The seed germination properties of two hyperaccumulator plant species with the potential for Ni agromining

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ABSTRACT:
The aim of this study is to investigate the effect of different nickel concentrations and light in combination with storage conditions and storage time on the seed germination ability of two serpentine-endemic nickel hyperaccumulating species (*Bornmuellera emarginata* and *B. tymphaea*). The seeds of both species were collected from natural populations in the Pindus Mountain range, Greece in early July and stored in a refrigerator (4°C) and in laboratory conditions (22°C). The seeds were exposed to a range of nickel concentrations typical of non-ultramafic – ultramafic gradient in two light environments (12 h photoperiod and continuous darkness). The nickel concentration only had a significant effect on *B. emarginata*, decreasing its seed germination rate with increasing Ni concentrations. The storage temperature significantly affected the germination percentage of both species and it was higher at 4°C compared to 22°C. A higher germination rate (> 60%) was observed for 5‒8-month-old seeds, but both species generally showed significantly higher germination rates in the tests conducted seven months after seed ripening in the field. A higher germination rate was observed in a 12-hour photoperiod than in continuous darkness only for *B. tymphaea*. This study provides guidelines on the germination capacity of two obligate nickel hyperaccumulators with a potential for use in agromining systems.

Keywords:
ultramafic, nickel, serpentinophytes, germinability, seed storage, *Bornmuellera*

INTRODUCTION

Ultramafic rocks are rich in ferromagnesian minerals (> 70%) with low silica content (< 45%) which give rise to soils generally characterised by high concentrations of nickel (Ni), chromium (Cr), cobalt (Co), iron (Fe) and manganese (Mn), a low Ca/Mg ratio and a lack of macronutrients such as nitrogen (N), potassium (K), and phosphorus (P) (Proctor & Woodell 1975; Kazakou et al. 2003; Kazakou et al. 2008; Echevarria 2021). Due to their geochemical peculiarities ultramafic soils are often characterised as plant biodiversity hotspots (Harrison & Inouye 2002). A high number of endemic species have evolved morphological and physiological adaptations, differentiating them from the flora of adjacent non-ultramafic soils (Proctor 1971; Brooks 1987; Stevanović et al. 2003; Kazakou et al. 2008; Bergmeier et al. 2009; Samojedny et al. 2022). Ultramafic flora includes unusual groups of plants, known as hyperaccumulators, which are able to accumulate extremely high concentrations of Ni in their aerial biomass, often 100–1000 times higher than those in non-accumulators without
showing any toxic effects (Bergmeier et al. 2009; van der Ent et al. 2013).

The unique capacity of Ni hyperaccumulators to accumulate metal in their aerial tissues implies technological applications, such as agromining (van der Ent et al. 2015; Nkrumah et al. 2016; Chaney et al. 2021; Nascimento et al. 2022). Due to their geochemical peculiarities, agricultural land found on ultramafic soils often becomes unproductive and unattractive for conventional agriculture. Nickel agromining represents a new form of agriculture which can generate income from low-productivity agricultural land derived from ultramafic bedrock. It consists of cultivating suitable hyperaccumulator species, the subsequent harvesting of the aerial biomass, its drying, incineration and processing, generating a bio-ore (ash) with up to 25% Ni (Chaney et al. 2007; van der Ent et al. 2015).

In Europe, ultramafic rocks cover large areas of the Balkans, more than in any other part of the continent. Furthermore, the Balkan Peninsula is a well-known region with numerous obligate and facultative serpentine-phytes (Stevanović et al. 2003; van der Ent et al. 2013; Jakovljević et al. 2022). Nickel-hyperaccumulators native to Europe are mostly distributed on the Balkan peninsula with a total of 27 taxa. The majority of them belong to the Brassicaceae family (24 taxa), mainly to the genus Odontarrhena (Kidd et al. 2018; Jakovljević et al. 2022). Therefore, this region is a potential target for nickel agromining activities (Brooks 1987; van der Ent et al. 2015).

