Optimizing proliferation and assessment of valak morpho-phenological traits; an endangered nutritious *Allium* endemic to Iran

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Abstract

Allium elburzense W. (subg. Melanocrommyum) is an endangered and endemic species to Iran, which is called “valak” and known as a valuable and pricy vegetable with high nutritional and medicinal properties. This study was carried out in order to domestication, removing barriers of sexual reproduction, micropropagation and investigation of phenological stages and growth cycle of the species. The results indicated that fresh seeds had better germination than one year old seeds and appropriate germination temperature in A. elburzense W. seed was 12 °C. According to ANOVA results, the triple interaction of scarification (Sc), stratification period (StP) and GA 3 had a significant effect on seedling length, seedling fresh and dry weight, bulb height and diameter ($p<0.01$). Comparison of means showed that the treatments in which the StP (45 days) and scarification was applied had better effects on seed germination (66.5% germination) than other treatments. Murashige and Skoog with 300 mg L -1 KH2PO4 (MSP) medium caused a significant increase in the diameter of the basal plate compared to MS medium. Efficient system for in vitro propagation and conservation of valak genetic resources were the use of MSP culture medium supplemented with 0.6 mg l -1 NAA, 4 mg l -1 BA and 120 g l -1 of sucrose, which resulted in a direct propagation coefficient of 26.83 seedlings and bulblets at 18 weeks. As regards its life cycle, the juvenile phase in a low proportion of individuals (13.33%) lasted 2 years from seeding, while 87.66% of individuals were capable of producing inflorescence in the third year. Results showed that this plant is a metamorphosis and cross pollinator species. Results of present study can be used in domestication, conservation of genetic resources and proper exploitation of valak species.

Key Message

In this study, a very valuable and widely used plant in Iran that was in danger of extinction due to excessive harvesting from wild and lack of cultivation was studied, and a rapid and low-cost micropropagation system was introduced. In addition, by reviewing life cycle and behavior of its various organs, the barriers to sexual reproduction were removed. According to the results obtained and high price of this species in Iran, not only will its extinction be prevented, but it will be a very good choice for cultivation and investment.

Introduction

The subgenus *Melanocrommyum* (Webb & Berthel.) Rouy is the second largest subgenus of the genus *Allium*, which consists of 10 section and has over 170 species. *Allium elburzense* W. belongs to the Asteroprason section and called “valak” in Iran and used as fresh vegetable and medical agent (Jafari et al. 2017). This species is found only in mountainous areas of northern Iran in sandy slopes of the Alborz mountain range (Frisch and Abbasi, 2013). Valak is a very expensive vegetable in Iran and its leaves uses for the preparation of a variety of special soups and “valak pilaw”. Its inflorescence uses to prepare pickles, spices and food decoration, and its bulb is consumed in the pharmaceutical industry (Jafari, 2017). This species is not cultivated, but collected from the nature (Akhavan et al. 2015). Indiscriminate harvesting of valak from natural habitats, harvesting pre-flower and before seed formation with bulb (permanent organ) and low percentage of seed germination has caused the endemic species is exposure to extinction. Reducing the number of individuals in a habitat has led to a decrease in genetic diversity, which in turn will increase the vulnerability of the species to environmental changes, genetic drift and natural selection. Domestication of these valuable species is a suitable way to preserve desirable species and prevent their extinction (Ebrahimi, 2014). One of the main steps in the process is to study the methods of reproduction and preservation of genetic resources of these species. Therefore, awareness and promotion of knowledge about the life cycle of a species, the effect of the environment on its reproduction and its phenological and genetic traits are of great importance. As such knowledge will provide a better understanding of their phenology in order to predict the most favorable periods for seedling

establishment in the field and facilitate the provision of nursery plants from seed and organ *in vitro* culture (Lentz and Johnson, 1998).

Low seed germination speed and rate, slow seedling growth and low energy storage of seeds are some of the germination problems of some *Allium* species. Seed dormancy has been reported in a large number of wild Alliums, so it seems necessary to study methods to eliminate it in this species (Dashti et al. 2012; Ebrahimi et al. 2014; Phillips 2010). Seed and bulb dormancy in wild allium species is an adaptation to environmental conditions to avoid germination and growth in inappropriate seasons, so, in order to eliminate the dormancy of these organs, their natural environment should be modeled. In addition to sexual reproduction, which has problems such as segregation of traits, time consuming and more costly, asexual methods such as tissue and organ culture can be used to produce these species. Tissue culture is the most effective virus-free and rapid propagation technology and provides an efficient tool for producing virus-free *Allium* plants (Gimenez et al. 2016; Taşkın et al. 2013).

There is no reliable information on the dormancy of seeds and bulbs and how to facilitate its propagation and conservation of *A. elburzense* W.. Therefore, due to the high nutritional, pharmaceutical and economic value of valak on the one hand, and its lack of cultivation on the other hand, this study was design and performed in order to domestication (evaluation of phenological behaviors of growth and reproduction stages by both bulb and seed pathway), elimination barriers to sexual and asexual reproduction and achievement a rapid and low-cost proliferation per unit of time.

Material And Methods

Plant material

Bulb and seed of species *A. elburzense* W. (Iranian Valak) were collected from the Kalha habitat (in Alborz province at an altitude 2454 m, 36°44 N and 51°17 E with average precipitation 411.2 mm and average temperature of 11.8°C (herbarium no. 006467).

Sexual propagation

Stratification period (StP), Germination temperature (GT) and seed age (SAg)

Seeds were disinfected into sodium hypochlorite 1% before use for 5 minutes and then were washed 3 times with distilled water. A factorial experiment was conducted in a completely randomized design (CRD) with 3 factors and 3 replications (each replicate containing 100 seeds). The factors were included stratification period (StP) (a1= 15, a2= 30, a3= 45 and a4= 60 days of moist chilling at 5 °C), seed age (SAg) (b1= fresh seed and b2= one year old seed) and germination temperature (GT) (c1= 5, c2= 12 and c3= 20 °C). Percent and rate of germination were measured in the test.

Scarification (sc) , stratification, GA_3 and KNO_3

A factorial experiment was conducted as a CRD design with 4 factors in 3 replications. The factors were included scarification (Sc) (a1= without scarification, a2= scarification with 95% sulfuric acid for 10 minutes and a3= scarification with 95% sulfuric acid for 20 minutes, and a4= sandpaper for 30 seconds), moist stratification (St) (b1= 0 and b2= 45 days at 5°C), GA_3 (immersed seeds in c1= 0 and c2= 500 ppm for 12 h) and KNO_3 (immersed seeds in d1= 0 and d2= 0.2% for 24 h). Percent and rate of germination, seedling length, seedling fresh and dry weight and bulb height and width were measured. The seeds and filter papers were kept wet with distilled water. The petri dishes were placed in a germinator at 20°C, 16 h light and 8 h dark conditions for 20 days. Seeds in which the tip of the root

appeared were considered germinated (radicle protrusion) (Albert et al. 2002). Treated seeds were planted in pots (25 seeds/plot) containing coco-peat and perlite (with a ratio 3:1).

Asexual propagation (*In vitro*)

Plant material

The collected bulbs were placed in a cool temperature (15-18 °C) for a week in order to drying the surface moisture and avoidance of rotting, and then placed in a refrigerator of 5 °C (June 2015). Bulbs were reviewed in November and explants prepared after observing the sprouts grown. The bulb central bud with basal plate were used as explant.

Disinfection test

In order to find the best disinfectant concentration and duration of treatment, a factorial experiment based on CRD was conducted with 3 factors in five replications included sodium hypochlorite (NaOCl), immersion time and type of explants (cut out before or after treatment (Fig. 1)).

