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Optimum Sterilization Method for In Vitro Cultivation of Dimorphic Seeds of the Succulent Halophyte *Suaeda aralocaspica*

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Abstract: *Suaeda aralocaspica* is an annual halophyte in the Amaranthaceae in the saline deserts of central Asia. This plant has succulent leaves and grape-like fruits and is a potential horticultural plant. To obtain the efficient sterilization method and optimal culture conditions, two types of seeds produced from a single plant of *S. aralocaspica* were treated with 75% ethanol for different time durations first, and then sodium hypochlorite (NaClO) or mercury chloride ($HgCl_2$), with five different timing treatments were used for second seed surface sterilization. Sterilized seeds were germinated on a Murashige and Skoog (MS) medium at different potential hydrogenation (pH) levels, to examine germination and seedling performance. The results showed that the highest germination percentage of brown seeds was 100% and that of black seeds was 17%. Thus, brown seeds were more suitable for further culture experiments than black seeds. For brown seeds, the sterilization effect of NaClO was better than that of $HgCl_2$, based on the results of seed germination, contamination, and seedling survival. Rinsing with 75% ethanol for 60 s, sterilizing with NaClO for 8 min, and cultivating at pH 8.0 MS for 7 days was the best of all sterilization procedures and cultivation methods tested, which has been successfully applied to *S. aralocaspica* in vitro culture. The optimized protocol described here can be used as the reference for the *Suaeda* genus.

Keywords: sodium hypochlorite; mercury chloride; sterilization time; seed germination

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1. Introduction

Suaeda aralocaspica is an annual halophyte (Amaranthaceae), with succulent leaves and grape-like fruits, distributed in the saline deserts of central Asia. In China, this plant is mainly distributed in the southern margin of the Junggar Basin in Xinjiang [1–3]. The study of *S. aralocaspica* is mainly focused on the leaf morphology and anatomical structure [4,5], germination characteristics [6–9], and photosynthetic type [10]. *S. aralocaspica* is a single-cell C_4 photosynthetic plant without Kranz anatomy and has two types of chloroplast, called 'Borszczowioid type' [10,11]. This plant can produce two distinct types of seeds on a single plant, which have obvious differences in seed coat color, seed size, dormancy and germination characteristics. The brown seeds have high salt tolerance and are not dormant, while the black seeds have low salt tolerance and non-deep physiological dormancy [12–14]. However, there is no significant difference in growth, mineral nutrient content, and salt tolerance at middle and late growth stages [12]. Besides, short time pre-soaking, with a low concentration of abscisic acid (ABA), promotes the germination and seedling growth of dimorphic seeds of *S. aralocaspica* [15].

There are few molecular studies about *S. aralocaspica*. Compared with four traditionally used reference genes, *GAPDH* and *β-TUB* are stable internal reference genes and more suitable for the subsequent gene research of *S. aralocaspica* under different experimental conditions [16]. The results of KEGG enrichment and gene expression analysis reveal that specific genes and miRNAs are regulated differently between black and brown seeds during germination, which may contribute to the different germination behaviors of dimorphic seeds of *S. aralocaspica* in unpredictable environments [17]. The sequencing and assembly of the *S. aralocaspica* whole genome are finished and the length is 425 Mb. In addition, a complete chloroplast genome is also assembled. *S. aralocaspica* is the first sequenced halophyte and single-cell C_4 plant [18].

Suaeda. aralocaspica can grow normally in typical saline soils (salt content of the topsoil exceeds 10%). Brown seeds can germinate at high salinity (over 1000 mmol/L NaCl). This plant is valuable for the study of salt tolerance, C_4 photosynthesis without Kranz anatomy, and seed heteromorphism. However, this plant is limited to saline deserts in central Asia, and the field sampling is also restricted by the season and the low density of persistent soil seed bank. These factors influence the supplement of *S. aralocaspica* material for experimental research. Thus, an efficient seed sterilization and culture method is essential for the effective supply of this research material.

Obtaining high-quality sterile seeds and seedlings is affected by various factors, such as the type of disinfectant, the time duration of sterilization, the pH of the medium, etc. [19]. Common surface disinfectants include ethanol, sodium hypochlorite, hydrogen peroxide, and mercury chloride [20–22]. As a commonly used medical disinfectant, 70–75% ethanol inactivates some bacteria by infiltrating through their cell membranes to denature various proteins. The bactericidal effect of 75% ethanol, when used together with other disinfectants, is better than that of using only ethanol as a disinfectant [22]. Mung bean seeds are disinfected with 75% ethanol for 30 s and then disinfected with 1.0% NaClO for 10 min [23]. *Salicornia europaea* seeds are treated with different concentrations of sodium hypochlorite (NaClO), mercuric chloride ($HgCl_2$), and hydrogen peroxide (H_2O_2) on a Murashige and Skoog (MS) medium, with different concentrations of the hormone. The optimal sterilization effect of *S. europaea* seeds is after they have been treated with mercuric chloride, with a quality fraction of 0.1% for 10–20 min [24].

