

Full Length Research Paper

In vitro* culture and medium-term conservation of the rare wild species *Gladiolus imbricatus

Elena Rakosy-Tican^{1*}, Barna Bors¹ and Ana-Maria Szatmari²

¹Department of Experimental Biology, Plant Genetic Engineering Group, Faculty of Biology and Geology, "Babeş-Bolyai" University, Clinicilor str. 5-7 RO-40006 Cluj-Napoca, Romania.

²Biological Research Center Szeged, Hungary.

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***Gladiolus imbricatus*, a rare species spread in the hill region of Europe, is resistant to abiotic and biotic stress being one of the most cold-tolerant in the genus. Moreover it contains high vitamin C and minerals in the leaves and the flowers are considered as edible. The aim of our study was to develop *in vitro* technologies for micropropagation, multiplication, corm development, somatic embryogenesis and medium-term storage of this endangered species, Red listed in South-Eastern and Central European countries. Initiation of *in vitro* cultures was started from wounded seeds or excised mature embryos. Micropropagation and multiplication was achieved on Murashige Skoog (MS) solid medium with 30 g L⁻¹ sucrose and 1 mg L⁻¹ N-6-benzyl adenine (BA). The callus developed from wounded seeds on a richer MS medium proved to be embryogenic. Increased concentrations of sucrose promote corm development as also shown for other genotypes of cultivated gladioli. Further on a two-step culture protocol, on solid and liquid shaken MS based media, each for 6 months was done to evaluate the effects of acetic acid and ancymidol on corm development. Acetic acid + sucrose 9%, alone or in combination with ancymidol stimulated corm formation and yield. For medium-term conservation maintenance of the *in vitro* cultures in low temperature in the dark proved to be the best in reducing the growth rate of the shoots after 3, 6 and 12 months. The recovery of plant growth was evaluated after 12 months, by the transfer to normal growth conditions. 25% of the plants were recovered after one year storage. Besides the importance for biotechnology, the *in vitro* techniques described here might be used, after molecular analysis of genetic stability, for the restoration of natural populations into the habitats where this species became extinct.**

Key words: Acetic acid, ancymidol, corm development, somatic embryogenesis.

INTRODUCTION

Gladiolus imbricatus is a rare wild species spread from the South Scandinavian hills to the coast of Mediterranean Sea but its origin is considered to be East Europe and Turkey. This species belonging to Iridaceae family is included on the Red List as endangered species in entire Eastern and Central Europe (Kässermann, 1999; Schnittler and Günther, 1999). It belongs to Euro-Asian species and together with *Gladiolus palustris* and *Gladiolus illyricus* are the three wild *Gladiolus* species spread in Romania (Cantor and Tolety, 2011). *G. imbricatus* with its magenta decorative flowers occurs in

dry ground, containing calcium carbonate and iron and grows in dry grasslands at the border of bushes or in clearings, at an altitude between 100 and 1450 m (Figure 1A and B). Both *G. imbricatus* and *G. palustris* are almost the most cold-tolerant species of the genus and their seeds and corms require a chilling period in order to germinate. *G. imbricatus* is a tetraploid species with 2n = 60 chromosomes (Krahulcová, 2003). Moreover this rare species, known in the past as the "victory flower", is a rich source of vitamin C and minerals and the flowers are edible having a taste similar to lettuce (www.squidoo.com/edible-flowers). *In vitro* culture of this species was, to the best of our knowledge, not previously reported.

The genus *Gladiolus* contains about 250 species and

*Corresponding author. E-mail: lrakosy@hasdeu.ubbcluj.ro or arina5744@yahoo.com. Fax: (40) 264 591906.

more than 10,000 cultivars. The majority of the species (150) are originally from South Africa, which is considered as the diversification center for the cultivars (Ohri and Khoshoo, 1985). Today's gladioli are hybrids of different wild species or hybrids of subsequently obtained hybrids. All of them belong to the class named *Gladiolus hybridus* and are tetraploids ($2n = 4x = 60$). The cultivated varieties are very sensitive to diseases such as fungi (*Fusarium*, *Sclerotinia* and *Botrytis* sp.) and viruses (Ziv and Lilien-Kipnis, 1990). *In vitro* culture - micropropagation, callus, corm development, cell suspension cultures, were reported for different cultivars of *G. hybridus* (Ziv et al., 1970; Bajaj et al., 1982; Ziv and Lilien-Kipnis, 1990; Steinitz and Lilien-Kipnis, 1989; Steinitz et al., 1991; Kamo, 1995; Remotti and Loffler, 1995; Sen and Sen, 1995; Misuk et al., 1998; Kumar et al., 1999; Rakosy-Tican et al., 2000; Kumar et al., 2001). Corm development was also previously reported in some wild African species (Bruyn and Ferreira, 1992). Recent studies include transformation for potential creation of a new genetically modified cultivar (Kamo, 2008). The wild species *G. imbricatus* represents a valuable reservoir of resistance genes to both biotic and abiotic stresses occurring in Europe, which may be used to improve gladiolus cultivars through biotechnological techniques such as embryo rescue or protoplast fusion (Rao et al., 1991b).