Culture conditions and establishment medium

MS medium (Murashige and Skoog, 1962) containing 3 % (w v^{-1}) sucrose and 0.7 % agar was used. According to past researches, MS is the most suitable medium for *in vitro* culture of *Alliums* (yan et al. 2009; Ebrahimi et al. 2014; Farhadi et al. 2017). The medium pH was adjusted to 6 before autoclaving at 121 ° C for 20 minutes. The cultivated explant were placed in a growth chamber at a light intensity of 40 µmol m^{-2} s^{-1} (a combination of cool fluorescent lamps with white and yellow light with a ratio of 50:50) at 25 ± 1 °C. The explants were cultured in MSP and MS media in six replicates to determination of establishment medium. The amount of phosphorus in MSP (with 300 mg L^{-1} KH2PO4) medium was twice the MS (170 mg L-1 KH2PO4) medium, approximately (Kahane et al. 1992). Diameter of basal plate was measured after 45 days with digital caliper and its increase was calculated as a percentage.

Effect of growth regulators on micro propagation indices

The explants were divided into two parts from the base plate after 45 days and then transferred to MS containing growth regulators. A factorial experiment based on CRD was conducted with two factors in six replications, including NAA (a1= 0, a2= 0.2, a3= 0.4, a4= 0.6 and a5= 0.8 mg l^{-1}) and cytokinin contains benzyl adenine and Kinetin (b1= 0, b2= 2BA, b3= 4BA, b4= 6BA, b5= 1Kin, b6= 2Kin and b7= 3Kin mg l^{-1}). Explants were placed in 14 hours light and 10 hours dark (based on pre-treatment) and characteristics of callus growth index (equation 1) (Fan et al. 2017), number of days to callus emergence, number of bulblet and shoot per explant were calculated.

Callus growth index= (final callus fresh weight - initial callus fresh weight)]/final callus fresh weight× 100

Effect of sucrose concentration on micro propagation indices

Effect of sucrose different concentrations (30, 60, 90, 120 and 150 g l^{-1}) on propagation indices of *A. elburzense* W. was evaluated in a completely randomized design with six replications. Explants were placed in 14 hours light and 10 hours dark. After 8 weeks, callus growth index, number of days to callus emergence, number of bulblet and shoot per explant were calculated.

Calculation of *in vitro* propagation coefficient (direct organogenesis)

Explant of *A. elburzense W.* was cultured in the best condition (establishment medium, BA, cytokinin, sucrose) based on the results of previous experiments and propagation coefficient was calculated for an explant after 18 weeks. Propagation coefficient was total number of shoot and bulblet composed of a complete explant, in a given period of time.

Acclimation and transfer to non-controlling condition

The formed bulblet were separated and cultured in pots containing perlite and cocopeat with a 3:1 ratio and were transferred to ambient temperature (non-controlling condition) in late February 2016.

Morpho-phenological evaluation of *A. elburzense* W.

This experiment was carried out in order to evaluation of growth phenology and stages of reproductive organs production of *A. elburzense* W., duration 3 years, at the Horticultural Sciences Research Station of the University of Tehran (long. 51° 10' 35" N, lat. 35° 42' 18" E, height 1297 m), in the both form of sowing seeds and bulbs. The dried and mature inflorescences (previously marked) and bulbs were collected from the Kalha habitat in May 2014 and kept in the shade at 25 °C. After separating the hollow and immature seeds, 30 seeds were planted in trays of 30 cells with a mixture combination of peat moss, coco peat and perlite bed in a proportion of 6:1:1 in 3 replications. Observations were recorded until the formation of inflorescences and seeds. The bulbs from the seedlings harvested and were kept at 17 °C in a place with low humidity and then planted in November 2015 10 cm pots (10 bulbs per pot) in mixture of soil and sand in proportion of 1:1. Simultaneously with sowing seeds, bulbs collected from this habitat were also planted in the field to study bulb behavior and asexual reproduction. Developmental phases from flower formation to seed maturity were examined using a stereomicroscope (S21, Berlin, Germany). After the end of the growing season and drying of the aerial part, the bulbs were harvested and weighed before planting at the beginning of the cold season in both planting methods (seed and bulb). In June 2016 percent of inflorescence formation, bulb diameter and weight, number of leaf and plant height were evaluated. In early autumn 2016 the harvested bulbs were planted. In June 2017, bulbs were harvested and morphological traits were measured.

Statistical analysis

Data on indices of seed germination and micropropagation were analyzed with the statistical analysis software SPSS v.21 and MSTATC (version 2.1 USA). Comparisons between establishment media were performed using independent t-test. All data were subjected to analysis of variance and means were compared using Duncan's multiple range test at $P < 0.01$.

Results And Discussion

Facilitate germination and remove obstacles

Effect of germination temperature, seed age and stratification period

The results showed that the highest germination rate was obtained in 45 and 60 day stratification (Fig. 2). Based on the comparison of means, the highest germination percentage was observed in treatments of 45 days StP × GT 12 °C (62.42%) and 60 days StP × GT 20 °C (61.1%) (Table 1).

Therefore, it can be said that the seeds of *A. elburzense* W. need at least 45 days StP at 5 °C for germination. In the treatment of SAg×GT, the highest germination percentage and rate were observed in fresh seed and GT 12 °C with 43.24 % and 4.31 s d^{-1}, respectively (Table 2).

Effect of scarification, stratification, GA_3 and KNO_3

According to the results of the previous experiment, the GT 12 °C and Stp 45 days were considered for this experiment. The use of GA_3 improved the studied traits compared to the control. KNO_3 had no significant effect on the studied traits. No seeds germinated in 10 minutes using of sulfuric acid without StP. The results showed that the interaction effect of GA_3 and KNO_3 led to a significant increase in germination percentage (19.98 %) and germination rate (2.17 s d^{-1}) (Table 3).

The results of ANOVA showed that the triple interaction of Sc, StP and GA_3 had a significant effect on seedling length, seedling fresh and dry weight, bulb height and diameter at the statistical level of 1%. Comparison of means showed that the treatments in which the StP (45 days) was applied had better effects on the studied traits than other treatments (Table 4). Although all *Allium* species follow the same germination pattern, the mechanisms of seed dormancy differ in taxonomic groups and in different habitats (Kamenetsky and Rabinoswitch 2006). Specht and Keller (1997) examined the germination temperatures of 94 *Allium* species and the appropriate seed germination temperature for *Allium* subspecies was 16 °C, for *Rhiziridium* subspecies between 16-26 °C, and 5-15 °C was also reported for species belonging to the *Melanocrommyum* section. The use of sulfuric acid removed the black seed coat, but had no significant effect on seed germination indices. However, sandpaper had a better effect than sulfuric acid and caused significant seed germination and improved seedling quality indices (Table 4). In the present study, it was observed that without using the appropriate StP, the seeds did not germinate significantly. These results were also reported by Ebrahimi et al. (2014) in shallot (*A. hirtifolium* Boiss). According to the obtained results, dormancy of *A. elburzense* W. seed can be considered as physiological and physical type. GA_3 increases the growth potential and strength of the embryo, overcoming resistant shells and mechanical barriers (pericarp, seed coat and endosperm) for germination and seedling growth (Finch-Savage and Leubner-Metzger 2006). Endosperm attenuation is regulated by phyto-hormones and environmental factors. Radicle emerging can be accelerated by GA_3 and inhibited by abscisic acid (Silva et al. 2004; Finch-Savage and Leubner-Metzger 2006; Linkies et al. 2010). The hard and thick endosperm in species of *Melanocrommyum* section is a type of plant acclimation to low temperatures in the main habitats, which protects the embryo during the winter cold.