Seed sterilization and aseptic seedling cultivation have played a crucial role in subsequent research. Sterile seedlings are the source of explants in the tissue culture system. However, the sterilization methods of *S. aralocaspica* seeds have not been reported. We hypothesized that dimorphic seeds had distinct responses to disinfectants, sterilization time, and pH value of the mediums. Therefore, the present study was conducted to compare different sterilizing protocols, employed for different types of seeds in vitro culture, and to find out the best and most efficient sterilization procedure, based on germination, contamination, and seedling survival, which can be used for the rapid propagation system of *S. aralocaspica*.

2. Materials and Methods

2.1. Seed and Pretreatment

Freshly matured fruits of *S. aralocaspica* were collected from Fukang, Xinjiang in October 2020. All fruits were manually rubbed to remove the fruit coat. Brown seeds and black seeds were hand-sorted before sterilization to ensure uniformity in type. The seeds were rinsed using running water for 30 s to remove the impurities and then dried naturally in the laboratory. Every group of 50 seeds was packed in a 2 mL Eppendorf tube for further use.

2.2. Preparation of Reagents

Precisely measured 36 mL 10% NaClO solution and 64 mL sterilized double distilled H_2O (ddH_2O) were poured into a 150 mL sterile conical flask. Then the mixture was the required solution (100 mL 3.6% NaClO) for this experiment. Accurately weighed 0.1 g $HgCl_2$ reagent powders (Analysis pure) using a calibrated and zeroed electronic balance were poured into a 100 mL sterile beaker. We slowly poured a small amount of sterile ddH_2O and stirred with a glass stick until the powder dissolved completely, and then poured the solution into a 100 mL volumetric flask. Next, took a small amount of the new sterile water rinse beaker and glass stirring bar, and combined the rinse solution into the volumetric flask. Repeated the rinsing step 3–4 times (the total volume of the liquid should be less than 100 mL). Finally, added an appropriate amount (depending on the situation) of sterile water to a constant volume of 100 mL. At this point, the 100 mL solution in the 100 mL volumetric flask was the 0.1% $HgCl_2$ solution required for the experiment. MS medium was poured into a disposable sterile bacterial petri dish of 90 mm (d) × 15 mm (h) after autoclave sterilization and solidified into a flat plate at room temperature. The preparation of all the above reagents was completed in an ultra-clean workbench. It is worth noting that mercuric chloride is toxic, so we should be careful when configuring and using it. Besides, adjusting the pH values of MS medium to 5.0, 6.0, 7.0, 8.0, 9.0 respectively was necessary before autoclave sterilization.

2.3. Sterilization and Germination Procedure

Both types of seeds were first sterilized with 75% ethanol, with a treatment time duration of 30 s, 1 min, 3 min, 5 min, and 8 min. Then seeds were rinsed with sterile distilled water 3 times. Following this, 3.6% NaClO or 0.1% $HgCl_2$ was selected as the secondary sterilizing agent. The soaking time duration of 3.6% NaClO was set to five gradients, including 3, 5, 8, 11, and 15 min and that of 0.1% $HgCl_2$ was also set to five gradients, including 1, 3, 5, 8, and 11 min. Then seeds were cultured on MS mediums with different pH values (Table 1). According to Orthogonal Table L25 (5^6), 3 columns were used in the test. The sterilization experiment for dimorphic seeds of *S. aralocaspica* was designed. To differentiate the treatments of 3.6% NaClO and 0.1% $HgCl_2$ for different types of seeds, 25 treatments of 3.6% NaClO for brown seeds were named N1–N25 (Table S1), and 25 treatments of 3.6% NaClO for black seeds were named n1–n25 (Table S2). Further, 25 treatments of 0.1% $HgCl_2$ brown seeds were named H1–H25 (Table S3), and the 25 treatments of black seeds treated with 0.1% HgCl2 were named h1–h25 (Table S4). Each experimental group in this study was repeated three times and contained 20 seeds. The above operations were completed in an ultraclean workbench.

