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Optimization of seed germination in an Iranian serpentine endemic, *Fortuynia garcinii*

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ABSTRACT

Fortuynia garcinii (Brassicaceae) is endemic to serpentine soils in central Iran. It has indehiscent silicles. The effects of its fruit pericarp on seed imbibition and germination were determined. The effects of moist chilling (15 days) and gibberellic acid (GA_3, four levels), both alone and combined, were also investigated. In addition, the effects of nickel (Ni) on germination and early seedling growth were evaluated. When inside the indehiscent silicles, imbibition of seeds was hampered and germination completely inhibited. Immediately after removing the pericarp, the seeds were able to imbibe as good as isolated seeds, but their germination rates were significantly lower when the removed pericarps were included in the Petri dishes. All the GA_3 concentrations, moist chilling, both alone and combined, significantly shortened mean germination time (MGT), and increased the germination index (GI) and the germination percentage (GP). Excess Ni did not affect GP, but inhibited seedling growth. In conclusion, the pericarp inhibits seed germination, both chemically and mechanically, through impeding imbibition, in *F. garcinii*. Moist chilling or GA_3 improves the speed and final percentage of germination. Seedling growth is much more sensitive to Ni excess than seed germination.

1. Introduction

Serpentine soils are characterized by physically and biologically stressful edaphic properties such as steep slopes, unstable substrates, coarse rocky textures and low water-holding capacities. They also have low Ca/Mg ratios, low contents of essential nutrient elements such as nitrogen, potassium and phosphorus, and relatively high, often toxic, concentrations of nickel (Ni), or occasionally Cr and Co (Ghaderian and Baker, 2007). Serpentine soils have a specific flora, often with considerable numbers of endemic species or specialized ecotypes. Most serpentinophytes are hypertolerant to Ni (Clemens, 2001; Asemaneh et al., 2006; Ghaderian and Baker, 2007). Most metal-hypertolerant species or ecotypes are 'excluders', which restrict the uptake and/or root-to-shoot transport of heavy metals (Baker, 1981), although at least part of their hypertolerance usually relies also on a superior capacity to sequester the metals inside their body, through chelation and subcellular compartmentalization, usually mainly in root cell vacuoles (Hall, 2002). A minority of metal-hypertolerant species are so-called 'hyperaccumulators', most of which hyperaccumulate Ni. These species accumulate metals at extremely high concentrations, usually 2–3 orders of magnitude higher than 'normal' plants, in their leaves (Baker and Brooks, 1989).

Fortuynia garcinii (Burm.f.) Shuttlew, also known as *Fortuynia bungei* Boiss. (Brassicaceae), is a non-Ni-hyperaccumulating, woody based branched perennial, endemic to serpentine soils in central Iran (Ghaderian and Baker, 2007). It usually flowers in late spring, and produces a large number of fruits. The fruit is an indehiscent silicle, containing one or two seeds (Rechinger, 1968). The species is propagated through seeds only. Several reports indicated that in Brassicaceae with dry indehiscent fruits germination within the fruit is prevented by the rigid pericarp, which physically prevents the embryo to expand and protrude. However, the pericarp might also inhibit germination through hampering the diffusion of water or oxygen to the embryo (Mekenian and Willemsen, 1975; Lu et al., 2010; Ohadi et al., 2011; Lu et al., 2015). The fruits ripen during late May–early June, and are then dispersed by wind. Germination occurs early in spring of the next year, after decay of the pericarp. Thus, in this species fruit indehiscence may be considered to represent an adaptation to avoid exposure in the seedling stage to extreme heat and drought in summer, or extreme cold and frost in winter, which are characteristic for the local climate of the natural habitat of the species.

