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Factors affecting dormancy of *Oncocyclus* iris seeds

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ABSTRACT

Oncocyclus irises (Iridaceae) are endangered plants in Israel, yet with high potential for cultivation as ornamental flowers. However, their high seed dormancy level prevents fast development of germplasm for horticultural reproduction. In this paper we describe in-vitro and in-vivo germination experiments with seeds of *Oncocyclus* irises from Israel. We examined the effects of (1) mechanical scarification and different growing media on in-vitro seed germination; and (2) soil type, covering, and water amount on in-vivo germination. Seeds showed high dormancy, as hardly any seed germinated in the first year after sowing, and only in the second growing season the germinating fraction was considerable (up to 37%), still only under high humidity conditions. We also report an effective in-vitro forced germination protocol, which employs seed scarification. Following these results for in-vivo germination, and based on the protocol developed for in-vitro germination, we recommend two methods for artificial seed germination. For fast germination, good results from a modest quantity of seeds can be obtained by an in-vitro forced germination. For mass seed propagation, when time is not a limiting factor, the in-vivo procedure can be used, using an artificial soil seed bank and treating those seeds during (at least) two seasons under shade and continuous watering.

INTRODUCTION

Iris species of the section *Oncocyclus* are characterized by an ariluous seed, with a very hard seed coat (Avishai, 1977; Blumenthal et al., 1986). This hard seed coat is developed by dehydration during the ripening process of the seeds at the end of the spring (Avishai, 1977). It has been shown that these seeds have high dormancy, which causes a slow rate of germination under natural conditions, usually no more than 30% in the first few years (Shimshi, 1967). Avishai (1977) reported 10–15% germination in the first year, while the rest of the seeds germinated throughout the subsequent 5–6 years. Blumenthal et al. (1986) found different germination rates among species, from 1% to 60% seed germination in the first year for *Iris lortetii* Barbey and *I. atropurpurea* Dinsmore, respectively. *Oncocyclus* iris species grow in the Middle East in herbaceous plant communities, usually in dry Mediterranean or semiarid climates (Avishai, 1977). It is plausible that seed dormancy in the *Oncocyclus* irises has evolved as an adaptation to these climates (Blumenthal et al., 1986).

All Israeli *Iris* species of the section *Oncocyclus* are rare and endangered, with high priority for conservation (Avishai, 1977; Sapir et al., 2003; Shmida and Pollak, 2007). Currently, many natural populations of these species in Israel are declining or under threat of being destroyed, causing the possible loss of unique genetic germplasm. Ex-situ maintenance of rhizomes from destroyed habitats is a potential method to preserve this genetic diversity, but relocation success is usually un-

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certain (Y. Sapir, unpublished data). Another option is species maintenance in cultivation. However, this has shown to be problematic because rhizomes obtained from natural populations tend to rot and die after a few years, probably due to viral infection (Shimshi, 1967; Achilles, 2007; Y. Sapir and S. Volis, unpublished data). Nonetheless, this infection seems to be limited to vegetative parts of the plant, and does not transfer through seeds. Therefore, an apparent solution to the problem is to produce ex-situ populations by germinating seeds and producing new generations iteratively (Shimshi, 1967).

One possible technique to overcome seed dormancy in *Iris* is scarification and low temperature stratification (Sun et al., 2006). Soaking the seed in NaOH solution dissolves the seed coat, exposes the endosperm, and possibly reduces the mechanical block that prevents the embryo from absorbing water. Possibly also, physiological inhibitors produced by the seed coat are removed in the process (Sun et al., 2006). Low temperature has been shown to alter the degree of dormancy in seeds (Vleeshouwers, 1995). This method of scarification and stratification had been shown to be successful in *I. lactea* Pallas (section *Limniris*), which is characterized mainly by physiological dormancy (Sun et al., 2006). Germination percentage in this species increased from 0% in untreated seeds to >80% in treated seeds (Sun et al., 2006). Volis et al. (2007) used a similar procedure on non-geminating seeds of *I. atrofusca* Baker (section *Oncocyclus*). There, keeping the seeds in a moist and low temperature (11 °C) environment did have a small positive effect on seed germination (2%). Nonetheless, this method was not examined in the present study, due to its preliminary failure.

Shimshi (1967) proposed an in-vitro germination protocol for *Oncocyclus* irises that includes removing the seed coat from unripe fresh seed, and then growing the embryo on a growth medium. He proposed that this method both prevents accumulation of germination-inhibiting substances acquired in the seed coat, and improves water absorption by the embryo. Blumenthal et al. (1986) found evidence supporting Shimshi's (1967) proposal. They showed that high mechanical resistance of the seed coat at the micropylar area is the main cause of seed dormancy. The pressure needed to pierce the seed coat of two species, *I. lortetii* and *I. atropurpurea*, was 135 and 77 atmospheres, respectively. It was also found that extracts of the outer integument of the seed coat inhibited embryo germination (Blumenthal et al., 1986).