Table 1. Sterilization programs and parameters of *Suaeda aralocaspica* seeds.

	Factor	Level 1	Level 2	Level 3	Level 4	Level 5
Seed sterilization program (I)	Ethanol	30 s	1 min	3 min	5 min	8 min
	NaClO	3 min	5 min	8 min	11 min	15 min
	pH	5.0	6.0	7.0	8.0	9.0
	Factor	Level 1	Level 2	Level 3	Level 4	Level 5
Seed sterilization program (II)	Ethanol	30 s	1 min	3 min	5 min	8 min
	$HgCl_2$	1 min	3 min	5 min	8 min	11 min
	pH	5.0	6.0	7.0	8.0	9.0

All Petri dishes were incubated in a growth chamber at 25/10 °C under a 14 h light/10 h dark photoperiod for 20 days. A seed was considered to be germinated when the radicle length reached 5 mm. Germinated seeds were recorded every day. The final germination percentage (Equation (1)), contamination percentage (Equation (2)) and seedling survival percentage (Equation (3)) were calculated after 20 days of cultivation.

$$\text{Final germination percentage (\%)} = \text{number of germinated seeds/numbers of tested seeds} \times 100\% \quad (1)$$

$$\text{Contamination percentage (\%)} = \text{number of seeds contaminated by microorganism/number of tested seeds} \times 100\% \quad (2)$$

$$\text{Seedling survival percentage (\%)} = \text{number of seedlings without contamination and browning/number of tested seeds} \times 100\% \quad (3)$$

2.4. Statistical Analysis

All data were expressed as mean ± s.e. Arcsine transformation was performed before statistical analysis to meet assumptions. Linear mixed models were used to test the significance of main effects (time duration of ethanol, pH, secondary disinfectant type, time duration of secondary disinfectant, and seeds type) on final germination percentage, contamination percentage, and seedling survival percentage. The statistical analysis was performed using SPSS version 16 (SPSS for Windows, Released 2007, Chicago, IL, USA, SPSS Inc.). One-way ANOVA was used to compare treatments. For comparison, least significance difference test (LSD) ($p < 0.05$) was employed. Independent samples T Test was used to analyze differences between brown and black seeds under different secondary disinfectant treatments.

3. Results

3.1. The Effect of Different Treatments on Final Germination Percentage of S. aralocaspica Seeds

Ethanol time and pH had no significant effect on the germination of dimorphic seeds ($p > 0.05$). Secondary disinfectant type ($p < 0.001$), secondary disinfectant time ($p < 0.001$), and seed type ($p < 0.001$) significantly affected the seed germination percentage (Table 2). Compared with the seeds without sterilization treatment (Figure S1), the NaClO treatment did not affect the germination percentage of *S. aralocaspica* seeds. With the increase in the sterilization time duration of 0.1% $HgCl_2$, the germination percentage of brown seeds decreased significantly. The germination percentage of black seeds was low under all treatments and the highest germination percentage was only 16.67%.

Table 2. A mixed model ANOVA on final germination percentage of dimorphic seeds of *Suaeda aralocaspica*.

Source	Numerator df	Denominator df	F	Sig.
Ethanol time	4	285	1.132	0.341
pH value	4	285	0.435	0.783
Secondary disinfectant type	1	285	93.649	0.000
Secondary disinfectant time	4	285	5.753	0.000
Seed type	1	285	1230.597	0.000

When 3.6% NaClO was used, the germination percentages of brown seeds in treatment N11, N15, N16, N17 were 100%, and the germination percentage of other treatments, except N5 and N21, was ≥90% (Table 3). The germination percentages of black seeds in treatment n10 and n23 were the highest (16.67%). When 0.1% $HgCl_2$ was used, the germination percentage of brown seeds in treatment H1 and H7 was the highest (93.33%). The germination percentage of brown seeds in the other groups was from 20% to 90%. In contrast, black seeds did not germinate under many treatments. The highest germination percentage of treatment h13 was only 13.33%, and the other groups were less than 10%. The toxicity of 3.6% NaClO on seed germination of *S. aralocaspica* was lower than that of 0.1% $HgCl_2$.

Table 3. Final germination percentages of *Suaeda aralocaspica* seeds cultured with different treatments after 20 days incubation.