At the European territory level, nickel agromining field trials have been conducted almost continuously in Albania focusing on the Mediterranean species Alyssum murale (syn. Odontarrhena chalcidica) since 2005 (Bani et al. 2010, 2015a, b, 2021; Bani & Echevarria 2019). Alyssum murale is probably the most studied Ni hyperaccumulator for phytomining activities to date (Zhang et al. 2014; Nkrumah et al. 2016; Zupfer 2017; Nascimento et al. 2022).

Concerning Greece, ultramafic soils account for 3–4% of the territory of the country which could justify the development of phytomining activities as an alternative to local agriculture on such unproductive land (Kidd et al. 2018). Recently, two Greek endemic Ni hyperaccumulators, Bornmuellera emarginata (Boiss.) Rešetnik & Ashraf (Boiss.) Rešetnik and B. tymphaea (Hauusskn.) Hausskn., native to the Pindus mountain range (Greece), have been studied for their potential in Ni agromining in the Mediterranean area (Chardot et al. 2005; Kidd et al. 2018; Pardo et al. 2018; Kyrkas et al. 2019a; Hipfinger et al. 2022). The agronomic practices followed so far in the Greek field trials have shown that B. tymphaea and B. emarginata are suitable as perennial crops through transplanting seedlings or cuttings (Kyrkas et al. 2019a, b). The nickel yields recorded from B. tymphaea (88.3 kg ha⁻¹) and B. emarginata (151 kg ha⁻¹) in field experiments in their natural environment in 2018 were exceptionally high (Kyrkas et al. 2019a, b). The highest foliar Ni concentrations observed up to date are 31.2 g kg⁻¹ DW in B. tymphaea and 34.9 g kg⁻¹ DW in B. emarginata when growing in their natural environment (Reeves et al. 1980, 1983; Jakovljević et al. 2022). Although Ni phytomining has proven to be an economically viable technology, appropriate soil and plant management practices, based on insights from laboratory and field trials are required to maximise the yields of the selected crops (Nkrumah et al. 2016; Chaney et al. 2021).

Seed germination is one of the most important stages in crop development which predetermines the establishment of a healthy crop, better growth, and yield (Ahmad & Ashraf 2011; Jóźwiak et al. 2016; Uçak & Bagdatlı 2017). Also, the planning of the seeding season and the establishment in the field is equally important. The seeds are the first into contact with the soil and the peculiarity of ultramafic soils, such as the presence of metals, the low Ca/Mg ratio and the low nutrient content, can be factors which impact on the germination rate (Pędziwiatr et al. 2020). Since high Ni levels exert toxic effects on seed germination, information on seed germination contributes to a better understanding of plant adaptation to habitats. However, studies investigating the germination of hyperaccumulators are quite rare (Pavlova et al. 2018). Nickel affects seed germination due to changes in protease and ribonuclease activity, where an increase of RNA is observed (Sethy & Ghosh 2013). The availability of seeds, as well as the recording of all the information from the seed collection sites are determining factors for the upgrading of the Ni agromining process in Greece and beyond (Bani et al. 2021). Finally, propagation and harvest management will be dependent upon the species being used for agromining and on the climate of the production site (Chaney et al. 2021).

Considering the factors which may influence seed germination, the aim of this study is to investigate the effects of different nickel concentrations and light in combination with storage conditions and storage time on this process in two serpentine-endemic nickel hyperaccumulating species, Bornmuellera emarginata and B. tymphaea as candidates for use in agromining.

**MATERIALS AND METHODS**

**Selected species and study site.** The plant material (seeds) was collected from Ni-hyperaccumulating species Bornmuellera emarginata and B. tymphaea, members of the Brassicaceae family. Both species are Greek endemics, native to the Pindus Mountains and closely related to ultramafic substrates (Dimopoulos et al. 2023). The seeds were collected from one population of each species distributed on ultramafic substrates from Pefki (Thessaly), (N 39.79724°, E 21.38595°, alt. 990 m a.s.l.) in July 2019.
The seeds were extracted manually from the fruits, and those of poor quality (damaged, empty and immature) were rejected. The vouchers were deposited in the Department of Agriculture of the University of Ioannina (Be/MD19,6; Bt/MD19,2).

