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ANALYSIS OF SEED VIABILITY FROM TWO *ALYSSUM* SPECIES, CANDIDATES FOR PHYTOMINING IN THE BALKANS

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Abstract: Two populations of the serpentine endemic and obligate Ni hyperaccumulator species *Alyssum markgrafii* and two populations of the facultative Ni hyperaccumulator *A. murale* were studied. To maximise the yields of the selected 'metal crop', suitable experiments on seed viability pattern are of importance. The aims of this study were to estimate the relation between the viability of the seeds of metallophyte *Alyssum* species - *A. markgrafii* and *A. murale* with their seed germinability. Tetrazolium (TZ) test for seed viability as an alternative quick method for seed's germinability testing was used. A positive tetrazolium result was concluded if seeds are stained in light pink as well as red. The germinability of both *Alyssum* species and their populations was significantly low. In general, *A. murale* demonstrated higher germinability compared to *A. markgrafii*. The results of the test showed that potential viability of the seeds was higher than germination percentage. The variation between viability and germination for the studied species is probably a result of seed dormancy. The variation in seed germination between the populations found for both *Alyssum* species could also be a result of an adaptive trait of plants to harsh environment provided by the serpentine soils.

INTRODUCTION

Serpentine (ultramafic or ophiolitic) substrates cover quite large areas in the Balkans, more than in other parts of Europe (Brooks, 1987; Tatić and Veljović, 1992). In Albania where they are ~10% of the territory of the country which could justify the development of phytomining activities as an alternative to local agriculture on such unproductive land (Bani et al., 2007; 2015a).

Some higher plants have developed heavy metal tolerance strategies that enable them to survive and reproduce in highly metal-contaminated soils. A very small number of such species (<0.2 % of flowering plants) are absolute metallophytes and are metal hyperaccumulators growing only on metal-enriched substrates such as serpentines (Baker et al., 2000). Species with such potential can be used in the restoration of areas following mining, remediation of metal contaminated soils and waters (phytoremediation; Baker et al., 2000), or phytoextraction of metals for economic returns (phytomining; Bhatia et al., 2005; Nkrumah et al., 2016). Metallophytes colonizing such soils have limited geographic distribution. The Balkan Peninsula is a well known region with numerous obligate and facultative *Alyssum* hyperaccumulator species (van der Ent et al., 2013). They are of potential utility for extracting Ni from soil, whether for remediation of contaminated soil or for phytomining from natural serpentine soils in temperate regions (Bani et al., 2010, 2015b; van der Ent et al., 2015). The obligate Ni hyperaccumultor and serpentine endemic *Alyssum markgrafii* O.E. Schultz. is one of the representatives of sect. *Odontarrhena* (now accepted as a separate genus by Španiel et al., 2015) of Brassicaceae family which is an herbaceous indicator of nickeliferous (ultramafic) soils in Albania (Bani et al., 2010). This species is morphologically close to the more widespread on the Balkans facultative Ni hyperaccumulator *Alyssum murale* Waldst. & Kit. and similarly to it suitable for phytomining (Bani et al., 2010; Nkrumah et al., 2016). Suitable species must be relatively easy to collect as bulk seed accessions and have high success rates of germination, establishment and growth (O'Dell and Claassen, 2011). Ni phytomining is a highly profitable agricultural technology but appropriate soil and plant management practices, based on insights from laboratory and field tests are required to maximise the yields of the selected 'metal crop' (Nkrumah et al., 2016).

Since the Ni hyperaccumulator species *A. markgrafii* and *A. murale* are plants with potential to be used in agricultural practice for phytoextraction and phytomining technologies in Albania (Bani et al., 2010; 2011) many specific questions about their production, commercialization and genetic improvement are of importance prior to inclusion the species in the agro technologies. Moreover, before exploiting any plant for industrial application, it is imperative to have complete information about its seed viability pattern so that the potential of the plant could be utilized maximally (Kumar et al., 2013). A viable seed is considered to be capable of producing normal seedling under favorable conditions. As emphasized by Tunes et al. (2009), the tetrazolium test is important on seed quality control because it allows a fast estimate of the seed germination capacity, including the dormant ones. The species may be propagated via direct seeding (Li et al., 2003) and therefore laboratory experiments on seed viability and germination are of interest.

The aims of this study were to estimate the relation between the viability of the seeds of both metallophyte *Alyssum* species with their germinability.

MATERIALS AND METHODS

Study sites

Two populations of the serpentine endemic and obligate Ni hyperaccumulator species *A. markgrafii* and two populations of the facultative Ni hyperaccumulator *A. murale* in Albania were selected for the experiments in order to compare the viability of seeds and their germinability. The plant material (seeds) was collected from *A. markgrafii* populations near to Gjegjan and Kukës while for *A. murale* the sites were Prrenjas-Domosdova and Pogradec, Pojska (Table 1).