Micropropagation

Disinfection of explants

The results showed that the lowest contamination (0%) was observed in the treatment of 3% active chlorine and 15 minutes immersion time, and the highest contamination in the explant (78%) belonged to the treatment of 1% active chlorine for 5 minutes in cut explants (Table 5). As a result, 3% active chlorine treatment and 15 minutes of immersion were used for uncut explants.

Establishment medium

The results of T-test showed that there was a significant difference between the two used establishment culture medium, basal plate in MSP medium indicated an increase with an average of 7.36% than explant cultured in MS medium (Table 6). The amount of phosphorus in MSP medium (300 mg l^{-1} KH_2PO_4) is nearly twice that of MS medium (170 mg l^{-1} KH_2PO_4). High phosphorus in medium causes the growth of the basal plate and with increasing the surface of the explant, its potential for organogenesis increases (Kahane et al. 1992).

Effect of growth regulators on micropropagation indices

Comparison of means showed that the best treatment in terms of callus production was 0.2 mg l^{-1} NAA with 1 mg l^{-1} Kin (47.14% callus growth) ($p< 0.01$) (Table 7). Farhadi et al. (2017) reported that the highest amount of callus in shallot explants (*A. hirtifolium*) was obtained in MS medium with 1.5 mg l^{-1} 2.4, D and 0.5 mg l^{-1} BAP. In the present study, it was observed that with increasing cytokinin content, callus growth decreased, which was reported by Toaima et al. (2003) in *A. ampeloprasum*, Tiwari et al. (2004) in *A. cepa* and Yan et al. (2009) in *A. sativum*,. Based on results, it was observed that much time is required for callus emergence. Callus induction in monocots requires more time than in dicotyledons (Zheng et al. 1998; Luciani et al. 2006). Therefore, in this study, direct organogenesis was targeted. The results of analysis of variance indicated that the effect of auxin and cytokinin on the number of seedlings per explant was significant ($p<0.01$). BA was better than Kin in direct seedling production. The highest number of seedlings per explant (7.17 per explant) was obtained in the treatment of 0.6 mg l^{-1} NAA in combination with 4 mg l^{-1} BA. Cytokinin plays a key role in shoot production in *in vitro* culture medium (Xu et al. 2008; Hailekidan et al. 2013; Fan et al. 2017). Kahane et al. (1992) reported that in the absence of cytokinin no shoots were formed in onion explants and the highest number of shoots obtained at 100 µM BA. However, in this study, some albino seedlings emerged from some cytokinin-free treatments (Fig. 3). Plant tissues contain varying amounts of different types of plant growth regulators that affect their response to external growth regulators in the culture medium. As a result, different species will react differently to regulators and even at different times (Winson et al. 2020).

Pelkonen and Kauppi (1999) were investigated the effect of different auxins on direct bulblet emerging of *Lillium Regale* Wil and was reported that among the various auxins (NAA, 2,4, D and IAA) used, NAA had the best results in terms of organogenesis and especially direct bulblet formation. The superiority of NAA over other types of auxins in organ regeneration in onion plants has been proven in many studies (Ghosh and Sen 1991; Wang et al. 1993; Buiteveld et al. 1993; Mizuguchi and Ohkawa 1994; Cid et al. 1994; Yan et al. 2009; Farhadi et al. 2017). Therefore, in this study only NAA was used and it was observed that alone (without the use of cytokinin) it is able to directly from bulblets and seedlings and caused high callus growth.

The highest number of bulblets per explant was observed in the treatment of 0.6 mg l^{-1} NAA and 4 mg l^{-1} BA (6.17 bulblets). Based on the results, the best combination of growth regulators for direct organogenesis (seedling and bulblet) in *A.elburzense* W. explants was 0.6 mg l^{-1} NAA and 4 mg l^{-1} BA (Table 7). Naik and Nayak (2005) investigated the direct organogenesis of *Ornithogalum virens* and reported that the highest number of bulblets (12-15 bulblets) was obtained in the treatment of 1 mg l^{-1} NAA, 2 mg l^{-1} BA and 60 g.l^{-1} sucrose during 8 weeks. Direct production of bulblets from bulb explants (basal plate and scales) in other *Allium* species including *A. hirtifolium* Boiss. (Ebrahimi et al. 2014), *A. sativum* (Yan et al., 2009) and other bulbous plant (Bach, 1992; Slabbert and Niederwieser, 1999) have been reported.

Carbohydrates effect

The results showed that no seedlings were formed at any of the concentrations of sucrose used. Concentrations of 120 and 150 g.l^{-1} sucrose caused the highest increase in the diameter of the basal plate and concentrations of 60 and 30 g.l^{-1} showed the lowest (Fig. 4 (left)). The highest number of bulblets per explant was obtained in 120 and 150 g.l^{-1} sucrose treatments (Fig. 4 (right)). Sucrose is stored as starch in the storage organs (such as bulb scales) of most bulbous plants and has been reported that sucrose to increase the formation of various storage organs (bulbs, corms, tubers and rhizomes) in plants with these organs (Arora et al. 1996; Nayak 2000; Naik and Nayak 2005).

Proliferation index

The explant multiplication coefficient was considered as the sum of seedlings and bulblets consisting of one explant at a certain period. Explants were cultured in the best treatments based on previous experiments (MSP medium completed

with 0.6 $m.l^{-1}$ NAA, 4 $m.l^{-1}$ BA, 120 $g.l^{-1}$ sucrose) and observed that an average of 26.83 seedlings and bulblets were obtained from an *A. elburzense* W. bulb during 18 weeks. The bulblets that formed the seedlings were transferred to the pot and were placed for 10 days at 20 °C, 10 hours of light and 14 hours of darkness, at a light intensity of 40 $\mu mol\ m^{-2}\ s^{-1}$ (a combination of cool fluorescent lamps with white and yellow light with a ratio of 50:50), then were transferred to the open space in March. Eighty two percent of the seedlings continued to grow and formed a leaf, after 19 days were dried. The leaves are the main organ used by humans, reaching full growth 3 weeks after emergence. It was observed that after transferring the seedlings to the pot, they acted like mother plants and reached their maximum growth after 2 to 3 weeks. In results, by using obtained protocol, the production of high-performance and low cost is possible. Fig. 5 shows images of direct organogenesis in valak explants.

Morpho-Phenology traits and life cycle

After the emergence of the germination hook in early march 2015 (seed method), it took between 10-14 days for the length of the seedling (root and aerial part) to reach a constant value (11-20 cm). After 3 weeks of germination and emergence of the radicle, a small bulb with a diameter of 3-4 mm and a length of 3-5 mm was formed at a depth of 6-8 cm (Fig. 6). After another 2 weeks, the bulb size increased (5-7 mm in diameter and 6-8 mm in length) and finally the aerial part dried (35-40 days from germination to aerial drying). Based on results in the first year, seedlings from seeds formed only one leaf and seedlings from bulb yielded 1.33 ± 0.02 leaves (Table 8).

Plants grown from seed did not form inflorescence in the first year and the average weight of the formed bulbs was 1.05 ± 0.04 gr. 73.33 ± 1.05 % of the plants from bulb cultivation formed inflorescences with an average diameter of 5.07 ± 0.11 cm. The weight of bulbs at the first year was 11.65 ± 0.42 gr. In early March 2016, plant grown from seed sowing formed 1.48 ± 0.05 leaves and 13.33 ± 0.11% of them formed inflorescences with a diameter of 4.4 ± 0.07cm. The average weight of bulbs obtained from seed cultivation in the second year (2015/2016) was 6.76 ± 0.13 gr. In the third year of growth, the plants obtained from seed cultivation reached full maturity and formed 1.71 ± 0.08 leaves, 87.66 ± 1.35 % formed inflorescences with a diameter of 5.85 ± 0.16 and the bulbs weighed 12.92 ± 0.21 gr. The mean of studied traits in plants from bulb cultivation in the second and third years were not significantly different (Table 8). Therefore, it can be concluded that with bulb cultivation, in the first and second year, the desired result (leaf and bulb harvest or seed production) was achieved, but from seed cultivation to seed production in *A. elburzense* W. taken 3 years. After the third year, was observed that the bulbs did not reproduce. While Kamenetsky and Rabinoswitch (2006) reported that the bulb of most *Allium* species is a permanent organ, which will be completely replaced by a new bulb after two years, the study found that one bulb per seed it was formed and the bulb remained stable and was not replaced by another bulb for 3 years.