Treatment	3.6% NaClO Brown Seeds	Treatment	3.6% NaClO Black Seeds	Treatment	0.1% $HgCl_2$ Brown Seeds	Treatment	0.1% $HgCl_2$ Black Seeds
N1	96.67 ± 3.33 aA	n1	10.00 ± 10.00 aB	H1	93.33 ± 3.33 aA	h1	3.33 ± 3.33 abB
N2	90.00 ± 5.77 aA	n2	3.33 ± 3.33 aB	H2	83.33 ± 8.82 abcA	h2	3.33 ± 3.33 abB
N3	93.33 ± 3.33 aA	n3	3.33 ± 3.33 aB	H3	86.67 ± 6.67 abA	h3	3.33 ± 3.33 abB
N4	96.67 ± 3.33 aA	n4	6.67 ± 6.67 aB	H4	53.33 ± 13.33 cdefA	h4	0.00 ± 0.00 bB
N5	76.67 ± 3.33 bA	n5	10.00 ± 0.00 aB	H5	26.67 ± 3.33 fghA	h5	6.67 ± 6.67 abA
N6	93.33 ± 6.67 aA	n6	10.00 ± 10.00 aB	H6	73.33 ± 8.82 abcdeA	h6	3.33 ± 3.33 abB
N7	90.00 ± 5.77 aA	n7	3.33 ± 3.33 aB	H7	93.33 ± 3.33 aA	h7	6.67 ± 6.67 abB
N8	93.33 ± 6.67 aA	n8	6.67 ± 3.33 aB	H8	90.00 ± 5.77 aA	h8	3.33 ± 3.33 abB
N9	90.00 ± 0.00 aA	n9	13.33 ± 3.33 aB	H9	50.00 ± 5.77 defghA	h9	6.67 ± 3.33 abB
N10	93.33 ± 3.33 aA	n10	16.67 ± 12.02 aB	H10	40.00 ± 15.28 fghA	h10	10.00 ± 0.00 abA
N11	100.00 ± 0.00 aA	n11	6.67 ± 3.33 aB	H11	76.67 ± 14.53 abcdeA	h11	6.67 ± 6.67 abB
N12	93.33 ± 3.33 aA	n12	10.00 ± 0.00 aB	H12	73.33 ± 12.02 abcdeA	h12	0.00 ± 0.00 bB
N13	96.67 ± 3.33 aA	n13	3.33 ± 3.33 aB	H13	80.00 ± 10.00 abcdA	h13	13.33 ± 6.67 aB
N14	90.00 ± 0.00 aA	n14	6.67 ± 6.67 aB	H14	23.33 ± 3.33 fghA	h14	3.33 ± 3.33 abA
N15	100.00 ± 0.00 aA	n15	0.00 ± 0.00 aB	H15	46.67 ± 6.67 efghA	h15	0.00 ± 0.00 bB
N16	100.00 ± 0.00 aA	n16	3.33 ± 3.33 aB	H16	73.33 ± 14.53 abcdeA	h16	6.67 ± 3.33 abB
N17	100.00 ± 0.00 aA	n17	6.67 ± 3.33 aB	H17	80 ± 11.55 abcdA	h17	3.33 ± 3.33 abB
N18	93.33 ± 3.33 aA	n18	10.00 ± 0.00 aB	H18	46.67 ± 6.67 efghA	h18	6.67 ± 3.33 abB
N19	93.33 ± 3.33 aA	n19	0.00 ± 0.00 aB	H19	50.00 ± 0.00 defghA	h19	0.00 ± 0.00 bB
N20	90.00 ± 5.77 aA	n20	3.33 ± 3.33 aB	H20	23.33 ± 6.67 ghA	h20	0.00 ± 0.00 bA
N21	70.00 ± 0.00 bA	n21	6.67 ± 3.33 aB	H21	83.33 ± 8.82 abcA	h21	0.00 ± 0.00 bB
N22	93.33 ± 3.33 aA	n22	10.00 ± 0.00 aB	H22	40.00 ± 5.77 fghA	h22	0.00 ± 0.00 bB
N23	96.67 ± 3.33 aA	n23	16.67 ± 3.33 aB	H23	56.67 ± 14.53 bcdefA	h23	0.00 ± 0.00 bB
N24	96.67 ± 3.33 aA	n24	6.67 ± 3.33 aB	H24	20.00 ± 0.00 hA	h24	0.00 ± 0.00 bA
N25	93.33 ± 6.67 aA	n25	10.00 ± 0.00 aB	H25	40 ± 17.32 fghA	h25	3.33 ± 3.33 abA

Different lowercase letters in each column indicate significant differences among different treatments; different uppercase letters indicate significant difference between dimorphic seeds treated with the same secondary disinfectant.

