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A Study on Endogenous Inhibitors of *Nitraria roborowskii* Kom. Seeds

Shangfu Ren [1,2,3], Lamei Jiang [1] and Guanghui Lv [1,4,5,*]

[1] College of Ecology and Environment, Xinjiang University, Urumqi 830017, China; renshangfu@163.com (S.R.); jianglam0108@126.com (L.J.)
[2] College of Life and Geographic Sciences, Kashi University, Kashi 844006, China
[3] Key Laboratory of Biological Resources and Ecology of Pamirs Plateau in Xinjiang, Kashi 844006, China
[4] Key Laboratory of Oasis Ecology of Education Ministry, Xinjiang University, Urumqi 830017, China
[5] Xinjiang Jinghe Observation and Research Station of Temperate Desert Ecosystem, Ministry of Education, Urumqi 830017, China
* Correspondence: ler@xju.edu.cn

Abstract: *Nitraria roborowskii* Kom. seeds have deep dormancy characteristics. Under natural conditions, the germination rate of the seeds is low, and the germination time is long. Therefore, exploring the reasons for seed dormancy is highly important. The results showed that the extracts of the methanol phase, ethyl acetate phase, petroleum ether phase and water phase of *N. roborowskii* seeds all had a significant inhibitory effect on the germination rate and germination index of *Brassica rapa* seeds, among which the extract of the methanol phase had the strongest inhibitory effect, and the inhibitory effect decreased in the following order from the strongest to the weakest: methanol phase > ethyl acetate phase > petroleum ether phase > water phase. The components of the methanol phase, ethyl acetate phase and petroleum ether phase ether extracts of *N. roborowskii* seeds were identified by gas chromatography–mass spectrometry (GC–MS). The experimental results showed that the organic phase extracts of *N. roborowskii* seeds contained a variety of inhibitory compounds, which included 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-dibutyl phthalate; 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol; 13-docosenamide, (Z)-; 3-hydroxy-4-methoxybenzoic acid; vanillin; 2,4-di-tert-butylphenol; and cyclohexane, ethyl-. The seeds of *N. roborowskii* contain a variety of endogenous inhibitors, which are the main reason for its seed dormancy.

Keywords: *Nitraria roborowskii* Kom.; seeds; endogenous inhibitor; GC–MS; extract

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1. Introduction

Nitraceae are Tertiary relict plants with Mediterranean–West to Central Asian distribution types. In China, they are mainly distributed in the northwestern and northern regions [1]. As saline plants, they not only have important ecological value, such as improving saline and alkaline land and preventing winds and fixing sands, but also have nutritional value, medicinal value and economic value. They are common shrubs in saline soil distribution areas worldwide; they have become the winning and top community builders in desert and arid zones and are often used as the preferred plants for the management of desert and saline and alkaline land in the arid areas of the northwest region of the country. The Nitraria seed oil content is approximately 12%, the unsaturated fatty acid content is up to 95%, the linoleic acid content is 60%–70% and their ability to lower blood lipids prevents atherosclerosis and other effects. Nitraria is an economic tree species for the development of special industries [2].

In 1908, Komapov published the Central Asian species *Nitraria roborowskii* Kom. based on the samples from Qiemo, Xinjiang [3]. Studies have shown that *N. roborowskii* fruits are the most abundant [4], with the smallest proportion of reproductive allocation (11.82%) [5]; the greatest fruiting rate is 63.55% [6]; the greatest nutrient content is present [7]; fruits

contain five essential macronutrients and ten essential trace elements [8]; the greatest growth benefit is achieved [9] and the greatest initial seed dormancy rate is 88% [9]. *N. roborowskii* has a greater fruit setting rate and greater ripeness.

N. roborowskii seeds exhibit dormancy characteristics [4,10]. Most *N. roborowskii* seeds that are collected in a particular year become dormant [10], resulting in a relatively low germination rate of only approximately 30% [11]. Therefore, studying the release of dormancy in the seeds of *N. roborowskii* is highly important. According to previous studies, the seed embryo of *N. roborowskii* is fully mature [12] and has the characteristic of sclerotization [13], so this paper focuses on whether the seeds of *N. roborowskii* contain endogenous inhibitors.

Seed dormancy is the temporary inability of viable seeds to germinate under conditions suitable for germination. Seeds in a dormant state cannot germinate because they receive internal hindrances, and they can only germinate when those internal inhibitors are removed. Due to the presence of genetic material (phenolics, caffeic acid, ferulic acid) in the pulp of *Lycopersicon esculentum* M. and *Cucumis sativus* L. fruits, their seeds do not germinate in the fruit but germinate easily if the seeds are removed and rinsed well [14]. Apple cotyledons contain inhibitors that are translocated to the radicle to inhibit its growth so that the degree of embryo dormancy in apples decreases progressively as the cotyledon tissue is removed [15]. Kentzer and Amen considered the presence of inhibitory substances to be one of the main causes of plant seed dormancy [16,17]. During seed germination, endogenous inhibitors can prevent water uptake, inhibit respiration, inhibit enzyme activity, hinder embryo growth or alter osmotic pressure [18]. Endogenous inhibitors are produced in plants, and there are a very large number of inhibitory substances, such as organic acids, phenols, alkaloids, coumarins and abscisic acid (ABA).

Therefore, this paper mainly explores whether *N. roborowskii* seeds contain endogenous inhibitors. The selection of nondormant seeds that are more prone to germination in response to extracts can determine whether the experimental seeds have endogenous inhibitors [19]. The existence and intensity of endogenous seed inhibitors are indicated by the germination rate of nondormant seeds (*Brassica rapa*) [20]. Generally, the methanol phase, ethyl acetate phase and ether phase extracts of plant seeds are used to explore whether plant seeds contain substances that inhibit germination and seedling growth [21]. To date, there have been no studies on endogenous inhibitors in the seeds of *N. roborowskii*, and to further investigate the causes of its seed dormancy, the present study raises two scientific questions for investigation: (1) whether *N. roborowskii* seeds contain endogenous inhibitors? and (2) what are the endogenous inhibitors in the seeds?

2. Materials and Methods

2.1. Experimental Materials

The plump and healthy seeds of *N. roborowskii* used in the experiment were purchased from the planting base of Linquan Ecological Seed Industry Co., Ltd., in Minqin County, Wuwei, China. *B. rapa* (Xiaoza 56) seeds with a purity $\geq$ 98.0% and a germination rate $\geq$ 85.0% were purchased from Shandong Denghai Seed Co., Ltd. (Yantai, China).