The aim of this paper was to establish reproducible *in vitro* culture techniques for the rare wild species *G. imbricatus*. In the first step initiation of *in vitro* culture from mature seeds or excised embryos was achieved as well as development of embryogenic callus. After initiation sustained micropropagation/multiplication and a two-step protocol for efficient corm development were established. Acetic acid (10 mM) alone or in combination with ancymidol (α -cyclopropyl- α -(4-methoxyphenyl)-5-pyrimidine methanol) an inhibitor of gibberellin biosynthesis, was used for the first time to improve corm development. For medium-term conservation maintenance at low temperature (4°C) or dim light for one year, the percentage of recovered plants after storage were evaluated. The results obtained allowed good micropropagation/multiplication (7 to 10 times/two months), corm development and regeneration of embryogenic callus. Medium-term conservation for one year, at low temperature, allowed 25% recovery of stored plants. The importance of these techniques, for the use of *G. imbricatus* in biotechnology, *in vitro* conservation and further exploitation in the restoration of natural populations are discussed.

MATERIALS AND METHODS

Plant material and *in vitro* cultures

G. imbricatus capsules with mature seeds have been collected from the field at Muntele Baişorii (1200 m altitude), West-Carpathian Mountains Romania. The seeds were thoroughly washed with tap

water, sterilized by immersion in 96% alcohol for 1 min followed by 'Domestos' 10% (v/v) (commercial bleach which contains about 5% sodium hypochlorite), for 30 min. Then the seeds were washed with sterile water 5 to 6 times in the laminar hood. The seeds were pretreated at 4°C, in the refrigerator, 18 days, for chilling. After sterilization the seeds were treated with 1 mg mL⁻¹ giberellic acid (GA3) for 1 h and soaked in sterile water for 24 h. The entire seeds, wounded seeds with a cut at the embryonic end or excised (rescued) mature embryos (under stereomicroscope) were inoculated. The seeds or mature embryos were germinated on ½ MS salt medium (Murashige and Skoog, 1962) with 30 g L⁻¹ sucrose and 3% (w/v) activated charcoal, solidified with 7 g L⁻¹ agar, pH = 5.8. Regenerated plants from *in vitro* germinated seeds were micropropagated on the same solid MS salt medium with 1 mg L⁻¹ barbituric acid (BA) and 30 g L⁻¹ sucrose, medium noted MS-3. The cultures were maintained in a growth room at 21 ± 2°C, a photoperiod of 16 h and 70 µmol m⁻² s⁻¹ daylight fluorescent illumination. Callus was formed from mature embryo root when the seeds with an incision at the embryonic end were cultured on solid MS salt medium enriched with: 1 mg L⁻¹ B1 vitamin, 5 mg L⁻¹ adenine sulfate, 80 mg L⁻¹ NaH₂PO₄, 1 g L⁻¹ casein hydrolysate and 2 mg L⁻¹ 2, 4-D (medium MS-K). The callus was grown on the same medium for 5 months and then it was transferred in 800 mL jars with 100 mL MS-3 medium. Two types of aeration systems were assessed, jars with one aeration vent (provided with a sponge cube), or 9 aeration vents (with 9 sponge cubes, at equal distances from each other). The callus was transferred three times on fresh MS-3 medium, at 4 weeks intervals, and callus biomass was weighed at the beginning and at each transfer on fresh medium. The growth curves of the callus in the two aeration conditions were compared.

In vitro corm development

For corm development, five groups of five-eight shoots were inoculated in jars containing 100 mL solid MS-3 medium for 20 days, then the same groups of plants were transferred on MS media with higher concentrations of sucrose (60 g L⁻¹ = MS-6 or 90 g L⁻¹ = MS-9). After 4 weeks, the number of cormlets was counted and the length of leaves and fresh weight of cormlets were determined. To MS-9 media either acetic acid or ancymidol were added, comparing the following: MS-9-0 (medium with addition of 90 g L⁻¹ sucrose), MS-9-AC (with 90 g L⁻¹ sucrose plus 10 mM acetic acid), MS-9-An (with 90 g L⁻¹ sucrose plus 2 mg L⁻¹ ancymidol) and MS-9-AC-An (with 90 g L⁻¹ sucrose plus both acetic acid and ancymidol at the same concentrations as above). The cultures were maintained in a growth room as mentioned above. Corm development was determined after 6 months on solid media (7 g L⁻¹ agar), followed by 6 months on liquid shaken media (orbital shaker Stuart Scientific, at 120 rpm). After 6 months on solid medium the corms were harvested, and the number of corms was counted and corm diameter and weight as well as leaf length were measured. Harvested corms with the leaves and roots discarded were then transferred to liquid medium having the same components: MS-9-0, MS-9-AC, MS-9-An, MS-9-AC-An. At the end of the experiment, the corms were harvested again, were counted and measured then dried at room temperature for further storage in the refrigerator. Such corms were used either to reinitiate *in vitro* culture or direct transfer to the soil. The experiments were repeated at least twice with three replicates per each experiment. Mean values and standard deviations were calculated.

Medium-term storage of *in vitro* cultures

For medium-term storage, three to five groups of ten shoots were transferred on MS-3 medium lacking BA. After growth for one

Table 1. The percentage of germination, mean leaf and root growth and mean leaf number per plant in *Gladiolus imbricatus* regenerated *in vitro* from wounded seeds or mature embryos (germination on MS ½ medium with 3% (w/v) activated charcoal; the growth of the shoots is recorded on MS-3 medium after one month).