3.2. The Effect of Different Treatments on Contamination Percentage of S. aralocaspica Seeds

Compared with the seeds that were not disinfected, almost all sterilization treatments had a good sterilizing effect. The effects of ethanol time ($p > 0.05$) and secondary disinfectant time ($p > 0.05$) on the contamination percentage were not significant. The effects of pH ($p < 0.05$), secondary disinfectant type ($p < 0.05$) and seed type ($p < 0.05$) on the contamination percentage were significant ($p < 0.05$) (Table 4).

Table 4. A mixed model ANOVA on contamination percentage of dimorphic seeds of *Suaeda aralocaspica*.

Source	Numerator df	Denominator df	F	Sig.
Ethanol time	4	285	1.182	0.319
pH value	4	285	5.936	0.000
Secondary disinfectant type	1	285	15.211	0.000
Secondary disinfectant time	4	285	0.264	0.901
Seed type	1	285	15.508	0.000

When using 3.6% NaClO as the secondary disinfectant, the brown seed contamination percentages were 56.67% and 53.33% in treatments N21 and N9, respectively, followed by 33.33%, 26.67% and 23.33% in treatments N19, N12 and N5, respectively (Table 5). The black seeds were most polluted in treatments n3 and n24, and the microbial contamination percentage was 6.67% (Table 5). When using 0.1% $HgCl_2$ as the secondary disinfectant, the sterilization effect was more significant, and only three treatments of both seeds were polluted. The highest contamination percentage of brown seeds was 23.33% in treatment

H20, and the contamination percentages in treatments H23 and H24 were 10% and 6.67%, respectively (Table 5). In treatment h1, 16.67% of the black seeds were polluted, which was the most serious, followed by treatment h17 and treatment h21, with the contamination percentage of 3.33%. Other treatment combinations were not polluted by microorganisms (Table 5).

Table 5. Contamination percentage of *Suaeda aralocaspica* seeds cultured with different treatments after 20 days incubation.

Treatment	3.6% NaClO Brown Seeds	Treatment	3.6% NaClO Black Seeds	Treatment	0.1% $HgCl_2$ Brown Seeds	Treatment	0.1% $HgCl_2$ Black Seeds
N1	6.67 ± 6.67 cA	n1	0.00 ± 0.00 aA	H1	0.00 ± 0.00 bA	h1	16.67 ± 16.67 aA
N2	0.00 ± 0.00 cA	n2	3.33 ± 3.33 aA	H2	0.00 ± 0.00 bA	h2	0.00 ± 0.00 bA
N3	6.67 ± 3.33 cA	n3	6.67 ± 6.67 aA	H3	0.00 ± 0.00 bA	h3	0.00 ± 0.00 bA
N4	0.00 ± 0.00 cA	n4	0.00 ± 0.00 aA	H4	0.00 ± 0.00 bA	h4	0.00 ± 0.00 bA
N5	23.33 ± 8.82 bcA	n5	0.00 ± 0.00 aA	H5	0.00 ± 0.00 bA	h5	0.00 ± 0.00 bA
N6	0.00 ± 0.00 cA	n6	0.00 ± 0.00 aA	H6	0.00 ± 0.00 bA	h6	0.00 ± 0.00 bA
N7	10.00 ± 10.00 cA	n7	0.00 ± 0.00 aA	H7	0.00 ± 0.00 bA	h7	0.00 ± 0.00 bA
N8	0.00 ± 0.00 cA	n8	0.00 ± 0.00 aA	H8	0.00 ± 0.00 bA	h8	0.00 ± 0.00 bA
N9	53.33 ± 8.82 abA	n9	3.33 ± 3.33 aB	H9	0.00 ± 0.00 bA	h9	0.00 ± 0.00 bA
N10	0.00 ± 0.00 cA	n10	0.00 ± 0.00 aA	H10	0.00 ± 0.00 bA	h10	0.00 ± 0.00 bA
N11	0.00 ± 0.00 cA	n11	0.00 ± 0.00 aA	H11	0.00 ± 0.00 bA	h11	0.00 ± 0.00 bA
N12	26.67 ± 26.67 abcA	n12	0.00 ± 0.00 aA	H12	0.00 ± 0.00 bA	h12	0.00 ± 0.00 bA
N13	13.33 ± 6.67 cA	n13	0.00 ± 0.00 aA	H13	0.00 ± 0.00 bA	h13	0.00 ± 0.00 bA
N14	0.00 ± 0.00 cA	n14	0.00 ± 0.00 aA	H14	0.00 ± 0.00 bA	h14	0.00 ± 0.00 bA
N15	0.00 ± 0.00 cA	n15	0.00 ± 0.00 aA	H15	0.00 ± 0.00 bA	h15	0.00 ± 0.00bA
N16	0.00 ± 0.00 cA	n16	0.00 ± 0.00 aA	H16	0.00 ± 0.00 bA	h16	0.00 ± 0.00 bA
N17	20.00 ± 0.00 cA	n17	3.33 ± 3.33 aB	H17	0.00 ± 0.00 bA	h17	3.33 ± 3.33 bA
N18	6.67 ± 3.33 cA	n18	0.00 ± 0.00 aA	H18	0.00 ± 0.00 bA	h18	0.00 ± 0.00 bA
N19	33.33 ± 33.33 abcA	n19	0.00 ± 0.00 aA	H19	0.00 ± 0.00 bbA	h19	0.00 ± 0.00 bA
N20	6.67 ± 6.67 cA	n20	3.33 ± 3.33 aA	H20	23.33 ± 23.33 aA	h20	0.00 ± 0.00 bA
N21	56.67 ± 14.53 aA	n21	3.33 ± 3.33 aA	H21	0.00 ± 0.00 bA	h21	3.33 ± 3.33 bA
N22	0.00 ± 0.00 cA	n22	0.00 ± 0.00 aA	H22	0.00 ± 0.00 bA	h22	0.00 ± 0.00 bA
N23	0.00 ± 0.00 cA	n23	0.00 ± 0.00 aA	H23	10.00 ± 0.00 aA	h23	0.00 ± 0.00 bA
N24	0.00 ± 0.00 cA	n24	6.67 ± 6.67 aA	H24	6.67 ± 3.33 bA	h24	0.00 ± 0.00 bA
N25	3.33 ± 3.33 cA	n25	0.00 ± 0.00 aA	H25	0.00 ± 0.00 bA	h25	0.00 ± 0.00 bA

