Postprint of a Study on Sterile Kiwi Fruit Seed Collection Methods

Authors: Sun Jingjie, Yang Di, Zou Tingting, Zhao Xinshi, Tang Juan, Shi Jiajie, Zhang Naiqun, Zhang Naiqun

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Abstract

Sterile kiwifruit seeds constitute fundamental materials for techniques such as kiwifruit endosperm culture and seedling micrografting; chemical disinfection represents a commonly employed approach for sterile seed collection, with mercuric chloride and sodium hypochlorite (NaClO) being the most widely utilized disinfectants. This study proposes a novel method for sterile kiwifruit seed collection—the sterile stirring method. To evaluate its reliability and applicability, the efficacy of three approaches for collecting sterile kiwifruit seeds was compared: 0.2% mercuric chloride sterilization for 20 min, 10% sodium hypochlorite sterilization for 20 min, and the sterile stirring method, along with their effects on seed germination and seedling development. The results demonstrated that the sterile stirring method and 0.2% mercuric chloride sterilization for 20 min represented stable and effective methods for sterile kiwifruit seed collection, whereas 10% sodium hypochlorite sterilization for 20 min yielded inconsistent collection results. Within the same timeframe, seeds treated with the sterile stirring method exhibited the highest germination rate at 89.90%, albeit with lower germination potential; nevertheless, all seeds developed into normal seedlings. The germination rate of seeds treated with 10% sodium hypochlorite sterilization for 20 min ranked second, showing no significant difference from the sterile stirring method; however, this treatment achieved the highest germination potential and seedling formation rate at 47.47% and 67.86%, respectively, and demonstrated a dormancy-breaking effect on kiwifruit seeds, with an overall effect similar to that of gibberellin (GA3) seed soaking treatment. The 0.2% mercuric chloride sterilization for 20 min exhibited inhibitory effects on kiwifruit seed germination, with all indices being significantly lower than those of seeds treated with the sterile stirring method, and resulted in retarded growth. Furthermore, the sterile stirring method constitutes a physical treatment that poses no harm to seeds, operators, or the environment. This study confirms the practicality and advantages of the sterile stirring method, reveals the role of sodium hypochlorite

in breaking kiwifruit seed dormancy, and provides a reference for sterile seed collection in other similar fruit species.

Full Text

Preamble

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Title: Study on Obtaining Aseptic Seeds of Kiwifruit
Authors: SUN Jing-Jie, YANG Di, ZOU Ting-Ting, ZHAO Xin-Shi, TANG Juan, SHI Jia-Jie, ZHANG Nai-Qun*
Affiliation: College of Life Science and Technology, Nanyang Normal University, Nanyang, Henan 473061, China

Abstract

Aseptic kiwifruit seeds are fundamental materials for techniques such as endosperm culture and seedling micrografting. Disinfection using chemical agents is a common method for obtaining aseptic seeds, with mercuric chloride (HgCl) and sodium hypochlorite (NaClO) being the most widely used disinfectants. This paper proposes a novel method for aseptic seed collection—the aseptic stirring method. To evaluate its reliability and applicability, we compared the effectiveness of three seed collection approaches: 0.2% mercuric chloride sterilization for 20 min, 10% sodium hypochlorite sterilization for 20 min, and the aseptic stirring method, along with their effects on seed germination and seedling development.

The results demonstrated that both the aseptic stirring method and 0.2% mercuric chloride sterilization for 20 min were stable and effective for aseptic seed collection, whereas 10% sodium hypochlorite sterilization for 20 min produced inconsistent results. Within the same timeframe, the aseptic stirring method achieved the highest germination rate at 89.90%, though with lower germination energy; however, all seedlings developed normally. Sodium hypochlorite sterilization for 20 min yielded the second-highest germination rate, which showed no significant difference from the aseptic stirring method, but achieved the highest germination energy (47.47%) and seedling formation rate (67.86%). Notably, this treatment also effectively broke seed dormancy, producing effects similar to gibberellin (GA) soaking. In contrast, 0.2% mercuric chloride sterilization for 20 min inhibited kiwifruit seed germination, with all metrics significantly lower than those of the aseptic stirring method, and resulted in slow growth. Furthermore, as a physical treatment, the aseptic stirring method is harmless to seeds, operators, and the environment. This study confirms the practicality and advantages of the aseptic stirring method, reveals the dormancy-breaking effect of sodium hypochlorite on kiwifruit seeds, and provides a valuable reference for aseptic seed collection in similar fruit crops.