Parameter	Wounded seed	Mature embryo
Percentage of germination (% ± SD)	75 ± 5	50 ± 6
Mean leaf length (cm ± SD)	3.26 ± 0.7	1.84 ± 0.4
Mean root length (cm ± SD)	4.8 ± 0.3	5.0 ± 0.2
Mean number of leaves (± SD)	2.66 ± 0.8	1.75 ± 0.7

month in the growth room having the same parameters as above, the leaves were cut at 1.5 cm height and the cultures on fresh MS-3 media (BA free) were transferred: in the same conditions (controls); in low light ($12 \mu\text{mol m}^{-2} \text{s}^{-1}$, daylight fluorescent illumination for 16 h, in the growth room having the same temperature as above; or in the refrigerator at 4°C, in the dark. The controls were transferred on fresh media each three months. The cultures in low light or temperature were maintained for 12 months, and the length of the leaves was measured at 3, 6 and 12 months. After one year, the shoots were transferred on fresh MS-3 media and maintained in normal growth conditions, as the controls. The percentage of recovered plants was determined. The experiments were repeated at least twice with three replicates per experiment. Mean values and standard deviations were calculated. The data were analyzed statistically for significance by applying student t test – p values are indicated.

RESULTS

In vitro culture initiation and micropropagation

In previous trials, all vegetative organs of *G. imbricatus* plants collected from the field (Figure 1 A) were heavily infected and could not be used to initiate *in vitro* cultures whatever sterilization was applied. Only the seeds and excised mature embryos could be sterilized and used as initial explants. When entire mature seeds were cultured on MS½ salt medium with the addition of activated charcoal, it took as long as 8 months for the seeds to germinate. In order to reduce germination time, the seeds were wounded (through an incision at the embryonic end) or the mature embryos were excised (rescued). In both cases, germination took place in 1 – 2 weeks (Figure 1C and D) and plants could be regenerated, grown and micropropagated *in vitro* through repeated transfers, at one-month interval, on fresh MS-3 medium (Table 1; Figure 1). At the first transfer on MS-3 medium the growth of the plants was slow. There were no significant differences between the growth curves of the plants originating from wounded seeds or mature embryos, although the height of the plants regenerated from excised embryos was lower (Figure 2). After repeated transfer of 3 to 5 groups of ten shoots in jars with 100 mL MS-3 medium, the shoots growth became constant with a propagation rate of 7 to 10 times/two months (Figure 1E). Some of the shoots also developed cormlets on this medium.

Callus cultures

When the seeds wounded by incision at embryonic end were cultured on a medium richer in organic components, MS-K, from the embryonic root a mass of callus was developed. The callus showed embryo-like structures in different stages of development from globular to torpedo (Figure 3A). The growth curve of the callus transferred on MS-3 medium was showing a slow ramp, and higher aeration (jars with 9 aeration vents) was causing not only stagnation in the callus growth but drying and loss of callus weight (Figure 3B). The transfer in larger vessels with aeration was not beneficial for the embryogenic callus and only the calluses maintained on MS-K medium in Petri dishes developed green somatic embryos (Figure 3A).

In vitro corm development

The initial assessment of the effect of sucrose concentration in the solid culture medium on corm development has shown that both corm number and fresh weight increased significantly with the increase of sucrose concentration up to 90 g L^{-1} . The leaf mean length was decreasing when sucrose concentration increased (Figure 4). Since the higher concentration of sucrose tested was the best for corm development, only MS-9 medium was used to assess the effects of acetic acid or/and ancymidol on corm development. The measurements after 6 months on solid media showed that the addition of acetic acid or ancymidol alone to the MS-9 media did not increase corm diameter, number or fresh weight but when added together, a significant increase in corm number could be noticed. After additional 6 months in liquid medium, the number of corms did not increase significantly, irrespective of media tested, but the gain in mean fresh weight was significantly higher in MS-9-An. The corms grown in MS-9-AC also had increased in their fresh weight as compared to MS-9-0. Interesting enough, the presence of both ancymidol and acetic acid in liquid medium does not have a synergistic effect on corm weight as it has on corm number in solid media (Tables 2 and 3; Figure 5). The two-step protocol consisting in the growth for 6 months



Figure 1. *Gladiolus imbricatus* growing in a clearing, in the habitat where plant material was harvested for *in vitro* culture initiation (Muntele Băișorii, 1200 m altitude) and *in vitro* culture: A, The plant in natural habitat; B, a closer view of inflorescence; C, wounded seed germination *in vitro*; D, excised mature embryos germinated *in vitro*; E, well-developed shoots on MS-3 medium. Bar = 1 mm (C and D) or 1 cm (A and E).

on solid media, followed by 6 months in liquid medium having the same composition, was efficient in producing a high number of well-developed corms (Figure 5). The corms could be germinated *ex vitro* for acclimatization or *in vitro* for new culture initiation in a percentage of 80 to 100%.