Different lowercase letters in each column indicate significant differences among different treatments; different uppercase letters indicate significant difference between dimorphic seeds treated with the same secondary disinfectant.

Medium pH ($p > 0.05$) at different levels had no significant effect on seed germination, but pH ($p < 0.05$) significantly affected microbial growth and reproduction. No matter whether NaClO or $HgCl_2$ was selected as the secondary disinfectant, almost no microorganisms could grow on the MS medium under acidic conditions (pH 5.0). A small amount of bacterial or fungal contamination was observed at pH 6.0 to 8.0.

3.3. The Effect of Different Treatments on Seedling Survival Percentage of S. aralocaspica

Only the seedlings without the growth of bacteria and browning can be used as the source of plant material. The available sterile seedlings were obtained after the seeds were sterilized and cultured for 20 days. There were significant differences in the number of available seedlings obtained from different types of disinfectants ($p < 0.001$), ethanol time ($p < 0.001$) and seed type ($p < 0.001$) (Table 6).

Table 6. A mixed model ANOVA on the seedling survival percentage of *Suaeda aralocaspica*.

Source	Numerator df	Denominator df	F	Sig.
Ethanol time	4	285	5.850	0.000
pH value	4	285	1.005	0.405
Secondary disinfectant type	1	285	79.494	0.000
Secondary disinfectant time	4	285	0.447	0.775
Seed type	1	285	45.029	0.000

Seedlings grown from brown seeds had a higher survival percentage than that from black seeds. The seedlings sprouted after sterilization with $HgCl_2$ were short, and most browning deaths cannot be included in the surviving available seedlings. Compared with NaClO, the $HgCl_2$ treatment significantly reduced the seedling survival percentage for brown seeds of *S. aralocaspica*. The survival percentage of seedlings produced by brown seeds in treatment H7 was only 10%. When NaClO was used as the main disinfectant, the highest survival percentage of seedlings from brown seeds in treatments N4 and N7 was 46.67%, and the survival percentage was 43.33% in treatments N3 and N10. Under the treatment of two secondary disinfectants, the survival percentage of seedlings from black seeds was low. The highest survival percentage was only 13.33% under the NaClO treatment n10 and 10% under the $HgCl_2$ treatment h10 (Table 7).

Table 7. Seedling survival percentage of *Suaeda aralocaspica* cultured with different treatments after 20 days incubation.