Keywords: seeds, kiwifruit, aseptic, germination, stirring

Introduction

Seeds are the reproductive units of seed plants and play a crucial role in species perpetuation. Kiwifruit (*Actinidia* spp.) is a perennial vine belonging to the family Actinidiaceae. Native to China, it is now widely cultivated worldwide. The fruit is delicious and highly nutritious, earning the title "king of fruits" due to its high vitamin C content, while its roots, stems, leaves, and flowers have medicinal value, making it a truly versatile crop. Kiwifruit seeds are numerous and small, embedded in the pulp, which affects fruit texture and processing for products such as juice. To develop seedless kiwifruit varieties, extensive research has been conducted. Endosperm culture in angiosperms is an important approach for obtaining sterile plants and has been widely applied in fruit trees including passion fruit, carambola, and loquat. Kiwifruit endosperm culture has also advanced significantly, with triploid plants successfully obtained. Kiwifruit germplasm resources exhibit rich diversity, but the species has a long juvenile period and highly heterozygous genes. Current propagation methods primarily rely on seedling grafting, which is time-consuming and susceptible to canker disease infection at wound sites. Micrografting, combining tissue culture with grafting techniques, has been widely used in grapes and apples. Using seeds to cultivate sterile seedlings in vitro followed by aseptic grafting of superior varieties can shorten the propagation cycle, improve survival rates, and prevent canker disease infection. Both endosperm culture and micrografting require aseptic kiwifruit seeds as fundamental materials. Conventional aseptic seed collection employs chemical disinfectants, with mercuric chloride and sodium hypochlorite being the most commonly used. However, the interior of a normal, intact kiwifruit is a sterile environment, with seeds enclosed by sterile pulp. To avoid exposing seeds to contamination during extraction and eliminate unnecessary work, we propose a novel method for direct aseptic seed collection—the aseptic stirring method—which avoids chemical disinfectants and optimizes the preliminary steps for endosperm culture and micrografting techniques.

Materials and Methods

1.1 Materials

Six kiwifruit cultivars were selected for this study: 'Guichang' , 'Hongyang' , 'Xuxiang' , 'Cuixiang' , 'Hort 16A' , and 'Hayward' . Fruits were purchased from supermarkets or collected from kiwifruit orchards in Xixia County.

1.2 Experimental Conditions and Procedures

1.2.1 Experimental Conditions All experiments used MS medium. The study was conducted in the plant culture room at Nanyang Normal University. Culture media, magnetic stir bars, deionized water, and jam jars were sterilized using an autoclave. Gibberellin was sterilized by filtration through a 0.2 μm micropore membrane. The temperature was maintained at approximately 25°C with a 12 h photoperiod and light intensity of 1500–2000 lx. Except for the

"stability assessment of aseptic seed collection methods" step, all experiments were initiated in December.

1.2.2 Aseptic Seed Collection (1) Aseptic Stirring Method

Mature, intact fruits of 'Guichang', 'Hort 16A', and 'Cuixiang' were used for aseptic seed collection. The procedure was as follows: In a laminar flow hood, kiwifruit were placed on sterile filter paper. The fruit surface was wiped with alcohol-soaked cotton balls, then peeled using sterile forceps and scalpels. The tools were repeatedly flamed over an alcohol burner during peeling to maintain sterility. The peeled pulp was transferred to another sterile filter paper, surface pulp was removed with a flamed scalpel, and the remaining pulp was placed in a sterile jam jar. The pulp was crushed with sterile forceps, sterile water was added to two-thirds of the jar volume, the lid was tightened, and the mixture was stirred on a magnetic stirrer for 3–5 min. After settling, seed separation from pulp was observed. Pulp debris was rinsed away with sterile water, leaving clean kiwifruit seeds. The collected seeds were directly inoculated onto MS medium in a laminar flow hood (6 seeds per plate, 10 plates per treatment, totaling 60 seeds). Contamination was observed at 20 d, and data were recorded. Observations ceased at 20 d after the first seed germinated, and whether germinated sprouts were contaminated was recorded. Seeds collected by the aseptic stirring method from 'Guichang', 'Hort 16A', and 'Cuixiang' were labeled A1, A2, and A3, respectively. Control groups were established by collecting seeds from the same three cultivars using the magnetic stirring method in a non-sterile environment, labeled CK1, CK2, and CK3, for comparison with the aseptic stirring method.

