a calibration curve ranging from 1 ng/mL to 4000 ng/mL. The LOQ values were acquired at 10 ppb for all isoflavones.

4.4.4. Preparation of Test Solutions

All seeds were ground using a ball mill. Seed meal powders (0.1 g) were mixed with 10 mL of 70% aqueous methanol followed by serial extraction via shaking for 1.5 min at room temperature by AGIA200 (Collomix, Milwaukee, WI, USA), sonication at 40 kHz for 60 min at 45 °C by CPX8800H-E (Branson, Danbury, CO, USA), and shaking for 1.5 min at room temperature. After extraction, each sample was centrifuged at 17,000 rpm for 1 min. Subsequently, 100 µL of supernatant was mixed with 900 µL of 70% methanol, vortexed for 30 s, and then re-centrifuged at 17,000 rpm (Dilution factor = 10×). The diluted sample solutions were filtered through a 0.22 µm PVDF membrane (Thermo Fisher Scientific, Waltham, MA, USA) for LC-MS/MS.

4.4.5. Analytical Conditions

The amounts of daidzin, daidzein, genistin, genistein, and glycitein in the sample extracts from soybean meal powders were qualified and quantified by LC-MS/MS (Agilent 1290 Infinity II liquid chromatography and 6470 series triple quadrupole tandem mass spectrometer). The mass spectrometer was operated under the negative electrospray ionization (ESI) mode under MS/MS conditions, and MS spectrum data were acquired under MRM mode consisting of the following transitions from negative ESI: (precursor ion m/z → product ion m/z) 415.1 → 252 (CE = 28 eV), 415.1 → 195 (CE = 72 eV) on 177 V of fragmentor for daidzin; 253.1 → 223 (CE = 36 eV), 253.1 → 132 (CE = 44 eV) on 162 V of fragmentor for daidzein; 431.1 → 269 (CE = 16 eV), 431.1 → 239 (CE = 48 eV) on 162 V of fragmentor for genistin; 269.0 → 133 (CE = 36 eV), 269.0 → 63.1 (CE = 40 eV) on 147 V of fragmentor for genistein; and 283.06 → 268 (CE = 16 eV), 283.06 → 211 (CE = 36 eV) on 122 V of fragmentor for glycitein. The conditions of ESI MS/MS settings were as follows: negative electrospray capillary voltage, 3500 V (4146 nA); nozzle voltage, 500 V; chamber current, 0.61 µA; gas temperature, 300 °C; gas flow, 5 L/min; sheath gas temperature, 250 °C; sheath gas flow, 11 L/min; nebulizer, 45 psi. Isoflavones (daidzin, daidzein, genistin, genistein, and glycitein) were separated on an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm; Waters, Milford, USA) at 35 °C, and the mobile phase consisted of distilled water (A) and acetonitrile (B) each containing 0.1% formic acid at a flow rate of 0.34 mL/min. A 3 µL aliquot of the sample was injected from an autosampler maintained at 7 °C, and the total running time was 10 min for analysis. The retention times of each peak on the total ion chromatogram were 1.248 min (nicotine) and 0.766 min (nornicotine). The gradient elution conditions were as follows: mobile phase B (acetonitrile + 0.1% formic acid) was set to 7% at 0 min and maintained for 3 min; a linear gradient from 7% to 100% mobile phase B for 4 min; and an isocratic mode of 100% mobile phase B from 4 min to 7 min. Subsequently, the initial condition (7% acetonitrile + 0.1% formic acid) was maintained from 7.1 min to 10 min.

Data acquisition was performed using Agilent MassHunter Workstation software (LC/MS Data Acquisition for 6470 Series Triple Quadrupole, Version B.08.02), and quantitation was performed using Agilent MassHunter Workstation software (Quantitative Analysis for QQQ, Version B.08.00).

4.5. Statistical Analysis

The germination rate and germination index of each treatment group from the preliminary experiment were averaged from 20 seeds per replicate and compared with the control group using Student's *t*-test. In the germination experiment, the germination rate, germination index, germination energy, germination vigor index, fresh weight, dry weight, water content, and root length were averaged from 30 seeds per replicate and statistically analyzed using Duncan's multiple range test ($p < 0.05$) and analysis of variance (ANOVA). Statistical analyses were performed using the SPSS Statistics program (IBM SPSS Statistics 20, IBM Co., Armonk, NY, USA).

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Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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