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RESEARCH PAPER

The effect of abiotic factors on *in vitro* seed germination in *Oxytropis chankaensis* Jurtz., a rare endemic species of the Russian Far East

Kseniya S. Berdasova*, Anastasiya S. Pianova & Liubov A. Kameneva

Kseniya S. Berdasova*
e-mail: k.berdasova@mail.ru

Anastasiya S. Pianova
e-mail: berdasova_as@mail.ru

Liubov A. Kameneva
e-mail: lubavar1188@mail.ru

Botanical Garden-Institute FEB RAS,
Vladivostok, Russia

* corresponding author

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ABSTRACT

The paper presents the results of different ways of overcoming dormancy of seeds of *Oxytropis chankaensis* Jurtz., a rare endemic species of the Far East characterized by small isolated populations. The peculiarities of seed germination under the influence of various abiogenic factors were studied: concentrated sulfuric acid, stratification, seed hilum puncture and abrupt temperature difference. A protocol for introducing the species studied to culture *in vitro* in order to preserve this endemic has been developed. The assessment of seedling viability after exposure to each of the factors was carried out. It was shown that the most effective and fastest way of scarification is the use of concentrated sulfuric acid. Stimulation of seed germination by this factor leads to 100 % seed germination and obtaining more than 90 % of viable plants capable of microclonal multiplication.

Keywords: rare species, seed reproduction, scarification, biodiversity conservation, endemic

РЕЗЮМЕ

Бердасова К.С., Пьянова А.С., Каменева Л.А. Влияние абиотических факторов на всхожесть *in vitro* семян *Oxytropis chankaensis* Jurtz., редкого эндемичного вида Дальнего Востока России. В работе представлены результаты различных путей преодоления покоя семян *Oxytropis chankaensis* Jurtz. – редкого эндемика Дальнего Востока, характеризующегося небольшими изолированными популяциями. Были изучены особенности прорастания семян под влиянием различных абиогенных факторов: концентрированная серная кислота, стратификация, прокол рубчика и резкий температурный перепад. Разработан протокол введения исследуемого вида в культуру *in vitro* с целью сохранения данного эндемика. Проведена оценка жизнеспособности проростков после воздействия каждого из факторов. Показано, что наиболее эффективным и быстрым способом скарификации является использование концентрированной серной кислоты. Стимуляция прорастания семян данным фактором приводит к 100 % всхожести семян и получению более 90 % жизнеспособных растений, способных к микроклональному размножению.

Ключевые слова: редкий вид, семенное возобновление, скарификация, сохранение биоразнообразия, эндемик

One of the effective strategies for the conservation of unique flora species is based on microclonal propagation methods as well as on the study of seed germination mechanisms. Understanding seed germination conditions and finding optimal *in vitro* cultivation protocols are essential for successful and rapid reproduction of the plant gene pool.

Specialist plant species with a narrow distribution range are more vulnerable compared to other species (Burlakova et al. 2011). One such plant in the flora of the Far East is *Oxytropis chankaensis* Jurtz., a perennial herbaceous plant of the family Fabaceae, occurring on the western shore of Lake Khanka (Primorye Territory, Russia) (Fig. 1A–D). It is endemic to the Far East and is listed in the Red Data Book of Primorye Territory (Pavlova 2008).

According to the International Legume Database and Information Service (ILDIS), *O. chankaensis* is considered a subspecies of *O. hailarensis* Kitag. L.I. Malyshev (2008) treated *O. chankaensis* as a synonym of *O. hailarensis*. Whereas in the Flora of China, *O. hailarensis* is synonymous with *O. oxyphylla* (Pall.) DC. However, according to Artyukova & Kozyrenko (2012), *O. hailarensis* and *O. oxyphilla* are independent species. Thus, the taxonomic position of *O. chankaensis* remains ambiguous. In this work, we share the viewpoint of Pavlova (1989) and accept *O. chankaensis* as an independent species.

The species is confined to open sandy habitats; the populations are isolated from each other. The area occupied by the species is subject to anthropogenic impact. In addition, in the past 20 years, nearly all habitats of *O. chankaensis* has disappeared or has been heavily transformed by the rising water level in Lake Khanka (Zhuravlev & Klyshevskaya 2015). Such factors could lead to the complete extinction of the unique Far Eastern species.

Although biotechnological methods are now widely used to conserve rare and endemic plants (Engelmann 2011, Reed et al. 2011, Corlett 2017, Coelho et al. 2020), there is little data on alternative ways to conserve *Oxytropis* species. *In vitro* cultivation of *Oxytropis triphylla* (Pall.) Pers., an endemic of the endangered Baikal flora, has been reported by Yurjeva et al. (2008). In 2015, He et al. (2015) demonstrated the possibility of successful *in vitro* cultivation of *Oxytropis glabra* (Lam.) DC. However, there are no data on microclonal propagation of *O. chankaensis*. The only known biotechnological method for conservation of this species is seed cryopreservation (Voronkova & Kholina 2017).