Analytical purity: methanol, ethyl acetate and petroleum ether. Chromatographic purity: methanol, acetonitrile and acetone. All chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Experimental Design and Research Methodology

2.2.1. Extraction of Inhibitors from *N. roborowskii* Seeds

A 10.0 g sample of *N. roborowskii* seeds was weighed using an electronic column (1/10,000) and then crushed in a triangular flask. Then, 50 mL of 80% methanol was added and extracted in a closed chamber at 4 °C with stirring at regular intervals, and the clarified filtrate was filtered after 24 h. The clarified filtrate was kept as a reserve, and the precipitate was extracted by adding 50 mL of 80% methanol again. The extraction was repeated three times, and the extracted solutions were combined and centrifuged at 4000 r/min for 10 min, after which the supernatant was retained (Figure 1).

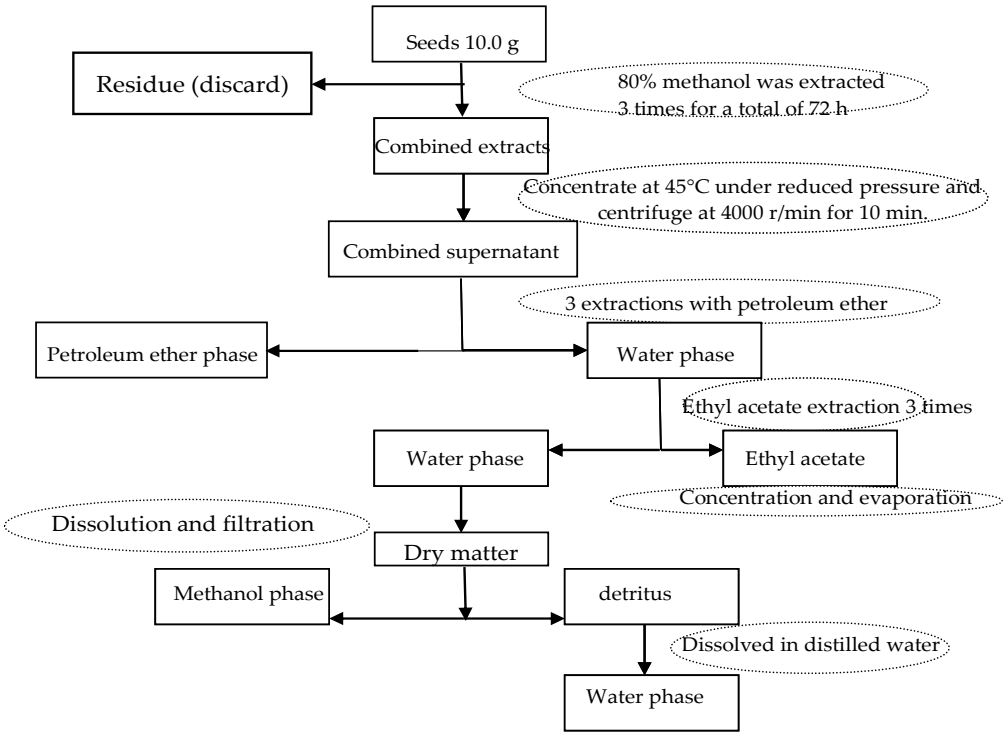

Figure 1. Flow chart of the extraction of endogenous inhibitors from the seeds of *N. roborowskii*.

2.2.2. Separation of Inhibitors from *N. roborowskii* Seeds

The methanol-phase extract of *N. roborowskii* seeds was separated using the systematic solvent method [22]. The extract was concentrated under reduced pressure at 56 °C to remove the methanol phase, and the remaining concentrated liquid was centrifuged at 4000 r/min for 10 min. Then, 50 mL of petroleum ether was added to the concentrated liquid of the seed inhibitors and extracted 3 times in succession, and the extracted liquid was combined to obtain the petroleum ether phase of the inhibitors of *N. roborowskii* seeds. Fifty milliliters of the ethyl acetate phase was added to the water phase for further extraction, which was repeated 3 times, and the extract liquid was combined to obtain the ethyl acetate phase of the inhibitors of *N. roborowskii* seeds. Finally, the water phase of the seed inhibitor extract was concentrated and evaporated under reduced pressure at 65 °C. The dry matter was dissolved in methanol and filtered to obtain the methanol phase and residue, and the residue was added to water to obtain the water phase.

2.2.3. Bioassay of the Inhibitory Activity of the Seeds of *N. roborowskii*

The inhibitory activity of *N. roborovskii* seeds was tested on nondormant seeds of *B. rapa* under laboratory conditions. Solutions with concentrations of 0%, 25%, 50%, 75% and 100% were prepared for the methanol phase, ethyl acetate phase, petroleum ether phase and water phase, respectively, of *N. roborowskii* seeds for bioassays. Three milliliters of each solution containing the inhibitors was added to separate the Petri dishes. After the organic solvents in each phase had completely evaporated [23], 40 *B. rapa* seeds were placed in Petri dishes with 5 mL of distilled water and the control with distilled water, with 3 replications for each treatment, and placed at 25 ± 1 °C, 80% humidity, 3000 l× light, and 12 h light/12 h dark for germination experiments. The germination and germination indices of the *B. rapa* seeds were determined after 24 h and 48 h, respectively.

2.2.4. GC–MS Identification of Inhibitors from *N. roborowskii* Seeds

A total of 100 mL of the organic phase extract of the seeds of *N. roborowskii* in an RE-2000A rotary evaporator with a circulating multipurpose vacuum pump (SHB-IIIA) was used for decompression and concentration, and concentrated dry matter with a light

brown color was obtained. Then, concentrated dry samples of the phases were made into concentrated samples of the various organic phase extracts of the seeds by using the corresponding organic solvents with shaking, solubilization and condensation to 2 mL.

GC–MS was conducted at the Physical and Chemical Testing Center of Xinjiang University. The instrument used was an Agilent 7890B/5977A gas chromatography–mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The ionization source was electron impact (EI), and the chromatographic column used was a DB-5ms5% phenyl-methyl polysiloxane capillary column (30 m × 0.25 mm). The chromatographic conditions were as follows: injection port temperature, 280 °C; injection method, 5:1; programmed temperature increase, 50 °C (10 min) raised to 280 °C at 5 °C/min (15 min); carrier gas, helium; flow rate, 1.0 mL/min and injection volume, 1.0 μL. The mass spectrometry conditions were as follows: ionization method: electron impact source (EI), ionization energy 70 eV; ion source temperature 230 °C, interface temperature 280 °C; scanning method: full scan (SCAN), scanning range 35 amu–650 amu, scanning time 0.45 s and solvent delay 3 min. After injection, the mass spectra of each component in the sample were compared with the stock signals of all compounds controlled by the computer to identify the sample components and verify them against standard spectra.

2.3. Data Statistics

This experiment used a completely random design, and the data were processed using SPSS 17.0 (IBM, Armonk, NY, USA). One-way ANOVA was performed at the $p < 0.05$ level, and the least significant difference method (LSD) was used to test for significant differences between means and for multiple comparisons. All charts and graphical processing were completed using GraphPad Prism 5.0.