A third method proposed for germination of *Iris* species is "enhanced germination treatment", which reduces the dormancy of seeds in-vivo by controlling their environmental conditions (e.g., light, temperature, and moisture; McAllister, 2007).

Here we present results of a study in germination of *Oncocyclus* iris seeds. Our specific objectives were to compare in-vivo and in-vitro germinating protocols and to develop a germination protocol which will overcome seed dormancy efficiently. This study is part of ongoing research aimed at conservation of the *Oncocyclus* irises by means of ex-situ solutions, alongside establishing new populations in the natural habitats.

MATERIALS AND METHODS

Source of seeds

Three Israeli *Iris* species were used in this study: *Iris mariae* Barbey, *I. atrofusca* Baker, and *I. petrana* Dinsmore. Seeds of *I. mariae* were obtained from plants that were rescued from a natural habitat in the northwestern Negev desert (Latitude: 31°10′N; Longitude: 34°19′E) that was destroyed due to agricultural development. Rescued plants were replanted in spring 2007 in a nethouse at the Institute for Applied Research, and seeds were collected from flowers that were pollinated naturally in situ before replanting. Seeds of the other two species, *I. atrofusca* and *I. petrana*, were collected in natural populations in 2007 and 2008, from Goral hills (31°18′N, 34°47′E) and Yeruham Iris Reserve (31°01′N, 34°58′E), respectively. The seeds were kept in paper bags at room temperature until the start of the experiment (January, 2008).

In-vitro forced germination

Seeds were kept in 60% H_2SO_4 for 8–10 min for sterilization, and then washed in tap water for 20–25 min, placed in 70% Ethanol for 3 min, and sterilized again with a 0.3% NaOCl solution (domestic-use bleach) for 6–8 min. Lastly, the seeds were rinsed in sterile water four times for 6–8 min. After sterilization, seeds were deposited on filter papers in U-shaped tubes with sterile water as the growing medium, one or two seeds per tube. Seeds were kept in 22–25 °C under short-day light regime (8 h daylight) in a growing chamber. Seeds contaminated by fungi were removed 10–14 days after sterilization. Two weeks after the start of the germination process, seeds were transferred to tubes with one of three media (see below), where the aril was cut just above the embryo for half of the seeds on each germination medium.

The following germination media were used: (1) MS20 [1/2 strength Murashige & Skoog + 20 g L^{-1} sucrose]; (2) A1 (MS20 + 1 mg L^{-1} GA_3); (3) A10 (MS20 + 10 mg L^{-1} GA_3). All media were solidified by agar. Seeds were incubated at 22–25 ºC and 8-h light regime in a growing chamber. Tubes were visually examined every third day for scoring germinated seeds and re-

moving contaminated ones. Seeds were determined as germinating when having a root of at least 5 mm in length. Seedlings with leaves of 1–2 cm height were transferred to glass tubes with 8–10 ml MS20 medium, and about 10 days later, when plants reached 3–5 cm in height, they were transferred to a pot with perlite, still within the same growing chamber. Most of the germination occurred within 4–6 weeks from start of the process. No germination was observed after the sixth week. Well-developed seedlings at 10–15 cm of height were transferred to a commercial potting mixture soil in the net-house.

Initially, this experiment was performed on seeds of *I. mariae* only. Following the results of this experiment (see Results), seeds of *I. atrofusca* and *I. petrana* were also used but treated only with the A10 medium.

In-vivo germination

In order to determine the ecological factors affecting seed germination, seeds of *I. atrofusca* and *I. mariae* were used in a common-garden experiment. One hundred seeds were spread uniformly at 1 cm depth in 1-L pots with either of two soil types, loess and sand, the soils found at the natural habitats of these species, respectively. Pots experienced one of two humidity treatments, either increased humidity by artificial irrigation in addition to the natural rainfall ("irrigated"), or natural rainfall only ("not irrigated"). Natural rainfall in Be'er Sheva was 110 mm in the winter of 2007–2008 and 137 mm in 2008–2009 (Israel Meteorological Service website, www.ims.gov.il). In the "irrigated" treatment, pots were watered daily. The third factor was shadowing the pots by covering. Pots were covered with 5 layers of a black net ("covered") or were simply mounted on a table in the net-house ("uncovered"). It should be noted that light and humidity conditions were probably strongly associated: the covered pots kept moisture longer than uncovered ones following rain events. Full-factorial experimental design was used, with 5 replications of each species/treatment combination, to a total of 8000 seeds. The experimental pots were placed in a net-house (30% reduction of solar radiation) at the Institute for Applied Research at Ben-Gurion University, Be'er Sheva (31°15′N, 34°48′E).