Treatment	3.6% NaClO	Treatment	3.6% NaClO	Treatment	0.1% $HgCl_2$	Treatment	0.1% $HgCl_2$
	Brown Seeds		Black Seeds		Brown Seeds		Black Seeds
N1	23.33 ± 8.82 abcdA	n1	10.00 ± 10.00 aA	H1	0.00 ± 0.00 cA	h1	0.00 ± 0.00 bA
N2	16.67 ± 12.02 cdA	n2	0.00 ± 0.00 aA	H2	3.33 ± 3.33 bcA	h2	0.00 ± 0.00 bA
N3	43.33 ± 3.33 abA	n3	3.33 ± 3.33 aB	H3	3.33 ± 3.33 bcA	h3	0.00 ± 0.00 bA
N4	46.67 ± 3.33 aA	n4	6.67 ± 6.67 aA	H4	0.00 ± 0.00 cA	h4	0.00 ± 0.00 bA
N5	23.33 ± 8.82 abcdA	n5	3.33 ± 3.33 aA	H5	0.00 ± 0.00 cA	h5	6.67 ± 6.67 abA
N6	26.67 ± 6.67 abcdA	n6	6.67 ± 6.67 aA	H6	6.67 ± 3.33 abA	h6	0.00 ± 0.00 bA
N7	46.67 ± 3.33 aA	n7	0.00 ± 0.00 aB	H7	10.00 ± 0.00 aA	h7	3.33 ± 3.33 abA
N8	20.00 ± 0.00 bcdA	n8	3.33 ± 3.33 aB	H8	0.00 ± 0.00 cA	h8	3.33 ± 3.33 abA
N9	23.33 ± 12.09 abcdA	n9	6.67 ± 3.33 aA	H9	0.00 ± 0.00 cA	h9	3.33 ± 3.33 abA
N10	43.33 ± 3.33 abA	n10	13.33 ± 13.33 aA	H10	0.00 ± 0.00 cA	h10	10.00 ± 0.00 aA
N11	6.67 ± 3.33 dA	n11	3.33 ± 3.33 aA	H11	3.33 ± 3.33 bcA	h11	0.00 ± 0.00 bA
N12	13.34 ± 8.82 cdA	n12	6.67 ± 3.33 aA	H12	3.33 ± 3.33 bcA	h12	0.00 ± 0.00 bA
N13	10.00 ± 10.00 cdA	n13	3.33 ± 3.33 aA	H13	0.00 ± 0.00 cA	h13	3.33 ± 3.33 abA
N14	20.00 ± 10.00 bcdA	n14	6.67 ± 6.67 aA	H14	0.00 ± 0.00 cA	h14	3.33 ± 3.33 abA
N15	26.67 ± 14.53 abcdA	n15	0.00 ± 0.00 aA	H15	0.00 ± 0.00 cA	h15	0.00 ± 0.00 bA
N16	13.33 ± 3.33 cdA	n16	3.33 ± 3.33 aA	H16	0.00 ± 0.00 cA	h16	3.33 ± 3.33 abA
N17	6.67 ± 3.33 dA	n17	0.00 ± 0.00 aA	H17	0.00 ± 0.00 cA	h17	0.00 ± 0.00 aA
N18	10.00 ± 10.00 dA	n18	0.00 ± 0.00 aA	H18	0.00 ± 0.00 cA	h18	6.67 ± 3.33 abA
N19	20 ± 11.55 bcdA	n19	0.00 ± 0.00 aA	H19	3.33 ± 3.33 bcA	h19	0.00 ± 0.00 bA
N20	20.00 ± 10.00 bcdA	n20	0.00 ± 0.00 aA	H20	3.33 ± 3.33 bcA	h20	0.00 ± 0.00 bA
N21	0.00 ± 0.00 dA	n21	3.33 ± 3.33 aA	H21	0.00 ± 0.00 cA	h21	0.00 ± 0.00 bA
N22	10.00 ± 5.77 cdA	n22	3.33 ± 3.33 aA	H22	0.00 ± 0.00 cA	h22	0.00 ± 0.00 bA
N23	13.33 ± 3.33 cdA	n23	3.33 ± 3.33 aA	H23	0.00 ± 0.00 cA	h23	0.00 ± 0.00 bA
N24	6.67 ± 3.33 dA	n24	3.33 ± 3.33 aA	H24	3.33 ± 3.33 bcA	h24	0.00 ± 0.00 bA
N25	13.33 ± 8.82 cdA	n25	3.33 ± 3.33 aA	H25	0.00 ± 0.00 cA	h25	0.00 ± 0.00 bA

Different lowercase letters in each column indicate significant differences among different treatments; different uppercase letters indicate significant difference between dimorphic seeds treated with the same secondary disinfectant.