3. Results

3.1. Bioassay of the Extracts of Various Organic Phases from the Seeds of N. roborowskii

Compared with the germination rate of CK (84.18%), the germination rate of *B. rapa* seeds treated with various organic phase extracts at different concentrations tended to decrease (Figure 2). After variance analysis, the inhibitory effects of various organic phase extracts at different concentrations on the germination of *B. rapa* seeds were significantly different ($p < 0.05$), indicating that there were substances in the methanol phase, ethyl acetate phase, petroleum ether phase and water phase of *N. roborowskii* seeds at different concentrations that inhibited the germination of *B. rapa* seeds, but the inhibitory effects of the various organic phase extracts on the germination of *B. rapa* seeds were different (Figure 2). The germination rate of *B. rapa* seeds treated with the 100% methanol phase extract was the lowest, at 61.68%. Through comparative experiments, it was found that the methanol phase extract of *N. roborowskii* seeds had the strongest inhibitory effect on the germination of *B. rapa* seeds (Figure 2A).

After treatment with 100% methanol, ethyl acetate, petroleum ether, or water for 48 h, the germination rates of the seeds decreased by 22.50%, 17.50%, 13.35% and 11.68%, respectively, compared with those of the control group (84.18%) (Figure 2), and the germination indices decreased by 11.84, 9.84, 6.67 and 5.67%, respectively, compared with those of the control group (44.84) (Figure 3). This proves that the extracts of each separation phase of *N. roborowskii* seeds had a very significant inhibitory effect on the germination rate and germination index of *B. rapa* seeds ($p < 0.05$). The 25% water phase and 25%, 50%, 75% and 100% methanol phase extracts had stronger inhibitory effects on the germination of *B. rapa* seeds than did the other organic phase extracts (Figures 2 and 3). The inhibition of *B. rapa* seeds by extracts from each isolated phase of *N. roborowskii* seeds at the same concentration differed (Figure 4), and the inhibitory effects of the various organic phase extracts on the germination rate and germination index of *B. rapa* seeds decreased in the following order: methanol phase > ethyl acetate phase > petroleum ether phase > water phase.

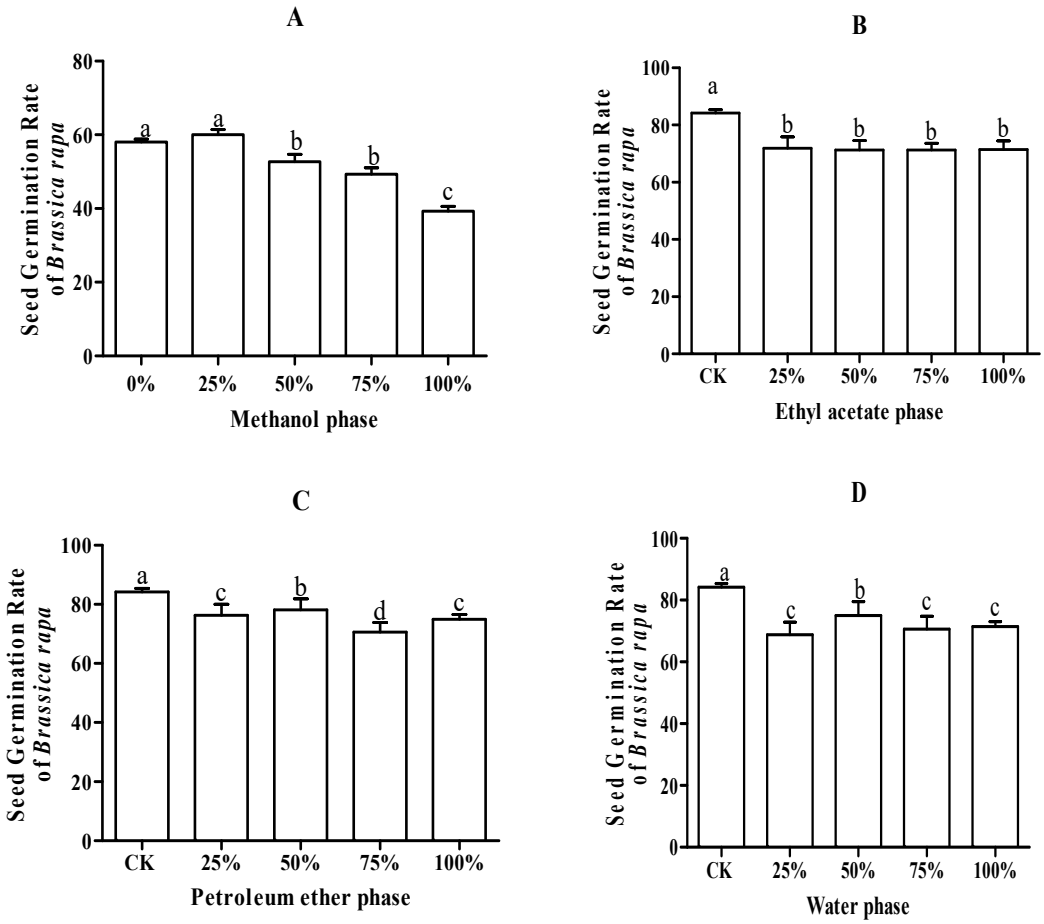

Figure 2. The effect of inhibitors from *N. roborowskii* seeds on the percentage of *B. rapa* that germinated (mean (M) ± standard error (SE)). (**A**): methanol phase; (**B**): ethyl acetate phase; (**C**): petroleum ether phase; (**D**): waterphase). Different lowercase letters indicate significant differences between different treatments ($p < 0.05$), $n = 3$.

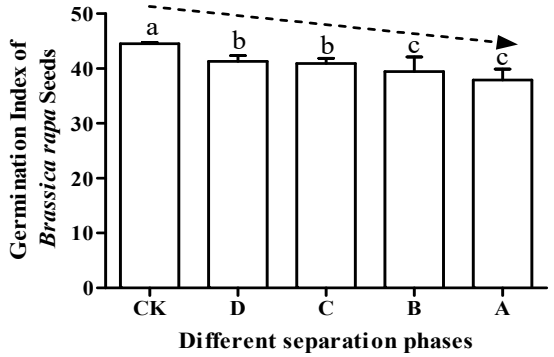

Figure 3. Effects of the same concentration of different phase inhibitors on the germination index of *B. rapa* seeds (mean (M) ± standard error (SE)). The inhibition of germination index of *B. rapa* seeds by control, water phase, petroleum ether phase, ethyl acetate phase and methanol phase extracts became increasingly significant. (A: methanol phase; B: ethyl acetate phase; C: petroleum ether phase; D: water phase). Different lowercase letters indicate significant differences between different treatments ($p < 0.05$), $n = 3$.