Table 1. Species populations sampled and characteristics of sampling locations.

Locality	Geographical coordinates	Altitude (m)	Data of collection	Site abbreviation
		Alyssum markgrafii		
Gjegjan	42°03'50"N, 20°01'26"E	400-600	July 2014	G
Kukës	42° 00 35"N, 20° 17.44" E	1300	July 2014	K
		Alyssum murale		
Prrenjas-Domosdova	41°00'08"N 20°33'11"E	400	June 2014	Pr
Pogradec, Pojska	40°59'55"N 20°38'09"E	500	September 2015	Po

Sampling was done by hand and the material was put in different in size envelopes with labels including the characteristics of the collected plant, inside and out. The material was collected and stored according to the international guidelines proposed by the European Native Seed Conservation Network (ENSCONET 2009). Vouchers are kept in the Agricultural University of Tirana (AB-2014001; AB-2014020; AB-2014002; AB-2015010). The seeds were checked for mechanical damages and only the mature ones with well-developed embryo were used for the experiment.

Viability test

Tetrazolium (TZ) assay is the fast evaluation for seed viability and an alternative quick method for seed's germinability (Wharton, 1955). The test is based on the capabilities of respiring tissues to convert a colourless compound, TZ (2, 3, 5 triphenyl tetrazolium chloride) to a carmine red coloured water-insoluble formazan by hydrogen transfer reaction catalysed by the cellular dehydrogenases (Verma and Majee, 2013). The viable seeds are colored in red, whereas the non-viable seeds remain unstained. The method consists in soaking seeds in a 1% solution of 2,3,5-triphenyl tetrazolium chloride for 48 hours at 30^{o}C (ISTA, 2009). The seeds of *A. markgrafii* were scarified by soaking approximately 100 seeds (for three replicates) in 1 ml scarification solution prepared as 20 ml commercial bleach and 100 µl Triton X-100 in 100 ml autoclaved distilled water

for 15 min under shaking conditions. The seeds were washed at least five times with distilled water to remove the bleach. After scarification excess water was removed and the seeds were colored by incubation in 1% TZ solution (1g 2,3,5 triphenyl tetrazolium chloride in 100 ml autoclaved distilled water) at 30°C for 48h in dark. The seeds treated at 100°C for 1h were used as negative control.

After staining the seeds were washed again 2-3 times with distilled water. To immerse the stained seeds they were put in clearing agent (mix lactic acid: phenol: glycerin: water in a ratio of 1:1:2:1) for 1-2 h. The whole experiment was observed every day under stereo microscope and the evaluation of the viability of the seeds was done on the basis of staining pattern and color intensity after removing the seed coat.

The classification of the seeds as viable, doubtful viable (producing normal or abnormal seedlings) and non-viable was based on the staining of the embryo and the endosperm. Three categories of seeds were calculated: entirely stained in red seeds were considered viable; partially red stained seeds that may produce either normal or abnormal seedlings were considered doubtful viable; unstained seeds and pink stained (indicating dead tissue) were considered non-viable. The percent of viability was calculated for all populations. The results are presented as mean values of three replications.

Seed germination

Seeds were germinated in sterile 9 cm Petri dishes on filter paper moistened with Ni solutions in a temperature-controlled growth cabinet set at 21/21° C (day/night cycle). Petri dishes were exposed to a 12 h light/12 h dark cycle. Illumination was provided by white fluorescent tubes with mean photon flux density of 220 $mmol^{-2}$ m^{-1} s^{-1} at seed level. The germinated seeds were counted every day. The seeds were considered germinated when 1 mm radicle appeared. The percent of germination (germinability) for each sample was calculated by the number of seeds germinated during the experiment in relation to the total initial seed number of 50.

RESULTS AND DISCUSSION

The results of the performed test show that 1% solution of TZ stain and the selected conditions for the testing of seed viability are suitable and give positive results (Fig. 1). The traditional TZ test revealed that all studied populations were viable and the rates varied between species and populations. The percentages of viable seeds ranged from 42% to 52% in *A. markgrafii-K*, from 19% to 28% in *A. murale-Pr*, and from 22% to 34% in *A. murale-Po*. No variation was found for *A. markgrafii-G* and the calculated percentage of viable seeds was 35%. The viability of the seeds of both *Alyssum* species is higher if doubtful viable seeds are added to viable and ranged from 90% to 95%.