Seed germination. The harvested seeds of *B. emarginata* and *B. tymbphaea* were stored in the refrigerator (4°C) and in laboratory conditions (22°C). Seeds from both groups were tested for their germinability without exposing them to any dormancy-relieving treatments. Prior to germination, all of the seeds were subjected to surface sterilisation in sodium hypochlorite solution (3%) for 10 min and then rinsed with deionized water. Standard solutions of 0, 4, 8 mM Ni as NiCl₂, 6H₂O were prepared with distilled water [Aldrich Nickel (II) chloride hexahydrate, P.N.: 654507]. The range of concentrations is based on metal concentrations commonly found in non-ultramafic soil to those measured in ultramafic soils (Pavlova et al. 2018). The seeds were sown in sterile 9 cm Petri dishes, lined with double filter paper moistened with the Ni solutions. They were kept in a temperature-controlled growth cabinet set at 22°C and tested for two light conditions (12 h photoperiod and continuous darkness) and 3 nickel concentration treatments. Illumination was provided by white fluorescent tubes with a mean photon flux density of 230 mmol m⁻² s⁻¹ at seed level (PAR). For each seed treatment, three independent replicates (each containing 20 seeds) were prepared (Fig. 1). Therefore, the number of seeds (n) used per treatment was 60. The total number (N) of seeds for all the plant species used in this study was 11,520. The germinated seeds were counted every 3-4 days. The seeds were considered germinated when a 1 mm radicle had emerged. The experiments were carried out for eight months in total (September 2019‒March 2020, and June 2020).

Data calculations and statistical analysis. The germination percentage (GP) was calculated by the number of seeds germinated (GS) during the experiment in relation to the total initial seed number (TS) of 20 (Equation) (Ranal et al. 2009).

\[
GP = \frac{GS}{TS} \times 100\ (%)
\]

The data was analysed with ANOVA using IBM SPSS software (version 28). Due to the hierarchical design of the experiments, Type I SS were used. The percentage of seed germination of each studied species was the response variable tested for significant differences between treatments. The design included the following factors: the seed storage temperature, the nickel concentration, the light conditions (nested at nickel concentration), the month in which the experiment was conducted, and all the two-way interactions between them. An overall ANOVA with data for both species was also compiled. In that case, an additional species identity factor was included. In cases when the ANOVA showed statistically significant differences (P < 0.05), pairwise mean comparisons of the variables, i.e. a least significant difference (LSD) test was performed.

RESULTS AND DISCUSSION

The species studied showed significant differences in their germination percentages (significant main effects of ‘species’, Table 1; P < 0.001) and in their responses to increased nickel concentrations (significant ‘species × nickel’ interaction, Table 1; P < 0.001). That was to be expected as both species are obligate serpentinitophytes and Ni-hyperaccumulating Greek endemics, closely related to ultramafic soils (Stevanović et al. 2003). Nickel concentrations seemed to have a significant effect only on *B. emarginata* (Table 2), decreasing its seed germination rate with increasing nickel concentrations (Table 2; P < 0.001; Fig. 2). Comparable decreases have been observed in *Alyssum murale* (syn. Odontarrhena chalcidica) and *A. markgrafii* (syn. O. markgrafii) populations exposed to peak nickel concentrations of 8 mM (Pavlova et al. 2018). The differences in germination ability between species can be attributed to seed physiology. Reduced energy production by the embryo leading to a loss of seed viability could serve as an explanation for decreasing seed germination rates (e.g. Moosavi et al. 2012; Pavlova et al. 2018). Elevated levels of nickel act as inhibitors of processes such as protein synthesis, enzyme activity, carbohydrate metabolism and the mobilisation of food reserves (Ahmad & Ashraf 2011), and strongly affect the uptake of nutrients (e.g. K and Mg) by germinating seeds (Maheshwari & Dubey 2007).