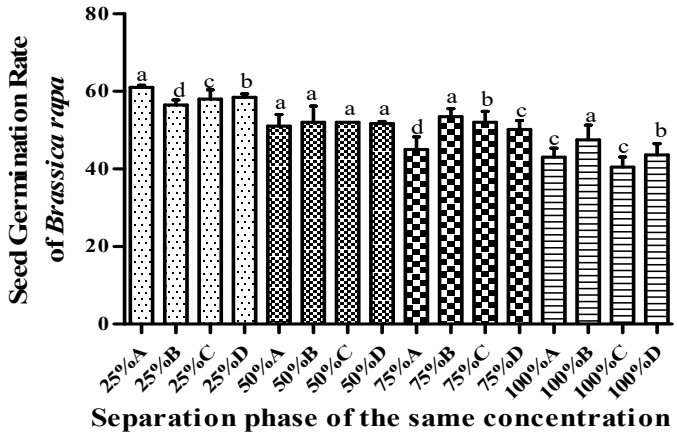

Figure 4. Effects of different endogenous phases at the same concentration on the germination rate of *B. rapa* seeds. (A: methanol phase; B: ethyl acetate phase; C: petroleum ether phase; D: water phase). Lowercase letters represent significant differences in comparisons at the 0.05 level.

3.2. Organic Compound Types in Extracts of N. roborowskii Seeds

3.2.1. Methanolic Phase Extract of *N. roborowskii* Seeds

The methanolic phase extract of *N. roborowskii* seeds was analyzed by GC–MS to determine the total ion flow, and eight peaks were separated (Figure 5). In this study, the relative content of each organic phase extract substance was calculated using the peak area normalization method, i.e., the sum of the peak areas of the separated phases was taken as 100%, and the ratio of the area of each peak to the total area represented the relative content of the component in the extract. A search through the mass spectrometry system and comparison with standard maps will result in substances with a good match (>80% similarity) [24]. Thirty-nine types of organic compounds were identified, twenty-eight of which were esters and ketones (Supplementary Materials). Eleven substances with good agreement were obtained (Table 1), and their relative contents decreased in order from high to low: 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-(34.48%), dibutyl phthalate (12.99%), triethyl phosphate (4.79%), 13-docosenamide, (Z)-(3.44%), 2-furancarboxaldehyde, 5-methyl-(3.12%), hexanedioic acid, bis(2-ethylhexyl) ester (2.95%), 1,2-cyclopentanedione (2.77%), furan (2.75%), formamide (2.67%), 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one (2.50%) and 2,5-dimethylfuran-3,4(2H,5H)-dione (2.10%).

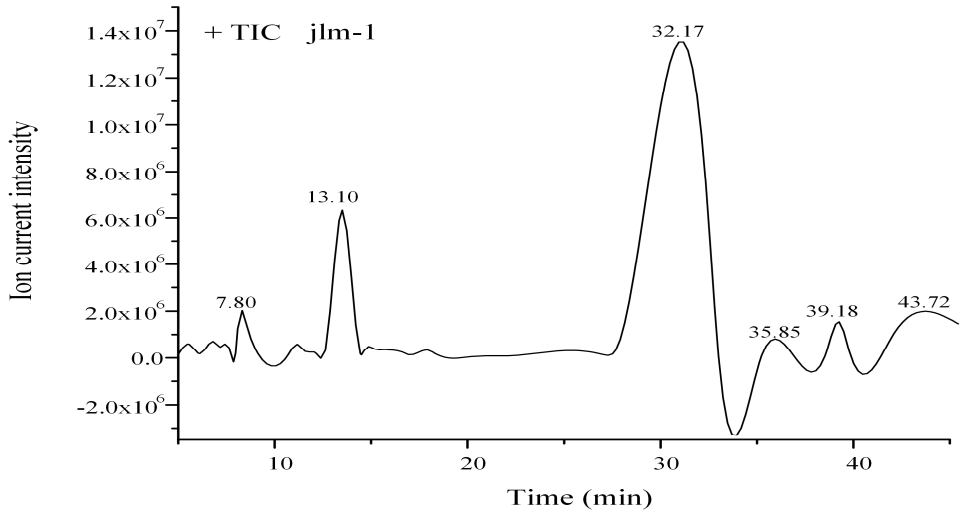

Figure 5. Chromatographs of methanol-phase inhibitors from *N. roborowskii* seeds.

Table 1. Types and relative contents of organic compounds extracted from the methanol phase of *N. roborowskii* seeds.

Peak Time (min)	Molecular Formula	Molecular Weight (Da)	Name	Peck Area Percentage Content (%)	Class Compounds
12.871	$C_6H_8O_4$	144	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	34.08	Ketone
32.097	$C_{16}H_{22}O_4$	278	Dibutyl phthalate	12.99	Salts
12.222	$C_6H_{15}O_4P$	182	Triethyl phosphate	4.79	Salts
45.431	$C_{22}H_{43}NO$	337	13-Docosenamide, (Z)-	3.44	Amides
7.616	$C_6H_6O_2$	110	2-Furancarboxaldehyde, 5-methyl-	3.12	Aldehydes
39.126	$C_{22}H_{42}O_4$	370	Hexanedioic acid, bis(2-ethylhexyl) ester	2.95	Salts
6.698	$C_5H_6O_2$	98	1,2-Cyclopentanedione	2.77	Ketone
7.157	C_4H_4O	68	Furan	2.75	Furans
6.604	CH_3NO	45	Formamide	2.67	Amides
8.012	$C_6H_8O_4$	144	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	2.50	Ketone
10.622	$C_6H_8O_3$	128	2,5-Dimethylfuran-3,4(2H,5H)-dione	2.10	Ketone

3.2.2. Extracts of the Ethyl Acetate Phase of *N. roborowskii* Seeds

The total ion flow of the ethyl acetate phase extract of *N. roborowskii* seeds was separated into 11 peaks by GC–MS analysis. By searching the computer mass spectrometry system and checking the sample spectrum obtained by mass spectrometry scanning with the standard spectrum, a total of 164 compounds were identified in the ethyl acetate phase of *N. roborowskii* seeds (Supplementary Materials), 26 of which were identified, mainly phenols, organic acids and aldehydes. Thirteen substances with good agreement were obtained (Table 2), and their relative contents decreased in order from high to low: 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol (20.05%), Unknown 1 (8.37%), Unknown 2 (4.92%), Unknown 3 (4.64%), 13-docosenamide, (Z)-(4.60%), Coniferyl aldehyde (3.30%), 3-hydroxy-4-methoxybenzoic acid (3.06%), vanillin (2.74%), Benzofuran, 2,3-dihydro-(2.29%), Unknown 4 (2.10%), Carbamic acid, methylphenyl-, ethyl ester (2.00%), Unknown 5 (1.94%) and Butanoic acid, 3-methyl-(1.83%) (Figure 6).