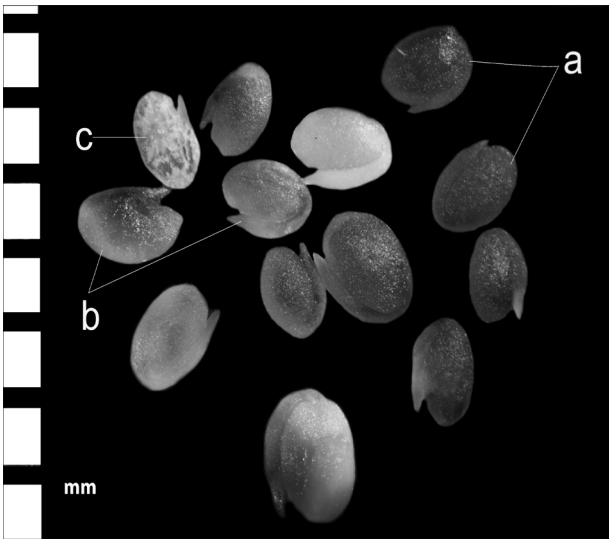

Fig. 1. Different pattern of TZ staining showing viable (a), doubtful viable (b) and non-viable (c) seeds.

The results from TZ test for viability presented as mean percent after three replications for both populations of *A. markgrafii* are shown on Fig. 2A. The populations of *A. markgrafii*-G demonstrate higher percentage of viable seeds (47%), compared to *A. markgrafii*-K where 35% were viable. The calculated percentages for the group of partially colored (doubtful viable) seed were 43% and 55%, respectively. The calculated percentage for seeds of this group was higher compared to the group of viable seeds in the population from Kukës. In both populations the percentage of non-viable seeds was equal (10%) and relatively high. The calculated percentages for the three groups of seeds for the negative control were 16%, 29% and 5%, respectively.

The results from the viability test for both *A. murale* populations are presented on Fig. 2B. The populations of *A. murale*-Po demonstrate higher percentage of viable seeds (27%), compared to *A. murale*-Pr (23%). The percentages of seeds from the second group were 70% and 68%, respectively for *A. murale*-Po and *A. murale*-Pr and higher than the calculated percentages for both *A. markgrafii* populations. The percentages of dead seeds for both *A. murale* populations were lower compared to *A. markgrafii*. The calculated percentages for the three groups of the colored seeds for the negative control were 19%, 25% and 6%, respectively.

The germinability of both *Alyssum* species and populations was quite low (Fig. 3). In general, *A. murale* seeds demonstrated higher germinability compared to *A. markgrafii* seeds. The highest percentage of germinability (germination) was calculated for *A. murale*-Po. The age of these seeds was about 10 months while the age of the other used for the experiment was about 18 months and this could be the reason for differences in germination.

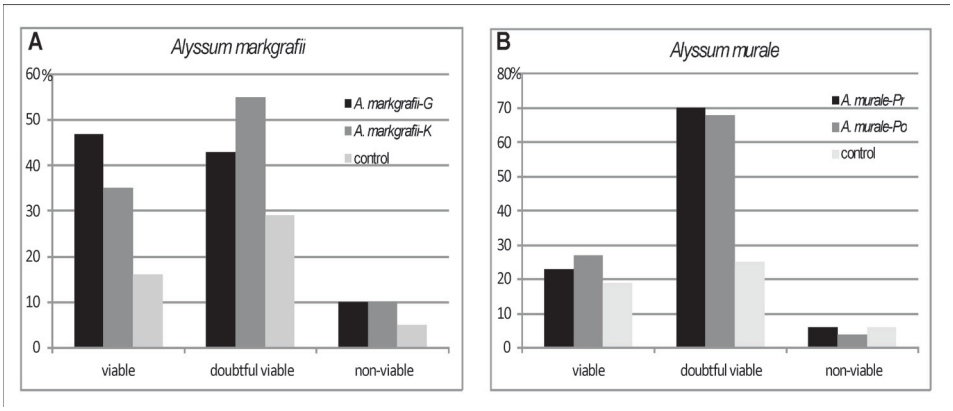

Fig. 2. The percentage of viable, doubtful viable and non-viable seeds tested with TZ test for: (A) *A. markgrafii* populations and (B) *A. murale* populations.

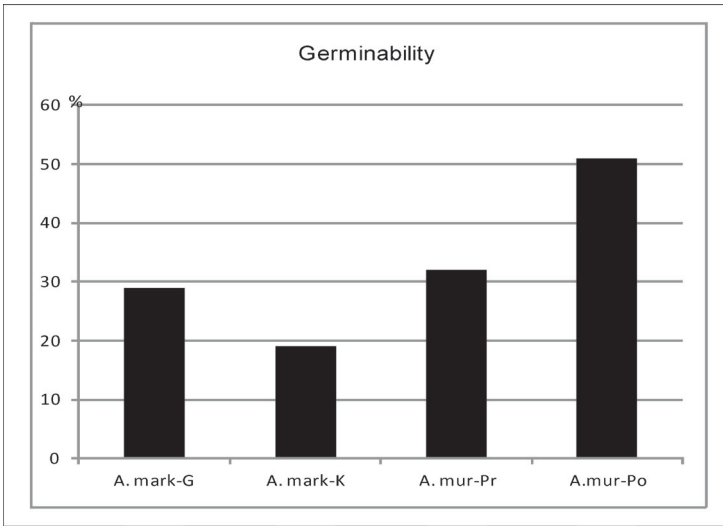

Fig. 3. The calculated percentage of germinability for the studied seeds and populations of *Alyssum* after TZ test.