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In many species seeds do not germinate when placed under conditions normally regarded as favorable to germination and are said to be dormant. Five types of seed dormancy have been recognized: physiological, morphological, morpho-physiological, physical and combinational dormancy (Baskin and Baskin, 2004). Seed (or fruit) coat-dependent dormancy is due to restriction of water uptake, gas exchange, mechanical resistance, or the presence of inhibitors such as abscisic acid (ABA) (Bewley and Black, 2012; Afroze and O'Reilly, 2016). Seed dormancy is one of the most important adaptive properties of wild species, and is a mechanism for optimizing the distribution of germination in time and place (Bhatia et al., 2005; Cousins et al., 2014). Various seed treatments, such as disruption of the seed coat (scarification), a period of dry storage (dry after-ripening), moist chilling (stratification), exposure to light (Finkelstein et al., 2008; Rouhi et al., 2010) and gibberellin (GA) treatments have been applied (Rouhi et al., 2010; Barreto et al., 2016; Mattana et al., 2016) to increase the speed and the percentage of germination, or to synchronize the germination day. Gibberellins stimulate seed germination in a wide range of plant species. However, the effectiveness of gibberellins depends on the species (Rouhi et al., 2010). Breaking dormancy by incubating seeds under moist and cold conditions, to simulate overwintering, is known as stratification (Finkelstein et al., 2008). The effectiveness of GA_3 as a germination promoter is believed to increase with stratification treatment (Yamauchi et al., 2004), and stratification has been reported to induce an increase in the GA_3 concentration in seeds (Yamauchi et al., 2004; Rouhi et al., 2010).

Nickel (Ni) is considered an essential micronutrient for plants (Brown et al., 1987), but becomes toxic at excess concentrations in most plant species. Typical symptoms of Ni toxicity in plants are inhibition of growth, chlorosis, wilting, foliar necrosis and root browning or die-back (Gerendás et al., 1999; Gajewska et al., 2013). Serpentine soils are often toxically Ni-enriched (Proctor and Woodell, 1975), and serpentine plant communities are largely composed of species or ecotypes with extreme levels of Ni tolerance, as compared to related non-serpentinophytic species or con-specific ecotypes (Baker and Brooks, 1989). Since seed germination is the first physiological process to be potentially affected by high Ni concentrations in the soil, the ability of a seed to germinate in a medium containing high Ni might be indicative of its level of tolerance to this toxic element, although Ni toxicity seems to impact more on seedling growth than on germination (Di Salvatore et al., 2008; Visioli et al., 2014).

Human activities and climate change, such as global warming, or changes in precipitation patterns can easily lead to extinction of rare species in restricted habitats, such as serpentine areas. Strengthening of populations using plant specimens that are propagated *ex situ* is regarded as a possibility to reduce the risk of local extinction of endangered species (Kirmizi et al., 2011). The germination requirements of *F. garcinii* have not previously been studied. Data on the germination conditions of this species could be employed in *ex situ* conservation management.

To better understand the germination ecology of *F. garcinii* and the potential role of pericarp-imposed seed dormancy therein, we determined the effects of pericarp removal and cold stratification on germination in *F. garcinii*. To check for a potential role for Ni in seedling establishment, we also investigated the effects of different concentrations of Ni on seed germination and early seedling growth.

2. Materials and methods

2.1. Plant material

Approximately 2000 mature fruits of *Fortuynia garcinii* were harvested from at least 50 randomly selected individual plants growing on serpentine soil at Anarak (33° 23′ N, 53° 41′ E and 33° 02′ N, 53° 03′ E), Iran, in June 2014. The fruits were mixed thoroughly, air-dried and stored at 4 °C for at least one month before starting the experiments. Seeds were isolated from the silicles and surface sterilized in 5% (v/v) sodium hypochlorite for 15 min and rinsed three times in distilled water.

2.2. Effect of pericarp (fruit parts) on germination

Experiments were performed with seeds in three states: within intact fruits, and separated from the silicles, either with or without the removed pericarps present in the Petri dishes. Seeds were placed about 1 cm apart from each other. The pericarp was removed and placed adjacent to its seed (at 0.5 cm from each other). The water permeability of seed coats and pericarps was determined by weighing 25 seeds before and after a 60-h imbibition period (maximum water absorption in isolated seeds), and the percentage weight increase (% Wr) was calculated as $Wr(\%) = [(Wi - Wd)/Wd] \times 100$, where Wi and Wd are mass of imbibed and air-dry dispersal units (seeds), respectively (Lu et al., 2015).