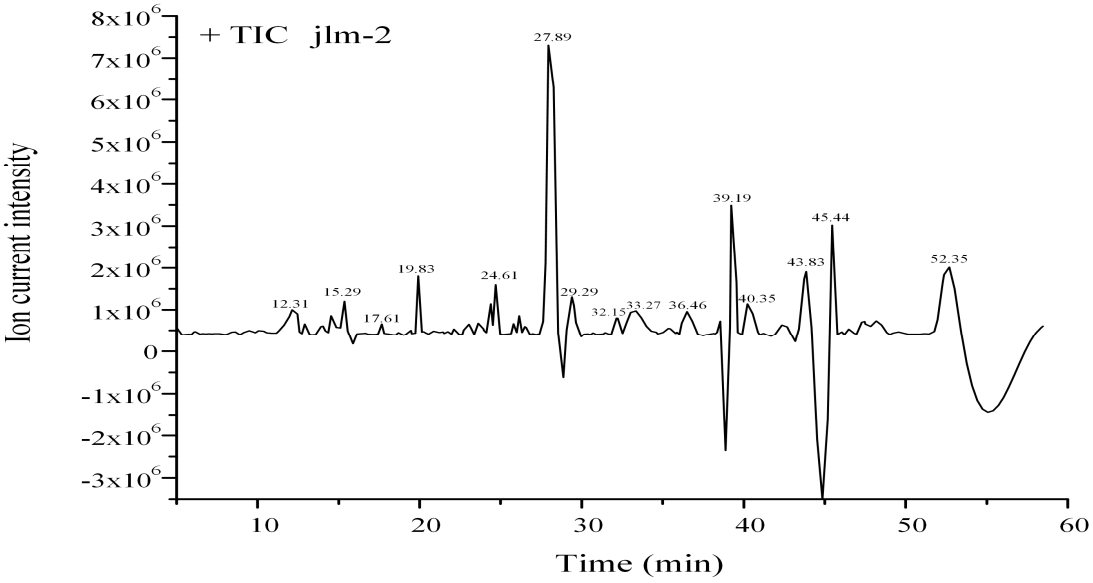

Figure 6. Chromatographs of the ethyl acetate phase inhibitors from *N. roborowskii* seeds.

Table 2. Types and relative contents of organic compounds extracted from the ethyl acetate phase of *N. roborowskii* seeds.

Peak Time (min)	Molecular Formula	Molecular Weight (Da)	Name	Peck Area Percentage Content (%)	Class Compounds
27.885	$C_{10}H_{12}O_3$	180	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	20.05	Phenols
12.034	-	-	Unknown 1	8.37	-
39.191	-	-	Unknown 2	4.92	-
52.354	-	-	Unknown 3	4.64	-
45.435	$C_{22}H_{43}NO$	337	13-Docosenamide, (Z)-	4.60	Amides
27.703	$C_{10}H_{10}O_3$	178	Coniferyl aldehyde	3.30	Aldehydes
24.309	$C_8H_8O_4$	168	3-Hydroxy-4-methoxybenzoic acid	3.06	Acids
19.827	$C_8H_8O_3$	152	Vanillin	2.74	Vanillin
15.257	C_8H_8O	120	Benzofuran, 2,3-dihydro-	2.29	Furans
29.462	-	-	Unknown 4	2.10	-
24.621	$C_{10}H_{13}NO_2$	179	Carbamic acid, methylphenyl-, ethyl ester	2.00	Salts
32.967	-	-	Unknown 5	1.94	-
4.714	$C_5H_{10}O_2$	102	Butanoic acid, 3-methyl-	1.83	Acids

3.2.3. Extracts of the Petroleum Ether Phase of *N. roborowskii* Seeds

The total ion flow of the petroleum ether phase extract of *N. roborowskii* seeds was separated into nine peaks by GC–MS analysis. By searching the computer mass spectrometry system and checking the sample spectrum obtained by mass spectrometry scanning with the standard spectrum, a total of 139 compounds were identified in the petroleum ether phase of *N. roborowskii* seeds (Supplementary Materials), 113 of which were identified, mainly alkanes. Fifteen substances with good agreement were obtained (Table 3), and their relative contents decreased in order from high to low: 13-docosenamide, (Z)- (16.31%), cyclohexane, ethyl-(5.13%), heptane, 2,2,4,6,6-pentamethyl-(3.20%), Unknown 1 (3.02%), 2,4-ddi-tert-butylphenol (2.64%), 1,5,9-uundradecatriene, 2,6,10-trimethyl-, (Z)- (2.63%), cyclohexane, 1,2-dimethyl-, cis-(2.02%), heptane, 2,6-dimethyl-(1.84%), nonane (1.83%), decane (1.74%), nonane, 3-methyl-(1.44%), heptane, 2,4-dimethyl-(1.44%), heptane, 2,3,4-trimethyl-(1.37%), Unknown 2 (1.37%) and Unknown 3 (1.34%) (Figure 7).

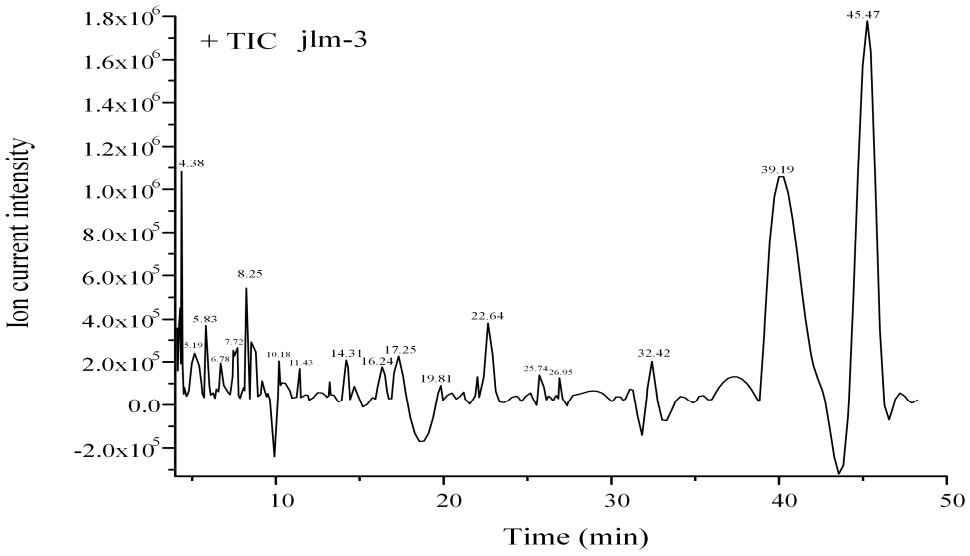

Figure 7. Chromatographs of petroleum ether phase inhibitors from *N. roborowskii* seeds.