To further verify the reliability of the aseptic stirring method, three additional cultivars ('Xuxiang', 'Hayward', and 'Hongyang') were processed using the same procedure, with identical observation and recording methods.

(2) Disinfectant Sterilization

Mercuric chloride (0.2%) and sodium hypochlorite (10%) were applied for 20 min, following established effective protocols from the literature. Seeds for disinfectant sterilization were collected using conventional methods: mature, intact fruits of 'Guichang', 'Hort 16A', and 'Cuixiang' were squeezed in a non-sterile environment, the pulp was wrapped in gauze and repeatedly rinsed to remove pulp and floating empty seeds, retaining only plump seeds. Seeds sterilized with mercuric chloride were labeled B1, B2, and B3, while those treated with sodium hypochlorite were labeled C1, C2, and C3. Sterilized seeds were inoculated onto MS medium in a laminar flow hood (6 seeds per plate, 10 plates per treatment, 60 seeds total). Contamination was assessed at 20 d, and observations continued until 20 d after the first germination to check for contamination in sprouts.

(3) Stability Assessment of Aseptic Seed Collection Methods

To determine the reliability of the aseptic stirring method and disinfectant ster-

ilization, 'Guichang' fruits were processed using both methods in December, January, February, and March, with observation protocols identical to those described above.

1.2.3 Seed Germination Culture Seed germination and seedling growth were observed to analyze the advantages and disadvantages of each aseptic seed collection method and further validate the practicality of the aseptic stirring method. 'Guichang' fruits were used for the following investigations:

(1) Direct Culture of Aseptic Seeds

Aseptic seeds collected by the aseptic stirring method, 0.2% mercuric chloride sterilization for 20 min, and 10% sodium hypochlorite sterilization for 20 min were inoculated onto MS medium and allowed to germinate naturally. Germination was observed every 10 d, and seedling formation (defined as emergence of true leaves) was assessed after 50 d. The experiment included three replicates of approximately 33 seeds each.

(2) Gibberellin Treatment

Aseptic seeds collected by the aseptic stirring method and 0.2% mercuric chloride sterilization for 20 min were soaked in 25 $mg \cdot L^{1}$ gibberellin solution for 0, 5, 8, 15, 20, and 24 h. Seeds from the aseptic stirring method were labeled D1–D6, while mercuric chloride-treated seeds were labeled D7–D12. Treated seeds were cultured on MS medium, and germination and growth were observed for 50 d. The experiment included three replicates of approximately 31 seeds each.

Evaluation Metrics: - Germination rate = (Final number of germinated seeds / Total seeds tested) × 100% - Germination energy = (Number of seeds germinated within first 20 d / Total seeds tested) × 100% - Seedling formation rate = (Number of germinated seeds developing into seedlings at experiment end / Total seeds tested) × 100%

1.2.4 Statistical Analysis Data were organized and graphed using Excel 2013. Statistical analysis was performed using SPSS 22.0 software, with one-way ANOVA and Duncan' s multiple comparison test.

Results

2.1 Effectiveness of the Aseptic Stirring Method

The aseptic stirring method successfully collected kiwifruit seeds [Figure 1: see original paper]. During stirring, clear separation of seeds from pulp was observed, with plump seeds settling at the bottom of the jar and empty seeds floating on the water surface, confirming the method' s reliability.