Flower and seed phenology

The time of flowering in Karaj farm varied from April 6 (the earliest specimen) to April 17 (the latest specimen). The growth stages of inflorescences and flowers in *A. elburzense* W. species can be seen in Figure 11. Like other alliums, *A. elburzense* W. seeds are initially flat and round, and as they matured, their surface becomes shrink and black due to the presence of phytomelanin (Rahn 1998) (Fig. 7).

Study of reproductive stages from flower bud formation to opening and seed formation revealed that *A. elburzense* W. is a protander plant (Fig. 8). According to protander nature of this species, it can be acknowledged that this plant is a metamorphosis and is pollinated by flowers from other plants.

Conclusion

The results showed that stratification period alone caused the germination of *A. elburzense* W. seeds. However, due to the hardness of the endosperm and the inhibition of radicle emerging, a combination of stratification (45 days at 5˚C) and scarification (sandpaper) led to better results. Based on results, it was determined that *A. elburzense* W. seeds had physiological and mechanical dormancy. Indiscriminate harvesting of valak from wild in the vegetative stage and before seed formation, lack of cultivation and low percentage of seed germination, it has made this valuable and endemic plant endangered. The results of the present study and the appropriate proliferation coefficient obtained, can be considered in 1) preserving the genetic resources of these species, 2) low cost production of virus-free plants, 3) reproduction facilitating, 4) Initiation and development of its cultivation and 5) preventing the extinction of these species. According to phenological evaluations, it takes 3 years from seed sowing to seed formation in *A. elburzense* W., while with bulb sowing, usable seeds and leaves can be obtained in the same year. Also, it was determined that *A. elburzense* W. is a protander and cross pollination species.

Declarations

Ethical statement

- The research meets all applicable standards with regard to the ethics of experimentation and research integrity, and the following is being certified true.
- As an expert scientist and along with co-authors of concerned field, the paper has been submitted with full responsibility, following due ethical procedure, and there is no duplicate publication, fraud, plagiarism, or concerns about animals or human experimentation.

A disclosure/ conflict of interest statement

- None of the authors of this paper has a financial or personal relationship with other people or organization that could inappropriately influence or bias the content of the paper.
- It is to specifically state that “No Competing interests are at stake and there is No Conflict of Interest” with other people or organization that could inappropriately influence or bias the content of the paper.

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Tables

Table 1 Effect of stratification period and germination temperature on germination (%) of *A. elburzense* W. seeds

StP (day)	GT (°C)	Germination (%)
15	5	3.33 g
15	12	1.11 h
15	20	0 i
30	5	18.88 ef
30	12	31.1 d
30	20	14.44 f
45	5	43.99 c
45	12	62.42 a
45	20	47.44 bc
60	5	45.55 bc
60	12	48.3 bc
60	20	61.1 a

Means followed by the same letter in column are not significantly different at 5 % (based on Duncan multiple range test)

Table 2 Effect of seed age and germination temperature on germination traits of *A. elburzense* W. seeds

SAg	GT (°C)	Germination (%)	Germination rate (s d^{-1})
fresh	5	27.03 de	2.58 e
fresh	12	43.24 a	4.31 a
fresh	20	37.31 b	3.99 abc
One year old	5	25.51 e	2.91 de
One year old	12	35.73 bc	3.62 bc
One year old	20	29.99 cde	3.2 cd

Means followed by the same letter in column are not significantly different at 5 % (based on Duncan multiple range test)

Table 3 Effect of GA_3 and KNO_3 on germination traits of *A. elburzense* W. seeds

Germination rate (s d^{-1})	Germination (%)	KNO_3 (%)	GA_3 (ppm)
1.4 b	12.91 b	0	0
1.28 b	11.63 b	0.2	0
1.45 b	12.33 b	0	500
2.17 a	19.98 a	0.2	500

Means followed by the same letter in column are not significantly different at 5 % (based on Duncan multiple range test)

Table 4 Effect of stratification period and germination temperature on germination traits of *A. elburzense* W. seeds

Sc	StP (day)	GA_3 (ppm)	Germination (%)	Germination rate (s/d)	Seedling length (cm)	Seedling FW (mg)	Seedling DW (mg)	Bulb height (mm)	Bulb width (mm)
Without Sc	0	0	2.6 k	0.23 h	4.95 hi	29.83 ef	2.31 f	1.33 d	1.14 e
Without Sc	0	500	19.42 gh	1.88 ef	11.85 cd	85.83 cd	6.64 cd	3.79 b	3.18 cd
Without Sc	45	0	55.14 c	5.69 b	16.76 b	107.66 b	8.52 a	5.43 a	4.65 a
Without Sc	45	500	60.08 b	6.34 a	17.11 b	110.81 ab	8.75 a	5.68 a	4.67 a
10 min H_2So_4	0	0	13.91 i	1.25 fg	6.01 g	30 e	2.42 f	2.01 cd	1.8 de
10 min H_2So_4	0	500	28.2 f	2.73 d	10.66 e	26.5 fg	2.03 f	2.18 c	1.99 de
10 min H_2So_4	45	0	59.7 b	5.6 b	17.01 b	105.74 b	7.61 bc	5.44 a	4.06 ab
10 min H_2So_4	45	500	64.35 a	5.93 ab	17.44 b	113.5 a	8.26 ab	5.82 a	4.57 a
20 min H_2So_4	0	0	3.46 k	0.29 h	4.35 i	21.13 g	1.62 g	1.42 d	1.35 e
20 min H_2So_4	0	500	16.33 hi	1.46 fg	6.93 g	33.55 e	2.12 f	2.71 bc	2.23 d
20 min H_2So_4	45	0	36.81 de	3.17 c	9.13 f	61.33 e	4.75 e	2.15 c	1.87 de
20 min H_2So_4	45	500	33.5 e	3.11 c	12.6 c	91.15 c	6.74 cd	2.98 b	2.12 d
Sandpaper	0	0	7.8 j	0.6 h	5.5 h	23.5 g	1.95 fg	1.6 d	1.33 e
Sandpaper	0	500	24.5 fg	2.11 de	10.36 e	82.66 d	5.28 de	3.2 b	2.64 d
Sandpaper	45	0	63.17 ab	5.82 ab	17.33 b	111 ab	8.32 ab	5.28 a	4.24 a
Sandpaper	45	500	66.5 a	6.17 a	18.75 a	114 a	8.89 a	5.79 a	4.81 a

Means followed by the same letter in column are not significantly different at 5 % (based on Duncan multiple range test)

Table 5 Percentage of explant contamination under the influence of 3 factors NaOCl, immersion time and explant type

NaOCl (% Cl active)	Immersion Time (Min)	Explant Type	Contamination (%)
1	5	Non cut	68.22 bc
1	5	Cut out	81.39 a
1	10	Non cut	49.16 e
1	10	Cut out	72.5 b
1	15	Non cut	37.8 fg
1	15	Cut out	59.17 d
2	5	Non cut	43.04 ef
2	5	Cut out	66.25 cd
2	10	Non cut	22.93 h
2	10	Cut out	41.31 f
2	15	Non cut	13.76 i
2	15	Cut out	34.67 g
3	5	Non cut	18.33 hi
3	5	Cut out	42.4 f
3	10	Non cut	2.67 j
3	10	Cut out	19.5 hi
3	15	Non cut	0 j
3	15	Cut out	3.14 j

Means followed by the same letter in column are not significantly different at 5 % (based on Duncan multiple range test)

Table 6 Comparing the mean of two established culture media on the percentage of increase in basal plate width of valak bulb based on the independent T-test

Trait	MS	MSP	T	Pr>t
Diameter of basal plate	4.58 ± 0.32	7.36 ± 0.89	2.93	0.013

Table 7 Mean comparison of the interaction of NAA, BA and Kin on micropropagation indices of *A. elburzense* W.