2.3. Effects of GA_3 and moist chilling on germination

Seeds without pericarp were used in the following experiments: germination tests in Petri dishes (9 cm diameter) with two layers of filter paper, moistened with 5 ml distilled water with different GA_3 concentrations (0, 200, 500 and 750 mg/L), and sealed with adhesive tape (Parafilm™) to avoid desiccation. To check the effects of cold stratification fully imbibed seeds were stratified at 4–5 °C in the dark for 15 days in the absence or presence of GA_3 at different concentrations (a pilot experiment showed that longer stratification periods did not further enhance germination, irrespective of the presence or absence of GA_3). Three replicates with 25 seeds each per GA_3 treatment level were then exposed to 25 ± 2 °C. Experiments were performed in the dark, because it appeared in pilot experiments that light did not affect germination. Seeds were considered germinated when the radicle protruded from the seed by one mm.

2.4. Effects of Ni concentration on germination and early seedling growth

Ni toxicity was evaluated by testing seed germination and hypocotyl and root elongation in a series of nickel sulfate ($NiSO_4$) concentrations in distilled water. The concentrations of Ni were: 0, 5, 10, 25, 50, 100, and 150 μM. To accelerate seed germination, based on the results obtained from preceding experiments, Petri dishes containing different concentrations of Ni were incubated at 4–5 °C for 15 days in darkness and then transferred to 25 ± 2 °C in dark. Root and hypocotyl length were measured after 8 days.

2.5. Germination characteristics

The germination percentage (GP) and the mean germination time (MGT) were calculated from the germination counts during 8 days (after 6 days, there was no further germination in pilot experiments).

The mean germination time (MGT) was calculated as:

$$MGT = \frac{\sum Dn}{\sum n}$$

in which n is the number of seeds germinated on day D, and D is the number of days after the start of the experiment (Dastanpoor et al., 2013).

Table 1
Effect of the pericarp on seed imbibition (% weight increase after 60 h), germination percentage (GP), mean germination time (MGT) and germination index (GI) in *Fortuynia garcinii* seedlings after 8 days.

State of seed	Weight increase (%)	GP (%)	MGT (days)	GI
Seeds within intact fruit	30.8 ± 1.9^{b}	0	>8	0
Seeds + pericarps	53.5 ± 2.7^{a}	12 ± 1.6^{b}	6.10 ± 0.25^{a}	0.22 ± 0.03^{b}
Seeds – pericarps	56.5 ± 2.1^{a}	46 ± 6.2^{a}	3.29 ± 0.09^{b}	4.30 ± 0.7^{a}

Values are means ± standard error (n = 4). Means in each column having the same letters are not significantly different using Duncan's test at $\alpha = 0.01$.

The germination index (GI) was calculated as:
$GI = G_1/T_1 + G_2/T_2 + \ldots + G_n/T_n$, in which G_1, G_2,. . .,G_n are the number of germinated seeds on the first count, second count, and so on until the last count (n), respectively, and T_1, T_2,. . ., T_n the number of days between sowing and the first count, between sowing and the second count, and so on until the last count (n), respectively (Dastanpoor et al., 2013).

2.6. Statistical analysis

Experiments were performed in a factorial randomized complete block design with at least three replicate Petri dishes with 25 seeds each. Data were analyzed using SPSS 18 and tested for homogeneity of variances and for normal distribution. Data were analyzed using one-way and two-way ANOVA. Duncan's test with $P < 0.05$ as significance threshold was used to compare individual means.

3. Results

3.1. Effects of pericarp on germination

None of the mature seeds of *F. garcinii* germinated while inside the indehiscent fruits. Their water content (%) was significantly lower than that of seeds separated from their pericarp in the vicinity of their pericarps and seeds without their pericarps, which were not significantly different from one another (Table 1). For seeds with detached pericarps in the Petri dishes (seeds + pericarp) GP and GI increased, and MGT decreased compared to seeds within intact fruits. The highest GP (46%) and GI (4.3) were recorded for seeds without pericarps. Thus the presence of the empty pericarps in the Petri dishes significantly decreased GP and GI, and increased MGT.