In addition to seed physiology, the abiotic conditions of species habitats (e.g. temperature, water or light regimes) may also affect seed germination rates (Vera 1997; Sethy & Ghosh 2013; Pavlova et al. 2018). Our results showed that the storage temperature had a significant effect on the germination percentages of both species (significant ‘species × temperature’ interaction, Table 1, P < 0.001), with the germination percentages being higher at 4°C compared to 22°C. This could be due to the better adaptation of these species to the unfavourable conditions.

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**Fig.1.** Experimental design. Three replicates per treatment were applied, each containing 20 seeds.
of the ultramafic soil in combination with the ecological conditions of the highlands. Although *B. emarginata* also occurs at lower altitudes, both species, especially *B. tymphaea*, are adapted to high mountain regions where the seeds used in the experiment were collected. The effect of the altitude where the species populations occur along with their habitat conditions have been found to explain the variation in the germination rates and mean germination time in nickel hyperaccumulators distributed in ultramafic soils in Albania (Pavlova *et al.* 2018). However, there was no difference in the effect of nickel on both species in response to the two seed storage temperatures (no significant ‘nickel × temperature’ interaction, Tables 1 & 2, *P* > 0.05; Fig. 2). This is probably due to the greater effect of the temperature (27.5%, defined as % of total SS; Table 1) in explaining the variance of the germination rate relative to nickel concentration (1.9%; Table 1), which in the case of *Bornmuellera tymphaea* amounts to 51% (Table 2).

The effect of the light conditions applied simultaneously with different concentrations of Ni was very significant only for *B. tymphaea* (*P* < 0.001). The *Bornmuellera tymphaea* seeds showed a higher germination rate in the 12-hour photoperiod than in continuous darkness. This suggests that the seeds of *B. tymphaea* should not be sown deeply in the soil. On the contrary, this factor seems irrelevant for *B. emarginata*, as is the case for *Stackhousia tryonii* (Bhatia *et al.* 2005).

Table 1. A summary of the ANOVA results for differences in the germination percentages of the seeds of both species between treatments. The sum of squares (SS), %SS, degrees of freedom (df), mean squares (MS), F-ratio, and *P*-values for each variable are shown. Significant values (*P* ≤ 0.05) are in bold.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Type I SS</th>
<th>%SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th><em>P</em>-value</th>
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<td>1.3</td>
<td>1</td>
<td>3047.960</td>
<td>17.739</td>
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<td>7</td>
<td>6192.702</td>
<td>36.041</td>
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<tr>
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<td>1</td>
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<td>2219.922</td>
<td>12.920</td>
<td>&lt;.001</td>
</tr>
<tr>
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<td>397.092</td>
<td>2.311</td>
<td>.075</td>
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<td>569.227</td>
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<td>1</td>
<td>5593.793</td>
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<tr>
<td>Error</td>
<td>91926.9</td>
<td>39.2</td>
<td>535</td>
<td>171.826</td>
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</tr>
<tr>
<td>Total</td>
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<td>575</td>
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Table 2. A summary of the ANOVA results for differences in the germination percentages of the seeds of (a) *Bornmuellera tymphaea* and (b) *B. emarginata* between treatments. The sum of squares (SS), %SS, degrees of freedom (df), mean squares (MS), F-ratio, and *P*-values for each variable are shown. Significant values (*P* ≤ 0.05) are in bold.

(a) *Bornmuellera tymphaea*  

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Type I SS</th>
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<th>MS</th>
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<td>2291</td>
<td>26.668</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Temperature</td>
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<td>51.0</td>
<td>1</td>
<td>54038</td>
<td>629.039</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Nickel</td>
<td>141.84</td>
<td>0.1</td>
<td>2</td>
<td>70.92</td>
<td>0.826</td>
<td>.439</td>
</tr>
<tr>
<td>Light (Nickel)</td>
<td>2885.677</td>
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<td>11.197</td>
<td>&lt;.001</td>
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<tr>
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<td>9652.691</td>
<td>9.1</td>
<td>7</td>
<td>1379</td>
<td>16.052</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Nickel * month</td>
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<td>14</td>
<td>118.04</td>
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<td>0.166</td>
</tr>
<tr>
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<td>2</td>
<td>25.26</td>
<td>0.294</td>
<td>0.745</td>
</tr>
<tr>
<td>Error</td>
<td>21562.41</td>
<td>20.3</td>
<td>251</td>
<td>85.906</td>
<td></td>
<td></td>
</tr>
<tr>
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</table>