Medium-term conservation of *in vitro* plants and post-conservation recovery

In vitro cultures of *G. imbricatus* showed growth stagnation only when exposed at 4°C in the dark, as illustrated by the growth curves under different storage

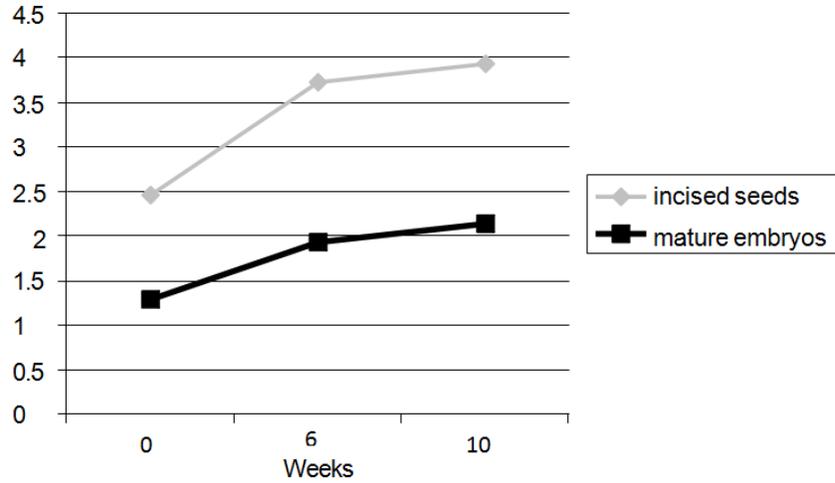


Figure 2. The growth curve of *Gladiolus imbricatus* plants on MS-3 medium, depending on their origin, from wounded seeds or excised mature embryos.

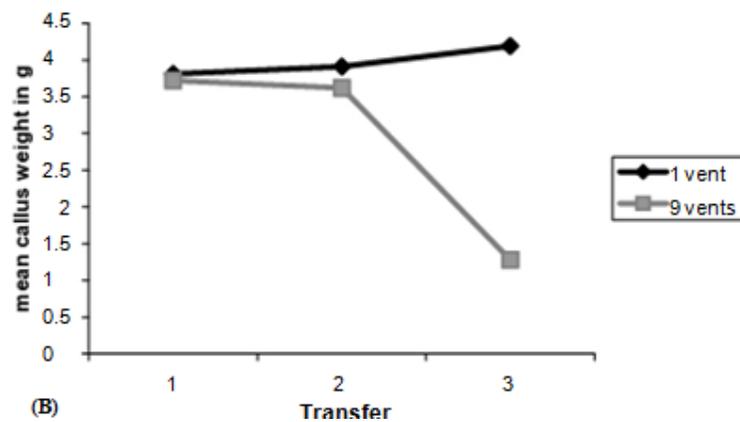
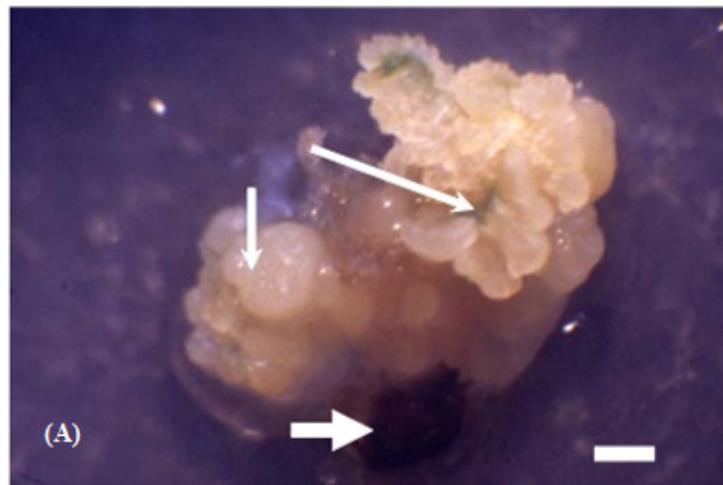


Figure 3. Callus development on zygotic embryo root of *Gladiolus imbricatus* cultured on MS-K medium: A, Embryogenic callus; white fat arrow – seed wounded at embryonic end; narrow arrows – somatic embryos arising from callus (bar = 1 mm); B, callus growth curve on MS-3 medium depending on the aeration system (jars provided with one or 9 aeration vents with sponge cubes).