NAA (mg l^{-1})	Cytokinin (mg l^{-1})	Callus (%)	Number of seedlings in explant	Number of bulblets in explant
0	0	0 l	0.33 k	0.33 i
	2 BA	0 l	0.66jk	0.67 hi
	4 BA	5.7 kl	1.17 ij	0.33 i
	6 BA	3.95 kl	0.33 k	0.17 i
	1 Kin	10.11 i-k	0.46 k	0.67 hi
	2 Kin	1.91 l	0.5 jk	0.33 i
	3 Kin	2.11 l	0.17k	0.17 i
0.2	0	33.98 b	0.66jk	0.83 hi
	2 BA	37.44 b	1.67 hi	1.33 gh
	4 BA	23.24 d-e	4.83 c	1.67 fg
	6 BA	8.79 i-k	2.17 gh	1.83 fg
	1 Kin	47.14 a	0.33 k	2 ef
	2 Kin	11.91 h-j	1.67 hi	0.67 hi
	3 Kin	8.98 i-k	0.83 ij	0.33 i
0.4	0	15.3 f-i	1.67 hj	1.67 fg
	2 BA	18.48 f-h	5.83 b	2.33 ef
	4 BA	9.6 i-k	5.66 b	1.83 fg
	6 BA	6.46 jk	2.17 gh	1.67 fg
	1 Kin	29.27 b-d	1.5 hi	1.33 gh
	2 Kin	17.19 f-h	1.83 gh	1.5 fg
	3 Kin	11.93 h-j	1 ij	0.5 i
0.6	0	30.29 b-d	0.17 k	1.83 fg
	2 BA	19.27 f-g	4.17 cd	3.67 c
	4 BA	3.56 kl	7.17 a	6.17 a
	6 BA	19.88 e-g	2.67 fg	3.17 cd
	1 Kin	37.89 b	0.83 ij	1.17 hi
	2 Kin	13.29 g-j	2.67 fg	2.67 de
	3 Kin	0 l	2.17 gh	1.67 fg
	0	27.86 c-e	0.5 jk	1.5 fg
	2 BA	32.21 bc	1.33 hi	2.17 ef
	4 BA	27.05 c-e	2.5 fg	3.33 cd
	6 BA	0 l	3.67 de	4.67 b

	1 Kin	12.37 g-j	2 gh	1.83 fg
	2 Kin	7.3jk	0.83 ij	2.17 ef
	3 Kin	0 l	0.5 jk	1.17 hi
0.8				

Means followed by the same letter in column are not significantly different at 5 % (based on Duncan multiple range test)

Table 8 Morphological properties of *A. elburzense* W. seedlings grown from seed and bulb over 3 years.

Propagation method	Traits	2014/2015	2015/2016	2016/2017
Seed	Leaf number	1 ± 0^{c}	1.48 ± 0.05^{b}	1.71 ± 0.08^{a}
	Inflorescence formation (%)	-	13.33 ± 0.11^{b}	87.66 ± 1.35^{a}
	Inflorescence diameter (cm)	-	4.4 ± 0.07^{b}	5.58 ± 0.16^{a}
	Bulb weight (gr)	1.05 ± 0.04^{c}	6.76 ± 0.13^{b}	12.92 ± 0.21^{a}
Bulb	Leaf number	1.33 ± 0.02^{b}	1.76 ± 0.03^{a}	1.67 ± 0.12^{a}
	Inflorescence formation (%)	73.33 ± 1.17^{b}	89.35 ± 1.3^{a}	84.2 ± 1.22a
	Inflorescence diameter (cm)	5.07 ± 0.11^{b}	5.63 ± 0.06^{a}	5.49 ± 0.18^{a}
	Bulb weight (gr)	11.65 ± 0.42^{b}	13.59 ± 0.24^{a}	12.69 ± 0.23^{ab}

Means followed by the same letter in row are not significantly different at $P < 0.05$ level

Figures

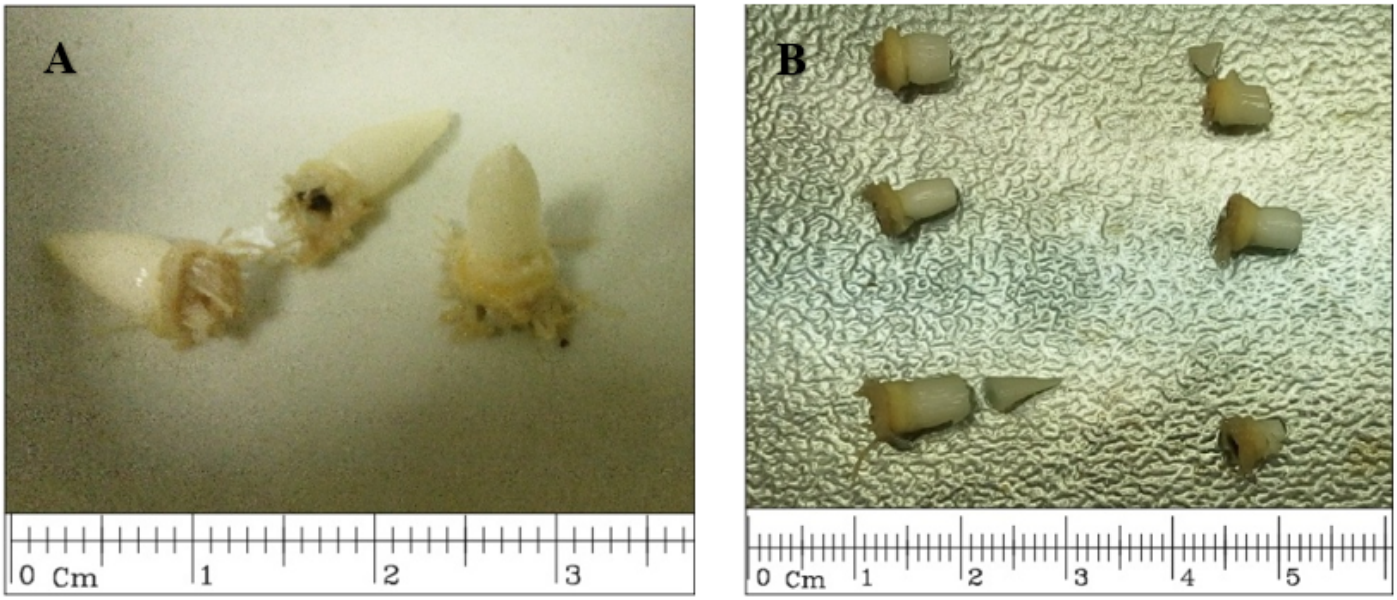

Figure 1

Explant of A. elburzense W. bulb, A without cutting the ends of bud and B cutting of the bud.