The main result of this experimental study was a step towards filling our knowledge about the relation between germinability (germination percentage) and viability assessment through 2,3,5 Triphenyl Tetrazolium chloride (TZ) test of seeds collected from different populations of these species in Albania. The results of the test show that the potential viability of the seeds is higher than the germination percentage for *A. markgrafii* and lower for *A. murale*. The variation between viability and germination for the studied species was probably a result of seed dormancy. Seeds at various maturity levels or of the same species harvested at varying positions on the mother plant may show differences in dormancy (Conklin and Sellmer, 2009; Baskin and Baskin, 2014). Gutormson and Patin (2002) considered seed dormancy is not always removed by applied procedures to break dormancy. Seeds of both species demonstrate different dormancy levels and some have remained dormant although they were stratified similarly to the data for some Norway maple cultivars (Conklin and Sellmer, 2009). The correlations between viability and germinability were presented for seeds of different species by many authors (Alvarez et al., 1992; Kumar et al., 2013) but lack of correlations was also reported (Conklin and Sellmer, 2009).

Although the tetrazolium test is a quick method that provides an accurate assessment of viability (Thompson et al., 2002), variations in staining of the seeds are of importance and should be taken into consideration more precisely. On the basis of the results obtained we can conclude that the variation in the group of partially stained seeds is higher for both species and populations. The reasons for this variation could be a result from different factors: external (conditions of the experiment) like temperature, time of incubation, crop storage conditions, and internal like seed age and dormancy. The relation between seed viability and temperature and humidity studied for different species showed that higher temperature and/or moisture accelerated the loss of viability (Gravina and Bellairs, 2000). Increasing the temperature during imbibition according to the same authors can reduce the length of time to staining seeds. Similarly to the data of Gravina and Bellairs (2000) we suggest that the temperature applied (30°C) in our study was factor of importance for the low amount of viable seeds for both species and this may need to be considered when checking the viability of *Alyssum* species.

Staining occurs for many species after approximately 24h and sometimes up to 48h imbibing in solution (Gravina and Bellairs, 2000) but it is species specific. In our study the seeds of *A. markgrafii* and *A. murale* need 48h for staining in tetrazolium and differ from the data presented by Ooi (2007) for *Leucopogon exolasius* (F.Muell.) Benth. and *L. esquamatus* R.Br.. The intensity of staining was variable between pink and red for viable seeds but all seeds from this group were viable and germinated normally. Variations in intensity of staining were found also for seeds included in the group of doubtful viable seeds where some seeds germinated normally and other abnormally. Thompson et al. (2002) also found that seeds of some Australian species stained only faintly but produced healthy germinants. Ooi (2007) considers low intensity of staining may be due to factors related to dormancy (low levels of respiration). These results shows that intensity of staining is insignificant when evaluate viability while the size of unstained

necrotic tissues in the embryo or endosperm are of importance for normal seed germination as this was mentioned in the guidelines of International Seed Testing Association (ISTA, 2009). No germination after staining with tetrazolium was also reported (Bell et al., 1995; Clarke et al., 2000). The lack of germination could also be due to the complex dormancy mechanisms, such as low levels of respiration. According to Ooi (2007) due to the long period of imbibition and the low intensity of staining, previous studies that have used tetrazolium to check viability of seeds may have erroneously attributed a lack of germination to high proportions of non-viable seeds. The relative low percentage of germination of seeds in this study can be related to the elevated number of seeds from second group where seeds are partly colored in the tetrazolium and remained in latent state. Similar results were reported from Pasqualini et al. (2016) for blueberry seeds.

The variation in seed germination between the populations found for both *Alyssum* species depends on their biology and edaphic factors (Bani et al., 2011) or can be a result of adaptive trait of plants to harsh environment similarly to the data provided by Li et al. (2015) for *Potamogeton pectinatus* L. The same authors consider relatively poor germination suggests that seeds are relatively unimportant in the short-term survival of populations and that it may be another adaptive trait allowing plants to take place in the right place and at the right time, especially in harsh environment. For more precise analysis of variation in seed germination in populations of the studied species and correlations with viability, further long-term detailed analyses are required.

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