The experiment was performed during two growing seasons, from January 10 to May 28, 2008, and from November 16 to April 12, 2009. During the dry season (May to October) the pots were kept in a dry place and were not irrigated. Last germination was observed in April 5, 2009, and the experiment was terminated a week later. During the growing period, the pots were inspected weekly for the number of emerging seedlings. Each germinated seed was immediately transferred from the pots, in order to reduce competition among seedlings.

Statistical analysis

The effects of growing medium and cutting treatments on in-vitro seeds germination of *I. mariae* were analyzed using Chi-Square test separately for each factor. Comparisons among all the three species for the success of in-vitro germination at each germination stage were also performed with Chi-Square tests.

In order to examine the effects of four environmental factors on in-vivo seed germination we used the Cox proportional hazards model to compare germination rate with time. We used only data from the second season, because germination in the first season was very low (see Results). Statistical analyses were performed in STATISTICA (StatSoft Inc., 2004).

RESULTS

In-vitro forced germination

Cutting the arils seems to improve germination rate, but the effect was only marginally significant ($\chi^2_7 = 3.12$, $p = 0.077$ for the combined media treatments; Fig. 1). Although not significant ($\chi^2_{76} = 2.32$, $p = 0.313$), the use of A10 medium slightly improved germination rates (Fig. 1). Following these results from *I. mariae*, we used A10 medium for seeds of *I. atrofusca* and *I. petrana* in consecutive experiments. For comparisons among species (below) we used only results from seeds grown on A10 medium.

Table 1 presents rates of germination success, as expressed by the fraction of seeds remaining through the three steps of the germination protocol. Overall, seeds

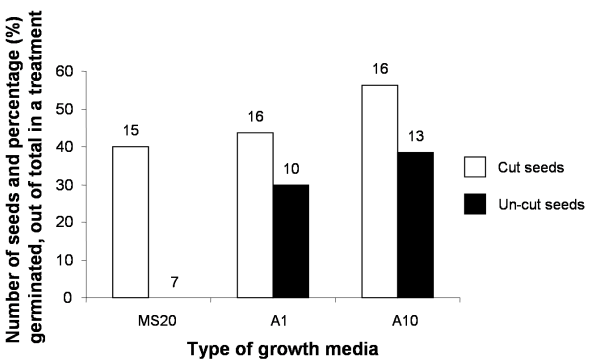

Fig. 1. Germination rates of *Iris mariae* seeds in an in-vitro forced germination experiment on three types of growing media, with two cutting treatments. Numbers above bars are sample sizes (n).

Table 1
Number of seeds used in each step of forced in-vitro germination of three *Oncocyclus* species. Fractions of seeds passed from the previous step/from the starting sample size are in parentheses

Step	*Iris mariae**	*Iris atrofusca*	*Iris petrana*
0. Sterilization and swelling	120	144	126
1. Germination	77 (64%/64%)	87 (60%/60%)	57 (45%/45%)
2. Seedlings	22 (29%*/18%)	36 (41%/25%)	26 (46%/21%)
3. Plants in-vivo	21 (95%/18%)	29 (81%/20%)	23 (88%/18%)

* For *I. mariae*, the germination-seedling steps were done using 3 different media and 2 different cutting treatments (see Fig. 1). Germination percentage on A10 medium and cutting is 56%—which we used when comparing among species—while the overall percentage is 29%. For the other two species, A10 medium and cutting were used for all seeds.

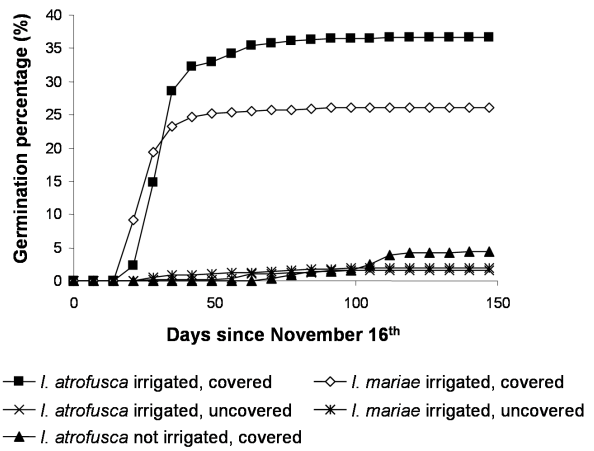

Fig. 2. Cumulative germination percentage in the second season of the in-vivo experiment, starting November 16, 2008, (start of irrigation). Five of the 8 possible combinations of three tested factors (species, water amount, and covering) are shown, because in the other three combinations cumulative germination did not exceed 0.2%. The data for each species combine two soil types, loess and sand.

of all three species showed similar success rates, ranging from 18 to 20%. The first two steps, seed sterilization and seed germination, had success rates of about half at each step (from 41 to 64%) while the final (third step) success rates were higher (81 to 95%). Significant differences were found among species in success for the first step ($\chi^2_{389} = 10.28$, $p = 0.006$), where *I. petrana* seeds had lower success. No significant differences were found among species in the second ($\chi^2_{168} = 0.69$, $p = 0.71$) and third ($\chi^2_{83} = 2.74$, $p = 0.25$) steps. All reductions in seed numbers between steps were due to contamination, which occurred in various steps of the experiment. Contaminated seedlings were counted as germinated, because we assume that seed germination and contamination are separate processes.