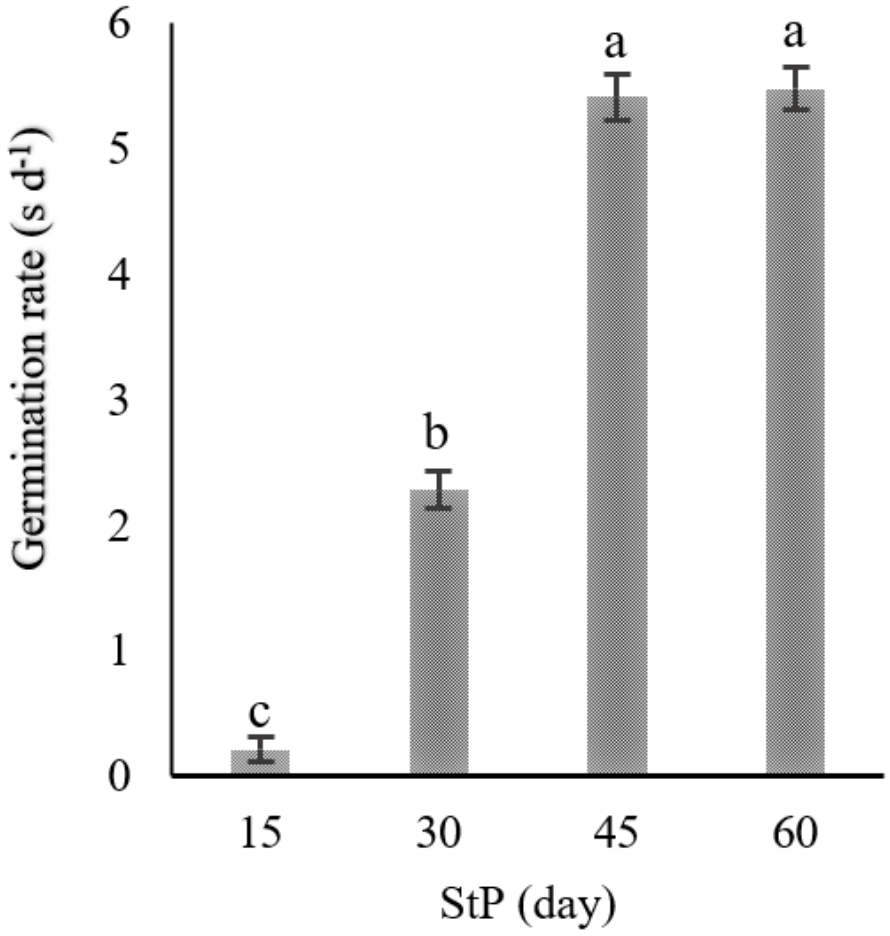

Figure 2

Effect of stratification period on germination rate of A. elburzense W. seeds (Bars followed by the same letter are not significantly different at 1 % (based on Duncan multiple range test))

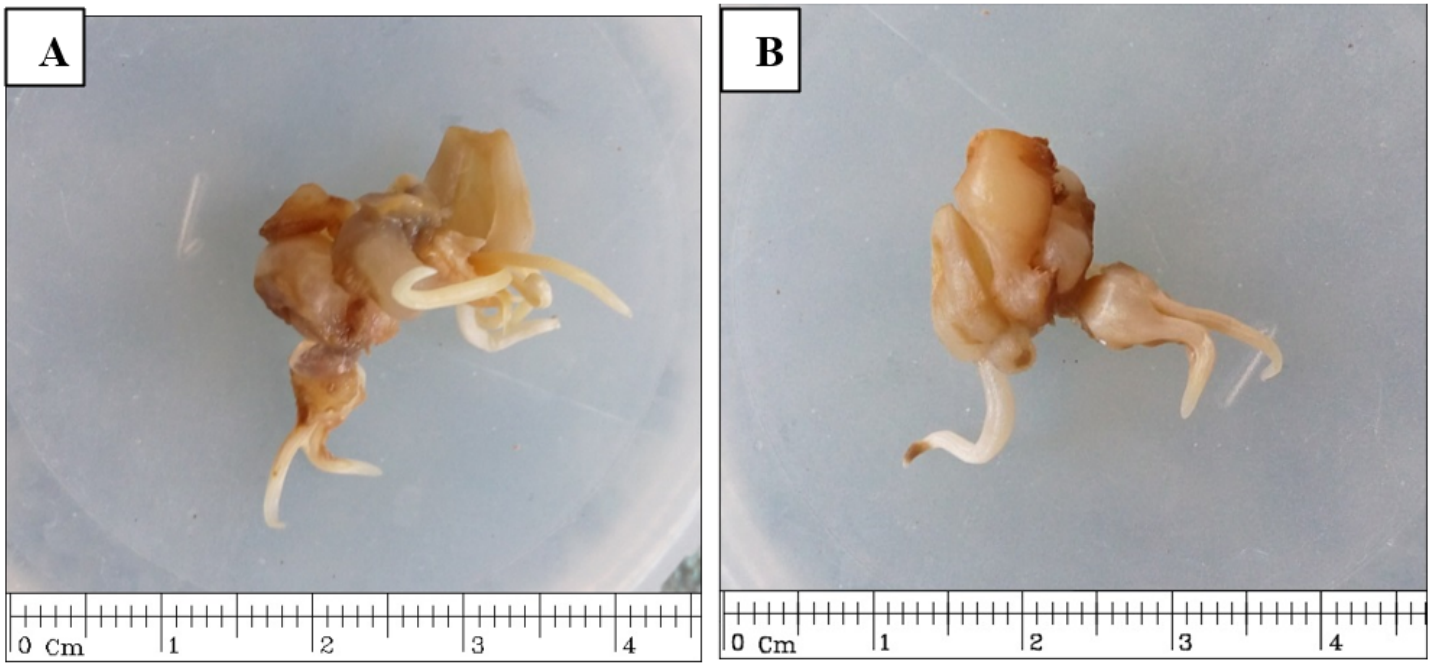

Figure 3

Albino seedlings of species A. elburzense W. A medium without growth regulators B medium containing 0.2 mg l-1 NAA.