Table 3. Types and relative contents of organic compounds extracted from the petroleum ether phase of *N. roborowskii* seeds.

Peak Time (min)	Molecular Formula	Molecular Weight (Da)	Name	Peck Area Percentage Content (%)	Class Compounds
45.467	$C_{22}H_{43}NO$	337	13-Docosenamide, (Z)-	16.31	Amides
4.381	C_8H_{16}	112	Cyclohexane, ethyl-	5.13	Alkanes
8.246	$C_{12}H_{26}$	170	Heptane, 2,2,4,6,6-pentamethyl-	3.20	Alkanes
39.185	-	-	Unknown 1	3.02	-
22.636	$C_{14}H_{22}O$	206	2,4-Di-tert-butylphenol	2.64	Phenols
46.025	$C_{14}H_{24}$	192	1,5,9-Undecatriene, 2,6,10-trimethyl-, (Z)-	2.63	Alkenes
4.293	C_8H_{16}	112	Cyclohexane, 1,2-dimethyl-, cis-	2.02	Alkanes
4.24	C_9H_{20}	128	Heptane, 2,6-dimethyl-	1.84	Alkanes
5.834	C_9H_{20}	128	Nonane	1.83	Alkanes
8.534	$C_{10}H_{22}$	142	Decane	1.74	Alkanes
7.716	$C_{10}H_{22}$	142	Nonane, 3-methyl-	1.44	Alkanes
4.115	C_9H_{20}	128	Heptane, 2,4-dimethyl-	1.44	Alkanes
7.457	$C_{10}H_{22}$	142	Heptane, 2,3,4-trimethyl-	1.37	Alkanes
4.987	-	-	Unknown 2	1.37	-
5.163	-	-	Unknown 3	1.34	-

The methanol phase extract contained 11 organic compounds in five categories, the ethyl acetate phase extract contained seven organic compounds in six categories, and the petroleum ether phase extract contained 12 organic compounds in three categories. The 30 compounds with high contents in each organic phase of *N. roborowskii* seeds were divided into nine classes (Table 4): alkanes, amides, ketones, esters, organic acids, phenols, aldehydes, furans and vanillin. Among them, there are 10 alkanes, accounting for 33.33% of the total number of organic compounds; four amides and ketones, accounting for 13.33% of the total; three esters and aldehydes, accounting for 10.00% of the total and two organic acids, phenols and furans, accounting for 6.67% of the total. Erucic acid amide was common and had a high content of organic matter in the three organic phase extracts (Tables 1–3).

Table 4. Distribution of the number of compounds in each organic phase extract.

Component	Number	Methanol	Ethyl Acetate	Petroleum Ether
Alkanes	10	0	0	10
Amides	4	2	1	1
Ketones	4	4	0	0
Esters	3	3	0	0
Organic acids	2	0	2	0
Phenols	2	0	1	1
Aldehydes	2	1	1	0
Furans	2	1	1	0
Vanillin	1	0	0	0
Number of categories	9	5	6	3
Number of organic compounds	30	11	7	12

4. Discussion

Many dormant plant seeds contain endogenous inhibitors of germination, which prevent seeds from germinating normally and are an important reason for seed dormancy. The degree of seed dormancy is altered in parent plants, and the maternal plant detects certain stimulants that affect seed dormancy and transports specific substances to the seeds to regulate their dormancy level [25]. None of the individual methanol, ethyl acetate or

petroleum ether organic solvents had a significant effect on *B. rapa* seed germination, so all the changes in *B. rapa* seed germination after treatment with the extracts in this study were caused by endogenous inhibitors in the extracts [26].

4.1. Bioassay of Endogenous Inhibitors in N. roborowskii Seeds

The studies have shown that the seed coat and embryo may contain different endogenous inhibitors. Due to the complex nature of *N. roborowskii* seeds, it is very difficult to separate the seed coat and embryo. Studies have shown that there is no significant difference in the inhibitory effect of the seed coat and embryo [27]. Therefore, in this study, organic phase extracts of intact *N. roborowskii* seeds were used to verify the presence of inhibitory substances.

After treatment with different organic phase extracts of *N. roborowskii* seeds at the same concentration, the germination rate, germination index and number of *B. rapa* seeds significantly decreased ($p < 0.05$). The order of the inhibitory effect on the germination rate and germination index of *B. rapa* seeds was methanol phase > ethyl acetate phase > petroleum ether phase > water phase, among which the methanol phase had the strongest inhibitory effect. Some studies have concluded that the methanol phase contains the most inhibitors [28]. An experiment also proved that *Nitraria tangutorum* Bobr. seeds also contain endogenous inhibitors, and the water extract of their seeds has a significant impact on the germination quality and growth of wheat plants [29].

4.2. Types of Inhibitory Substances in N. roborowskii Seeds

The compounds in the methanol phase, ethyl acetate phase and petroleum ether phase were separated and identified by GC–MS, which confirmed that the methanol phase of *N. roborowskii* seeds mainly contained 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-dibutyl phthalate, triethyl phosphate, 13-docosenamide, (Z)-furan carboxaldehyde, 5-methyl-hexanedioic acid, bis(2-ethylhexyl) ester and 1,2-cyclopentanedione. Some scholars have shown that 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl- may be a germination inhibitor in *Taxus chinensis (Pilger)* Rehd. seeds [12] and can inhibit the germination and seedling growth of *Stipa capillata* [30]. Dibutyl phthalate can inhibit and affect the germination of *Paeonia lactiflora* [31], *Glycine max* [32] and *B. rapa* seeds [33]. 13-Docosenamide (Z) has broad-spectrum antibacterial properties and can have a certain inhibitory effect on seed germination [34]. It has a significant inhibitory effect on the germination and growth of *Pinellia ternata* seeds [35].

The main substances identified in the ethyl acetate phase were esters, including phenol, benzoic acid and erucamide. The most abundant compounds identified were 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol, 13-docosenamide, (Z)-, coniferyl aldehyde, 3-hydroxy-4-methoxybenzoic acid, vanillin, benzofuran and 2,3-dihydro-. Phenolic compounds are widely present in nature and have certain antibacterial and antioxidant activities. These compounds may be converted into polyphenolic substances through oxygen in the seed coat, resulting in oxygen consumption by the seed coat and a lack of oxygen supply to the embryo or interference with cell oxidation [11,36]. Seeds containing phenolic compounds can inhibit their own germination or penetrate into the soil to inhibit the germination of neighboring seeds [11]. Root bark extract of *Michelia macclurei* Dandy (REMMD) containing 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol can significantly inhibit the growth of NIH/3T3 cells, and the inhibitory effect is dependent on its concentration [37].