Table 2
Results of Cox proportional hazards model for germination over time with four independent factors

Factor	Wald Statistics
Species	39.7 ***
Water	109.9 ***
Covering	46.1 ***
Soil	2.3 ns

***$p < 0.001$; ns—not significant.

In-vivo germination

During the first growing season (2007–2008) only 20 seeds (0.25%) germinated, 13 seeds of *I. mariae* and 7 seeds of *I. atrofusca*, all in the irrigated pots. During the second growing season (2008–2009), 709 (8.9%) seeds germinated, most of them during a two-week period, about a month from the start of irrigation (Fig. 2). The number of germinated seeds was unequally distributed among treatments. In the unirrigated/uncovered treatment none of the seeds germinated, while in the irrigated/covered treatment 31.4% of the seeds germinated. In the other two combinations, germination was similarly low: 2.3% in unirrigated/covered and 1.8% in irrigated/uncovered treatment. The effect of moisture and covering on germination rate is significant, tested by Cox proportional hazard models, describing the germination process over time (Table 2). Species identity also had a significant effect on germination: *I. atrofusca* had a higher percentage of germination than *I. mariae* (36.6% and 26.1%, respectively, in the irrigated/covered treatment; Fig. 2). Soil type had no significant effect on germination.

DISCUSSION

The protocol used here for in-vitro germination of seeds of three *Oncocyclus* species, namely, *I. mariae*,

I. atrofusca, and *I. petrana*, appears to be a promising method for conservation and propagation of these irises. It has been shown that innate seed dormancy can be broken, to get a relatively high percentage of germination (56, 41, and 46%, for *I. maria*, *I. atrofusca*, and *I. petrana*, respectively). Cutting the arils and exposing the embryo seems to give a slight improvement to germination success. Such scarification improves seed germination probably because it removes the seed coat, which prevents penetration of water and air, and physically inhibits germination, resulting in physical seed dormancy (Blumenthal et al., 1986). Modifications of the Murashige & Skoog media in our experiment had no significant effect on germination, although the use of A10 media produced the highest germination rate. An unsolved problem in our experiment is seed contamination by fungi. Despite sterilization procedures, about half of the seeds got infected and either failed to germinate (first step) or died soon after germination (second step). Fungal contamination reduced the percentage of seeds that reached the seedling stage to 18% (*I. mariae* and *I. petrana*) or 20% (*I. atrofusca*). Differences among the three studied species were minor, and found only at the first step. This might reflect environmental differences in the natural habitats of these species, or differences in the time of collecting the seeds. Thus, for mass germination in *Oncocyclus* irises, one should use fresh seeds, i.e., immediately upon maturation (ca. six weeks after flowering).

The second experiment, of seed germination in vivo, with seeds of *I. mariae* and *I. atrofusca*, sheds light on the importance of environmental factors, namely, soil type, shade, and soil humidity. In the first season (2007–2008), germination was very low and only under humid ("irrigated") conditions. In the second season (2008–2009), we found no effect of soil type (loess vs. sand) on germination percentage for both species. Shading had a strong positive effect on germination, relative to the uncovered treatment, perhaps due to reduced evaporation, which increased overall soil humidity. *I. mariae* started germination before *I. atrofusca*, but the latter had a higher overall germination rate. We conclude that seed dormancy is probably similar among *Oncocyclus* iris species, while its exact pattern may depend on morphological characteristics of the seed (Blumenthal et al., 1986).

Various methods and protocols are available for forced germination of seeds for horticulture. Two such methods were used in the present study. As shown here, these two methods differ in the time demands, and the efficiency of obtaining quantity or quality seedlings. For small quantities of seedlings that are needed in a short time, the in-vitro method is preferable, as it provides seedlings in a few months, and can be performed at any time of the year. However, this method requires a high investment, because it requires attention to each seed separately. Also, precautions should be taken against fungal contamination. When a large quantity of seeds of unknown quality and/or age is available, and time is not a limiting factor, these seeds can be germinated in-vivo with much less effort (simply sowing and irrigating). By this method, a fairly high germination rate can be obtained. This approach can be useful for population restoration. Overall, the results presented in this paper may be of use for management of population restoration or establishment, which can benefit conservation strategy. Further development of an effective protocol of germinating *Oncocyclus* irises seeds for conservation purposes can be based on our findings reported here.

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