Seeds of the studied species have a relatively large embryo, a curved hypocotyl, and a rather small endosperm. Due to the water-repellent shell of the seeds, they are characterized by physical dormancy (PY). Physical dormancy, also known as seed hardness, is due to the complete water resistance of the seed coat and, less frequently, the pericarp. Water impermeability, in turn, is determined by some features of the seed coat structure, i.e. the presence of cuticle and highly developed palisade epidermis (Nikolaeva et al. 1985).

Thus, evaluation of the influence of various abiotic factors on seed germination in *O. chankaensis* and on its further microclonal propagation is of great interest. The overall objective of this study was to determine the optimal conditions for stimulating mass seed germination in *O. chankaensis* when cultured *in vitro*.

MATERIAL AND METHODS

Mature seeds of *O. chankaensis* collected in natural habitats on the sandy shores of Lake Khanka (Khanka Region, Primorye Territory) served as the material for this study.

Seed morphology and quality were studied using a Stemi DV4 stereomicroscope (Carl Zeiss, Germany).

Seed pretreatment was performed according to the following schemes:

1) thermal exposure (seeds were placed first in cold (8°C) and then in hot (+80°C) water for 5 seconds, in 5 repetitions);

2) treatment of seeds with concentrated sulfuric acid for 25 minutes;

3) puncturing the seed hilum with a needle;

4) freezing the dry seeds in the freezer at -18°C for 7 days.

Sterilization and germination of seeds was carried out in the following sequence:

1. Seeds were sterilized with 1 % silver nitrate solution for 20 minutes, then washed with 1 % sodium chloride solution.

2. Sterilized seeds were washed three times with sterile distilled water.

3. Seeds were germinated on Murashige & Skoog (1962) hormone-free nutrient medium.

4. Seed efficiency and germination rate were evaluated under different pretreatment methods during the first week of cultivation at 3000 lux and 16/8 h light/dark illumination.

5. Germination was recorded by the appearance of a 3 mm long rootlet. Germination and seed viability were monitored daily for all treatment types. Seed viability is a measure of the percentage of seeds that remain alive and produce plants after germination.

Experiments were performed in 3 replications, 30 seeds each. Statistical processing and data analysis were performed using Microsoft Office Excel (2013). All data are presented as mean and error bars indicate standard deviation.

RESULTS

Seed morphology

The average seed size of *O. chankaensis* is 1.55 mm. The shape is reniform, the surface is smooth, matte, sometimes slightly shiny. The color is brown-olive (Fig. 1B).

In vitro germination

During incubation on the nutrient medium, seeds swelled rapidly, and their *in vitro* germination rate depended on the type of their pretreatment. When seeds were exposed to concentrated sulfuric acid, mass germination was registered on the second day, with 93 % of the sprouts being viable and later forming additional shoots. Seed hilum puncture also proved to be efficient – the first sprouts emerged on the third day, and resulting microplant viability reached 66 %, which is one-third lower than when using a chemical agent. The lowest values were obtained during thermal exposure, when the seeds were first put into cold and then into hot water. When applying this method, the first sprouts appeared on the fourth day. Only 13 % of all germinated microplants were viable. The experiment with seed pre-freezing showed low viability of the resulting sprouts (46 %). Moreover, this method is inferior to the previous three in terms of germination rate (the first sprouts emerged on the fifth day after sowing).

DISCUSSION

Seed dormancy in *O. chankaensis* is defined by the dense pericarp and water resistance. It is known that reactive oxygen species (ROS) are involved in plant metabolism regulation (Mittler 2017, Mhamdi & Breusegem 2018) and act as signaling molecules that regulate normal plant growth and response to stress of various genesis. It is moisture absorption