3.2. Effects of GA_3, moist chilling and their interaction on germination

In the absence of moist chilling, GP was significantly increased by GA_3, independent of its concentration (Fig. 1A). Moist chilling enhanced GP to the same level as GA_3 did, both in the control and all the GA_3 treatments, thus completely removed the GA_3 effect. MGT was significantly decreased with increasing GA_3 concentration in the non-stratified seeds, whereas all the GA_3 concentrations similarly decreased MGT in the stratified seeds compared to 0 mg GA_3/L. Thus the lowest MGT was observed with GA_3 at different concentrations, in combination with moist chilling (Fig. 1B). GI was significantly increased by moist chilling and, in a concentration-independent way, by GA_3 (Fig. 1C). Analysis of variance showed that the effects of GA_3 and moist chilling were significant for GP, MGT and GI ($P < 0.01$). Interaction between GA_3 and moist chilling was significant for GP and GI, but not for MGT (Table 2).

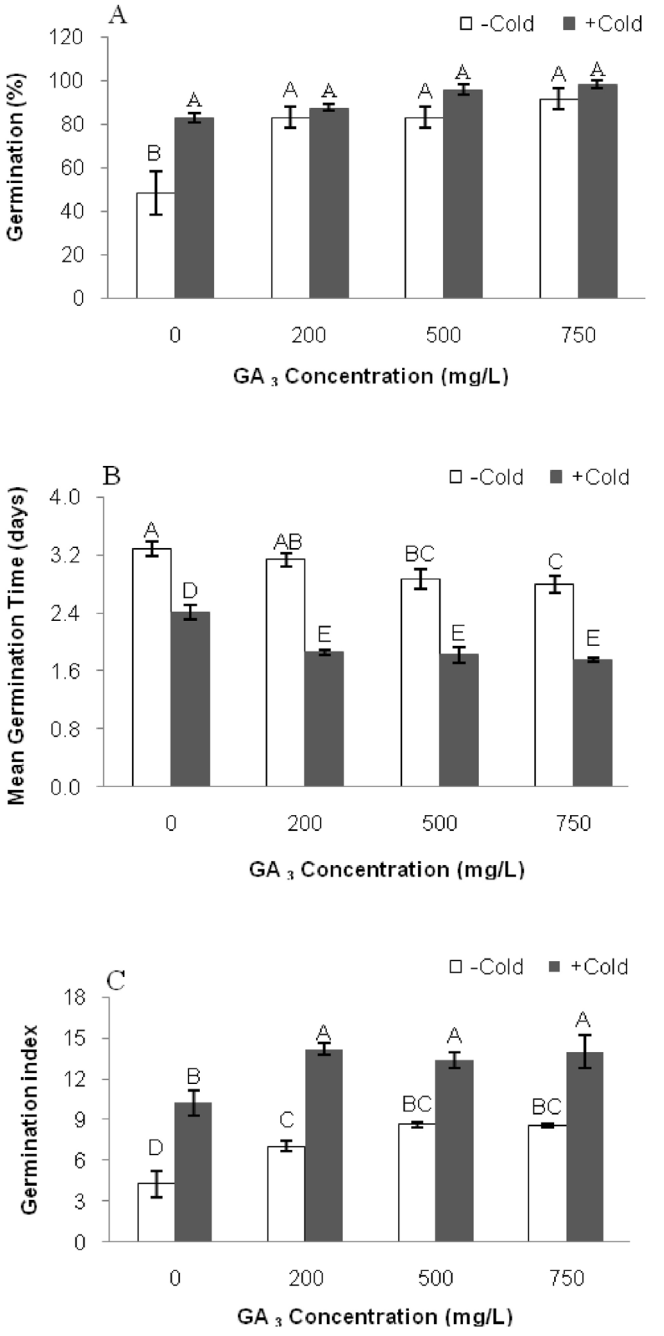

Fig. 1. Effect of GA_3 and moist chilling and their interaction on germination percentage (A), mean germination time (B) and germination index (C) in *Furtuynia garcinii* (mean ± SE, n = 3). Different letters indicate statistically significant differences based on Duncan's test ($p < 0.05$).

Table 2
Analysis of variance of the effect of moist chilling and GA_3 (results in Fig. 1) and their interaction on germination in *Fortuynia garcinii*.