The effectiveness of the aseptic stirring method for 'Guichang' , 'Hort 16A' , and 'Cuixiang' is shown in . All control group seeds were contaminated within 3–10 d. In contrast, seeds collected by the aseptic stirring method showed

minimal contamination: one 'Guichang' seed contaminated on day 7, one 'Hort 16A' seed on day 6, and no contamination in 'Cuixiang' seeds. Since control groups were completely contaminated, no germination occurred. Germination initiation times for the three cultivars were not significantly different (13–16 d), so contamination in sprouts was monitored until day 36, with no contamination detected. These results demonstrate that the aseptic stirring method is suitable for collecting aseptic kiwifruit seeds; the two contaminated seeds likely resulted from operational error.

To confirm that this contamination was not inherent to the method, 'Xuxiang' , 'Hongyang', and 'Hayward'were processed using the aseptic stirring method . No contamination was observed in any of the 180 seeds during the 20 d observation period. Germination began around day 14 for 'Xuxiang' and 'Hayward' , and day 16 for 'Hongyang' , with sprout contamination monitored until days 34, 36, and 34, respectively. The absence of contamination confirms that the two contaminated seeds in the previous trial were unrelated to the method itself, establishing the aseptic stirring method as a reliable technique for aseptic seed collection.

2.2 Effectiveness of Disinfectant Sterilization

Disinfectant sterilization results are presented in . Seeds of 'Guichang' , 'Hort 16A' , and 'Cuixiang' treated with 0.2% mercuric chloride or 10% sodium hypochlorite for 20 min showed no bacterial or fungal contamination within 20 d, with all 60 seeds per treatment remaining aseptic. Mercuric chloride-treated seeds germinated later (approximately days 16–18), while sodium hypochlorite-treated seeds germinated earlier (approximately days 11–12). No contamination was detected in sprouts from either treatment. Thus, disinfectant sterilization can also produce aseptic kiwifruit seeds.

2.3 Stability of Different Aseptic Seed Collection Methods

Stability assessment results for 'Guichang' seeds collected at different times are shown in . The aseptic stirring method produced only one contaminated seed in December, with no contamination in the other three months. Mercuric chloride treatment (0.2% for 20 min) yielded no contamination in any of the four collections. Sodium hypochlorite treatment (10% for 20 min) showed no contamination in December and January, but 7 contaminated seeds in February and 13 in March. As previously established, the single contaminated seed from the aseptic stirring method was not method-related. Therefore, both the aseptic stirring method and 0.2% mercuric chloride sterilization for 20 min represent stable methods for aseptic seed collection, while 10% sodium hypochlorite sterilization for 20 min showed significant instability in February and March.

2.4 Effects of Different Collection Methods on 'Guichang' Seed Germination and Seedling Formation

As shown in [Figure 2: see original paper], germination of 'Guichang' seeds occurred between 10–40 d, with no germination in the first 10 d and minimal change after 40 d. Seeds treated with 10% sodium hypochlorite for 20 min began germinating around day 11, reaching 47.47% germination by day 20 and 53.53% by day 30, with an additional 29.80% germinating between days 30–40. Seeds collected by the aseptic stirring method began germinating around day 15, with 57.81% germinating between days 30–40 and only 32.09% in the first 30 d. Mercuric chloride-treated seeds germinated latest (around day 17), with 19.19% germination in the first 30 d and a final rate of 46.46% at day 40, significantly lower than the other two methods [TABLE:5, $P < 0.05$].

After 50 d, significant differences in seedling formation rates were observed among treatments [Figure 3: see original paper]. Sodium hypochlorite-treated seeds achieved the highest seedling formation rate (67.86%), with cotyledons appearing approximately 3 d after germination initiation and most developing true leaves by day 22. Root length at day 30 ranged from 1.5–3.0 cm. Mercuric chloride-treated seeds showed the lowest seedling formation rate (10.33%), with slow growth, cotyledons appearing around day 26, and thin, short roots with few branches. Seeds from the aseptic stirring method developed cotyledons around day 19 and true leaves from day 24, with root lengths of 1–2 cm at day 30 and a seedling formation rate of 40.07%. Overall, sodium hypochlorite treatment produced the most vigorous growth, while the aseptic stirring method yielded healthy seedlings with robust, well-branched roots, though with delayed germination. Mercuric chloride treatment significantly inhibited germination and growth.