(b) *Bornmuellera emarginata*  

<table>
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<th>Source of variation</th>
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<th>df</th>
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<td></td>
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been observed in *Alyssum murale* populations (Pavlova *et al.* 2018), in *Alyssum bertolonii* (syn. *Odontarrhena bertolonii*) and *A. argenteum* (syn. *Odontarrhena argentea*) where seeds of 8–10 months of age exhibited a high germination ability (Pancaro *et al.* 1980, 1981), as well as in *Stackhousia tryonii* where the germination percentage increased after a storage period of up to 18 months (Bhatia *et al.* 2005).

**CONCLUSIONS**

In this study, we provide guidelines on the germination capacity of two obligate nickel hyperaccumulators, *B. tymphaea* and *B. emarginata*, which have the potential for use in agromining systems. Differences in the effects of storage temperature on the seed germination of both species are demonstrated for the first time. After collecting the seeds in the field, they must be stored in a refrigerator to achieve the highest germinability. The seeds of both species ripen in early July, so the best sowing time is January, seven months after collection. This is very important because seedlings ready for transplanting are required in March when the conditions are ideal to start cropping.

An important parameter in the preparation of the seedlings is the sowing depth, which also depends on the lighting conditions. In this experiment it was shown that continuous darkness produced better results for *B. tymphaea*, while *B. emarginata* was indifferent to changes in light.

The highest Ni levels (8mM), although not significantly different between the storage conditions, appeared to have a negative effect on the germination of *B. emarginata*. Commercial peat and perlite seedbeds can be used for the preparation of the seedlings for transplanting as the presence of nickel in the seeding substrate for both plant species is not necessary.

Future studies should focus on the causes behind the observed patterns, possible differences among the populations of each species, as well as studying more species with the potential for use in agromining.

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REZIME

Klijavost semena dve hiperakumulatorske biljne vrste sa potencijalom za fitorudarenje Ni

Dimitrios Kyrkas, Nikolaos Mantzos, George Patakoutas, Guillaume Echevarria, Evaggelos Filis, Panayiotis G. Dimitrakopoulos i Maria Konstantinou

Cilj ove studije je da se ispitati uticaj različitih koncentracija nikla i svetlosti u kombinaciji sa uslovima i vremenom skladištenja na sposobnost klijanja semena dve serpentinofanske endemite i hiperakumulatora nikla (*Bornmuellera emarginata* i *B. tymphaea*). Semena obe vrste su sakupljena iz prirodnih populacija u planinskom lancu Pindus, Grčka, početkom jula i čuvana u frižideru (4°C) i u laboratorijskim uslovima (22°C). Semena su bila izložena rasponu koncentracija nikla tipičnom za neultramafitsko-ultramafitski gradijent, i dva tipa osvetljenja (fotoperiod od 12 h i neprekidna tama). Koncentracija nikla je imala značajan uticaj samo na *B. emarginata*, smanjujući njenu klijavost semena sa povećanjem koncentracije Ni. Uticaj temperature skladištenja značajno je uticao na procenat klijanja obe vrste i bio je veći na 4°C u odnosu na 22°C. Veća stopa klijanja (> 60%) je primećena za seme staro 5–8 meseci, ali su generalno obe vrste pokazale značajno veće stope klijanja u ogledima sprovedenim sedam meseci nakon sazреваnja semena u polju. Veća stopa klijanja u fotoperiodu od 12 sati nego u neprekidnoj tami primećena je samo za *B. tymphaea*. Ova studija daje smernice o kapacitetu klijanja dva obligatna hiperakumulatora nikla sa potencijalom za upotrebu u fitorudarenju.

Ključne reči: ultramafiti, nikl, serpentinofite, klijavost, čuvanje semena, *Bornmuellera*