Source of variance	df	F ratio		
		GP	MGT	GI
GA_3	3,16	15.15^{**}	90.50^{**}	51.67^{**}
moist chilling	1,16	14.23^{**}	9.39^{**}	13.06^{**}
GA_3 × moist chilling	3,16	5.04^{*}	2.83^{ns}	3.45^{*}

GP, germination percentage; MGT, mean germination time; GI, germination index. ns, * and **, non-significant, and significant at $P < 0.05$ and $P < 0.01$, respectively.

Table 3
Effect of different concentrations of Ni on germination percentage (GP), mean germination time (MGT) and germination index (GI) in *Fortuynia garcinii* seedlings after 8 days.

Ni concentration (μM)	GP (%)	MGT (days)	GI
0	81.3 ± 3.5^{a}	2.2 ± 0.33^{b}	10.2 ± 1.50^{a}
5	78.7 ± 4.8^{a}	2.2 ± 0.13^{b}	8.7 ± 2.70^{ab}
10	85.3 ± 5.8^{a}	2.7 ± 0.26^{ab}	8.9 ± 1.34^{ab}
25	92.0 ± 2.3^{a}	2.4 ± 0.13^{ab}	8.6 ± 0.89^{ab}
50	82.7 ± 3.5^{a}	2.4 ± 0.05^{ab}	7.5 ± 0.11^{ab}
100	85.3 ± 3.5^{a}	2.7 ± 0.03^{ab}	7.1 ± 0.22^{b}
150	90.7 ± 4.8^{a}	2.9 ± 0.04^{a}	7.0 ± 0.28^{b}

Values are means ± standard error (n = 3). Means in each column having the same letters are not significantly different using Duncan test at α = 0.05.

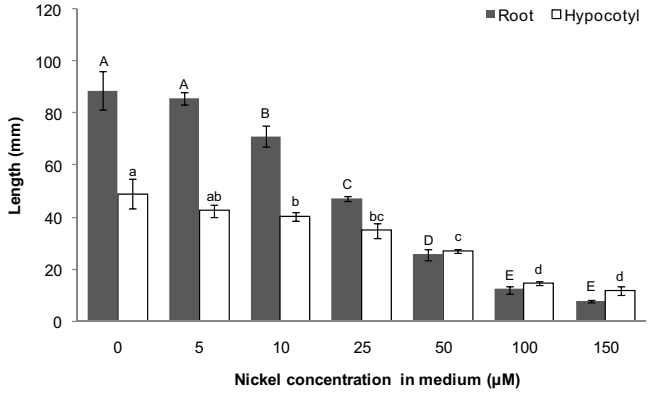

Fig. 2. Effect of Ni on root and hypocotyl length in *Fortuynia garcinii* seedlings (mean ± SE, n = 3). In each series, different letters (lowercase letters for hypocotyls and uppercase letters for roots) indicate statistically significant differences based on Duncan's test ($p < 0.05$).

3.3. Effect of Ni concentration on germination and early seedling growth

Germination percentage was not affected by Ni (Table 3). However, Ni caused a slight increase in MGT, which was only significant at the 150 μM exposure level (30% increase; $P < 0.05$). GI was decreased by Ni, though only significantly at the 100 μM or higher exposure levels (30% decrease).

Root and hypocotyl length were not significantly affected by Ni at the 5 μM exposure level (Fig. 2; $P > 0.05$). At higher Ni concentrations, both root length and hypocotyl length were significantly reduced, down to 9 and 32% of the control values at the 150 μM exposure level, respectively.