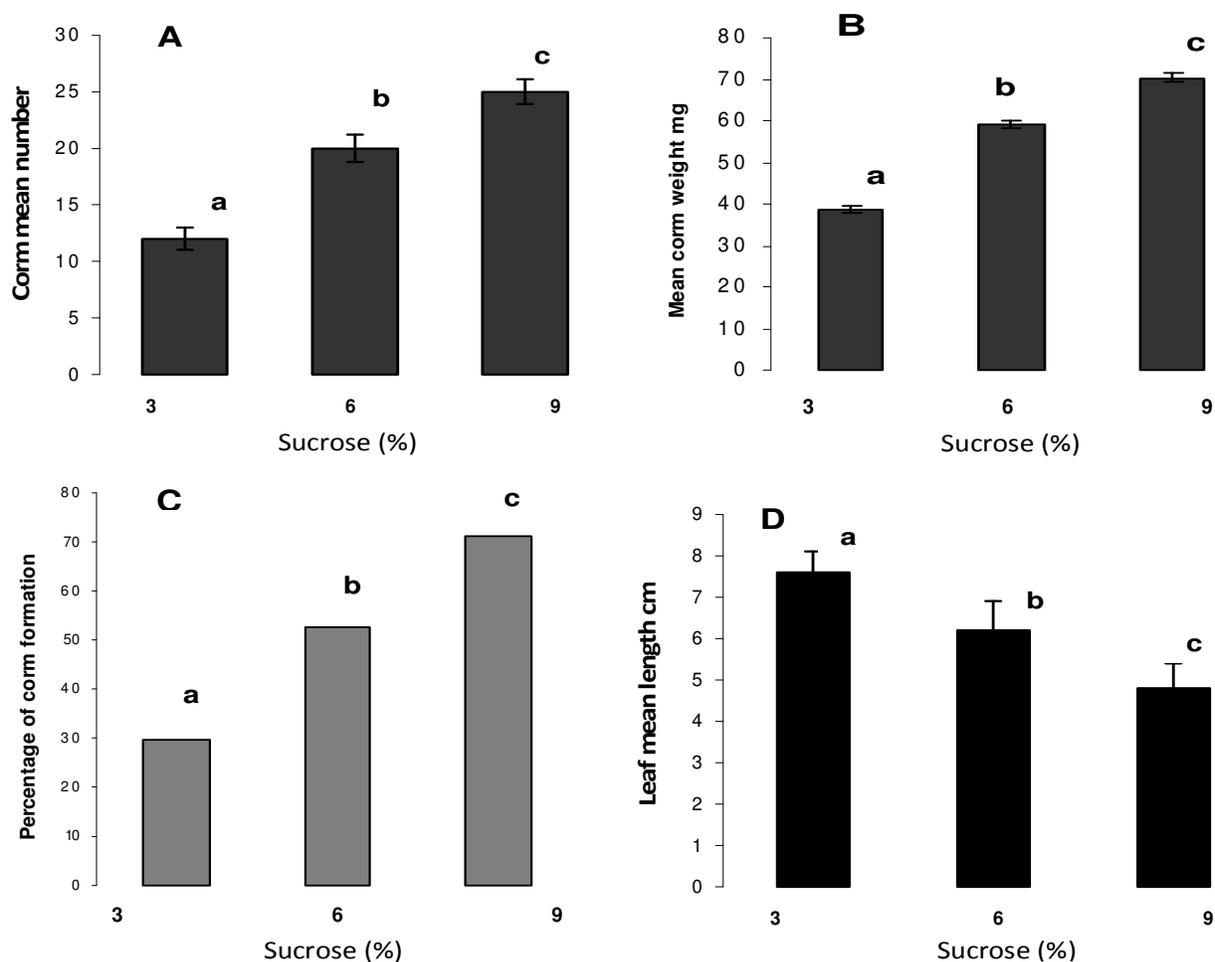


Figure 4. The effect of sucrose concentration on corm development after 4 weeks *in vitro*: A, Mean number of corms; B, mean corm fresh weight (mg); C, the percentage of shoots developing corms; D, mean leaf length (cm) (\pm S.D; different letters means significant at $p > 0.05$).

Table 2. The mean number, diameter and fresh weight of corms developed on different solid media after 6 months and their mean leaf length ($n = 15$; mean values \pm SD – for culture media see materials and methods).

Culture media	Mean number of corm	Mean corm diameter (cm)	Mean leaf length (cm)	Mean corm fresh weight (g)
MS-9-0	18 \pm 1.0	0.7 \pm 0.1	15 \pm 0.5	5.3 \pm 0.3
MS-9-AC	17 \pm 3.0	0.8 \pm 0.1	21 \pm 1.0	4.4 \pm 0.2
MS-9-An	15.3 \pm 2.5	0.6 \pm 0.2	13.7 \pm 3.2	2.5 \pm 1.8
MS-9-AC-An	27 \pm 8.5	1.0 \pm 0.0	10.5 \pm 0.5	5.4 \pm 0.4

MS-9-0, Medium with addition of 90 g L⁻¹ sucrose; MS-9-AC, 90 g L⁻¹ sucrose plus 10 mM acetic acid; MS-9-An, 90 g L⁻¹ sucrose plus 2 mg L⁻¹ ancymidol; MS-9-AC-An, 90 g L⁻¹ sucrose plus 10 mM acetic acid and plus 2 mg L⁻¹ ancymidol.

conditions (Figure 6). The cultures maintained in low light did grow a bit faster but were also etiolated and unable to recover after 12 months. On the contrary, few shoots (25%) were recovered and developed new shoots, from the cultures maintained 12 months at 4°C in the dark (Figure 7).

DISCUSSION

The wild rare species *G. imbricatus* was for the first time successfully cultured *in vitro*, multiplied and induced to form both well-developed corms and embryogenic callus. The results can be compared with those previously

Table 3. The mean number, diameter and fresh weight of corms developed on different liquid media for additional 6 months ($n = 15$; mean values \pm SD— for culture media see materials and methods).

