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Original Scientific Paper

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*

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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.* 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 serpentinophytes (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 and *B. tymphaea* (Hausskn.) 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; Ucak & Bagdatli 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. tymphaea* 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 $NiCl_2$ $6H_2O$ 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).

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

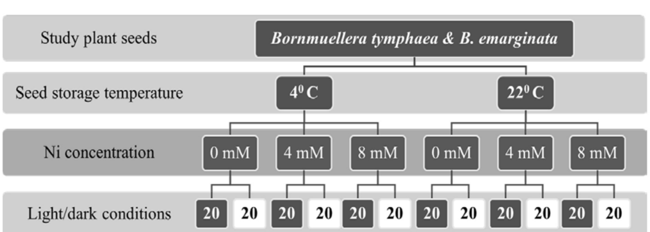

Fig.1. Experimental design. Three replicates per treatment were applied, each containing 20 seeds.

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 serpentinophytes and Ni-hyperaccumulating Greek endemics, closely related to ultramafic soils in the Pindus Mountains (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

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 ($P \leq 0.05$) are in bold.

Source of variation	Type I SS	%SS	df	MS	F	*P*-value
Species	3047.960	1.3	1	3047.960	17.739	**<.001**
Month	43348.915	18.5	7	6192.702	36.041	**<.001**
Temperature	64494.835	27.5	1	64494.835	375.350	**<.001**
Nickel	4439.844	1.9	2	2219.922	12.920	**<.001**
Light (Nickel)	1191.276	0.5	3	397.092	2.311	.075
Temperature * month	9161.762	3.9	7	1308.823	7.617	**<.001**
Nickel * month	7969.184	3.4	14	569.227	3.313	**<.001**
Temperature * Nickel	158.420	0.1	2	79.210	.461	.631
Species * Nickel	3213.6	1.4	2	1606.814	9.351	**<.001**
Temperature * species	5593.8	2.4	1	5593.793	32.555	**<.001**
Error	91926.9	39.2	535	171.826		
Total	234546.48		575			

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 ($P \leq 0.05$) are in bold.

(a) *Bornmuellera tymphaea*							**(b) *Bornmuellera emarginata***					
Source of variation	**Type I SS**	**%SS**	**df**	**MS**	**F**	***P*-value**	**Type I SS**	**%SS**	**df**	**MS**	**F**	***P*-value**
Month	16036.72	15.1	7	2291	26.668	**< 0.001**	47314	37.7	7	6759.1	48.365	**< 0.001**
Temperature	54038.28	51.0	1	54038	629.039	**< 0.001**	16050	12.8	1	16050	114.85	**< 0.001**
Nickel	141.84	0.1	2	70.92	0.826	0.439	7511.6	6.0	2	3755.8	26.875	**< 0.001**
Light (Nickel)	2885.677	2.7	3	961.89	11.197	**< 0.001**	1035.9	0.8	3	345.31	2.471	0.062
Temperature * month	9652.691	9.1	7	1379	16.052	**< 0.001**	8644.1	6.9	7	1234.9	8.836	**< 0.001**
Nickel * month	1652.604	1.6	14	118.04	1.374	0.166	9385.6	7.5	14	670.4	4.797	**< 0.001**
Temperature * Nickel	50.521	0.0	2	25.26	0.294	0.745	458.51	0.4	2	229.25	1.64	0.196
Error	21562.41	20.3	251	85.906			35078	28.0	251	139.75		
Total	106020.8		287				125477.8		287			

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* (Table 2, $P < 0.001$). 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).

The age of the seeds had a significant effect on their germination rate (significant effects of 'month'; Tables 1 & 2; Fig. 3) although differences across nickel treatments and seed storage temperature were also observed (Table 2; Fig. 3). A higher germination rate (> 60%) was observed for seeds of 7–10 months old, but both species generally showed significantly higher germination rates in the trials conducted in January, seven months after the seed ripening in the field (LSD test, $P < 0.001$; Fig. 3). Differences in germinability related to seed age have also

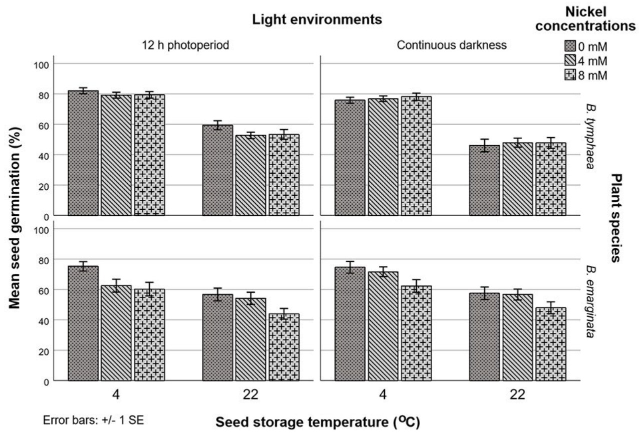

Fig. 2. The mean germination rate for *Bornmuellera tymphaea* and *B. emarginata* seeds stored at two different temperatures under increased nickel concentrations and different light regimes (12 h photoperiod and continuous darkness). All the results are the means of the original data ± SE.

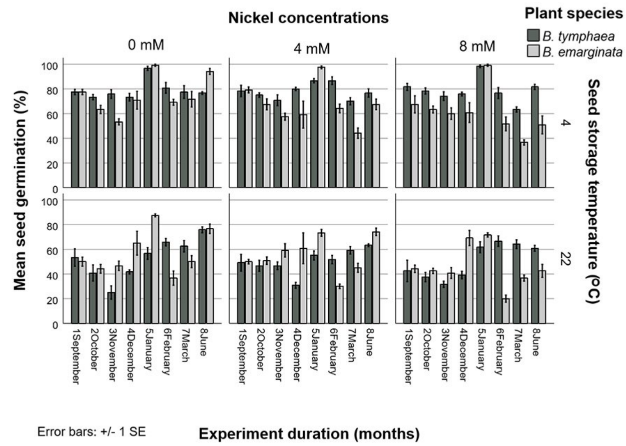

Fig. 3. The mean germination rate of *Bornmuellera tymphaea* and *B. emarginata* seeds as a function of their age (in months) under conditions of two different storage temperatures and increased nickel concentrations. All the results are the means of the original data ± SE.

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

Botanica SERBICA

Klijavost semena dve hiperakumulatorske biljne vrste sa potencijalom za fitorudarenje Ni

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Cilj ove studije je da se ispita uticaj različitih koncentracija nikla i svetlosti u kombinaciji sa uslovima i vremenom skladištenja na sposobnost klijanja semena dva serpentinska endemita 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 sazrevanja 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*