4. Discussion

Our results demonstrate that the indehiscent pericarp of *Fortuynia garcinii* diminished seed imbibition and completely inhibited germination as long as the seeds were inside the fruits. Seeds incubated with their pericarps separated were able to absorb water at the same rate as seeds without pericarps, but their germination rate was significantly lower. This result supports the hypothesis that the pericarp acts through providing both mechanical resistance to seed imbibition as well as chemical inhibitors, such as demonstrated in other species of Brassicaceae, including *Raphanus raphanistrum* (Mekenian and Willemsen, 1975), *Diptychocarpus strictus* (Lu et al., 2010), *Rapistrum rugosum* (Ohadi et al., 2011), and *Lachnoloma lehmannii* (Lu et al., 2015). Thus, the pericarp has the potential to delay germination over an extended period of time (Lu et al., 2015). In the natural habitat of the species, such a delay might serve to prevent seedling mortality owing to desiccation in the first summer after fruit shed. The pericarp might also contribute to a further delay of germination until after winter, which can be extremely cold in the mountains of central Iran. However, the clear-cut stimulating effect of cold-stratification suggests that physiological dormancy, rather than mechanically-imposed mechanisms or, more likely, a combination of both, may be responsible for delaying germination until next spring.

In this study stimulatory effects of GA_3 on GP were exclusively observed in non-stratified seeds. Stimulatory effects of GA_3 on MGT and GI, however, were also apparent in stratified seeds. In any case, our results confirm that GA_3 is involved in the dormancy-breaking effect of cold stratification (Rouhi et al., 2010). It has been reported that moist chilling increases GA biosynthesis, and at the same time suppresses GA catabolism (Yamauchi et al., 2004). Germination enhancing effects of GA_3 have been reported in many species (Leubner-Metzger et al., 1996; Rouhi et al., 2010; Erickson et al., 2016).

The relatively low rate of germination of non-stratified *F. garcinii* seeds may be due to a high concentration of abscisic acid (ABA) and a low concentration of GAs within the seeds (Bewley and Black, 2012). Moist chilling (stratification) often improves the sensitivity to other treatments to overcome dormancy (Bubel, 1988; Yamauchi et al., 2004). In particular, cold stratification in combination with GA_3 was successful at breaking seed dormancy in *F. garcinii*, which was also found in *Prunus spinosa* (Afroze and O'Reilly, 2016), *Senecio olympicus* (Kirmizi et al., 2011), *Stachys germanica* (Güleryüz et al., 2011), *Morus nigra* (Koyuncu, 2005) and *Jeffersonia dubia* (Rhie et al., 2015).

Nickel is an essential micronutrient for higher plants (Furini, 2012). In the present study, GP was not significantly influenced by high Ni concentrations. Our results are in contrast with those of several other studies (Seregin and Kozhevnikova, 2005; Furini, 2012; Visioli et al., 2014). The ineffectiveness of Ni in preventing seed germination in our study may be explained by the fact that Ni may not have penetrated the seed coat, at least not in amounts sufficient to damage the growing radicle (Di Salvatore et al., 2008). However, MGT was significantly increased, and GI decreased by elevated Ni concentrations. Enhancement of MGT in response to heavy metal toxicity has been reported in various species (Muccifora and Bellani, 2013). These effects might be related to Ni interference with the water balance of the germinating seed (Poschenrieder and Barceló, 1999), or suggest that the seed coat is slightly permeable to Ni already during early imbibition. Alternatively, in a later phase of the imbibition period, when water uptake slows down, the seed coats may become much more permeable to the metal, thus seedling growth will be restricted due to Ni toxicity (Muccifora and Bellani, 2013). In our study Ni inhibited root length more strongly than hypocotyl length, suggesting that the effects of Ni on shoot growth can be secondary, merely reflecting the loss of root functions such as water and nutrient uptake (Rout et al., 2000; Gajewska et al., 2013). The Ni-imposed effect on root elongation is almost certainly caused by Ni accumulation in the root itself, which eventually causes inhibition of cell elongation and cell division (Di Salvatore et al., 2008; Visioli et al., 2014), the more so because in *F. garcinii*, as in other non-Ni-hyperaccumulators, Ni primarily accumulates in roots (Rout et al., 2000; Gajewska et al., 2006).

5. Conclusions

Our results show that the pericarp plays a dominant role, through mechanically inhibiting imbibition and chemical inhibition, in seed dormancy of *Fortuynia garcinii*, preventing precocious germination. Removal of the pericarp, followed by moist chilling at 4–5 °C for 15 days in the presence of GA_3 is an effective strategy to improve seed germination. In *F. garcinii*, which is native to serpen-

tine soil, seedling growth is much more sensitive to Ni than seed germination.

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