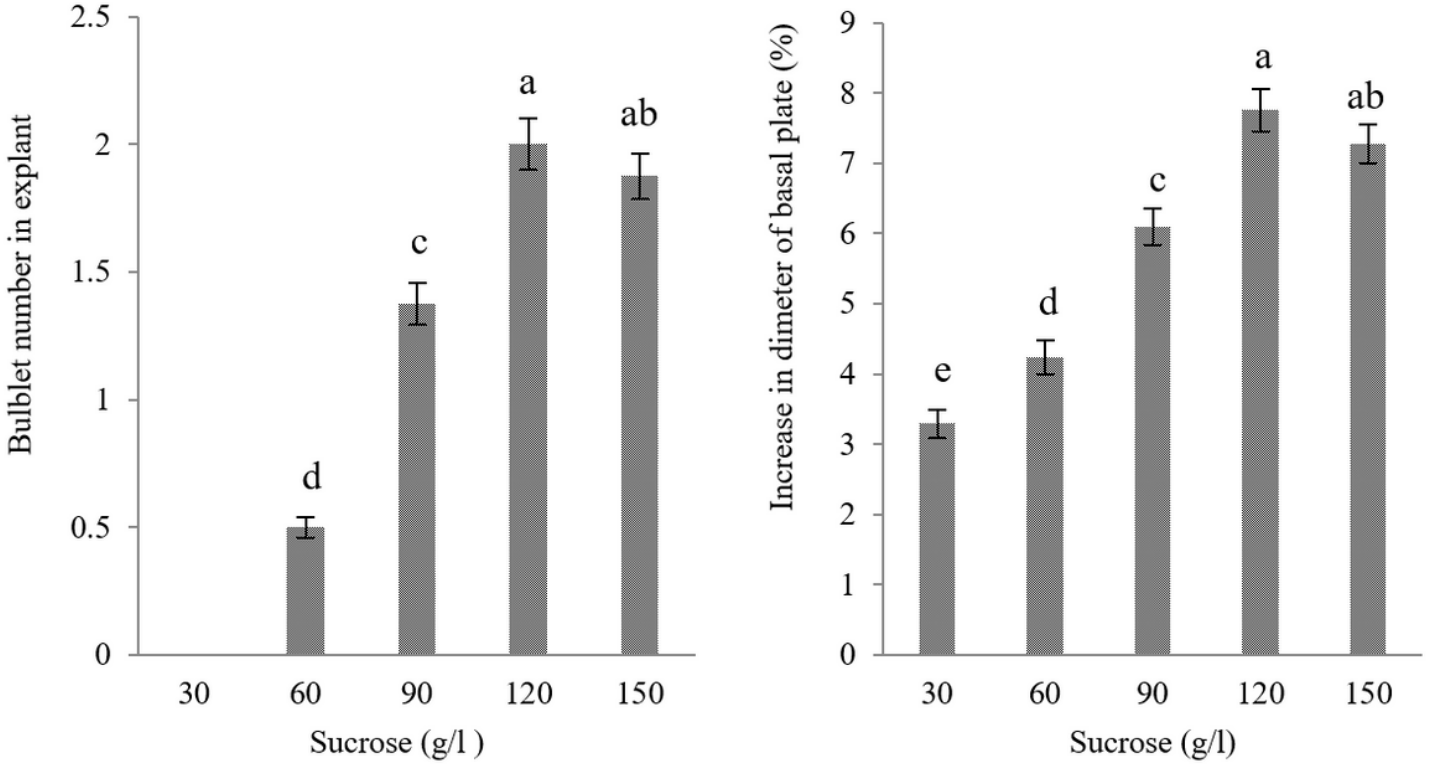

Figure 4

The effect of sucrose different concentrations on the percentage increase in diameter of basal plate (left) and the number of regenerated bulblet per explant (right) in A. elburzense W. (Bars followed by the same letter are not significantly different at 5 % (based on Duncan multiple range test))

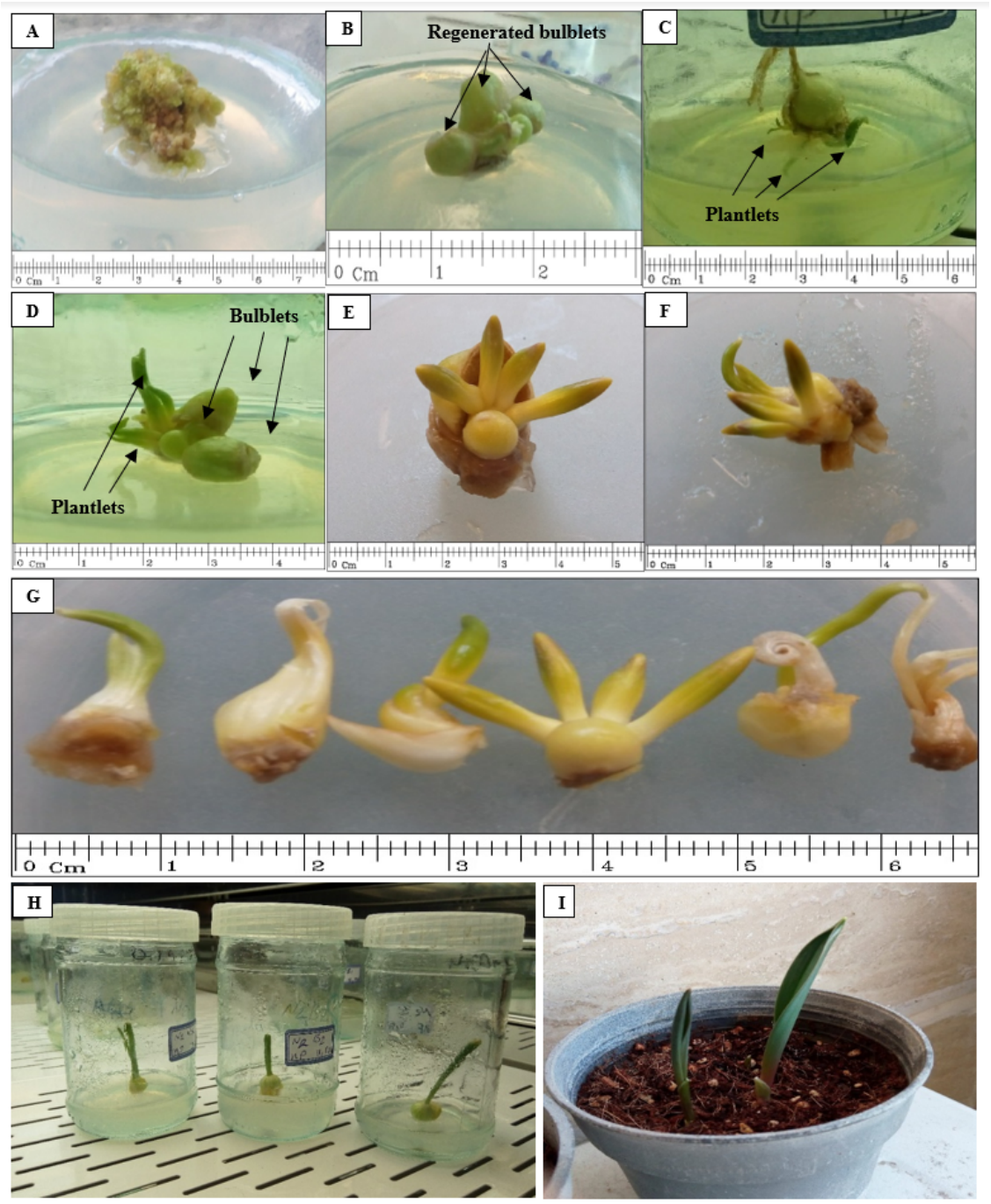

Figure 5

A callus proliferation from basal plate, B production of bulblets and emergence plantlets, C plantlet direct production from scales and basal plate, D bulblets and plantlets grown from an explant, E plantlets formation from a bulblet, F Plantlet production from sub cultured bulblet, G Regenerated plantlets and bulbs from a complete explant (one basal plate), H In vitro acclimatization of A. elburzense W. (pretreatment of bulblet on ½ MS + 0.6 m.l-1 NAA for 10 days) and I 3 week-old acclimatized plantlet in peatmoss, cocopeat and perlite (3:1:1)

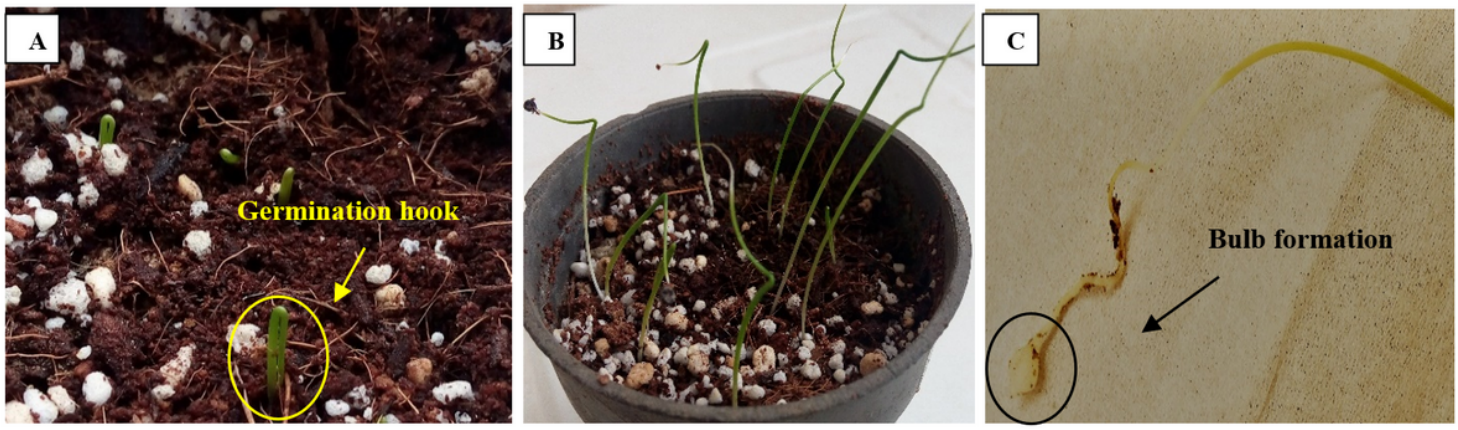

Figure 6

A Germination hook, after 11 days B leaf, after 18 days C bulb formation, after 20 days from seed sowing in A. elburzense W.