The organic compounds identified in the petroleum ether phase extract mainly belonged to the alkane class, and the compounds with the highest contents were 13-docosenamide (Z)-; cyclohexane, ethyl-; heptane, 2,2,4,6,6-pentamethyl-; 2,4-di-tert-butylphenol; 1,5,9-undidecatriene, 2,6,10-trimethyl-, (Z)-; cyclohexane, 1,2-dimethyl-,cis-; heptane, 2,6-dimethyl-; nonane and decane. The results showed that the compounds in the petroleum ether phase had a relatively low inhibitory effect on the germination of *B. rapa* seeds, indicating that the compounds in the petroleum ether phase had relatively weak activity or a lower content of

germination inhibitors. 2,4-Di-tert-butylphenol has an inhibitory effect on the growth of *Nicotiana benthamiana* and Ramie [38,39].

This study revealed that the endogenous inhibitors in the seeds of *N. roborowskii* mainly included amides (13-docosenamide, (Z)-), esters (dibutyl phthalate; trimethyl phosphate; hexanedioic acid, bis(2-ethylhexyl) ester), ketones (4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-; 1,2-ccyclopentanedione), aldehydes (2-furancarboxaldehyde, 5-methyl-; coniferyl aldehyde), alkanes (cyclohexane, ethyl-; heptane, 2,2,4,6,6-pentamethyl-; cyclohexane, 1,2-dimethyl-, cis-; heptane, 2,6-dimethyl-; nonane; decane) and phenols (2,4-ddi-tert-butylphenol; 4-(1E)-3-hydroxy-1-propenyl)-2-methoxyphenol). The seeds of *N. roborowskii* may contain one or several inhibitors that interact with each other to inhibit seed germination. The water phase also has a certain inhibitory effect on the germination of *B. rapa* seeds, but due to instrument limitations, specific inhibitors in the water phase have not yet been identified [26,27]. These issues require further investigation.

Chemical treatment, lamination, flushing, low-temperature treatment and other physical methods are commonly used for the elimination of inhibitory substances [18]. Many plant extracts inhibit seed germination in Petri dishes, but this effect often disappears in soil because the leaching, adsorption and degradation in the soil can reduce the effectiveness of toxins [40]. Most natural seed germination inhibitors are water soluble, so plants can be rinsed with rainwater or soaked in warm or flowing water to remove inhibitors from the pericarp or seed coat and accelerate seed germination [41]. *N. roborowskii* generally grows in arid desert areas with extremely rare precipitation, which reduces the possibility of its seeds naturally breaking dormancy to a certain extent. Therefore, it is of great practical significance to investigate the causes of seed dormancy in *N. roborowskii*, especially through the study of its endogenous inhibitors, which can guide the artificial reversal of seed dormancy. Plant inhibitors have a certain autotoxic effect and can prevent seeds from being consumed and infected by bacteria, ensuring the survival and reproduction of their natural populations [42]. The deep dormancy of the seeds of *N. roborowskii* severely restricts the natural renewal rate of its population, and artificial cultivation has become an important way to compensate for the shortage of wild resources, while the release of its seed dormancy is one of the key problems of *N. roborowskii* that urgently needs to be solved. The present study demonstrated for the first time the existence of endogenous inhibitors with high activity in the seeds of *N. roborowskii*, which lays a foundation for explaining the mechanism of seed dormancy in *N. roborowskii* and for decreasing the dormancy of the seeds of *N. roborowskii*.

5. Conclusions

The methanol phase, ethyl acetate phase, petroleum ether phase and water phases of extracts isolated from the seeds of *N. roborowskii* had certain inhibitory effects on the germination of *B. rapa*. The order of the inhibitory effects of the four separation phase extracts was methanol phase > ethyl acetate phase > petroleum ether phase > water phase. GC–MS of the organic phase extract of the seeds of *N. roborowskii* revealed 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-dibutyl phthalate; 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol; 13-docosenamide, (Z)-; 3-hydroxy-4-methoxybenzoic acid; vanillin; 2,4-di-tert-butylphenol; cyclohexane and ethyl and other inhibitors. In this study, we investigated the causes of dormancy in the seeds of *N. roborowskii* from the perspective of endogenous inhibitors, and identified that it is still necessary to explore the role of inhibitors by determining the effective effective half-inhibitory concentration (IC_{50}).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15050773/s1.