Culture media	Mean number of corm	Mean corm diameter (cm)	Mean corm fresh weight (g)	Increase in mean corm fresh weight per 6 month (g)
MS-9-0	20 \pm 1.0	0.9 \pm 0.15	6.22 \pm 0.2	0.92 \pm 0.25
MS-9-AC	20 \pm 1.0	0.95 \pm 0.5	7.19 \pm 1.2	2.79 \pm 0.7
MS-9-An	21 \pm 8.7	0.98 \pm 0.4	8.75 \pm 1.5	6.25 \pm 0.8
MS-9-AC-An	27 \pm 8.5	1.1 \pm 0.1	8.59 \pm 0.7	3.19 \pm 0.6

MS-9-0, Medium with addition of 90 g L⁻¹ sucrose; MS-9-AC, 90 g L⁻¹ sucrose plus 10 mM acetic acid; MS-9-An, 90 g L⁻¹ sucrose plus 2 mg L⁻¹ ancymidol; MS-9-AC-An, 90 g L⁻¹ sucrose plus 10 mM acetic acid and plus 2 mg L⁻¹ ancymidol.

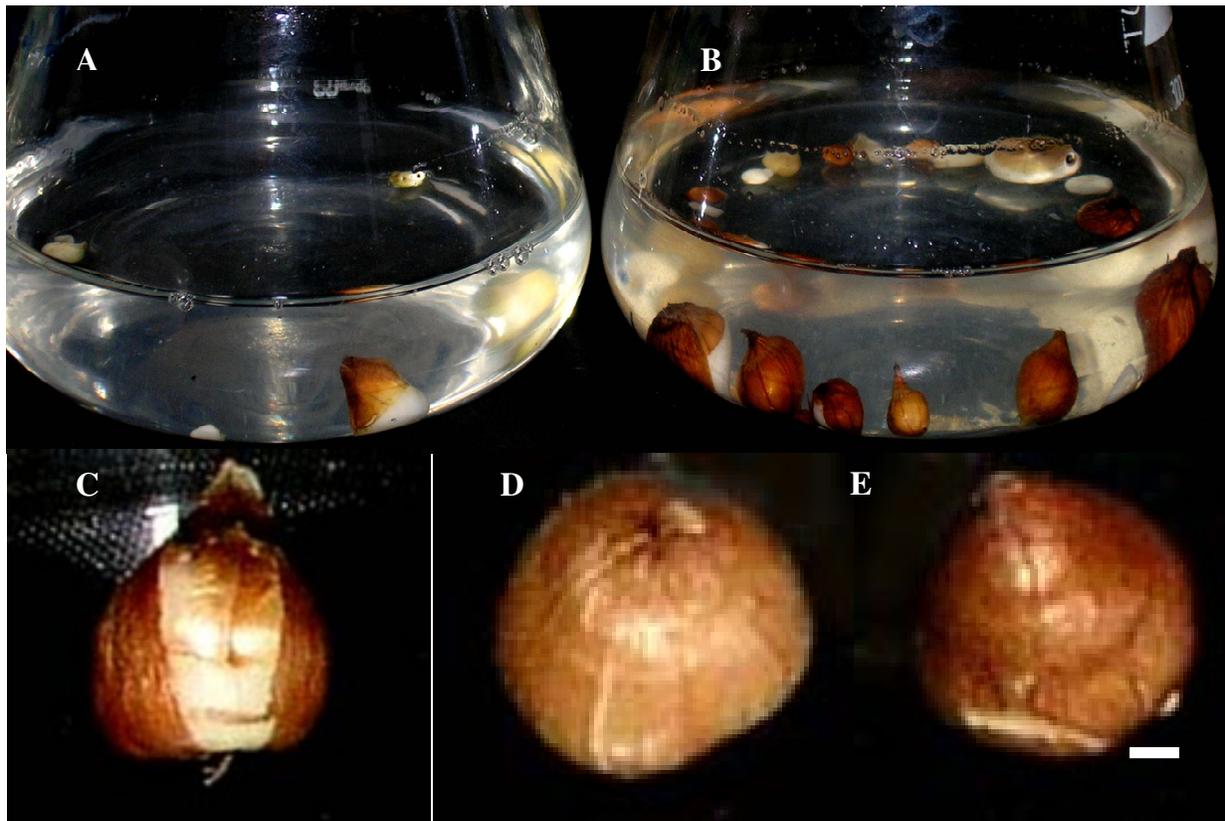


Figure 5. Corms of *Gladiolus imbricatus* developed after 6 month on solid media followed by additional 6 month in shaken liquid media: A, Medium MS-9-0; B – medium MS-9-AC - An; C, detail of a well-formed corm on MS-9-0 medium; D, detail of a corm developed in MS-9-An or in MS-9-AC (E) (bar = 1 cm in A and B, and 1 mm in C, E).

reported for *Gladiolus* cultivars or other wild species (Ziv et al., 1970; Ziv and Lilien-Kipnis, 1990; Steinitz and Lilien-Kipnis, 1989; Steinitz et al., 1991; Bruyn and Ferreira, 1992; Kamo, 1995; Remotti and Löffler, 1995; Sen and Sen, 1995; Misuk et al., 1998; Kumar, 1999; Rakosy-Tican et al., 2000; Kumar et al., 2001). The plant material collected from the field in late summer was heavily contaminated but mature seeds proved to be a suitable starting material for *in vitro* culture initiation. This was in contrast to gladioli cultivars where usually corms and corm buds were giving good results (Ziv et al., 1970;