4. Discussion

Although the ecology, physiology, and molecular biology of *Suaeda* species have been studied extensively, there is no culture system in vitro for further study of the molecular mechanism. The effective acquisition of high-quality sterile explant material is the key to the subsequent tissue culture [19]. Our study takes the first step of this process by comparing the sterilization effects of different disinfectants and their effects on seed germination percentage.

Compared with the brown seeds, the black seeds were not easily contaminated by microorganisms, which might be due to the protective effect of the black and dense seed coat on the surface of the black seeds. 75% ethanol needs to be used with other disinfectants, for using it solely has an incomplete and unsatisfactory sterilization effect [21,22], and 0.1% $HgCl_2$ has a good sterilizing effect because Hg^{2+} can combine with negatively charged proteins to desaturated bacterial proteins and inactivate enzymes. NaClO solution is much milder than mercury chloride and is often used to sterilize tissue culture explants [20–28]. When the same disinfectant is used, the contamination rate will decrease, and the death rate will increase with the extension in sterilization time. Under natural conditions or abiotic stresses, the germination percentages of brown seeds of *S. aralocaspica* were much higher than that of black seeds [7,9,15], which was consistent with the germination results after our sterilization treatment. The seed type had a significant effect on the three evaluation indexes of germination percentage, bacterial growth percentage, and survival percentage. When treating explants, different sanitizer and sterilization time was used for sterilization, and the effect was obviously different. The results showed that with the prolongation of ethanol infiltration time, the browning number of seedlings from brown seeds of *S. aralocaspica* increased and the survival percentage decreased.

In our study, mercury chloride and sodium hypochlorite were used to disinfect with 75% ethanol. Sodium hypochlorite has strong oxidation, and long sterilization time means it is easy to cause plant browning. When 3.6% NaClO was used as the main disinfectant, N8 had the best comprehensive effect on brown seeds, which were soaked in 75% ethanol for 60 s, and then sterilized with 3.6% NaClO for 8 min, and finally inoculated into a pH 8.0 MS medium. Black seeds grew well under treatment 6 (n6), which was disinfected with 75% ethanol for 1 min and then treated with 3.6% NaClO for 3 min. Although mercury chloride can be effectively sterilized, it also has strong toxicity, causing irreversible browning damage to plants [19,20,22]. When 0.1% $HgCl_2$ was used as the main disinfectant, H7 had the best comprehensive effect on brown seeds (75% ethanol for 1 min + 0.1% $HgCl_2$ for 5 min + pH 8.0 MS). h6 had the best comprehensive effect on black seeds, which was 75% ethanol for 1 min + 0.1% $HgCl_2$ for 1 min + pH 6.0 MS.

It was found that a large number of browning seedlings appeared on the 8th day of culture, and the whole germination and growth process was completed on the 7th day. Therefore, the culture time can be shortened to 7 days to reduce energy and costs. Some studies found that adding anti-browning agents, such as vitamin C, activated carbon to the medium, or improving the activity of polyphenol oxidase and the antioxidant system enzyme Mars could effectively inhibit seedling browning [29–31]. In this experiment, we did not take special measures to prevent seedling browning, which could be optimized in further research.

5. Conclusions

In summary, this study shows that brown seeds with a high germination percentage should be chosen as a source of sterile explants. The best sterilization method entailed 75% ethanol 60 s + 3.6% NaClO 8 min, placed in an MS medium with pH 8.0 for 7 days. At present, this method has been successfully applied to the seed sterilization of *S. aralocaspica* in vitro, and the pollution-free percentage can reach 100%, when ignoring the pollution caused by improper operation. The sterilization and cultivation method was successful for *S. aralocaspica* and may be also applied to other *Suaeda* species. In the future, other

combinations of sterilization methods should be tested for *S. aralocaspica* seeds, and the exact mechanism of the sterilization effects needs to be fully understood.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8040289/s1, Figure S1: The final germination percentage and contamination percentage of dimorphic seeds that washed with sterile water and incubated at different pH conditions, Table S1: 25 different treatments with NaClO as the secondary disinfector to sterilize brown seeds of *Suaeda aralocaspica,* Table S2: 25 different treatments with NaClO as the secondary disinfector to sterilize black seeds of *Suaeda aralocaspica*, Table S3: 25 different treatments with 0.1% $HgCl_2$ as the secondary disinfector to sterilize brown seeds of *Suaeda aralocaspica*, Table S4: 25 different treatments with 0.1% $HgCl_2$ as the secondary disinfector to sterilize black seeds of *Suaeda aralocaspica.*

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