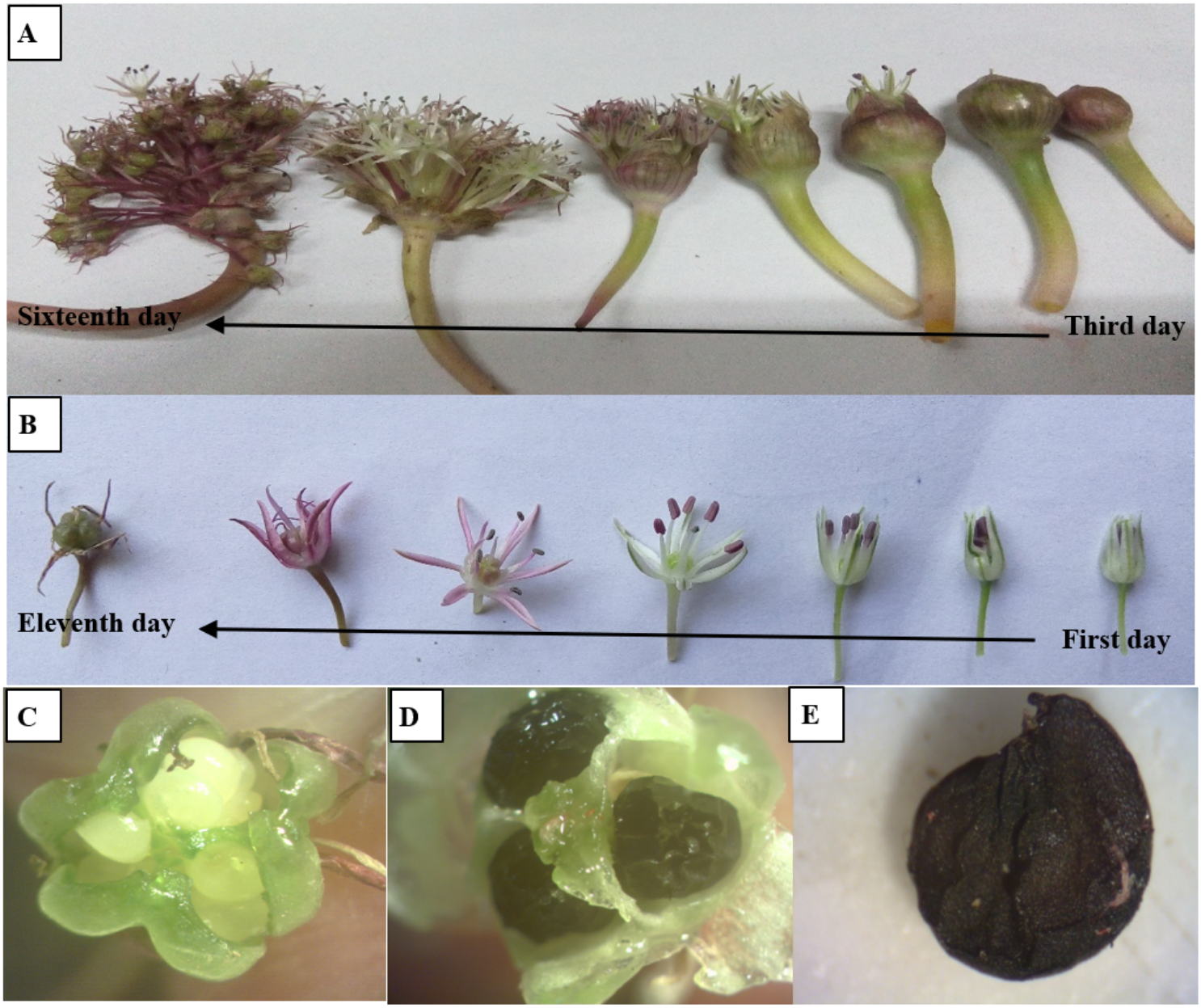

Figure 7

A Inflorescence development stages from the time of emergence B flower development stages from the time of inflorescence opening C, D and E seed (C, D and E using stereomicroscope) of A. elburzense W.

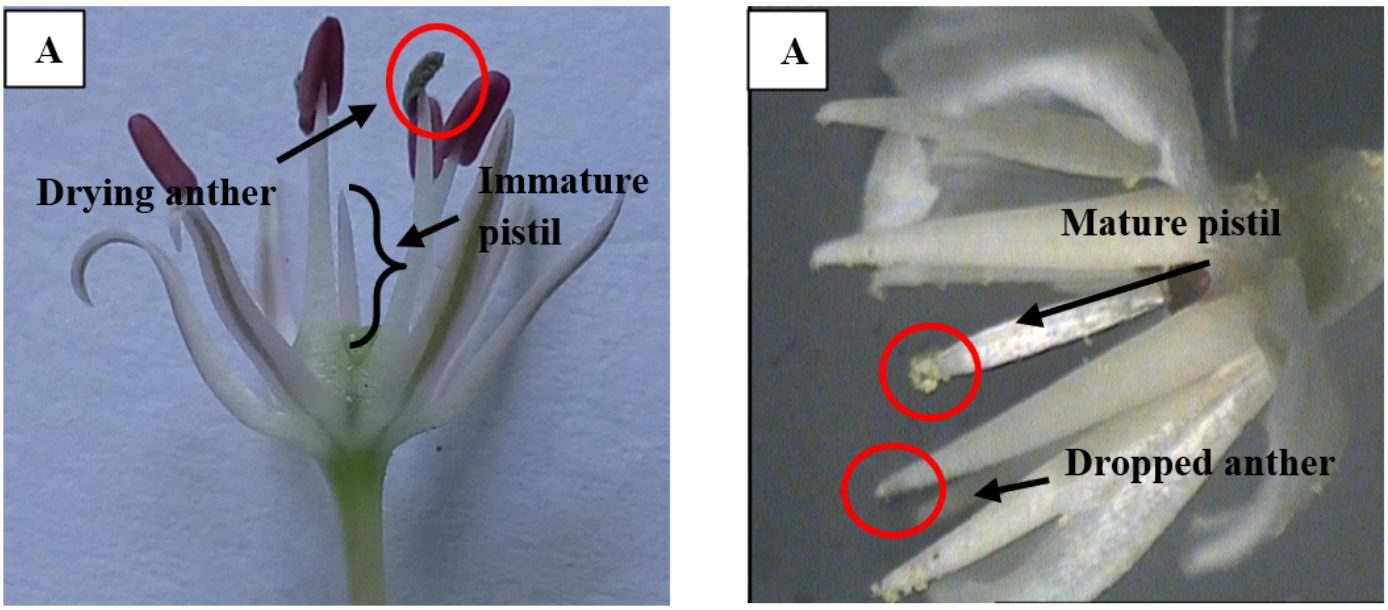

Figure 8

Protandry in A. elburzense W. A mature anthers and immature pistil B flowers with mature pistil and anthers dried and dropped.