Bajaj et al., 1982; Ziv and Lilien-Kipnis, 1990; Steinitz and Lilien-Kipnis, 1989; Steinitz et al., 1991; Kamo, 1995; Kumar et al., 1999; Rakosy-Tican et al., 2000). Being a cold-tolerant species with vegetative propagation through development of corms and cormlets, *G. imbricatus* seeds need chilling, and a long time to germinate. This is why besides a chilling period of 18 days the seeds should be wounded or mature embryos excised in order to reduce the time to their germination. Without such treatments, it took 8 months to break seed dormancy *in vitro* or even the germination was not achieved, like previously

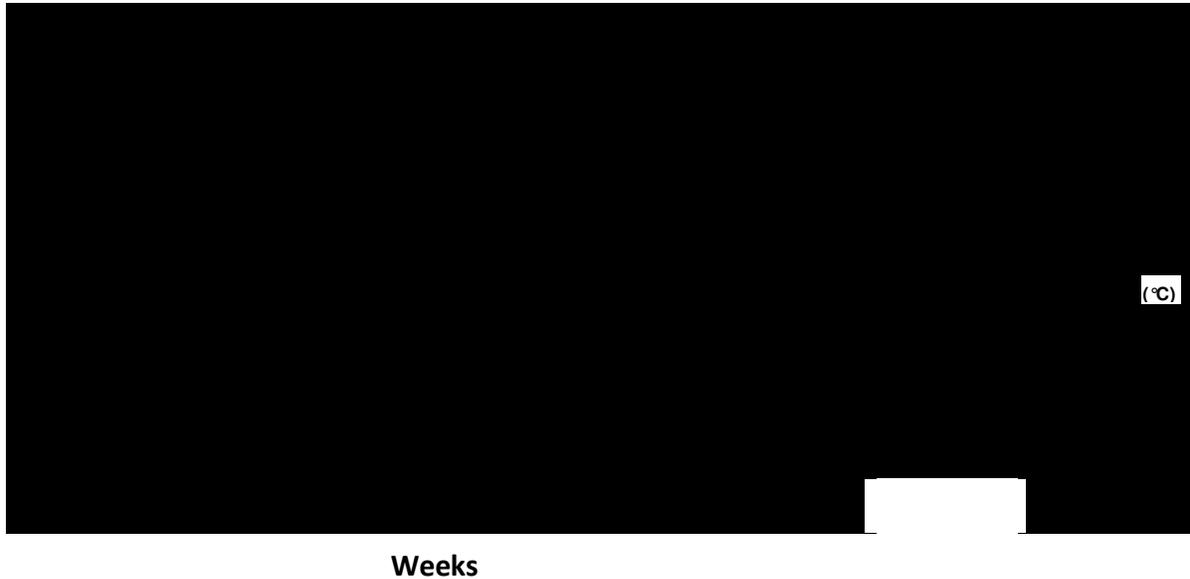


Figure 6. The growth curve of *G. imbricatus* shoots maintained *in vitro* in the growth room at 21°C, 16 h photoperiod and $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ (controls), in low light or in the dark at 4°C.