2.5 Effects of Gibberellin Soaking on Germination of Aseptic Seeds Collected by Different Methods

Gibberellin treatment effects are summarized in . Compared to controls (D1, D7), all gibberellin treatments promoted 'Guichang' seed germination, shifting the germination peak to the early (11–20 d) and middle (20–30 d) periods. For aseptic stirring method seeds (D2–D6), germination rates reached 100% for D3–D6 and 95.70% for D2. D3 treatment (8 h soaking) showed the highest germination energy (82.79%) and a seedling formation rate of 94.62%, while D5 treatment (20 h) achieved 81.72% germination energy and 96.77% seedling formation, with no significant difference between them. However, D3 required 12 h less soaking time, making it the optimal treatment. The shortest soaking time (D2, 5 h) produced moderate results, while the longest (D6, 24 h) showed the lowest germination energy and seedling formation rate, indicating that gibberellin' s dormancy-breaking effect is effective only within a limited time range.

For mercuric chloride-treated seeds, the best gibberellin treatment (D12, 24 h

soaking) achieved 94.62% germination, 50.54% germination energy, and 70.05% seedling formation—significantly lower than most aseptic stirring method treatments. This confirms that mercuric chloride sterilization inhibits seed germination and growth even after dormancy is broken.

In summary, gibberellin soaking effectively breaks kiwifruit seed dormancy, with the optimal treatment identified as 25 mg · L ^{1} gibberellin for 8 h.

Discussion

When collecting aseptic seeds, three critical factors must be considered: collection method effectiveness, seed viability maintenance, and associated problems and solutions. This study demonstrates that both the aseptic stirring method and disinfectant sterilization can effectively collect aseptic kiwifruit seeds, but sodium hypochlorite sterilization showed unstable efficacy—performing well in colder months (December, January) but poorly in warmer months (February, March). Previous research on kiwifruit stem segments found that seasonal transitions affect contamination rates, with warming temperatures increasing contamination. Mercuric chloride maintained good sterilization effects due to high concentration and extended treatment time, but such conditions typically inhibit explant viability in other applications, where 0.1% concentration for less than 15 min is standard. The 0.2% mercuric chloride for 20 min protocol, while common in kiwifruit endosperm culture literature, presents significant drawbacks. Mercuric ions (Hg^{2}) bind to negatively charged proteins, causing denaturation and microbial death, but residues remain on explant surfaces and are difficult to remove, affecting survival. Additionally, mercury is a heavy metal that poses environmental risks and potential health hazards to operators, requiring careful waste disposal.

The aseptic stirring method, as a physical approach, offers a simpler operation without harming seeds or creating environmental and safety concerns, making it suitable for laboratory applications. Seed dormancy is an ecological adaptation that enables plants to withstand adverse conditions and optimize germination timing. Kiwifruit seeds exhibit dormancy characteristics. While cold stratification is commonly used to break kiwifruit seed dormancy, this method is time-consuming (2–3 months) and requires careful control of moisture and air flow. Gibberellin is a standard dormancy-breaking agent, and this study found that its promoting effect is effective only within a specific time window, consistent with previous research on delicious kiwifruit. The optimal treatment identified was 25 mg · L ^{1} gibberellin for 8 h.

Interestingly, sodium hypochlorite sterilization also demonstrated dormancy-breaking effects, likely by corroding the seed coat and facilitating water and nutrient absorption. The 10% sodium hypochlorite for 20 min treatment showed similar germination timing to the optimal gibberellin treatment, though with 35.32% lower germination in the 11–20 d period. While gibberellin treatment was superior, sodium hypochlorite' s advantage lies in its much shorter treat-

ment time (20 min vs. 8 h). Although this study only examined the 20 min treatment, the dormancy-breaking potential of sodium hypochlorite warrants further investigation.

In conclusion, mercuric chloride sterilization has numerous drawbacks and should be avoided. Sodium hypochlorite sterilization is unreliable for aseptic collection but shows promise for dormancy breaking. The aseptic stirring method circumvents the limitations of both chemical disinfectants and provides a reliable, practical approach for collecting aseptic kiwifruit seeds. This study confirms the method' s reliability and utility, offering a new efficient and safe technique with significant practical value and expanding possibilities for aseptic seed collection in similar plant species.

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Note: Figure translations are in progress. See original paper for figures.