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References

1. Wu, Z.Y. Distributional types of seed plant genera in China Yunnan Plant Research. *YunnanBotanicalResearch* **1991**, *4* (Suppl. IV), 1–139.
2. Suo, Y.R. *Research and Development of Nitraria in the Qaidam Basin*; Science Press: Beijing, China, 2010.
3. Wang, J.Q. Taxonomy, Distribution and Bioecological Characteristics of Nitraria. *Arid Zone For. Res.* **1989**, *2*, 47–63.
4. Zeng, Y.J.; Wei, J.P.; Yu, L.; Wang, Y.R. Seed viability, germination and dormancy of *Nitraria roborowskii* (Nitrariaceae). *Seed Sci. Technol.* **2016**, *44*, 647–653. [CrossRef]
5. Li, Q.H.; Xin, Z.M.; Gao, T.T.; Wang, S.X.; Xu, J.; Sun, F. Reproductive allocation in four desert species of the genus *Nitraria* L. *J. Ecol.* **2012**, *32*, 5054–5061.
6. Wang, S.; Li, Q.; Xu, J.; Gao, T.; Xin, Z. Experimental research on the pollination characteristics of 4 plant species of genus *Nitraria* L. *J. Biol.* **2012**, *29*, 49–51.
7. Liu, L.P. Analysis and Appraisal on Nutritional Components of the Four Plants of Nitraria in Inner Mongolia. Ph.D. Thesis, Inner Mongolia Agricultural University, Hohhot, China, 2009.
8. Yuan, Y.Y.; Zhou, Y.B.; Sun, J.; Deng, J.; Bai, Y.; Wang, J.; Lu, X.F. Determination and principal component analysis of mineral elements based on ICP–OES in Nitraria roborowskii fruits from different regions. *Chin. J. Tradit. Chin. Med.* **2017**, *42*, 2334–2338.
9. Gao, S.W. Study on Seed Dormancy, Germination Resistance to Adverse Stresses and Soil Seed Persistence in Genus *Nitraria*. Master's Thesis, Lanzhou University, Lanzhou, China, 2021.
10. Zeng, Y.J.; Wang, Y.R.; Zhang, J.; Li, Z.B. Germination responses to temperature and dormancy breaking treatments in *Nitraria tangutorum* Bobr. and *Nitraria sibirica* Pall. *Seed Sci. Technol.* **2010**, *38*, 537–550. [CrossRef]
11. Commander, L.E.; Merritt, D.J.; Rokich, D.P.; Dixon, K.W. Seed biology of Australian arid zone species: Germination of 18 species used for rehabilitation. *J. Arid Environ.* **2009**, *73*, 617–625. [CrossRef]
12. Decraene, L.; Smets, E.F. Morphological studies in Zygophyllaceae. I. The floral development and vascular anatomy of *Nitraria retusa*. *Am. J. Bot.* **1991**, *78*, 1438–1448. [CrossRef]
13. Ren, S.F.; Lv, G.H. Effects of different pretreatments on seed germination of *Nitraria roborowskii* Kom. *Seed* **2017**, *36*, 10–14.
14. Teng, Y.Z. *Dormancy and Germination in Plants*; Science Press: Beijing, China, 1980.
15. Thevenot, C.; Come, D. Inhibition of the germination of the embryonic axis by the cotyledons in apple. *CR Acad. Sci. Ser.* **1973**, *D277*, 1873–1876.
16. Kentzer, T. Gibberellin-like sub stance and growth inhibit ors in relation to the dormancy and after-ripening of ash seeds (*Fraxinus excelsior* L.). *Acta Soc. Bot. Pol.* **2015**, *35*, 575–585. [CrossRef]
17. Amen, R.D. A model of seed dormancy. *Bot. Rev.* **1968**, *34*, 1–31. [CrossRef]
18. Sun, J.; Guo, J.F.; Wei, S.N. Overview on Inhibitors of Plant Seed Germination. *Seeds* **2012**, *31*, 57–61.
19. Singh, M.; Tamma, R.V.; Nigg, H.N. HPLC identification of all elopathic compounds from *Lantana camara*. *J. Chem. Ecol.* **1989**, *15*, 81–90. [CrossRef]
20. Fu, J.R. *Seed Physiology*; Science Press: Beijing, China, 1985.
21. Fu, Q.C.; Liu, C.; Wang, Y.; Li, C.H.; Hu, Q. Study on germination inhibiting substances in methanol extract of drupes of *Sinojackia sarcocarpa*. *North. Hortic.* **2015**, *2*, 6–9.
22. Zhang, Y.J. Studies on the Dormancy Mechanism of *Taxus chinensis* var. mairei Seeds. Ph.D. Thesis, Nanjing Forestry University, Nanjing, China, 2007.
23. Liu, W.Y. Studies on the Dormancy and Release Mechanism of *Medicago truncatula Gaertn.* Seeds. Ph.D. Thesis, Gansu Agricultural University, Lanzhou, China, 2015.
24. Li, L.J.; Zhao, T.T.; Qian, C.M.; Li, S.X.; Zhai, J.T. Effect of Endogenous Inhibitors on Seed Germination of *Sapium sebiferum*. *J. Northeast For. Univ.* **2022**, *50*, 9–14.
25. Fenner, M.; Thompson, K. *The Ecology of Seeds*; Cambridge University Press: Cambridge, UK, 2005.
26. Zou, Y.T.; Chen, L.; Fan, X.J.; Li, X.S. Preliminary identification of endogenous inhibitors in Crataegus viridis seeds. *Seed* **2018**, *37*, 23–28.
27. Zhu, M.W.; Zou, Y.T.; Li, Y.R.; Li, S.X. Endogenous inhibitors in seeds of oil peony cultivar 'Feng Dan'. *J. Southwest For. Univ. (Nat. Sci.)* **2019**, *39*, 64–70.
28. Yan, F. Study on the Dormancy Mechanism of *Daphne giraldii* Seeds. Ph.D. Thesis, Gansu Agricultural University, Lanzhou, China, 2017.
29. Cheng, H.B.; Li, X.R.; Guo, F.X. Inhibitory effects of *Nitraria tangutorum* Bobr. Effects of seed extracts on wheat seed germination and growth. *J. Gansu Agric. Univ.* **2010**, *45*, 58–61.

30. Kamble, S.T.; Pawar, A. Allelopathic potential of petal leachates of *Cassia fistula* L. against an invasive weed *Alternanthera tenella* L. *Allelopath. J.* **2020**, *49*, 165–176. [CrossRef]
31. Zhang, S.Q. Bioassay and analysis of inhibitors in *Paeonia lactiflora* seed germination. *Anhui For. Sci. Technol.* **2015**, *41*, 17–20.
32. Li, C.F.; Chen, M.; Ma, F.M.; Gai, Z.J.; Guo, J.; Wang, Y.B. Study on the allelopathic effect of root exudates of *Beta vulgaris* on *Soybean*. *J. Northeast Agric. Univ.* **2016**, *47*, 21–30.
33. Ge, J.; Cheng, J.; Li, Y.; Li, Q.X.; Yu, X. Effects of dibutyl phthalate contamination on physiology, phytohormone homeostasis, rhizospheric and endophytic bacterial communities of *Brassica Rapa Var. Chinensis*. *Environ. Res.* **2020**, *189*, 109953. [CrossRef]
34. Tamilmani, E.; Radhakrishnan, R.; Sankaran, K. 13-Docosenamide release by bacteria in response to glucose during growth—Fluorescein quenching and clinical application. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 6673–6685. [CrossRef]
35. Tang, C.L.; Lei, Q.; Luo, F.L.; Zhao, Z.; Hang, Y.; Wang, H.L.; Luo, C.L.; Chen, S.H. Chemosensory effects of the chemosensory substance extracts of *Semen Armeniacae* on 8 crops. *Guizhou Agric. Sci.* **2019**, *47*, 31–37.
36. Khan, A.A. *The Physiology and Biochemistry of Seed Dormancy and Germination*; North-Holland Publishing Company: Amsterdam, The Netherlands; New York, NY, USA; Oxford, UK, 1977.
37. Song, X.K. GC–MS analysis of volatile components in root bark of *Michelia macclurei Dandy* and its inhibitory effect on the growth of NIH/3T3 cells. *Chin. J. Mod. Appl. Pharm.* **2011**, *28*, 1122–1125.
38. Wang, Z.W.; Bai, Y.C.; Fu, H.Y.; Zhao, L.; Cui, G.X.; She, W.; Yang, R.F. Effects of the water extract of Ramie straw on the physiological and biochemical characteristics and rhizosphere microorganisms of Ramie straw. *Mol. Plant Breed.* **2022**, *1*, 1–16.
39. Li, S.S.; Tan, X. Research on the influence of light intensity on continuous cropping obstacles of plants. *Anhui Agric. Sci. Bull.* **2022**, *28*, 12–15+42.
40. Krogmeier, M.J.; Bremner, J.M. Effects of phenolic acids on seed germination and seedling growth in soil. *Biol. Fertil. Soils* **1989**, *8*, 116–122. [CrossRef]
41. Yan, Q.C. *Seed Science*; China Agriculture Press: Beijing, China, 2001.
42. Bewley, J.D.; Bradford, K.J.; Hilhorst, H.W.M. *Seeds: Physiology of Development, Germination and Dormancy*, 3rd ed.; Springer: New York, NY, USA, 2013.