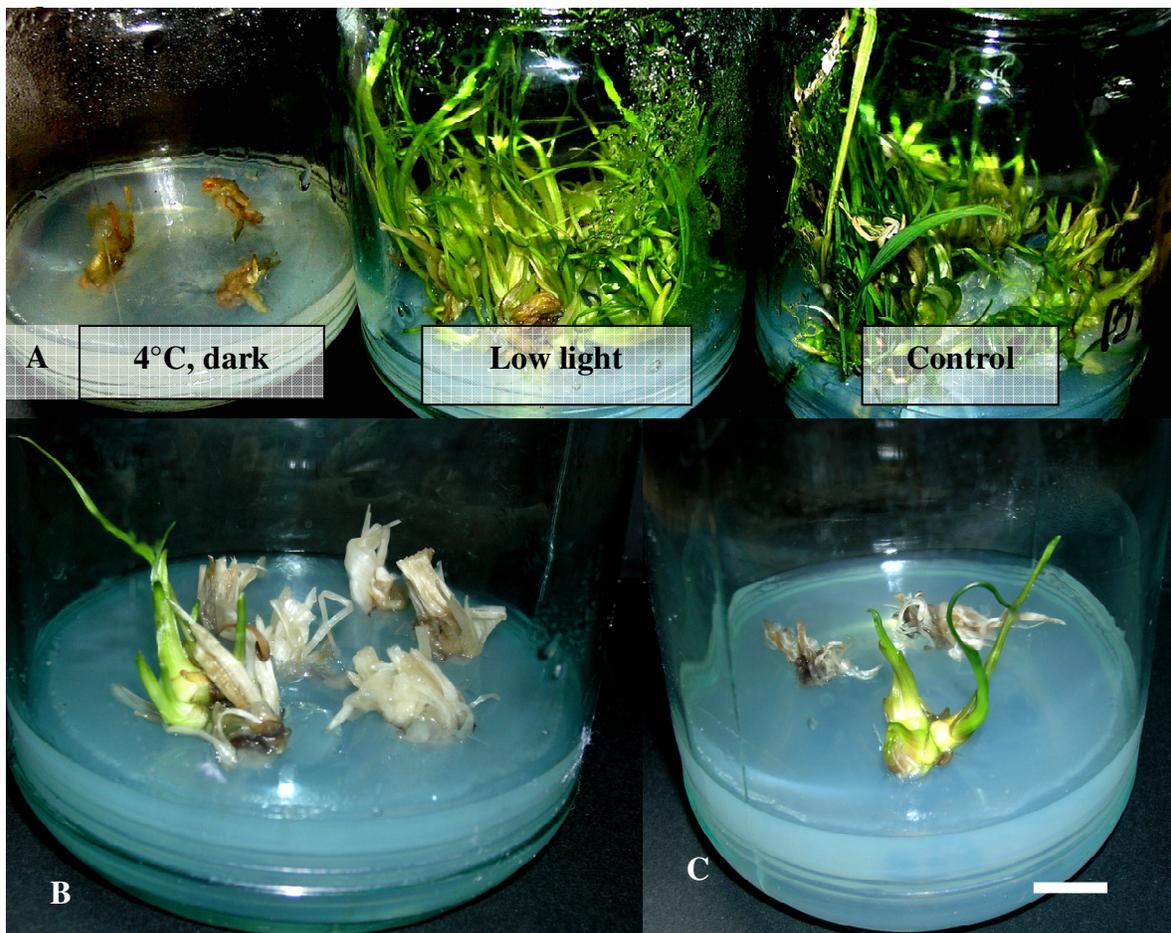


Figure 7. *In vitro* short term conservation of *G. imbricatus*: A. Comparative illustration of plant growth, after 3 month, for low temperature, low light or normal growth conditions – control; B and C, recovery of few plants in the cultures maintained for 12 month at 4°C in the dark and then transferred to normal growth conditions (bar = 1 cm).

reported in attempts for germplasm conservation (Dembicz et al., 2011). Although, lacking the nutritious reserves of the endosperm, excised mature embryos gave comparable results with wounded seeds when cultured on a poor mineral medium (MS $\frac{1}{2}$). Moreover the presence of activated charcoal had a beneficial influence on both seeds and mature embryo germination. For efficient micropropagation of *G. imbricatus* only the medium MS-3, containing 1 mg L⁻¹ BA sustained a good growth of the shoots along with their multiplication and few cormlets formation, in a similar way to the results described for two *Gladiolus* varieties (Rao et al., 1991a). Corm development was stimulated by increased concentrations of sucrose as it was demonstrated for some cultivars of *G. hybridus* (Goo and Kim, 1994). A two-step procedure consisting of solid media followed by transfer of corms in liquid shaken media also stimulated corm development in gladiolus cultivars (Sen and Sen, 1995), as it was after the addition of acetic acid and ancymidol in our previous experiments. This is why the same concentrations of acetic acid and ancymidol were evaluated for this wild species. For *G. imbricatus*, the best treatment was the solid medium MS-9-AC-An that improved corm number. Further on the gain in corm, fresh weight was stimulated by the growth retardant ancymidol plus 9% sucrose (MS-9-An). Additional six months of culture on liquid media did only slightly stimulate accumulation of fresh weight (MS-9-An and MS-9-AC). Ancymidol is known to influence the shoot growth by its inhibitory effect on gibberellins biosynthesis (Coolbaugh et al., 1982), but recently its effect on cell extension by the inhibition of cellulose biosynthesis was also demonstrated (Hofmannova et al., 2008). It is interesting to note here that acetic acid was not used previously to stimulate corm development in gladioli, excepting the work done in our group. There is only one report on its effect on *in vitro* tuber development in potato where it improved dry matter accumulation (Sharma et al., 2004). Since acetic acid is cheaper in comparison to ancymidol, it can be also used alone to stimulate corm development in gladiolus micropropagation. All corms obtained after the two-step growth protocol were well formed and had a good germination potential allowing direct transfer into soil. That way, we were able to develop an efficient *in vitro* technology for the micropropagation and multiplication of the rare species *G. imbricatus*. Such a technology might be used, after molecular analysis of genetic stability of *in vitro*-clones, for the restoration of natural populations into the habitats where this species became extinct.

Our results also demonstrate that *in vitro* cultures can be used for medium-term conservation of *G. imbricatus*, inducing growth stagnation by maintenance for 12 month at 4°C in the dark in the absence of phytohormones and subsequent recovery of a third of the plants when transferred at normal temperature and light conditions. Since phytohormones are thought to induce genetic changes it has to be expected that true to type clones are

regenerated but only by molecular analysis the genetic stability could be confirmed. Such analysis is planned in near future. The development of embryogenic callus represents a starting point for further research on rapid cloning and long-term conservation of this rare species by cryopreservation.

Conclusions

In vitro technologies reported here for the first time for *G. imbricatus* guarantee clonally propagation and multiplication for restoration of natural populations of this rare, Red listed species. The first results on medium-term conservation will help the stable maintenance *in vitro* with lower costs along with further possible use of embryogenic callus for cryopreservation.

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