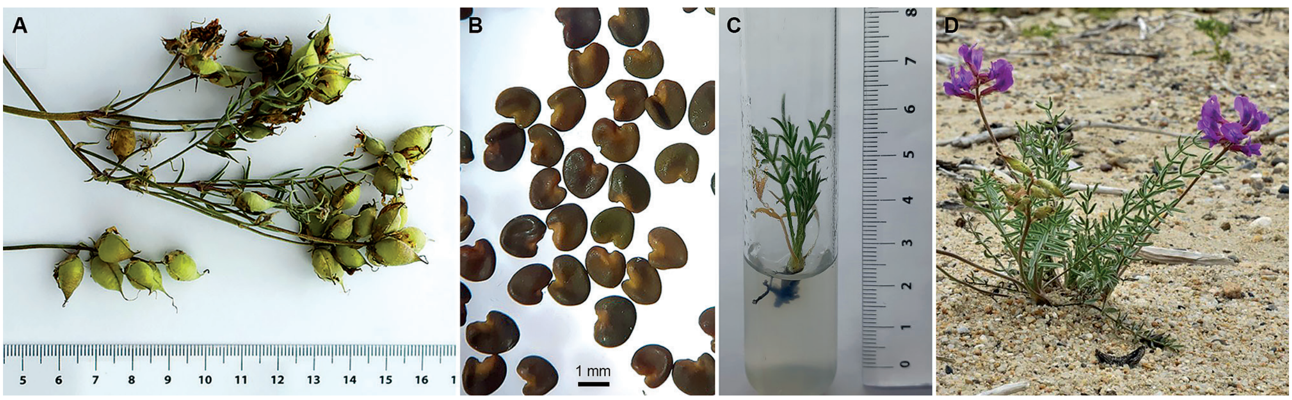

Figure 1 *Oxytropis chankaensis* Jurtz. fruits (A) (photo by K.S. Berdasova); seeds (B) at full maturity collected on the shore of Lake Khanka (Primorye Territory, Russia); microplant obtained in vitro (C) (photo by A.S. Pianova) and growing plant in natural conditions (D) (photo by L.V. Kraynik)

that contributes to ROS accumulation, which is the result of metabolism resumption in seeds with physiological dormancy (Bailly et al. 2008). Thus, we have studied the impact of 4 abiotic stress factors on seed germination in *O. chankaensis* and identified the most efficient approach to obtain a large number of viable sprouts of the rare Far East endemic. In our opinion, concentrated sulfuric acid acts as the most impactful factor to overcome seed hardness and trigger metabolic reactions in cells. Using this agent resulted in 100 % seed germination as well as minimal losses during their cultivation on the Murashige-Skoog nutrient medium free of growth regulators (Fig. 2).

Furthermore, the microplants obtained retained their viability and formed additional shoots (Fig. 1C).

Seed hilum puncture also facilitates an easy intake of water and oxygen into the seed. At the germination stage, only 10 % of seeds did not develop, that is 3 seeds out of 30. During further cultivation, 7 more sprouts are lost, hence 20 out of 30 plants remain viable. A decline in viability may be attributed to the embryo damage caused by seed hilum puncture and further abnormal development or to the launch of an apoptosis programme due to ROS high reactivity.

When modeling extreme natural conditions and studying factors such as seed freezing and temperature contrast almost equal germination was noted – 18 and 12 sprouts respectively. It was expected that after using these stimulation methods sprout viability would be high. However, when freezing dry seeds, only 14 sprouts retained their viability and developed into robust microplants during further *in vitro* cultivation. Therefore, this method can also be used for preserving *O. chankaensis*, although it is unlikely that many viable microplants will be obtained.

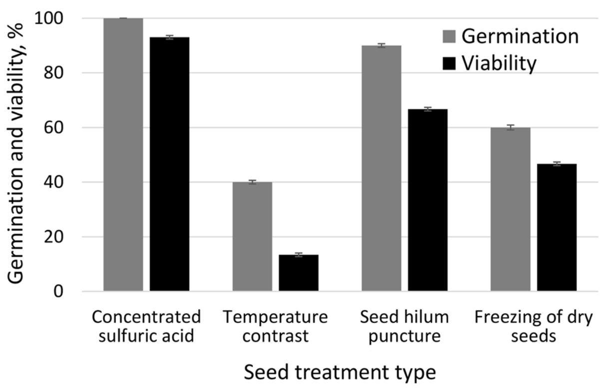

Figure 2 Pretreatment impact on germination and viability of *Oxytropis chankaensis* Jurtz. when introduced to *in vitro* culture

The experiment based on a temperature contrast proved to be the least efficient – only 4 microplants out of 30 remained viable (Table 1). As early as at the germination stage, over half of the samples were lost. Such losses might be caused by the thermal breakdown of enzymes triggering oxidation-reduction reactions in the embryo, when the seed coat dehisces. This part of the experiment demonstrates low resilience of the rare Far East endemic *O. chankaensis* and further confirms the urgent need to develop various strategies for its reproduction and conservation.

CONCLUSION

The optimal conditions for stimulating mass seed germination in *O. chankaensis* when cultivated *in vitro* were indented. Exposure of *O. chankaensis* seeds to concentrated sulfuric acid is the most favourable and time-efficient way of pretreatment since it triggers rapid destruction of the seed coat, opening access to water and oxygen without damaging the embryo. The majority of viable sprouts were obtained when using a chemical agent – concentrated sulfuric acid.

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Table 1. Pretreatment impact on germination and viability of *Oxytropis chankaensis* Jurtz. when introduced to *in vitro* culture.

Parameters	Concentrated sulfuric acid	Seed hilum puncture	Freezing of dry seeds	Temperature contrast
Number of non-developing seeds	0	3	12	18
Number of germinated seeds	30	27	18	12
Number of lost sprouts	2	7	4	8
Number of viable sprouts	28	20	14	4
Total number	30	30	30	30

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